INVESTIGATION OF THE STRUCTURE, FUNCTION, AND REGULATION OF QUINOLINATE SYNTHASE: THE IRON–SULFUR CLUSTER ENZYME INVOLVED IN PROKARYOTIC NAD⁺ BIOSYNTHESIS

A Dissertation in
Biochemistry, Microbiology, and Molecular Biology

by

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Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

May 2011
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ABSTRACT

Nicotinamide adenine dinucleotide (NAD⁺) is an essential biological cofactor known primarily for its role in oxidation–reduction reactions, but is also involved in ADP ribosylation, adenylation and histone deacetylation. The key intermediate in NAD⁺ biosynthesis is quinolinic acid. The pathway to form quinolinic acid differs between most eukaryotes and prokaryotes, making the prokaryotic pathway a possible target for design of antibacterial agents. In eukaryotes, quinolinic acid is formed via the degradation of L-tryptophan by a series of enzymes, of which three require molecular oxygen for catalysis. In prokaryotes, quinolinic acid is synthesized via the action of two enzymes, L-aspartate oxidase (NadB) and quinolinate synthase (NadA). NadB oxidizes L-aspartate to form iminoaspartate by a two–electron oxidation utilizing the cofactor, flavin adenine dinucleotide (FAD). The iminoaspartate is then condensed with dihydroxyacetone phosphate by NadA to form quinolinic acid. In contrast to the eukaryotic pathway, the prokaryotic pathway can function under strictly anaerobic conditions.

NadA from Escherichia coli was initially proposed to contain an iron–sulfur (Fe/S) cluster, due to its instability in the presence of molecular oxygen and the presence of a CysXXCysXXCys motif, which contains the cysteines that commonly ligate a [4Fe-4S] cluster. This enzyme was later confirmed to contain one [4Fe-4S] that is essential for catalysis. Through further investigation of the cysteine variants of NadA, we have demonstrated that the cluster is ligated by three cysteines that are conserved in all quinolinate synthases, of which only one lies in the CysXXCysXXCys motif. Interestingly, we found that the other two cysteines in the CysXXCys motif form a redox–active disulfide bond, which regulates the rate of formation of quinolinic acid. Disulfide bond formation is regulated by thioredoxin, a disulfide bond reductase, and we have determined the redox potential of NadA to be – 264 mV using the thioredoxin redox couple.
Despite the lack of a CysXXCys motif, we have demonstrated that NadA from *Mycobacterium tuberculosis* is also regulated by disulfide bond formation. Our results suggest that the redox potential is higher than that of the *E. coli* enzyme because optimal activity is obtained with DsbA, a periplasmic oxidoreductase more oxidizing than thioredoxin. Site–directed mutagenesis studies demonstrate that the disulfide bond is formed between two cysteines in a CysXCys motif. The role of the disulfide bond *in vivo* has also been investigated using an *E. coli* strain in which the *nadA* gene has been replaced with a kanamycin cassette (ΔnadA). A variant with a single substitution of the disulfide bond forming cysteine is unable to complement the ΔnadA strain unless the level of expression of the variant is increased and the strain is grown in the absence of oxygen. These results suggest that the disulfide bond may play a role in protecting the Fe/S cluster from oxidative stress.

The only published crystal structure of NadA is from *Pyrococcus horikoshii* and is lacking the essential Fe/S cluster. Utilizing the apo–structure bound to the substrate mimic, malate, and the knowledge of the three conserved cysteines ligating the Fe/S cluster, we have modeled the Fe/S cluster and quinolinic acid precursor into the structure. Using this model, we identified eight conserved active site residues positioned to interact with substrate and changed each of these residues by site–directed mutagenesis. We then characterized the resulting variant proteins for their ability to form both quinolinic acid and inorganic phosphate, a second product of the reaction. Through these studies we have identified three variants that are completely inactive for formation of both products and five others with varying reaction rates. In future experiments, these variants will be used to build up intermediates to determine the order of events during the condensation reaction.
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<thead>
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<tr>
<td>AI</td>
<td>as–isolated</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>DHAP</td>
<td>dihydroxyacetone phosphate</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5’-dithiobis-(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>Ec</td>
<td><em>Escherichia coli</em></td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ENDOR</td>
<td>electron nuclear double resonance</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FDR</td>
<td>fumarate reductase</td>
</tr>
<tr>
<td>Fe/S</td>
<td>iron–sulfur</td>
</tr>
<tr>
<td>G3P</td>
<td>glyceraldehyde-3-phosphate</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione (reduced)</td>
</tr>
<tr>
<td>GSSG</td>
<td>glutathione (oxidized)</td>
</tr>
<tr>
<td>HEPES</td>
<td>(N)-(2-hydroxyethyl)piperazine-(N^\prime)-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>HPLC</td>
<td>high–performance liquid chromatography</td>
</tr>
<tr>
<td>IA</td>
<td>iminoaspartate</td>
</tr>
<tr>
<td>IMAC</td>
<td>immobilized metal affinity chromatography</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>IS</td>
<td>internal standard</td>
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<tr>
<td>LB</td>
<td>Luria–Bertani</td>
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<tr>
<td>LC–MS</td>
<td>liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>MALDI–TOF</td>
<td>matrix–assisted laser desorption/ionization time–of–flight</td>
</tr>
<tr>
<td>Mtb</td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propane-sulfonic acid</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>nicotinamide adenine dinucleotide</td>
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<tr>
<td>NadA</td>
<td>quinolinate synthase</td>
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<td>NadA&lt;sub&gt;ox&lt;/sub&gt;</td>
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<td>NadB</td>
<td>L-aspartate oxidase</td>
</tr>
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<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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<td>NaMN</td>
<td>nicotinic acid mononucleotide</td>
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<tr>
<td>Ni-NTA</td>
<td>nickel nitrilotriacetic acid</td>
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<td>nicotinamide mononucleotide</td>
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<td>oxaloacetate</td>
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<tr>
<td>P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>inorganic phosphate</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>Ph</td>
<td><em>Pyrococcus horikoshii</em></td>
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<tr>
<td>PMSF</td>
<td>phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PNC</td>
<td>pyrimidine nucleotide cycle</td>
</tr>
<tr>
<td>QA</td>
<td>quinolinic acid</td>
</tr>
<tr>
<td>RCN</td>
<td>reconstituted</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>----------------------------------------------------</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SDH</td>
<td>succinate dehydrogenase</td>
</tr>
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<td>SDS-PAGE</td>
<td>sodium dodecylsulfate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>TB</td>
<td>terrific broth</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
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<tr>
<td>TCEP</td>
<td>tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TIM</td>
<td>triosephosphate isomerase</td>
</tr>
<tr>
<td>Trx</td>
<td>thioredoxin</td>
</tr>
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<td>Trx&lt;sub&gt;x&lt;/sub&gt;</td>
<td>oxidized thioredoxin</td>
</tr>
<tr>
<td>Trx&lt;sub&gt;red&lt;/sub&gt;</td>
<td>reduced thioredoxin</td>
</tr>
<tr>
<td>WT</td>
<td>wild–type</td>
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ACKNOWLEDGEMENTS

I first want to dedicate this dissertation to my daughter, Maisie. You have motivated me to work hard in the hours that we are apart so that I can come home and enjoy time with you. I look forward to watching you grow and hope that you can learn from my experiences and obtain all your future goals. Next, I need to thank my husband Gregg, since I would not be here today if it was not for you. You have supported me throughout the years, from the time that I decided to pursue this degree, through all of the hard work, and especially in this last year as I was juggling being a new mom and finishing up my thesis. We have had so many life experiences here in State College, PA: we got married, bought our first home, and had our first child. We will always look back and remember these days. I also want to thank my parents, who have encouraged me to obtain my goals and stressed the importance of my education.

For my success as a researcher, I need to thank the current Booker lab members. Lauren Sites, you are brave to take on the NadA project and I wish you luck. It has been a pleasure training you as we have grown to be good friends and workout buddies. Kyung-Hoon Lee, my molecular biology go-to man, you taught me numerous techniques that I use daily and would not have made it through without your help. Nick Lanz, Art Arcinas, and Brad Landgraf, thank you for your help and for making lab fun. Tyler Grove, we’ve been here from the start together and are the last two standing. We’ve had our disagreements, but I think we helped keep each other on our toes and I thank you for that. Monica McLaughlin, a talent high school senior, for setting up numerous NadA crystal trials. As for previous lab members, Amy Griffiths, my NadA partner in crime, and Liz Bilgren, I really enjoyed working with both of you and have missed you these last
years. Dr. Robert Cicchillo and Dr. David Iwig, for talking me into joining this lab and teaching me in the very early stages of my graduate career.

Thank you to other labs at Penn State that have significantly contributed to my work. Dr. Marty Bollinger for providing the pTrx strain used for my E. coli NadA disulfide experiments. Dr. Carsten Krebs for your Mössbauer expertise and guidance in a number of experiments. Sarah Barchinger and Dr. Sarah Ades for numerous strains, plasmids, and microbiology related discussions for my studies of the nadA knockout. Neela Yennawar, thank you for your help initiating my modeling studies and also for sharing your expertise as we set up our crystal trials.

Thank you to my committee members, Dr. Marty Bollinger, Dr. Carsten Krebs, Dr. Ming Tien, and Dr. Philip Bevilacqua, for your insight and suggestions through the years. Last, but not least, thank you to my advisor, Dr. Squire Booker, for whom none of this would be possible without. Your vast knowledge and ability to motivate students to be excited about science is inspiring. Thank you for supporting my ideas and allowing me to grow into the independent scientist I have become.
Chapter 1

Introduction

Nicotinamide adenine dinucleotide (NAD\(^+\)) is an essential biological molecule found in all living cells and is involved in many cellular processes, such as general metabolism, signaling and transcription (Figure 1.1). NAD\(^+\) is known primarily for its role in oxidation–reduction reactions, in which it accepts a hydride at carbon 4 of the nicotinamide ring.

![Nicotinamide adenine dinucleotide (NAD\(^+\))]({fig1.1.png)

Figure 1.1: Nicotinamide adenine dinucleotide (NAD\(^+\))

NAD\(^+\)/NADH and the phosphorylated forms nictinamide adenine dinucleotide phosphate (NADP\(^+\)/NADPH), are required for the reactions of over 300 different kinds of enzymes (/). Unlike reactions that utilize a cofactor, NAD\(^+\)/NADH most often are not returned to the original form after the reaction is complete and are therefore a cosubstrate. In some enzymes, such as urocanate hydratase, UDP–galactose epimerase, and S-adenosylhomocysteine hydrolase, NAD\(^+\) acts as a true cofactor because there is no net change to the molecule in the reaction. In
contrast to the other common organic redox cofactor, flavin adenine dinucleotide (FAD), there are no known enzymes that bind NAD$^+$ covalently.

A number of cellular processes, such as metabolism, signaling and transcription are impacted by the oxidation/reduction potential of the cell. The cell requires sufficient reducing equivalents to prevent damage that occurs from oxidative stress, often due to the presence of reactive oxygen species or reactive nitrogen intermediates. The oxidation/reduction potential is directly related to the NAD$^+$/NADH ratio, which is approximately 8 in *Escherichia coli*, while the NADP$^+$/NADPH ratio is lower (~1) (2). In prokaryotes, the concentrations of pyrimidine dinucleotides have been estimated to be 0.8 mM NAD$^+$, 0.02 mM NADH, 0.05 mM NADP$^+$, and 0.15 mM NADPH (3). When cells are exposed to increasing concentrations of oxygen, the NAD$^+$ concentration increases while the NADH concentration remains the same (4). Also, the rate of NAD$^+$ turnover is greater under aerobic conditions, with a 90 min half-life compared to 333 min under anaerobic conditions (5). In summary, cells require more NAD$^+$, especially upon oxygen exposure when cells are producing and using more NAD$^+$.

### 1.1 NAD$^+$ Metabolism, Recycling and Biosynthesis

#### NAD$^+$ Metabolism

In addition to acting as a cosubstrate in oxidation/reduction reactions, NAD$^+$ is also required as a substrate in myriad other reactions. One such reaction is the modification of proteins by adenosine diphosphate (ADP) ribosylation, in which nicotinamide is cleaved from NAD$^+$ and the ADP–ribose unit is transferred to a variety of target molecules by ADP–ribose transferases (ARTs) or poly(ADP) polymerases (PARPs) (Figure 1.2). In response to DNA–strand breaks, PARP-1 catalyzes the covalent attachment of ADP–ribose units on the γ-carboxyl group of glutamate residues of acceptor proteins, such as histones, transcription factors and PARP-1 itself (6). The over–activation of PARPs in response to oxidative stress can lead to consumption of NAD$^+$, and the inhibition of PARP has been shown to protect against cardiac, inflammatory and
neurodegenerative conditions (7). Also, nicotinamide is an inhibitor of this enzyme, and future therapies may target these NAD$^+$–depleting enzymes to increase cellular NAD$^+$ concentrations (8).

![Figure 1.2: ADP–ribosylation reaction](image)

The SIRT family of NAD$^+$–dependent histone deacetylases, also known as sirtuins can transfer the ADP–ribose group to an acetyl group (9). This reaction often occurs in the deacetylation of lysine residues of histones. The proteins have been investigated to be involved in regulation of metabolism and the physiological response to calorie restriction, a notable method for extending lifespan in yeast, worms, flies and mammals (9). Also, the bacterial cholera and diptheria toxins act to infect their host by the ADP ribosylation of eukaryotic host proteins resulting in the interference of their signal transduction pathways (10).

NAD$^+$ is required for the prokaryotic DNA–ligase reaction, which catalyzes the formation of phosphodiester bonds between adjacent 5’-phosphate and 3’-hydroxyl groups of nicked double–stranded DNA. In most eukaryotes and archaea, this reaction is energetically driven by the hydrolysis of adenosine triphosphate (ATP) to adenosine monophosphate (AMP) and pyrophosphate (11). In bacteria, the cleavage of NAD$^+$ to nicotinamide mononucleotide (NMN) and AMP drives the reaction (Figure 1.3). Despite the similarity in the reactions, there is
no sequence similarity between the ATP– and NAD$^+$–dependent ligases, yet both utilize an adenylated enzyme intermediate on the lysine that resides in the KXDG sequence, which is common to all DNA ligases (12). Despite the lack of sequence similarities, recent structural comparisons have unveiled conserved domains between the two families of proteins. NAD$^+$–dependent DNA–ligase is essential for many species of bacteria but is not present in eukaryotes, making this enzyme an attractive antibiotic target.

![Figure 1.3: NAD$^+$–dependent DNA ligase reaction](image)

In the enzyme urocanase, it is proposed that the nicotinamide ring of NAD$^+$ forms a transient covalent adduct with the substrate urocanate to facilitate catalysis via an electrophilic mechanism (13). In this reaction, NAD$^+$ acts as a true cofactor and is not consumed during the reaction. It is proposed that a transient covalent intermediate is formed between NAD$^+$ and the substrate, urocanate (13). The biosynthesis of thiamin thiazole in *Saccharomyces cerevisiae* has been shown to begin with the cleavage of the $\text{N}$-glycosyl bond of NAD$^+$ (14). These reactions are important to many functions in the cell, all requiring sufficient levels of NAD$^+$.

**NAD$^+$ Recycling**

In addition to the *de novo* synthesis of NAD$^+$, many organisms utilize the breakdown products of NAD$^+$–consuming reactions to reconstruct the molecule via the pyrimidine nucleotide cycle (PNC) (Figure 1.4). Nicotinamide often enters this recycling or salvage pathway where it is...
deamidated by a nicotinamidase to form nicotinic acid. Nicotinic acid is then transferred to a phosphoribosyl group by nicotinic acid phosphoribosyl transferase to form nicotinic acid mononucleotide (NaMN). The last two steps to form NAD$^+$, adenylation by NaMN adenyltransferase and subsequent amidation by NAD$^+$ synthetase, are identical to the de novo synthesis pathway. Nicotinamide mononucleotide (NMN), a product of the NAD$^+$–consuming enzyme prokaryotic DNA–ligase, can also be recycled by a number of alternative routes: it can be used to reform NAD$^+$ by the enzyme NaMN/(NMN) adenyltransferase; it can undergo deamidation to form NaMN by NMN deamidase; or it can be further broken down to nicotinamide by NMN glycohydrolase.
The recycling pathway is energetically favorable, and therefore when functional is the preferred method to synthesize NAD$^+$ in the cell over de novo synthesis (15). There are some organisms that lack the de novo pathway and depend solely on the environment to acquire NAD$^+$. This is true in the bacteria *Haemophilus influenzae*, which requires NAD$^+$, NMN, or nicotinamide riboside to grow, and the yeast *Candida glabrata*, which is auxotrophic for nicotinic acid (16).
The lethal infectious bacterium, *Mycobacterium tuberculosis* (Mtb), was originally believed to have a nonfunctional recycling pathway (17). Instead, the organism actually prefers to acquire nicotinamide exogenously from its host to produce NAD\(^+\) utilizing a nicotinamidase (encoded by *pncA*). The salvage pathway has been the target for the development of new drugs to kill this lethal bacterium, and the nicotinamide analog, pyrazinamide, is currently used as a component in Mtb chemotherapy. Recent research shows that the best line of defense is via simultaneous inhibition of both the *de novo* and salvage pathways (18).

**NAD\(^+\) Biosynthesis**

NAD\(^+\) is consumed in a multitude of cellular reactions; therefore, available pools of NAD\(^+\) need to be replenished either through salvage or *de novo* biosynthesis. The biosynthesis of NAD\(^+\) is a target for drug discovery, because the pathway in most eukaryotes is different from that in most prokaryotes. The first common intermediate in both pathways is quinolinic acid (QA). After its formation, the subsequent steps in both pathways to form NAD\(^+\) are virtually identical. QA is decarboxylated and then appended to ribose 5-phosphate via the action of QA phosphoribosyltransferase (NadC), yielding NaMN (Figure 1.5). This S\(\text{N}\)\(_1\) reaction is facilitated by coordination of the pyrophosphate moiety of the substrate phosphoribosyl pyrophosphate by two Mg\(^{2+}\) ions to activate it as a leaving group (19). The resulting oxocarbenium is then trapped by the positively charged nitrogen of QA and is stabilized by the carboxylate at C2 of QA. The positively charged nitrogen then facilitates the decarboxylation reaction to form the resulting NaMN and the by–product CO\(_2\). The proposed S\(\text{N}\)\(_1\) reaction is supported by studies of the closely related reaction catalyzed by orotic acid phosphoribosyltransferase (20).

The next step is the adenylation of NaMN to form nicotinic acid dinucleotide (NaAD) by the enzyme NaMN adenylytransferase (NadD). This Mg\(^{2+}\)–dependent reaction occurs via nucleophilic attack of the 5’-phosphate of NaMN on the \(\alpha\)-phosphorus of ATP to form NaAD and pyrophosphate. In addition to the *de novo* reaction, NadD is also able to catalyze formation of
NAD\(^+\) from NMN via salvage, albeit at a rate that is 20-fold slower (21). The final step in the de novo pathway is amide transfer to NaAD to form NAD\(^+\), catalyzed by NAD\(^+\) synthetase (NadE).

In most bacteria and archaea, the amide donor is ammonia, while all eukaryotes and a few archaea and bacteria, such as *M. tuberculosis* and *Synechocystis sp.*, utilize glutamine (22-24).

![Figure 1.5: De novo NAD\(^+\) biosynthesis from QA](image)

1.2 Quinolinic acid Biosynthesis in Eukaryotes

In 1938, it was discovered that nicotinic acid was an effective treatment for dogs suffering from “canine black tongue”, which was later discovered to be due to a niacin–deficient diet, known as pellagra (25). Subsequent studies found that rats overcame pellagra when fed a nicotinamide–deficient diet supplemented with tryptophan (26). Isotope feeding experiments in a
*Neurospora* mutant strain showed that QA synthesis proceeds solely through tryptophan (27). The first committed step in this reaction is the cleavage of L-tryptophan to form formylkynurenine by the oxygen–dependent reaction catalyzed by tryptophan 2,3-dioxygenase (TDO) (28) (Figure 1.6). This enzyme utilizes a histidine–ligated heme to carry out oxygen activation and insertion into the substrate, which recent structural modeling studies suggest proceeds via a Criegee mechanism rather than a dioxetane intermediate (29). The second enzyme in the pathway, N-formylkynurenine formamidase (KFA), removes the formyl group from formylkynurenine to leave kynurenine (30). The kynurenine is then hydroxylated to form 3-hydroxy-kynurenine by a NADPH and O₂-dependent reaction catalyzed by the FAD–containing enzyme kynurenine-3-monooxygenase (KMO) (31). The hydrolytic cleavage of the amino acid side chain by kynureninase (KYN) results in 3-hydroxyanthranilate and L-alanine. KYN is a PLP–dependent enzyme, which also converts L-alanine to pyruvate in a slower transamination reaction (32). The final enzyme in the pathway to form QA is 3-hydroxyanthranilate-3,4-dioxygenase (HAD), which converts 3-hydroxyanthranilate to α-amino-β-carboxymuconate-ε-semialdehyde (ACMS), which is unstable and spontaneously cyclizes to QA (33). HAD is in a class of intramolecular dioxygenase enzymes with non–heme ferrous iron and is located in the cytosol.
1.3 Quinolinic acid Biosynthesis in Prokaryotes

The tryptophan to QA pathway described above is also known as the aerobic pathway due to the requirement of molecular oxygen for three of the five enzymatic reactions. This pathway has been best characterized in mammals, yet it has also been studied in yeast, molds (Neurospora) (34), some higher plants (35), and the actinomycetes Xanthomonas pruni (36). Additionally, the genes involved in this biosynthesis pathway were recently discovered in myriad
other bacteria (37). Early nutritional studies found that L-tryptophan was not a precursor to nicotinic acid in E. coli (38). The discovery that QA could support growth of an NAD$^+$ auxotroph in E. coli and B. subtilis led to the proposal that QA is a common intermediate in the two pathways (39). Metabolic feeding studies performed by Ortega and Brown showed that the precursors for the pyridine ring of NAD$^+$ in E. coli are derived from a 4-carbon dicarboxylic acid and glycerol, or a compound easily made from glycerol (Figure 1.7) (40). This was demonstrated in a series of experiments that showed that cells supplemented with $[1,4\text{-}^{14}\text{C}]$-succinic acid and unlabeled glycerol produced nicotinic acid with $^{14}\text{C}$ in the carboxyl group but not in the pyridine ring. Cells supplemented with $[2,3\text{-}^{14}\text{C}]$-succinic acid and unlabeled glycerol produced nicotinic acid that was labeled in the pyridine ring but not the carboxylate. Finally, cells supplemented with $[1,3\text{-}^{14}\text{C}]$-glycerol and unlabeled succinate also produced nicotinic acid labeled only in the pyridine ring.

![Chemical structures](https://example.com/structures.png)

Figure 1.7: Chemical structures of succinic acid, glycerol, and nicotinic acid

### 1.4 Identification of the nadA and nadB Genes

In 1967, nicotinic acid–requiring E. coli mutants, designated nicA, were identified and the genes causing the mutations were mapped close to the galactose operon on the standard genetic map (41). A few years later, when the locus for nadB was identified, it was suggested that NAD$^+$ biosynthetic mutants were to be designated nad rather than nic (42). In depth studies on the biosynthesis of QA in E. coli continued in 1970 by Chandler and Gholson, who first showed that QA is formed via the direct condensation of a 3-carbon unit and a 4-carbon unit, which they
suggested to be a triosephosphate and L-aspartate (43). This was demonstrated by incubating the crude extract from a nadC mutant E. coli strain with radioactive precursors, then isolating and analyzing the QA produced. Similar to the findings of Ortega and Brown, when they used \(^{14}\text{C}\)-labeled glucose or glycerol, radioactive incorporation was found to be mainly at C4 and C6 of QA (Figure 1.8). They also obtained radioactively labeled QA upon incubation of the crude extract with DL-[1-\(^{14}\text{C}\)]-aspartate. Upon chemical decarboxylation of the isolated QA, 97.5% of the radioactivity was lost, indicating that C1 of aspartate is incorporated directly into the carboxylate of QA (C7) without significant randomization of the carbon skeleton (Figure 1.8).

![Figure 1.8: Prokaryotic pathway to QA formation](image)

Two proteins, designated NadA and NadB, were later found to be required for QA synthesis in E. coli and were partially purified. In addition, dihydroxyacetone phosphate (DHAP) was established as the triosephosphate substrate (44). The NadB protein was purified from a nadA
mutant of *E. coli* and determined by gel–filtration chromatography to have an apparent molecular mass of 85,000 Daltons (Da). The NadA protein was purified from a *nadB* mutant of *E. coli* and similarly determined to have an apparent molecular mass of 35,000 Da. When the two proteins were analyzed simultaneously, no evidence of complex formation was observed. Both proteins, DHAP, and FAD were found to be required for \(^{14}\text{C}\) incorporation into QA in an assay containing L-[\(^{1-14}\text{C}\)]aspartic acid. Glyceraldehyde-3-phosphate (G3P) was found to substitute for DHAP as a substrate with equal rates of QA formation; however, given that both compounds are products of the enzyme triosephosphate isomerase (TIM), it was hypothesized that purified NadA and NadB might be contaminated with TIM. This premise was consistent with the observation that an inhibitor of TIM, chloroacetol phosphate, had no effect on QA production when assays were conducted using DHAP, but strongly inhibited QA production when G3P was used as substrate, indicating that the G3P must be converted to DHAP prior to forming QA.

Experiments using \(^{14}\text{C}\)-labeled DHAP demonstrated that C3 of DHAP condenses with C3 of aspartate to form QA (Figure I.8) (45). These results were similar to those observed when analyzing QA formed in *Nicotiana rustica* using labeled G3P, which is converted to DHAP by TIM, suggesting the pathway for QA synthesis is the same in *E. coli* and some plants (46)

**Determination of the First Enzyme in QA Biosynthesis**

Subsequent to the determination that QA is formed from L-aspartate and DHAP, efforts were turned to the identification of any intermediates formed prior to QA and the order of action by the two enzymes in the pathway. Kerr and Tritz found that some *nadA*, *nadB*, and *nadC* mutants were able to grow in minimal media supplemented with casamino acids without nicotinic acid, yet at rates much lower than those obtained with nicotinic acid supplementation (47). When the *nadA* and *nadB* mutants were incubated together, higher growth rates were observed, but not when either was incubated alone with the *nadC* mutant. It was known that NadC is a quinolinate phosphoribosyl transferase that catalyzes the conversion of QA to NaMN. These studies
confirmed that the NadA and NadB proteins are required to form QA, which is then a substrate for the NadC protein. When the concentrations of the nadA and nadB mutants were monitored by growth on selective media, it was found that the number of nadA mutant cells increased with the optical density of the growing cultures while that of the nadB mutant did not. This observation led to the presumption that the nadA mutant was being fed by the nadB mutant, and therefore the NadA enzyme precedes the NadB enzyme in the QA biosynthesis pathway.

Wicks et al. reinvestigated the proposed order of action of the enzymes in a series of experiments using nadA and nadB mutants. Cell extracts were treated with toluene to permeabilize the E. coli membrane, which allowed for diffusion of small molecules while macromolecules remained in the cell (48). In a reaction mixture containing L-[U-14C]-aspartate, DHAP, FAD and the supernatant from toluene–treated extracts of both nadA and nadB mutants, radioactive QA was detected, while little or no QA was detected in reactions containing supernatants of either of the single mutants only. This suggested that an intermediate, formed by one mutant with the supplemented substrate(s), is then subsequently converted to QA by the other mutant. In order to test which mutant formed the intermediate, QA was quantified from reaction mixtures constructed in the following manner. Each of the two mutants was separately incubated in the reaction mixture, which was subsequently toluenized and centrifuged to remove the cells, and their resulting supernatants were then added to the supernatant of the other mutant. It was observed that 4.5 times more QA is produced when the nadA mutant reaction (functional NadB) was added to the nadB mutant reaction (functional NadA) than the reverse. This observation implied that the NadB protein forms a diffusible intermediate that is subsequently converted to QA by the NadA protein. The discrepancy in the order of the enzymes in the pathway from these findings (NadB followed by NadA) and those by Kerr and Tritz (NadA followed by NadB) was proposed to be due to the release of niacin into the medium during niacin starvation as previously observed by Lundquist and Olivera (49). If for some unknown reason the nadA mutant cells were
more resistant to niacin starvation, then the growth increase of the \textit{nadA} mutant observed by Kerr and Tritz may have been due to the presence of niacin released by the \textit{nadB} mutant cells.

\textbf{Enzyme and Substrate Investigations}

Following the determination that \textit{NadB} produces an intermediate that is acted upon by \textit{NadA}, the nature of the intermediate and the substrate that gives rise to it, L-aspartate or DHAP, was investigated. These studies were performed using \textit{NadA} purified from the \textit{E. coli nadB} mutant and a protein purified from calf liver that fully replaced the \textit{NadB} protein, which was later determined to be D-aspartate oxidase (48, 50). In these studies, L-aspartate was used as the substrate in the reaction and activity was observed, which was later determined to be due to contaminating D-aspartate present in the commercially prepared substrate (50). \textit{NadA} was incubated with D-aspartate oxidase in a dialysis apparatus such that each occupied a different chamber separated by a semi–permeable membrane that allowed diffusion of only small molecules. It was anticipated that the addition of the correct substrate in the chamber occupied by \textit{NadB} would result in the diffusion of the intermediate across the membrane to react with \textit{NadA} and the other substrate to form QA. If instead the incorrect substrate were added to the chamber containing \textit{NadB}, then the intermediate would not be formed until the correct substrate added to the chamber containing \textit{NadA} diffused to the chamber containing \textit{NadB}, reacted, and then the product diffused back to the chamber containing \textit{NadA}. The difference between one and two diffusion events was expected to be determined by the amount of QA formed in a specified period of time, given that more QA should be formed in a one diffusion situation than two. Results showed that more QA was formed when L-aspartate and \textit{NadB} were incubated together in one compartment while DHAP and \textit{NadA} were present in the other, than when L-aspartate and \textit{NadA} were incubated together in one compartment while DHAP and \textit{NadB} were present in the other. These experiments demonstrated that \textit{NadB} forms the intermediate from L-aspartate, which is then condensed with DHAP to form QA by \textit{NadA}.
As mentioned above, these experiments were performed with D-aspartate oxidase purified from calf liver, which was previously shown to tightly bind and retain FAD during the purification procedure (51). In the same publication, similar diffusion experiments were performed across an Amicon membrane filter cone using NadB from E. coli. This enzyme is larger in size than D-aspartate oxidase from calf liver, and does not bind FAD as tightly, requiring that FAD be included in its reaction mixtures in order to observe QA production. Because of the FAD requirement, it was proposed that the intermediate formed could be a dehydrogenation product of L-aspartate, such as iminoaspartate (IA), which would be unstable and hydrolyze to oxaloacetate (OAA). This hypothesis was confirmed by Nasu and Gholson, who showed that NadB and L-aspartate could be replaced by oxaloacetate (OAA) and an ammonia source, which undergo Schiff’s base formation to generate IA (52). The rates for QA formation with chemically–generated IA were approximately the same as those observed with NadB and L-aspartate. This demonstrated that IA is the intermediate formed by NadB and the substrate for NadA in the condensation reaction with DHAP to form QA.

**Quinolinic Acid Regulation**

It was first hypothesized that NAD⁰ biosynthesis may be regulated by the synthesis of QA when it was observed that the addition of increasing concentrations of nicotinic acid (NA) to growing E. coli nadC mutant cultures lead to decreased production of QA (53). Later studies confirmed these results and found that in addition to repression of QA synthesis, feedback inhibition was also observed when nicotinic acid, nicotinamide, NAD⁰ and structural analogs of those molecules were added to resting cultures (54). In crude extracts, the only inhibitor of QA synthesis was NAD⁰, suggesting that the other additives were inhibitory after their conversion to NAD⁰ in the cell. The authors also observed complete inhibition of QA synthesis at high concentrations of NAD⁰, only minor inhibition at NAD⁰ concentrations of 0.5 mM (approximately the concentration in the cell (55)), and no inhibition by NADP⁰ (56). This
indicated the ability of the cell to regulate the amount of NAD$^+$ present at the level of QA synthesis through either the repression of nadB or nadA gene expression, or inhibition of the activity of the corresponding enzymes.

Regulation of QA synthesis was established to be governed by the regulatory gene, nadR, which was discovered in putative nadB mutants that were unable to support growth of nadA mutants (57). Because the nadR mutant had no detectable NadA or NadB activity, two possibilities were proposed: a tight, inactive complex between NadA and NadB is formed in the nadR mutant or the expression of the nadA and nadB genes is regulated by nadR (58). NadR has since been characterized as a bifunctional regulatory protein that regulates the nad regulon consisting of nadA, nadB and pncB, which encodes the nicotinic acid phosphoribosyltransferase of the PNC. Under favorable concentrations of NAD$^+$, NadR represses the transcription of nadA and nadB to prevent accumulation of NAD$^+$ through the de novo synthesis pathway. When NAD$^+$ concentrations are low, it is involved in the transport of exogenous NMN into the cell to allow for production of NAD$^+$ via the salvage pathway (59). This observation demonstrates the direct relationship between the expression of nadA and nadB and NAD$^+$ concentration in the cell. The expression of the nad genes is also dependent on physiological changes in the cell, such as the conversion from aerobic to anaerobic growth conditions in which a two to three fold increase in expression was observed using nad–lac gene fusions (60). In addition to regulation by NadR, QA biosynthesis is also regulated by the competitive inhibition of NAD$^+$ with the native cofactor, FAD, of NadB (58, 61).

1.5 L-Aspartate oxidase (NadB)

The first step in NAD$^+$ biosynthesis in prokaryotes is the oxidation of L-aspartate to IA, catalyzed by L-aspartate oxidase, or NadB (61). NadB from E. coli is a 60 kDa protein that contains one non-covalently bound molecule of FAD per polypeptide ($K_d$ FAD = 3.8 µM) (62). During the reaction, L-aspartate is converted to IA via a two–electron oxidation, in which the
electrons are transferred from L-aspartate to FAD, producing the reduced form of the cofactor, FADH$_2$. In order to perform subsequent rounds of turnover, FADH$_2$ requires reoxidation back to FAD. Under aerobic conditions, FADH$_2$ can be reoxidized by molecular oxygen, affording hydrogen peroxide. Under strictly anaerobic conditions, FADH$_2$ can be reoxidized by fumarate, forming succinate (63) (Figure 1.9). The fairly unstable product, IA, hydrolyzes to OAA and ammonia with a half life of 144 s at 37 °C and pH 8.0 (61), and is also susceptible to β-decarboxylation, affording iminopyruvate and CO$_2$. This instability in addition to the observation that the enzyme binds IA very tightly with a $K_d$ of 1.4 µM (63), has led to the proposal that NadB and NadA may form a complex to channel this substrate to the next enzyme. Studies to provide evidence for this complex have been limited.

![Figure 1.9: Oxidation of L-aspartate to IA catalyzed by NadB](image)

In the absence of oxygen, fumarate is used as the electron acceptor and shares the same binding site as aspartate and IA. Consistent with this common binding site, steady-state kinetic analyses show that fumarate and IA are competitive inhibitors of each other (63). The anaerobic reaction proceeds via a ping–pong mechanism wherein NadB binds L-aspartate first, converts it to
IA, and releases IA prior to binding fumarate to reoxidize the flavin, and finally releases succinate. Under aerobic conditions, oxygen acts as the electron acceptor forming hydrogen peroxide. Turnover numbers under anaerobic conditions with fumarate serving as the oxidant are comparable to those under aerobic conditions (63).

**Structural and Mechanistic Studies of NadB**

Mechanistic implications have been inferred from the analysis of the two crystal structures of NadB from *E. coli*. The first structure, of the wild–type (WT) protein, was solved at a resolution of 2.2 Å resolution by Mattevi et al., but was lacking flavin and substrate (64). A substitution of Arg386 to Leu allowed subsequent determination of the structure of the holo–enzyme (FAD bound) in the presence and absence of succinate (2.5 and 2.6 Å, respectively) (65). Both the apo and holo structures showed a three–domain protein, consisting of a typical FAD–binding domain (blue), a capping domain with an irregular α+β topology (orange), and a helical C–terminal domain (green) (Figure 1.10). In the Arg386Leu variant, FAD (yellow) is bound in the center of the protein in the elongated conformation with no exposure to solvent. The major difference between the holo and apo structures is that the hinged capping domain covers the active site when in the FAD–bound state. Crystals of the holo protein were obtained only with the Arg386Leu substitution. This Arg residue is located at the interface of the FAD–binding domain and capping domain; therefore, the closing of the capping domain may be due to the hydrophobic patch created by the substitution of a charged amino acid residue with one containing an aliphatic side chain and may not derive from a native conformation of the enzyme. However, results from limited proteolysis studies on the variant were identical to those of the WT protein, which provided rationale to claim that the substitution does not cause a large conformational change.
NadB is a member of the succinate dehydrogenase (SDH)/fumarate reductase (FDR) family of flavoproteins and displays approximately 30% sequence identity with SDH and FDR (62, 66). These enzymes all act on dicarboxylic acid substrates, although NadB differs from the others in its use of a flavin cofactor rather than iron–sulfur (Fe/S) and/or heme cofactors, its
inability to oxidize succinate, and its cytoplasmic location rather than a constituent of a multi−subunit membrane−bound complex (67). The low midpoint potential (−216 mV) of the FAD/FADH$_2$ couple of NadB compared to that of succinate:quinone oxidoreductases (−70 to −20 mV) (67), has been hypothesized to be the reason that it does not have succinate oxidase activity (68).

The substrate for NadB, L-aspartate, is an asymmetric molecule, unlike the fumarate/succinate substrates for FDR/SDHs, which are unable to oxidize L-aspartate. Analysis of the active sites of these proteins showed that a glutamate, corresponding to Glu121 in _E. coli_ NadB, is strictly conserved in all NadB proteins, but replaced by an amino acid with an aliphatic side chain in FDR/SDHs. This residue is positioned in the structure of NadB to interact with the α-amino group of L-aspartate and is proposed to be involved in substrate recognition and binding. This was confirmed by recent studies that showed that changing Glu121 to a series of other residues (Ala, Gln, Asp, or Lys) results in retention of the protein’s fumarate reductase activity but elimination of L-aspartate oxidase activity (69).
In the active site of the Arg386Leu structure, succinate is bound with the oxygen atoms of the C1 carboxylate hydrogen bonded to the side chains of His244 and Thr259 and the backbone nitrogens of Gly51 and Glu260 (Figure 1.11). At the opposite end of the molecule, the C4 carboxylates are directed towards Leu386, which is replaced with Arg in the WT protein, and hydrogen bonded to the side chain of His351 and backbone nitrogen of Ser389. The central carbons are sandwiched between Glu121, Arg290, and the flavin ring.

The mechanism of fumarate reduction by NadB is hypothesized to be the same as the general mechanism for FDR/SDHs (70); specifically, hydride transfer from flavin N5 to fumarate...
C2 is coupled to proton donation by Arg290 of NadB. The oxidation of L-aspartate proceeds via the reverse reaction; hydride transfer from L-aspartate to FAD is coupled to proton abstraction by Arg290. This mechanism distinguishes NadB from other flavin–dependent amino acid oxidases, such as D-amino acid oxidase, in which the reaction proceeds via abstraction of a proton from the α-amino group by an active site base to allow for formation of the imine and hydride transfer from the Cα atom to the flavin N5 (71).

1.6 Quinolinate synthase (NadA)

The second enzyme in the *de novo* pathway for NAD⁺ biosynthesis in prokaryotes is quinolinate synthase (NadA). NadA catalyzes the condensation of DHAP and IA formed by NadB to release QA, inorganic phosphate (P₄) and two equivalents of water (Figure 1.8). Following the work described above by Gholson and coworkers, more groups began to study NadA with some initial difficulty due to the oxygen sensitive nature of the enzyme. Gardner and Fridovich showed that NadA is the oxygen–sensitive enzyme in the NAD⁺ biosynthesis pathway in *E. coli* by showing that the bacteriostatic effect of hyperoxia was relieved by the addition of QA (72). Additionally, they found that NadA activity is decreased in the presence of molecular oxygen and paraquat, and that the enzyme is reactivated when oxygen is removed. This reactivation was prevented by the addition of Fe(II)–chelating agents, such as α,α’-dipyridyl and 1,10-phenanthroline. The amino acid sequence of *E. coli* NadA contains a CysXXCysXXCys motif, often found in the ferredoxin class of [4Fe-4S]–containing proteins (73), in which three cysteines that act as ligands to three of the iron atoms of the cluster are each separated by two nonconserved amino acids (X). The oxygen–sensitive behavior and the purported cluster–binding motif led to the proposal that NadA contains an Fe/S cluster. This seemed plausible chemically given that NadA catalyzes a dehydration reaction, similar to those of the aconitase family of dehydratases, which contain non–redox active Fe/S clusters (74). It was confirmed that oxygen
poisons NadA catalysis, but not NadB catalysis, given that the same decrease in QA production was observed using OAA and ammonia to form IA (75).

Ceciliani et al. reported the first isolation of recombinant E. coli NadA produced via the homologous overexpression of the nadA gene (76). The protein was present as insoluble inclusion bodies, and was therefore resolubilized and refolded. Analysis of NadA by matrix–assisted laser desorption/ionization time–of–flight (MALDI–TOF) mass spectrometry was used to confirm the molecular mass (38,240 Da) predicted by its primary structure. The same study also described the first routine assay for NadA–dependent QA production based on the phosphorylation of glycogen by phosphorylase a, using the free P\text{i} formed by NadA. In the presence of P\text{i}, glucose-6-phosphate dehydrogenase, NADP\text{+} and glycogen, 6-phosphogluconolactone and NADH are formed. This reaction can be monitored by the change in absorbance at 340 nm, assumed to be proportional to the P\text{i} formed by NadA. The activity reported for NadA using NadB to form IA was 0.6 U/mg, with a unit (U) defined as the amount of protein that catalyzes the production of 1 mmol of NADPH per minute. Given the assay conditions, 60 µg NadA and 1 mM DHAP, this activity would afford formation of 1.62 mM QA over the 45 min time frame of the assay. This number is greater than the amount of limiting substrate (DHAP) present, and is most likely derives from a typographical error in the definition of a unit.

**Biophysical Characterization of NadA**

The finding of a [4Fe–4S] cluster associated with E. coli NadA was first reported by Cicchillo et al. in 2005. The E. coli nadA gene was co-expressed with a plasmid encoding the isc operon from Azotobacter vinelandii, which allowed for the overproduction of NadA in the presence of Fe/S cluster assembly proteins. This strategy afforded protein produced exclusively in the soluble state, in contrast to that observed by Ceciliani et al. (76). The subsequent isolation in the anaerobic chamber via immobilized metal affinity chromatography (IMAC) resulted in protein that was brown in color, suggesting the presence of an Fe/S cluster. Further spectroscopic
characterization of the cluster confirmed its configuration to be $[\text{4Fe-4S}]^{2+}$. The UV–visible spectrum of the purified enzyme showed a broad absorbance at 400 nm in addition to the typical peak at 280 nm that is diagnostic of protein. The as–isolated (AI) protein is electron paramagnetic resonance (EPR) silent, consistent with the presence of a diamagnetic $[\text{4Fe-4S}]^{2+}$ cluster, which has a net spin of zero. In the presence of sodium dithionite, the cluster is reduced to the $[\text{4Fe-4S}]^{+}$ paramagnetic ($S = 1/2$) state and exhibits an axial EPR spectrum with $g$–values that are typical for this type of cluster (2.05, 1.92, 1.87). EPR is not an exact determinant of cluster configuration and is not an accurate method for determination of cluster stoichiometry. The Mössbauer spectrum, in combination with analytical quantification of iron and sulfide content of the protein, 5.0 and 2.8 equivalents of iron and sulfide, respectively, allowed for the determination that NadA contains one $[\text{4Fe-4S}]^{2+}$ cluster per protein.

The activity of NadA was determined by monitoring QA formation by high–performance liquid chromatography (HPLC) following incubation of the protein with DHAP and IA (generated from L-aspartate by NadB), and a specific activity of $0.015 \mu\text{mol/min/mg}$ ($V_{\text{max}}/\lbrack E \rbrack_T$ of 0.01 s$^{-1}$) was obtained for the reconstituted (RCN) protein.

Simultaneously, similar studies were performed by the Fontecave group, who also demonstrated that NadA contains a $[\text{4Fe-4S}]^{2+}$ that is essential for activity (77). Initial attempts to purify NadA in the presence of oxygen resulted in protein that was slightly pink in color and contained a small amount of iron and sulfide (0.15 to 0.2 equivalents). Subsequent purifications were then performed in the same manner but inside an anaerobic glove box, which resulted in protein that was brown in color, displayed the UV–visible feature typical for a $[\text{4Fe-4S}]^{2+}$ and contained 3.1 irons and 3.0 sulfides per polypeptide. The iron content of the as–isolated (AI) NadA is slightly lower than that observed by Cicchillo et al., which may be due to over–expression in the absence of the plasmid encoding the Fe/S cluster assembly genes.
Characterization via EPR and Mössbauer spectroscopies confirmed that the anaerobically isolated enzyme contains a [4Fe-4S]$^{2+}$, which degrades upon exposure to oxygen.

The activity of NadA was determined by correlation of QA formation to DHAP consumption during the reaction. DHAP was quantified using the enzyme α-glycerophosphate dehydrogenase, which reduces DHAP to α-glycerophosphate while oxidizing NADH to NAD$^+$. The conversion of NADH to NAD$^+$ can be monitored by the decrease in absorbance at 340 nm. Specific reaction rates were not determined, but no DHAP was consumed using the apo protein, while 56% was consumed after 20 minutes with the cluster–containing enzyme (77). The majority of the DHAP (94%) was consumed by 60 min.

**Structural Studies of NadA**

Despite the findings by two independent groups that clearly demonstrated that NadA from *E. coli* contains a [4Fe-4S]$^{2+}$ and that the cluster is absolutely required for activity, in 2005 Sakuraba *et al.* published a 2.0 Å resolution crystal structure of NadA from the hyperthermophile archaeon *Pyrococcus horikoshii* that was lacking the Fe/S cluster (Figure 1.12). The structure of this 35-kDa enzyme is clover–leaf in shape, displaying three distinct domains in pseudo–3-fold symmetry. Each domain consists of a 4-stranded parallel β sheet (purple) with two α helices (blue) on each side. There are three surface loops connecting each domain, corresponding to amino acids 79–90, 165–175, and 257–260, that were not visible in electron density maps. A bioinformatic analysis of the amino acid sequence performed by Saunders *et al.* (78) and Murthy U.M. *et al.* (79) indicates that three cysteines are conserved throughout over 700 annotated NadA proteins. Two of these disordered loops contain two of the three conserved cysteines, Cys83 and Cys170. The third conserved cysteine, Cys256, is visible in the structure, but adjacent to one of the loops that is not visible.
The protein was purified and subsequently crystallized aerobically in the presence of malate, a substrate mimic of IA. The malate is bound at the central region of the protein where the three domains converge (displayed in green, Figure 1.12). The active site is approximately 10 Å deep, with one wide and one narrow opening. The inner pocket of the active site is lined with amino acids that are conserved in all species. Attempts were made to model the second substrate, DHAP, into the active site along with malate, but suitable space to accommodate the phosphate moiety of the molecule was not available. It was also noted the NadA crystals quickly
deteriorated when soaked with DHAP, an indication of a possible conformational change upon DHAP binding. Despite the lack of the essential Fe/S cluster, the group also reported an activity of 2.2 μmol/min/mg for NadA from *P. horikoshii*, about 150-fold higher than that reported for the holo enzyme from *E. coli*. The report made no mention of an Fe/S cluster: neither the lack of the cofactor in the structure nor the previous characterization of the cluster in publications.

1.7 Aconitase: Model Hydro–lyase Enzyme

NadA has been purported to be a member of the hydro–lyase class of enzymes, which catalyze dehydration reactions across a carbon–carbon bond. A number of these enzymes utilize a protein–bound Fe/S cluster as a Lewis acid for catalysis (74). The model enzyme for this class is aconitase, although two additional members have been studied in moderate detail. Dihydroxy-acid dehydratase, one of the key enzymes involved in the biosynthesis of branched chain amino acids, catalyzes the dehydration of 2,3-dihydroxy-3-methylpentanoate, affording 3-methyl-2-oxopentanoate (80). L-serine dehydratase catalyzes the dehydration of L-serine to pyruvate and ammonia, and in *E. coli* is responsible for maintaining proper levels of intracellular L-serine, which interferes with cell wall synthesis when present at high concentrations (81). These enzymes, as well as others in this class, have been proposed to utilize their [4Fe-4S] cluster cofactors in the dehydration reaction (74).

Aconitase catalyzes the second reaction in the citric acid cycle, the interconversion of citrate and isocitrate through a cis–aconitate intermediate (74, 82). The heart mitochondrial enzyme has been thoroughly characterized and crystallized in the presence of the product, isocitrate, or substrate inhibitors (82). More recently, the crystal structure of a Ser642Ala mutant, the base that abstracts a proton from Cα of citrate, was solved displaying both the citrate and isocitrate binding modes to a resolution of 1.8 and 2.0 Å, respectively (Figure 1.13) (83). The enzyme is a single polypeptide with a molecular weight of 83 KDa (84). The polypeptide forms
four domains, of which three are associated with a [4Fe-4S] cluster while the fourth is on the opposite side of the active site.

Figure 1.13: Crystal structure of Ser642Ala variant of porcine mitochondrial aconitase with citrate (cyan) bound to the [4Fe-4S] cluster (orange and yellow) in the active site.

The enzyme requires the [4Fe-4S]$^{2+}$ cluster for optimum activity. It displays less than 30% activity with the [4Fe-4S]$^+$ form and no activity in its apo–form (85). Three iron of the
cluster are ligated to three cysteines on the protein, while the fourth iron (Fe₄) is coordinated to the carboxyl and hydroxyl oxygen atoms of the substrate and a molecule of solvent (82, 86). The function of the Fe/S cluster is to act as a Lewis acid to activate the hydroxyl group for elimination during the dehydration reaction, forming the cis–aconitate intermediate. The stable intermediate is released from the active site, rebound with the Cα and Cβ positions flipped and then rehydrated at C2, forming the isocitrate product. The structure of the Ser642Ala variant in the citrate binding mode displays the interaction of the Cβ carboxyl and hydroxyl oxygens with the Fe₄, positioned 2.37 and 2.59 Å away (Figure 1.14). The enzyme in complex with isocitrate displays the interaction of the Cα carboxyl and hydroxyl oxygens with the Fe₄, consistent with the inversion of the Cα–Cβ bond during the reaction.
Figure 1.14: Active site of Ser642Ala variant of aconitase displaying the substrate citrate (cyan) bound to the [4Fe-4S] cluster (yellow and red) and key active site residues (green).

1.8 NadA Mechanistic Investigations

Although the reaction catalyzed by NadA is more complex than those catalyzed by characterized members of the hydro–lyase class of enzymes, it is believed to incorporate similar mechanistic features. The mode of substrate binding to the cluster has not been demonstrated, but it is proposed that the Fe/S cluster binds to the hydroxyl group that is removed in the second dehydration reaction (Figure 1.15) (74).
Studies of the mechanism of NadA have been hindered by the initial difficulties associated with purifying the oxygen–sensitive enzyme and the instability of IA. In 1982, Gholson published the first proposed mechanism that was consistent with experimental findings. In this mechanism, the first step is a nucleophilic attack by C3 of the enamine form of IA on C3 of DHAP, resulting in release of Pi and formation of the C–C bond (Figure 1.16A) (61). Subsequent enolizations allow for Schiff–base formation, which results in ring formation upon loss of the first equivalent of water. A second proton abstraction and subsequent dehydration step, hypothesized to be facilitated by the Fe/S cluster, leads to formation of QA.
Figure 1.16: Proposed Mechanisms for the NadA Reaction: A) Gholson mechanism, B) Begley mechanism, and C) Booker mechanism.
A distinctly different mechanism was proposed by Begley et al. in 2001, which begins with isomerization of DHAP to G3P to allow for Schiff–base formation between the amino group of IA and C3 of G3P. Elimination of phosphate via an $E_{1cb}$ mechanism affords a species that undergoes electrocyclic ring closure, which is followed by tautomerization to the predicted intermediate that undergoes Fe/S cluster–assisted dehydration to QA (Figure 1.16B) (87). A third, unpublished mechanism that also begins with the isomerization of DHAP to G3P can be envisaged. The phosphate is then released from G3P via an $E_{1cb}$ elimination, affording an $\alpha,\beta$-unsaturated aldehyde (Figure 1.16C). This Michael acceptor then reacts with C3 of the enamine form of IA to form the C–C bond. Ring closure and subsequent production of QA are then suggested to follow the mechanism proposed by Gholson.

**Substrate Channeling**

In early studies of NadA and NadB, the inability to isolate the intermediate, IA, in QA biosynthesis led to the proposal that these two enzymes may function as a complex (44). The ease of separation of the two proteins during purification suggested that if they do exist as a complex, they are not tightly bound (58). The product of NadB, IA, is fairly unstable and hydrolyzes to OAA with a half–life of 144 s at 37 °C, pH 8.0 (61). IA is also susceptible to $\beta$-decarboxylation, forming iminopyruvate. Channeling of IA would therefore protect this unstable intermediate prior to binding to NadA.

Studies in *Arabidopsis thaliana* demonstrated that *old5* (*Arabidopsis onset of leaf death5*) encodes for a NadA, and variants that lack a function copy of this gene exhibit early developmental senescence along with an increase in cellular concentrations of pyrimidine nucleotides due to higher activity of the enzymes in the NAD$^+$ salvage pathway (88). Yeast two–hybrid studies detected an interaction between the OLD5 protein and CpNiS and CpNiS3, confirming that it acts as a part of a cysteine desulfurase complex as determined by Murthy et al. (79). Interestingly, an interaction of OLD5 with aspartate oxidase from *A. thaliana* was also
detected. Subsequent studies by Marinoni et al. provided *in vitro* evidence for an interaction between NadA and NadB from *Bacillus subtilis* using an affinity capture protocol (89). In these studies, GST– or hexahistidine–tagged NadA was immobilized on resin that bound the specific tag and then incubated with either purified NadB or cell homogenate of *E. coli* overexpressing NadB. After washing, NadA was eluted from the resin and bound proteins were detected by SDS-PAGE. The investigators observed a band corresponding to NadB using both purified NadB or NadB overexpressed in crude cell extracts. Moreover, the same results were obtained when *E. coli* NadB was substituted for *B. subtilis* NadB, implying that NadA and NadB interact in the absence of substrate, and that this interaction is not species specific.

The results of an independent set of studies suggest that NadA and NadB either do not form a complex to allow for substrate channeling or the complex is transient and the two proteins only briefly interact to transfer the iminoaspartate. When the first partial purification of these two proteins from *E. coli* was reported, the proteins were subsequently run on a size–exclusion column and the experiment showed no evidence to suggest formation of a complex (44). Additionally, NadB and L-aspartate can be replaced by OAA and an ammonia source to form IA chemically in assays of NadA with similar rates for QA formation (52). In these studies it was also shown that when using L-[14C]-aspartate and NadB to form IA, as expected radioactively labeled QA was formed. When the reaction was run in the presence of unlabeled OAA and ammonia, the amount of radioactively labeled QA was greatly reduced. These experiments demonstrate that a portion of the radiolabeled IA is released by NadB into solution and not all directly transferred to NadA. Yet the experiment was performed in the presence of excess unlabeled OAA and ammonia in solution, which allows for the formation of IA that may be bound by NadB and then transferred to NadA. The suggested interaction between NadA and NadB needs to be more thoroughly investigated to determine if substrate channeling is really occurring.
1.9 Discussion

There exists a conflict of evidence in the literature for the requirement of the Fe/S cluster cofactor of NadA. Although two groups independently demonstrated that *E. coli* NadA harbors a [4Fe-4S] cluster that is required for activity (77, 90), the published crystal structure of the enzyme from *P. horikoshii* lacks the Fe/S cluster. Moreover, not only was activity of this apo enzyme stated to be robust, Ceciliani *et al.* also reported robust activity of apo–NadA from *E. coli*. The work described in this dissertation seeks to clarify this discrepancy and provide evidence that, similarly to aconitase, all quinolinate synthases contain a [4Fe–4S] cluster required for activity that is coordinated by three conserved cysteinyln residues, and that one iron atom of this cluster provides an open coordination site for substrate binding.

In *E. coli* NadA, these three cysteines correspond to Cys113, Cys200, and Cys297, and only Cys297 exists in the CysXXCysXXCys motif purported to contain the cysteines ligating the Fe/S cluster as described by Saunders *et al.* and Rousset *et al.*, and also detailed in Chapter 2 of this dissertation (91, 92). Further studies by Saunders *et al.* also showed that NadA from *P. horikoshii* and *Mycobacterium tuberculosis* (Mtb), both lacking the canonical CysXXCysXXCys motif, each contain a [4Fe-4S] cluster that is required for activity (91), while Marinoni *et al.* provided similar evidence for *Bacillus subtilis* NadA (89). These results demonstrate that all annotated NadAs most likely contain an essential Fe/S cluster that is ligated by these three conserved cysteines.

The non-cluster–ligating cysteines in this motif exist in a CysXXCys motif, which is a conserved sequence in the class of thioredoxin fold proteins in which these two cysteines form a disulfide bond (93). We found this to be true for *E. coli* NadA and the disulfide bond plays a role in regulating the activity of the protein (Chapter 3). This disulfide bond has a midpoint reduction potential of approximately -264 mV and can be oxidized/reduced by thioredoxin, suggesting that it may play a role in regulating QA biosynthesis *in vivo* (94).
The result of our biochemical studies provided evidence that the *P. horikoshii* NadA structure is incomplete. We then used this apo structure to model in the essential Fe/S cluster and the missing loops containing the cluster–ligating cysteines (Chapter 4). Additionally, we also modeled a QA precursor into the active site that is proposed to bind to the Fe/S cluster in the final step of the dehydration reaction. This allowed us to identify possible key conserved residues in the active site to change by site–directed mutagenesis and gain further information on the mechanism of the NadA reaction. Finally, we broadened our studies on the role of the NadA disulfide bond by looking at NadA from another organism, *M. tuberculosis*, and showed that it too has a redox–active disulfide bond despite the lack of a CysXXCys motif (Chapter 5).
1.10 References


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Chapter 2

The $[4\text{Fe}-4\text{S}]^{2+}$ Cluster in Quinolinate Synthase is Ligated by Conserved Cysteines

This chapter was reproduced from Saunders, A. H., Griffiths, A. E., Tu, L., Lee, K. H., Cicchillo, R. M., Stromberg, J. A., Krebs, C., and Booker, S. J. (2008) Characterization of quinolinate synthases from *Escherichia coli*, *Mycobacterium tuberculosis*, and *Pyrococcus horikoshii* indicates that $[4\text{Fe}-4\text{S}]$ clusters are common cofactors throughout this class of enzymes, *Biochemistry* 47, 10999-11012. The cloning of the *nadA* and *nadB* genes and construction of the Cys → Ser variants was performed by L. Tu; all other experiments described in this chapter was performed by A. Saunders.

2.1 Abstract

Quinolinate synthase (NadA) catalyzes a unique condensation reaction between iminoaspartate (IA) and dihydroxyacetone phosphate (DHAP), affording quinolinic acid (QA), a central intermediate in the biosynthesis of nicotinamide adenine dinucleotide (NAD$^+$). IA is generated via the action of L-aspartate oxidase (NadB), which catalyzes the first step in the biosynthesis of NAD$^+$ in most prokaryotes. NadA from *E. coli* was hypothesized to contain an iron–sulfur (Fe/S) cluster as early as 1991 (1), because of its observed labile activity, especially in the presence of hyperbaric oxygen, and its primary structure, which contained a motif (CysXXCysXXCys) that is commonly found in iron–sulfur proteins (2). Using analytical methods in concert with Mössbauer and electron paramagnetic resonance (EPR) spectroscopies,
the protein was shown to harbor a [4Fe–4S] cluster (3, 4). Following the characterization of the Fe/S cluster of NadA from *E. coli*, the X-ray structure of NadA from *P. horikoshii* was solved to 2.0 Å resolution (5). This protein does not contain a CysXXCysXXCys motif, and no iron–sulfur cluster was observed in the structure or even mentioned in the report. Moreover, rates of quinolinic acid production were reported to be 2.2 µmol min⁻¹ mg⁻¹, significantly greater than that of *E. coli* NadA containing an Fe/S cluster (0.10 µmol min⁻¹ mg⁻¹), suggesting that the [4Fe-4S] of *E. coli* NadA may not be necessary for catalysis. In the study described herein, *E. coli* NadA, which contains nine cysteine residues, is shown to require only three for turnover (Cys113, Cys200, and Cys297), of which only Cys297 resides in the CysXXCysXXCys motif. Parallel studies performed by Griffiths showed that NadA from both *Mycobacterium tuberculosis* and *P. horikoshii* contain [4Fe–4S] clusters that are absolutely required for activity despite the absence of a CysXXCysXXCys motif in their primary structures (6, 7). These results are consistent with a bioinformatics analysis of NadA sequences, which indicates that three cysteines are strictly conserved across all species. This study concludes that all currently annotated quinolinate synthases most likely harbor a [4Fe–4S] cluster, that the crystal structure reported by Sakuraba *et al.* does not accurately represent the active site of the protein, and that the “activity” reported does not correspond to quinolinate formation.

2.2 Introduction

NAD⁺ is an essential and ubiquitous cofactor known primarily for its role as a co-substrate in a multitude of biological oxidation–reduction reactions that involve hydride transfers (8). It is also involved in myriad non–redox reactions such as adenylation, ADP-ribosylation, and histone deacetylation (9, 10), and in enzymes such as urocanase, in which its nicotinamide ring is proposed to form a transient covalent adduct with the substrate urocanate, facilitating catalysis via an electrophilic mechanism (11). QA serves as the first common precursor to NAD⁺ and its derivatives in all organisms that synthesize NAD⁺ de novo; the subsequent steps that lead to
NAD$^+$ from QA are identical (12). By contrast, there are two distinctly different pathways by which QA is formed. In most eukaryotes, QA is generated from the oxidative degradation of tryptophan in a series of five enzymatic steps, of which three involve molecular oxygen as a cosubstrate (12). The requirement for molecular oxygen in this pathway has led to its designation as the aerobic pathway for QA production, which distinguishes it from an alternative pathway (anaerobic pathway) found predominantly in prokaryotes that does not display an obligate requirement for molecular oxygen. In the anaerobic pathway, L-aspartate is first converted to IA by NadB; IA is then condensed with DHAP to form QA in a reaction catalyzed by NadA. The byproducts of the reaction are 2 moles of water and 1 mole of inorganic phosphate (P$_i$). (Figure 2.1) (13).

Figure 2.1: Reactions catalyzed by L-aspartate oxidase and quinolinate synthase

NadB is a 60 kDa flavoprotein that contains one noncovalently bound molecule of flavin adenine dinucleotide (FAD) per polypeptide (14). During catalysis, L-aspartate is transiently oxidized to IA with concomitant reduction of FAD to FADH$_2$. Under oxic conditions, FADH$_2$ is reoxidized to FAD by molecular oxygen, with generation of hydrogen peroxide as a byproduct
(Figure 2.1). Under anoxic conditions, fumarate serves as the electron acceptor, being reduced to succinate (15). IA is an unstable intermediate; it hydrolyzes to ammonia and oxaloacetate (OAA) at pH 8.0 with a half-life of 144 s (16) and is also susceptible to decarboxylation to form iminopyruvate. In vitro the NadB protein can be replaced by reacting OAA and an ammonia source, such as ammonium sulfate or ammonium chloride, which undergo Schiff–base formation to afford an equilibrium concentration of IA (17).

NadA from *E. coli* has served as the prototype for the study of quinolinate synthases; however, detailed characterization and mechanistic analysis of the enzyme was originally hampered by its observed instability (16). The protein contains a CysXXCysXXCys motif, which is found often in the primary structures of [4Fe-4S]–containing proteins of the ferredoxin class (2). This motif, as well as its reported tendency to lose catalytic competence in the presence of hyperbaric oxygen, led to the suggestion that *E. coli* NadA is an iron–sulfur (Fe/S) protein (18). This hypothesis was later confirmed. The work of two independent showed that the protein binds and requires one [4Fe-4S]$^{2+}$ cluster per polypeptide by analytical and spectroscopic techniques (19, 20). NadA has been purported to be a member of the Fe/S cluster–dependent subclass of the hydro–lyase class of enzymes (21), which includes aconitase, the prototypical member (22). Aconitase contains a [4Fe-4S] cluster in which three of the iron ions are ligated by three cysteine residues contributed by the protein scaffold. The fourth iron (Fe₄) provides a site for bidentate coordination by the substrate or product with a corresponding change in its coordination geometry from tetrahedral to octahedral (23-26). Similarly, it is expected that the [4Fe-4S] cluster of NadA will also be ligated by three cysteinate protein ligands, allowing one iron site to be available for direct involvement in the reaction (21).

The X-ray crystal structure of quinolinate synthase from the hyperthermophilic archaeon *P. horikoshii* was solved to 2.0 Å in 2005. Surprisingly, the structure showed no evidence of an Fe/S cluster or any other type of cofactor, nor was mention of an Fe/S cluster made in the
published report (27). The protein was stated to catalyze formation of QA with a specific activity of 2.2 µmol min⁻¹ mg⁻¹, an activity that is significantly greater than that reported for the Fe/S–containing *E. coli* enzyme (19). However, the structure contained three regions of poorly defined electron density, in which three cysteine residues that are strictly conserved among NadA proteins are found (Figure 2.2). In our studies described herein we show that the three corresponding cysteines in *E. coli* NadA are absolutely required for turnover *in vitro*, and most likely act as ligands to the [4Fe-4S] cluster. When Cys→Ser substitutions were made at each of the nine cysteine residues present in the primary structure of *E. coli* NadA, only substitutions at Cys113, Cys200, and Cys297 afforded inactive proteins when analyzed in their as–isolated (AI) or reconstituted (RCN) forms. Importantly, Cys297 is the only one of the three present in the CysXXCysXXCys motif (Figure 2.2). These results are consistent with those obtained in the characterization of NadAs from *M. tuberculosis* and *P. horikoshii*, both of which were shown to contain a similar [4Fe-4S] cluster despite the lack of a CysXXCysXXCys motif (6, 7). We argue that all currently annotated quinolinate synthases are Fe/S proteins, and require [4Fe-4S] clusters in catalysis.
4.9

Figure 2.2: Sequence alignment of NadA from *Escherichia coli*, *Erwinia carotovora*, *Shigella flexneri*, *Yersinia pestis*, *Mycobacterium tuberculosis*, and *Pyrococcus horikoshii*. The conserved cysteines are highlighted in cyan, the CysXXCysXXCys motif is outlined in the purple box and the disordered loops missing in the *P. horikoshii* X-ray crystal structure are highlighted in red.

2.3 Materials and Methods

Materials. All DNA modifying enzymes and reagents, and Deep Vent DNA Polymerase and its associated 10× ThermoPol reaction buffer were purchased from New England Biolabs (Ipswitch, MA). PfuUltra High Fidelity DNA Polymerase and its associated 10× reaction buffer were obtained from Stratagene (La Jolla, CA). Oligonucleotide primers for cloning were obtained
from Integrated DNA Technologies (Carlsbad, CA). *Escherichia coli* genomic DNA (strain W3110) was obtained from Sigma Corp (St. Louis, MO). *E. coli* strain BL21(DE3) were obtained from Novagen (Madison, WI), as was vector pET-28a. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Biosynth International (Naperville, IL). Coomassie blue dye–binding reagent for protein concentration determination and the bovine serum albumin (BSA) standard (2 mg mL⁻¹) were obtained from Pierce (Rockford, IL). Nickel nitrilotriacetic acid (Ni–NTA) resin was purchased from Qiagen (Valencia, CA). Sephadex G-25 resin and PD-10 pre-poured gel filtration columns were purchased from GE Biosciences (Piscataway, NJ). 2,3-Pyridinedicarboxylic acid (quinolinic acid) was obtained from Aldrich (St. Louis, MO). Dihydroxyacetone phosphate (dilithium salt), L-(+)-arabinose, ferric chloride, and α-glycerophosphate dehydrogenase were obtained from Sigma (St. Louis, MO). All other buffers and chemicals were of the highest grade available. $^{57}$Fe (97-98%) metal was purchased from Isoflex USA (San Francisco, CA). It was washed with CHCl₃ and dissolved with heating in an anaerobic solution of 2 N H₂SO₄ (1.5 mol of H₂SO₄ per mole of $^{57}$Fe).

**General Procedures.** High performance liquid chromatography (HPLC) was conducted on a Beckman System Gold unit (Fullerton, CA), which was fitted with a 128 diode array detector and operated with the System Gold Nouveau software package. Iron and sulfide analyses were performed as previously described (28-30). Sonic disruption of *E. coli* cells was carried out with a 550 sonic dismembrator from Fisher Scientific (Pittsburgh, PA) in combination with a horn containing a ½ in. tip. The horn was threaded through a port in an anaerobic chamber to allow the process to be conducted under anoxic conditions. The polymerase chain reaction (PCR) was performed with a Robocycler temperature cycler from Stratagene (La Jolla, CA). DNA sequencing was carried out at the Pennsylvania State University Genomics Core Facility (University Park, PA).
**Spectroscopic Methods.** UV–visible spectra were recorded on a Cary 50 spectrometer (Varian; Walnut Creek, CA) using the associated WinUV software package. Low–temperature X-band EPR spectroscopy was carried out in perpendicular mode on a Bruker (Billerica, MA) ESP 300 spectrometer equipped with an ER 041 MR microwave bridge and an ST4102 X-band resonator (Bruker). The sample temperature was maintained with an ITC503S temperature controller and an ESR900 liquid helium cryostat (Oxford Instruments; Concord, MA).

Mössbauer spectra were recorded on a spectrometer from WEB research (Edina, MN) operating in the constant acceleration mode in transmission geometry. Spectra were recorded with the temperature maintained at 4.2 K. The sample was kept inside an SVT-400 dewar from Janis (Wilmington, MA), and a magnetic field of 40 mT was applied parallel to the γ-beam. The quoted isomer shift is relative to the centroid of the spectrum of a metallic foil of α-Fe at room temperature. Data analysis was performed using the program WMOSS from WEB research.

*Cloning of E. coli nadA and nadB Genes.* The *nadA* gene was amplified from *E. coli* (strain W3110) genomic DNA by PCR technology using primers nadAfor and nadArev (Table 2.1). Each amplification reaction contained the following in a volume of 50 μL: 0.4 μM of each primer, 0.2 mM of each deoxynucleoside triphosphate, 100 ng of *E. coli* genomic DNA, 2.5 U of PfuUltra High Fidelity DNA Polymerase, and 5 μL of 10× PfuUltra™ reaction buffer. The reaction mixture was overlaid with 30 μL of mineral oil. After a 2 min denaturation step at 95 °C, 30 cycles of the following program were initiated: 30 s at 95 °C, 30 s at 70 °C, 1 min at 72 °C. Following the cycling program, the reaction was incubated further for 10 min at 72 °C. The PCR product was digested with *NdeI* and *EcoRI* and then ligated into similarly digested pET-28a by standard methods (31). The *nadB* gene was amplified from *E. coli* (strain W3110) genomic DNA by PCR technology using primers nadBfor and nadBrev (Table 2.1). Each amplification reaction contained the same concentrations of reagents as described above for cloning the *nadA* gene, except that DMSO was added to a final concentration of 4%. After a 2 min denaturation step at
95 °C, 30 cycles of the following program were initiated: 30 sec at 95 °C, 30 sec at 63 °C, 3 min at 72 °C. Following the cycling program, the reaction was incubated further for 10 min at 72 °C.

The PCR product was digested with NdeI and HindIII and then ligated into similarly digested pET-28a by standard methods. All other procedures were carried out by standard methods. The correct constructs were verified by DNA–sequencing and designated pET28-NadA and pET28-NadB, respectively.

### Table 2.1: Sequence of Primers used in Cloning and Mutagenesis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
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<tbody>
<tr>
<td>nadAfor</td>
<td>5'-CGC GCG TCC ATA TGA GCG TAA TGT TTG ATC CAG ACA CGG CG-3'</td>
<td>Forward Primer for cloning E. coli nadA&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>nadArev</td>
<td>5'-GCC GGA ATT CTT ATC CAC GTA GTG TAG CCG CAA AAT CCA GC-3'</td>
<td>Reverse primer for cloning E. coli nadA&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>nadBfor</td>
<td>5'-CGC GCG TCC ATA TGA ATA CTC TCC CTG AAC ATT CAT GTG ACG TGT TGA TTA TCG G-3'</td>
<td>Forward Primer for cloning E. coli nadB&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>nadBrev</td>
<td>5'-CGC CGA AGC TTT TAT CTG TTT ATG TAA TGA TTG CCG GGG GAA AGG ATC GAC GG-3'</td>
<td>Reverse primer for cloning E. coli nadB&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>nadAcs291for</td>
<td>5'-GGT GAG GGT GCA ACC AGC AGC TGC GC-3'</td>
<td>Forward Primer for E. coli C291S</td>
</tr>
<tr>
<td>nadAcs291rev</td>
<td>5'-CGC CAG CTG CGG CTG GTT GCA CCC TCA CC-3'</td>
<td>Reverse Primer for E. coli C291S</td>
</tr>
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<td>nadAcs294for</td>
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</tr>
<tr>
<td>nadAcs294rev</td>
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<td>Reverse Primer for E. coli C294S</td>
</tr>
<tr>
<td>nadAcs297for</td>
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</tr>
<tr>
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<td>nadAcs113For</td>
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<td>Reverse Primer for E. coli C119S</td>
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</table>
Construction of the NadA Variants. E. coli NadA variants were constructed using the QuikChange II Site–directed Mutagenesis Kit (Stratagene) according to the manufacturer’s specifications, and as described previously (32), in which the cycling protocol is adapted for the Stratagene Robocycler thermocycler (33). Plasmid pET28-NadA was used as the template in conjunction with the appropriate primers for each respective amino acid substitution (Table 2.1). All mutations were verified by DNA–sequencing of the entire gene.

Expression of the Genes Encoding WT E. coli NadA and Variants. Plasmid pET28-NadA, encoding WT or variant NadA proteins, was cotransformed into E. coli BL21(DE3) with plasmid pDB1282 as described previously (34). A single colony was selected and used to inoculate 100 mL of Luria–Bertani (LB) media containing 50 µg/mL kanamycin and 100 µg/mL ampicillin, and

<table>
<thead>
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<th>Primer Name</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
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</tr>
<tr>
<td></td>
<td>CCA TCC CGA TCG-3'</td>
<td>Reverse Primer for E. coli C128S</td>
</tr>
<tr>
<td>nadAcs128Rev</td>
<td>5'-CGA TCG GGA TGG GCA TCG CTA AAT GCG TTA TAT</td>
<td>Forward Primer for E. coli C128S</td>
</tr>
<tr>
<td></td>
<td>TCT TCA ACA GGG-3'</td>
<td>Reverse Primer for E. coli C128S</td>
</tr>
<tr>
<td>nadAcs195For</td>
<td>5'-GGT GGA GAC ATT CTA AGC TGG CAG GGT GCC-3'</td>
<td>Forward Primer for E. coli C195S</td>
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<tr>
<td>nahAc113afor.</td>
<td>5'-GCC GAC ACT TCA GGC TGA AGC TTC ACT GGA TCT</td>
<td>Reverse Primer for E. coli C200S</td>
</tr>
<tr>
<td>AHS</td>
<td>5'-CCG AGA TCC AGT GAA GCT TCA GCC TGA AGT GTC</td>
<td>Forward Primer for E. coli C113A</td>
</tr>
<tr>
<td>nahAc113arev.</td>
<td>5'-GCT GGC AGG GTG CCT CTA TTG TGC ATG ATG AAT</td>
<td>Reverse Primer for E. coli C113A</td>
</tr>
<tr>
<td>AHS</td>
<td>TTA AGA CTC AGG-3'</td>
<td>Forward Primer for E. coli C200A</td>
</tr>
<tr>
<td>nahAc200afor.</td>
<td>5'-CCT GAG TCT TAA ATT CAT CAT GCA CAA TAG AGG</td>
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</tr>
<tr>
<td>AHS</td>
<td>5'-GCC GAC ACT TCA GGC TGA AGC TTC ACT GGA TCT</td>
<td>Forward Primer for E. coli C200S</td>
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<tr>
<td>nahAc297afor.</td>
<td>5'-CGC AGC TGC GCG CAT GCT CCG TGG ATG GCC-3'</td>
<td>Reverse Primer for E. coli C297A</td>
</tr>
<tr>
<td>AHS</td>
<td>5'-GCC CAT CCA CGG AGC ATG CGC GCA GCT GCC GCG-3'</td>
<td>Forward Primer for E. coli C297A</td>
</tr>
</tbody>
</table>

*Underlined bases represent NdeI restriction site.
*Underlined bases represent EcoRI restriction site.
*Underlined bases represent HindIII restriction site.
Bolded bases represent amino acid changes.
was cultured for ~7 h at 37 °C with shaking (180 rpm). A 60 mL portion of the culture was then evenly distributed among four 6 L Erlenmeyer flasks to inoculate 16 L of LB media containing 50 µg/mL kanamycin and 100 µg/mL ampicillin, and the bacteria were cultured further at 37 °C with shaking. At an optical density at 600 nm (OD$_{600}$) of 0.3, solid L-(+)-arabinose was added to each flask at a final concentration of 0.05 % (w/v). At an OD$_{600}$ of 0.6, the cultures were cooled in an ice–water bath and solid IPTG and ferric chloride were added to each flask at final concentrations of 200 and 50 µM, respectively. The cultures were then allowed to incubate further at 18 °C with shaking for 16 h. Cells were harvested by centrifugation at 10,000 × g for 10 min at 4 °C, and the resulting cell paste was frozen in liquid N$_2$ and stored at −80 °C until ready for use. Typical yields were 50-60 g of frozen cell paste per 16 L of culture.

**Purification of E. coli NadA Proteins.** All steps of the purification were conducted inside of an anaerobic chamber from Coy Laboratory Products, Inc. (Grass Lake, MI) under an atmosphere of N$_2$ and H$_2$ (95%/5%) with an O$_2$ concentration maintained below 1 ppm by the use of palladium catalysts. Steps using centrifugation were performed outside of the anaerobic chamber in centrifuge tubes that were tightly sealed before removal from the chamber. All buffers were prepared using distilled and deionized water that was boiled for at least 1 h and then allowed to cool with stirring uncapped in the anaerobic chamber for 48 h. All plastic ware was autoclaved and brought into the chamber hot, and allowed to equilibrate overnight before use.

Protein purification was carried out by immobilized metal affinity chromatography (IMAC) using a nickel-nitrilotriacetic acid (Ni-NTA) matrix. In a typical purification, 25 g of frozen cells were resuspended in 80 mL of buffer A (50 mM HEPES, pH 7.5, 0.3 M KCl, 20 mM imidazole, and 10 mM 2-mercaptoethanol). Solid egg white lysozyme was added to a final concentration of 1 mg mL$^{-1}$ and the mixture was stirred at room temperature for 30 min. After the mixture was allowed to cool in an ice–water bath to <8 °C, it was subjected to four 1 min bursts of sonic disruption (setting 7). Cellular debris was removed by centrifugation at 50,000 × g for 1
h, and the resulting supernatant was loaded onto a Ni-NTA column (2.5 × 7 cm) equilibrated in buffer A. The column was washed with 100 mL of buffer B (50 mM HEPES, pH 7.5, 0.3 M KCl, 40 mM imidazole, 10 mM 2-mercaptoethanol, and 20% glycerol), and subsequently eluted with buffer B containing 250 mM imidazole. Fractions that were brown in color were pooled and concentrated in an Amicon stirred cell (Millipore, Billerica, MA) fitted with a YM-10 membrane (10,000 Da MW cutoff). The protein was exchanged into buffer C (50 mM HEPES, pH 7.5, 0.1 M KCl, 10 mM DTT and 20% glycerol) by anaerobic gel filtration (Sephadex G-25), concentrated, and stored in aliquots in a liquid N₂ dewar until ready for use, or immediately reconstituted (vide infra). All buffers were chilled on ice prior to use and ice packs were used to jacket the Amicon stirred cell during protein concentration steps.

Reconstitution of NadA. Reconstitution of E. coli NadA proteins with iron and sulfide was carried out on ice in a Coy anaerobic chamber using anaerobic buffers and solutions. A typical reconstitution was conducted in a final volume of 20 mL on 100 µM NadA that was initially treated with 5 mM DTT for 20 min. Next, an 8-fold molar excess of FeCl₃ was added and the solution was allowed to sit on ice for 20 min. Finally, an 8-fold molar excess of Na₂S was added over a period of 3 to 4 h, and then the mixture was allowed to incubate on ice for 5-13 h. The mixture was then concentrated in an Amicon stirred cell and subjected to centrifugation at 14,000 × g for 2 min to remove precipitate. The supernatant was removed and exchanged into buffer C by gel filtration, reconcentrated, and then stored in aliquots in a liquid N₂ dewar.

Expression and Purification of NadB. Plasmid pET28-NadB was transformed into E. coli BL21(DE3). A single colony was used to inoculate 100 mL of LB media containing 50 µg/mL kanamycin and was cultured for 7 hours at 37 °C with shaking. A 60 mL portion of the culture was evenly distributed among four 6 L Erlenmeyer flasks to inoculate 16 L of LB media containing 50 µg/mL kanamycin, and was cultured further at 37 °C. At an OD₆₀₀ of 0.6, the cultures were cooled in an ice–water bath, and solid IPTG and FAD were added to each flask to
final concentrations of 200 and 10 µM, respectively. The cultures were then incubated at 18 °C with shaking for 16 h. Cells were harvested at 10,000 × g for 10 min at 4 °C, and the resulting frozen cell paste was frozen in liquid N₂ and stored at −80 °C until ready for use. Typical yields of frozen cell paste were between 50 and 60 g per 16 L growth.

In a typical purification, 25 g of frozen cells were resuspended in 80 mL of buffer D (50 mM HEPES, pH 7.5, 0.2 M NaCl, 10 mM imidazole, 10 mM succinate, pH 8, and 20 µM FAD). Solid egg white lysozyme and phenylmethanesulfonfyl fluoride (PMSF) were added to a final concentrations of 1 mg mL⁻¹ and 1 mM, respectively, and the mixture was stirred at room temperature for 30 min. After the mixture was allowed to cool in an ice–water bath to <8 °C, it was subjected to four 1 min bursts of sonic disruption (setting 7). Cellular debris was removed by centrifugation at 50,000 × g for 1 h, and the resulting supernatant was loaded onto a Ni-NTA column (2.5 × 7 cm) equilibrated in buffer D. The column was washed with 175 ml of buffer E (50 mM HEPES, pH 7.5, 0.2 M NaCl, 20 mM imidazole, 10 mM succinate, pH 8, 20 µM FAD, and 10% glycerol) and subsequently eluted with buffer E containing 250 mM imidazole. Protein–containing fractions were pooled and concentrated in an Amicon stirred cell fitted with a YM-10 membrane (10,000 Da MW cutoff). The protein was exchanged into anaerobic buffer F (50 mM HEPES, pH 7.5, and 10% glycerol) by anaerobic gel filtration (Sephadex G-25), reconstituted, and stored in aliquots in a liquid N₂ dewar until ready for use.

**Determination of Protein Concentrations.** Protein concentrations were determined by the Bradford dye staining procedure with BSA as the standard (35). Quantitative amino acid analysis on parallel samples of *E. coli* NadA, conducted at the University of Iowa Molecular Analysis Facility, confirmed that the Bradford assay was accurate without a correction factor.

**Activity Determinations.** The activity of WT and variant NadA proteins was determined by monitoring the formation of QA over a 20 min time period at 37 °C under anaerobic
conditions. The substrate IA was generated enzymatically via the NadB reaction using fumarate as the electron acceptor, or chemically by inclusion of OAA and ammonium sulfate in assay mixtures (36). Activity determinations conducted in the presence of NadB contained, in a final volume of 1300 µL, 200 mM HEPES, pH 7.5, 25 mM L-aspartate/fumarate solution (titrated to pH 7), 25 µM FAD, 1 mM DHAP, 0.1 M KCl, 0.3 mM L-tryptophan (internal standard), and either 5 µM NadA (RCN enzyme) and 5 µM NadB or 15 µM NadA (AI enzyme) and 15 µM NadB. When IA was generated chemically, the aspartate/fumarate solution, FAD, and NadB were replaced with 30 mM OAA and 30 mM ammonium sulfate. The reactions were initiated by addition of either NadB or OAA after incubation of the other components of the assay mixture at 37 °C for 5 min. At designated times, 200 µL aliquots of the assay mixture were removed and added to 40 µL of 2 M trichloroacetic acid (TCA) to quench the reaction. The precipitated protein was pelleted by centrifugation, and the supernatant was analyzed by HPLC with UV detection (268 nm) using a Zorbax SB-C18 column (4.6 × 250 mm) from Agilent (Foster City, CA). The column was equilibrated in 100% Solvent A (1% trifluoroacetic acid (TFA)) at a flow rate of 1 mL min⁻¹. These initial conditions were maintained for 10 min after injection, upon which a gradient of 0-45% Solvent B (acetonitrile) was applied over 10 min. At 20 min, a gradient of 45-90% Solvent B was applied over 3 min. Finally, at 25 min, a gradient of 90-0% Solvent B was applied over 3 min to re-establish the initial conditions until the end of the run at 32 min. Using this method, QA eluted at 8.3 min, and the tryptophan internal standard (IS) eluted at 20.3 min. The concentration of QA was determined from a calibration curve of known concentrations of the compound using the IS to correct for volume changes between sample injections.

Activity determinations were also performed by quantifying the amount of DHAP remaining as a function of time as described previously (20). Assay mixtures were as described above; however, at designated times, 50 µL aliquots of the quenched assay mixture were added to
a cuvette containing 100 mM HEPES, pH 7.5, and 250 µM NADH, and the absorbance at 340 nm was measured. α-Glycerophosphate dehydrogenase (5.6 U) was added to the cuvette, and the resulting decrease in absorbance at 340 nm (ε = 6440 M⁻¹ cm⁻¹) was recorded and then used to calculate the concentration of DHAP present. The concentration of QA was then assumed to be the difference between the starting DHAP concentration and the concentration measured at any given time point.

Preparation of Mössbauer and EPR Samples. Samples to be analyzed by Mössbauer and EPR spectroscopies contained 425-950 µM NadA and were all prepared inside the anaerobic chamber. For characterization by Mössbauer, bacterial growth and gene expression were carried out in M9 minimal media, and ^57FeSO₄ was added at induction. Samples (400 µL) were placed in small plastic cups and frozen in liquid N₂. For characterization by EPR, samples (250 µL) were treated with 2 mM sodium dithionite at room temperature for 5 min, placed in EPR tubes (2 mm i.d.), and frozen in liquid N₂.

2.4 Results

Analytical and Spectroscopic Characterization of WT and Variant E. coli NadA Proteins. The primary structure of P. horikoshii NadA contains only five cysteines, of which three (Cys83, Cys170, and Cys256) are conserved among all verified NadA proteins (Figure 2.2). Interestingly, these three conserved cysteines in P. horikoshii NadA are found in surface loops that are not observed in the X-ray structure (residues 79–90, 165–175, and 257–260) because of their disordered nature (27). This observation, in concert with the bioinformatics analysis, suggests that these cysteines are those that coordinate the required Fe/S cluster, and allows prediction that Cys→Ser or Cys→Ala substitutions made at equivalent positions in E. coli NadA should render the protein inactive. E. coli NadA contains a CysXXCysXXCys motif, which is often found in Fe/S proteins, especially of the ferredoxin type. The cysteines in this motif typically contribute three ligands to the Fe/S cluster, while the fourth is provided by a cysteine that can be
considerably further away in the primary structure (2). This motif is found in a large fraction, if not a majority, of NadA proteins, and was one of the initial indications that these proteins might contain Fe/S clusters (18). However, *P. horikoshii* and *M. tuberculosis* NadA proteins do not contain the motif, and only the last cysteine within it is strictly conserved (Figure 2.2). Therefore, serine substitutions at the cysteines in this motif were also made to assess their effect on cluster formation and turnover.

Wild–type (WT) and variant *E. coli* hexahistidine–tagged NadA proteins were overproduced in the presence of the plasmid pDB1282, which contains the *Azotobacter vinelandii* *isc* operon (3). This operon includes the genes *iscS, iscU, iscA, hscA, hscB*, and *fdx*, which are important in Fe/S cluster assembly (37-39). Expression of those genes is induced at an OD$_{600}$ of 0.3 by adding arabinose to a final concentration of 0.05%. Expression of the *nadA* gene was then induced at an OD$_{600}$ of 0.6 by adding IPTG to a final concentration of 200 µM IPTG. In typical growths, the expression of the Fe/S cluster assembly genes of plasmid pDB1282 appears to be low compared to the expression of the *nadA* gene cloned into pET28a (Figure 2.3a, lanes 3 & 4). Following sonication and centrifugation, there was a small amount of NadA in the pellet (Figure 2.3b, lane 3) migrating as a protein at of 45 kDa by SDS-PAGE. However, the IscS protein migrates similarly, making it difficult to determine the amount of insoluble NadA in the pellet. After centrifugation, the supernatant was loaded onto the Ni-NTA resin and subsequently washed. A portion of the soluble NadA elutes during the loading and washing of the column, but the amount of protein lost decreases with increasing volume of Ni-NTA resin (Figure 2.3b, lanes 4 & 5). Typical purifications from 20 g of cell paste yielded 200-250 mg of protein that was ≥95% pure. All of the *E. coli* NadA variants were produced and purified in a manner similar to that of the WT protein with similar yields.
The UV–visible absorption spectrum of as isolated (AI) WT E. coli NadA has been reported (19). It is shown again here in Figure 2.4A (solid line) to allow visual comparison with UV–visible spectra of variant proteins containing targeted Cys→Ser substitutions. The AI WT protein contains 3.0 ± 0.11 irons and 2.3 ± 0.07 sulfides (average and standard deviation based on four independent determinations), and the ratio of absorbance at 280 nm to that at 400 nm, a qualitative characterization of cluster content (lower numbers indicate better cluster incorporation), is 4.5 (Table 2.2). After reconstitution with an 8-fold excess of iron and an 8-fold excess of sulfide, the protein is found to contain 10 ± 0.72 equiv of Fe and 8.2 ± 1.2 equiv of S²⁻.
per polypeptide, indicating the presence of adventitiously bound iron and sulfide, as has been reported in a previous Mössbauer analysis of *E. coli* NadA (19). Reconstitution with a 3-fold excess of iron and an 8-fold excess of sulfide allows for more reasonable Fe/S numbers (3.5 ± 0.52 equiv of Fe and 3.6 ± 0.30 equiv of S²⁻ per polypeptide), which are in the range of the expected values for a [4Fe-4S] cluster. Reconstitution also increases the amplitude of the peak at 400 nm on the UV-visible spectrum (Figure 2.4A, dashed line), as well as the activity of the protein (Table 2.2, and vide infra).
Figure 2.4: UV–visible spectra of purified Al (solid line) and RCN (dashed line) WT *E. coli* NadA, normalized to $A_{280}$ (A), Cys291Ser (B), Cys294Ser (C), Cys113Ser (D), Cys200Ser (E), and Cys297Ser (F).
Table 2.2: Properties of *E. coli* Wild–Type and Variant NadA Proteins
d

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<thead>
<tr>
<th>Sample</th>
<th>Iron per polypeptide</th>
<th>Sulfide per polypeptide</th>
<th>Ratio</th>
<th>Turnover number (U)</th>
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<th>Reconstituted</th>
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<td>WT</td>
<td>3.0 ± 0.11</td>
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<td>1.8</td>
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<td>C64S</td>
<td>1.4 ± 0.11</td>
<td>0.73 ± 0.15</td>
<td>5.5</td>
<td>0.15</td>
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<td>C113A</td>
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<td>C113S</td>
<td>1.3 ± 0.39</td>
<td>0.42 ± 0.05</td>
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<td>C119S</td>
<td>0.98 ± 0.42</td>
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<td>0.20</td>
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<td>C128S</td>
<td>2.7 ± 0.07</td>
<td>1.1 ± 0.07</td>
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<td>C195S</td>
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<td>C200A</td>
<td>1.5 ± 0.29</td>
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<td>C200S</td>
<td>0.97 ± 0.32</td>
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<td>C294S</td>
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<td>1.8 ± 0.16</td>
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<td>1.1</td>
<td>2.0</td>
<td>4.2</td>
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<tr>
<td>C297A</td>
<td>0.33 ± 0.04</td>
<td>0.50 ± 0.04</td>
<td>15.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>C297S</td>
<td>0.29 ± 0.21</td>
<td>0.14 ± 0.12</td>
<td>19.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND, activity not detected. Reported errors reflect one standard deviation. Unit (U) defined as µM quinolinic acid per µM NadA, per min.

Defined as \(\frac{A_{280\text{nm}}}{A_{400\text{nm}}}\).”

In the presence of chemically generated IA, RCN WT NadA catalyzed formation of QA with a \(V_{max}/[E_1]\) of 4.1 min\(^{-1}\), a 1.9-fold increase from that of the AI enzyme (Figures 2.5A and 2.5B, closed circles). The activity determined using chemically generated IA is significantly greater than that obtained when using NadB and L-aspartate to generate the unstable substrate (Table 2.2, and Figures 2.5A and 2.5B, open circles). The lower activity may result from the finding that fumarate, the electron acceptor used to re-oxidize the flavin cofactor of NadB under anaerobic conditions, displays competitive inhibition with respect to IA, and that IA binds tightly to NadB (14). Therefore, the IA must be released from the enzyme prior to fumarate binding to allow re-oxidation of the flavin for subsequent rounds of turnover. To substantiate the accuracy of QA determination by HPLC, a second, independent method for activity determination was also
employed, in which the amount of DHAP consumed was quantified spectrophotometrically (20).

As shown in Figure 2.6, these two different methods for activity determination correlate well.

Figure 2.5: Time–dependent formation of QA catalyzed by WT *E. coli* NadA. The reaction was carried out as described in Materials and Methods and contained either 15 µM AI NadA (top panel, A) or 5 µM RCN NadA (bottom panel, B). The open circles represent assays with NadB and L-aspartate as the IA source, and the closed circles represent assays with OAA and AS as the IA source.
Figure 2.6: Correlation of two methods for the time–dependent determination of quinolinic acid (QA). HPLC method (open circles); Enzymatic method (closed circles).

The Cys113Ser, Cys200Ser, Cys291Ser, Cys294Ser and Cys297Ser variants were purified anaerobically—under the conditions described for WT NadA—and characterized. Cysteines 291 and 294 are the first two cysteines in the CysXXCysXXCys motif, while cysteines 113, 200, and 297 are those that are strictly conserved among NadA proteins (Figure 2.2). The Cys291Ser and Cys294Ser NadA variants were brown in color and have UV-visible spectra that are similar to that of the WT protein (Figures 2.4B & 2.4C, respectively). The $A_{280}/A_{400}$ ratios, 10.2 and 4.6, respectively, indicate varying degrees of cluster incorporation, which is consistent with the amount of iron and sulfide found associated with the AI proteins. Although the Cys291Ser variant appears to be seriously deficient in cluster content, the AI protein is capable of catalyzing formation of QA, displaying a $V_{\text{max}}/[E_T]$ that is ~3-fold lower than that of the WT protein using NadB to generate IA, and ~7-fold lower using chemically generated IA. Upon
reconstitution of this variant, its activity increased to rates comparable to that of WT NadA. The AI and RCN Cys294Ser variant displayed greater activity than the WT protein when NadB was used to generate IA, and comparable activity in the presence of chemically generated IA.

The UV–visible spectra of the AI Cys113Ser, Cys200Ser, and Cys297Ser variants are displayed in Figures 2.4D, 2.4E, and 2.4F, respectively. Their $A_{280}/A_{400}$ ratios varied from 8.6 to 19.4, and paralleled the amount of iron and sulfide found associated with the particular protein, which was consistently lower than that of WT NadA (Table 2.2). In addition, none of these three variants, in its AI or RCN state, was capable of catalyzing formation of QA regardless of the method used to generate IA, suggesting that they are prime candidates for ligands to the Fe/S cluster. After reconstitution, all three variants showed an increase in the peak at 400 nm, yet there was no concomitant gain in observed activity (vide infra).

In addition to the five cysteine residues that are conserved and/or present in the CysXXCysXXCys motif, the four remaining cysteines in the E. coli protein (Cys64, Cys119, Cys128, and Cys195) were also changed to serines, and the corresponding variants characterized. All of these AI variants, except for Cys119Ser, had only slightly lower iron and sulfide content, and displayed UV–visible spectra with slightly greater $A_{280}/A_{400}$ ratios (Table 2.2). The Cys119Ser variant had significantly lower iron and sulfide content and displayed a significantly greater $A_{280}/A_{400}$ ratio; however, the enzyme displayed catalytic competence, though significantly low, in both the AI and RCN forms, suggesting that this residue does not ligate the cluster (Table 2.2). The activities of the Cys64Ser, Cys128Ser, Cys195Ser, Cys291Ser and Cys294Ser variants varied considerably, depending on the particular variant and the conditions of the assay. Only one of the variants responded similarly (Cys291Ser), while most of the others displayed comparable activities under both conditions. The RCN Cys294Ser variant, however, displayed slightly lower activity in the presence of chemically generated IA (Table 2.2).
EPR and Mössbauer Spectroscopy of the Cys200Ser variant of E. coli NadA. As shown in Table 2.2, iron and sulfide analyses, and UV–visible spectra of E. coli NadA variants Cys113Ser, Cys200Ser, and Cys297Ser (Figure 2.4), those hypothesized to act as ligands to the Fe/S cluster, suggest the presence of small amounts of Fe/S clusters despite having one of the purported ligands to the cluster removed. The assignment as true [4Fe-4S] clusters rather than adventitiously bound iron and sulfide was verified by further spectroscopic studies on the Cys200Ser variant. The 4.2-K/53-mT Mössbauer spectroscopy spectrum of the RCN Cys200Ser E. coli NadA variant is shown in Figure 2.7 (top spectrum). In addition to the broad and featureless absorption extending from −5 mm/s to +5 mm/s (~40 % of total Fe), there is a pronounced quadrupole doublet with parameters typical of a [4Fe-4S]^{2+} cluster: isomer shift, δ, of 0.44 mm/s and quadrupole splitting parameter, ΔE_Q, of 1.13 mm/s. This quadrupole doublet accounts for 55% of total Fe. Together with the ratio of 1.0 Fe per protein (Table 2.2), this result indicates the presence of 0.14 [4Fe-4S] clusters per NadA polypeptide. The EPR spectrum of RCN Cys200Ser NadA, which is reduced in the presence of 2 mM sodium dithionite corroborates the presence of reduced [4Fe-4S]^+ clusters (Figure 2.7, solid line, bottom spectrum). The spectrum, obtained at 13 K and 5 mW power, shows an axial signal with approximate g–values \( g_1 = 2.04 \) and \( g_2 = 1.93 \), which are nearly identical to the g–values observed for the WT protein (19).
Figure 2.7: (A) 4.2-K/53-mT Mössbauer spectrum of Cys200Ser *E. coli* NadA. The solid line is a quadrupole doublet ($\delta = 0.44$ mm/s and $\Delta E_Q = 1.13$ mm/s, 55% of total intensity) representing the fraction of $[4\text{Fe-4S}]^{2+}$ clusters. (B) EPR spectrum of RCN Cys200Ser NadA (final concentration 581 µM, solid line) overlaid with the spectrum of RCN WT *E. coli* NadA (final concentration 300 µM, dashed line), both reduced in the presence of 2 mM sodium dithionite, obtained at 5 mW power and 13 K with a modulation amplitude of 10 G.
The results of EPR and Mössbauer spectroscopies suggest that despite the substitution of one of the Fe/S cluster ligands with a serine, a substoichiometric amount of [4Fe-4S] cluster is still formed; however, the resulting enzyme is inactive. To assess whether the ability to form a cluster on this variant protein derived from possible serine ligation, variants containing Cys→Ala substitutions at Cys113, Cys200, and Cys297 were also constructed. As expected, the resulting alanine variants were also inactive, but displayed similar UV–visible spectra and iron and sulfide content, suggesting that they too were able to support formation of a [4Fe-4S] cluster (Table 2.2).

2.5 Discussion

Quinolinate synthase has suffered a sinuous history with respect to its cofactor requirement in catalysis. The first indication that it might require an Fe/S cluster arose from studies by Gardner and Fridovich, in which the protein was shown to be inactivated by exposure to hyperbaric oxygen (1). In E. coli cell extracts, this oxygen–dependent inactivation was partially reversed upon anaerobic incubation of the extracts; however, reversal was blocked in the presence of iron chelators such as a,a’-dipyridyl or 1,10-phenanthroline (18). In addition, it was noticed that the primary structure of E. coli NadA contained a CysXXCysXXCys sequence. This motif is often found in proteins that contain Fe/S clusters, especially ferredoxins, and the cysteines lying in the motif contribute protein–thiolate ligands to the cluster (2). Additional evidence in support of an Fe/S cluster associated with E. coli NadA came from genetic studies in E. coli, wherein an iscS deletion mutant was found to be auxotrophic for nicotinic acid when grown on minimal medium (40). The iscS gene encodes a cysteine desulfurase, a pyridoxal 5’-phosphate–dependent enzyme that liberates elemental sulfur in the form of a cysteine persulfide to be used in Fe/S cluster biosynthesis as well as the synthesis of other important biological compounds (41). E. coli NadA was subsequently shown by two independent groups to contain [4Fe–4S] clusters. In one study the protein was overproduced in the presence of proteins that participate in various stages of Fe/S cluster biosynthesis, and was found to contain 5.0 ± 1.4 irons and 2.8 ± 0.3 sulfides per
polypeptide (19). In another study it was overproduced in the absence of accessory proteins, and contained 3.1 irons and 3.0 sulfides per polypeptide (20). Upon exposure of the protein to oxygen, UV–visible signatures that indicate the presence of Fe/S clusters bleached, with concomitant loss of activity (20).

Results from the Fontecave and Booker laboratories were in contrast to those of Ceciliani et al., who reported the first purification of recombinant NadA to homogeneity, in which the \textit{E. coli} enzyme was isolated from inclusion bodies obtained during overproduction (42). The purification included solubilization in the presence of 4 M urea and 50 mM EDTA, and dialysis in the presence of 10 mM EDTA, conditions not typically conducive to maintaining Fe/S clusters within a protein scaffold. Moreover, their reported specific activity (600 µmol min\(^{-1}\) mg\(^{-1}\)) was significantly greater than that reported by Cicchillo \textit{et al.} for the Fe/S–containing enzyme (0.015 µmol min\(^{-1}\) mg\(^{-1}\)) (3), or that reported in this study for RCN \textit{E. coli} NadA (0.10 µmol min\(^{-1}\) mg\(^{-1}\)). It is possible that there was a typographical error in their definition of a unit, which was 1 mmol min\(^{-1}\) instead of 1 µmol min\(^{-1}\), the accepted international unit definition. However, their reported activity would still be considerably greater than that observed for the enzyme containing a [4Fe-4S] cluster.

Surprisingly, the X-ray structure determination of NadA from \textit{P. horikoshii}, solved in 2005, seemed to lend support to the results of Ceciliani \textit{et al.} (5). The structure was solved to 2.0 Å resolution, and described as monomeric, but exhibiting pseudo-3-fold symmetry involving three repeating \(\alpha\beta\alpha\) sandwich domains. The protein was crystallized in the presence of malate, which is found in the structure at the interface of the three domains, suggesting the location of the active site. Interestingly, no metal or metallocofactor is found in the structure, and the possibility that the enzyme might contain an Fe/S cluster was not even addressed. Most surprisingly, however, this enzyme was stated to catalyze formation of QA with a specific activity of 2.2 µmol min\(^{-1}\) mg\(^{-1}\) (5).
Given the inconsistencies listed above we initiated efforts to conduct a comprehensive analysis of the requirement for Fe/S clusters in several quinolinate synthases. In light of the suggestion that NadA is a member of the Fe/S–dependent class of the hydro–lyase family of enzymes (43), we expected that, similarly to aconitase, three of the four iron atoms in the [4Fe-4S] cluster would be ligated by a Cys residue, allowing the non-cysteinate–ligated iron to participate directly in the reaction. This premise allows prediction that NadA should contain at least three conserved Cys residues in its primary structure. Indeed bioinformatics analyses conducted by us (44) and others (45) indicate that three cysteines are conserved throughout over 700 annotated NadA proteins. Using site–directed mutagenesis, we changed each of the nine Cys residues in the primary structure of *E. coli* NadA to Ser or both Ser and Ala, and isolated and characterized each of the variant proteins. Only six of the variants (Cys113Ser, Cys113Ala, Cys200Ser, Cys200Ala, Cys297Ser, Cys297Ala) displayed complete loss of activity, suggesting that Cys113, Cys200, and Cys297 are viable candidates for ligands to the cluster. Initially we had predicted that removal of one of three Cys ligands to a [4Fe-4S] cluster would yield a protein that was completely devoid of cluster. However, iron and sulfide analyses in concert with UV–visible spectroscopy suggested the presence of small amounts of bound cluster, which was verified by Mössbauer spectroscopy on the $^{57}$FeSO$_4$-RCN Cys200Ser variant. We would predict, therefore, that the [4Fe-4S] cluster in the Cys200Ser variant is ligated by only two Cys residues as has been shown previously for the RCN Cys30Ala variant of the activase protein of the anaerobic ribonucleotide reductase (46). Interestingly, only one of the three conserved Cys residues (Cys297) is found in the CysXXCysXXCys motif that was predicted to house the ligands to the cluster.

Finally, we believe that our findings and the report of Sakuraba *et al.* can be brought into accord. Sakuraba *et al.* note that there are three regions that are not visible in electron density maps due to their disordered nature (5). These regions constitute three surface loops that separate
each of the domains, spanning amino acids 79–90, 165–175, and 257–260. Our bioinformatics analysis indicates that two of these disordered loops contain Cys83 and Cys170, two of the strictly conserved cysteine residues postulated to ligate a [4Fe-4S] cluster. The third conserved cysteine (Cys256) is visible in the structure; however, it is immediately adjacent to the disordered loop spanning amino acids 257–260. It is likely that the absence of the [4Fe-4S] cluster, to which the three cysteines coordinate, renders the loops mobile, giving rise to their disordered nature.

The observed increase in activity displayed by *P. horikoshii* NadA can also be rationalized. In the report by Sakuraba *et al.*, an HPLC method was used to quantify QA, employing the NadB reaction to generate the required IA. We use a similar HPLC method to quantify QA, which is described herein. QA displays a maximum absorbance at 268 nm; however, in the report by Sakuraba *et al.*, formation of QA was monitored at 254 nm. OAA displays a $\lambda_{\text{max}}$ at 255 nm, and we routinely observe time–dependent formation of this compound when conducting assays in which the NadB reaction is used to generate IA, and observe it instantaneously in assays in which IA is generated chemically. Therefore, it appears that the numbers reported by Sakuraba *et al.* reflect formation of OAA from IA ($t_{1/2} = 144$ s) generated in the NadB reaction, rather than turnover by NadA. We believe, therefore, that crystallization of *P. horikoshii* NadA in the presence of its [4Fe-4S] cofactor will give a much clearer picture as to the organization of the active site of the enzyme, and the manner in which this enzyme catalyzes its reaction.
2.6 References


Chapter 3

Redox Regulation of *Escherichia coli* NadA by Disulfide Bond Formation

This chapter was reproduced from Saunders, A. H., and Booker, S. J. (2008) Regulation of the activity of *Escherichia coli* quinolinate synthase by reversible disulfide–bond formation, *Biochemistry* 47, 8467-8469.

3.1 Abstract

Quinolinate synthase (NadA) catalyzes a unique condensation reaction between dihydroxyacetone phosphate (DHAP) and iminoaspartate (IA), yielding inorganic phosphate (P$_i$), 2 moles of water, and quinolinic acid (QA), a central intermediate in the biosynthesis of nicotinamide adenine dinucleotide (NAD$^+$) and its derivatives. The enzyme from *Escherichia coli* contains a C$^{291}$XXC$^{294}$XXC$^{297}$ motif in its primary structure. Through characterization of cysteine variants along with a bioinformatics analysis, we and others have shown that three conserved cysteines, Cys113, Cys200, and Cys297, serve as ligands to a [4Fe-4S] cluster that is required for turnover, only one of which resides in the motif (1, 2). In this report we show that the two remaining cysteines, Cys291 and Cys294, undergo reversible disulfide–bond formation, which regulates the activity of the enzyme. This mode of redox regulation of NadA appears physiologically relevant, because disulfide–bond formation and reduction is effected by oxidized and reduced forms of *E. coli* thioredoxin (Trx). A midpoint potential of $-264 \pm 1.8$ mV is estimated for the redox couple.
3.2 Introduction

QA is a key intermediate in the biosynthesis of NAD$^+$ and its derivatives in all organisms that synthesize the cofactor *de novo*. The mechanism by which this common precursor is biosynthesized, however, differs between most, but not all, eukaryotes and prokaryotes (3). Vertebrates, and most other eukaryotes, synthesize QA via the degradation of L-tryptophan. By contrast, prokaryotes and a small number of eukaryotes synthesize QA via a unique condensation reaction between DHAP and IA, which is catalyzed by NadA (3). IA is generated by the action of L-aspartate oxidase (NadB), a flavin adenine dinucleotide (FAD)–requiring enzyme. During turnover, FAD is transiently reduced by two electrons to FADH$_2$ (4). Under aerobic conditions the flavin is reoxidized by molecular oxygen, affording production of hydrogen peroxide. Under anaerobic conditions fumarate acts as the oxidant, and is converted to succinate (5).

IA is labile, and has been reported to hydrolyze to oxaloacetate (OAA) and ammonia with a half–life of 140 s (4); it is also susceptible to β-decarboxylation yielding iminopyruvate and CO$_2$. Studies by Nasu and Gholson have shown that the NadB protein can be replaced by addition of OAA and an ammonia source, such as ammonium sulfate, which gives rise to an equilibrium concentration of IA via Schiff base formation (6).

In early mechanistic studies of NadA from *E. coli*, the enzyme was reported to be unstable, especially in the presence of hyperbaric oxygen (7, 8). This characteristic, as well as a CysXXCysXXCys motif found in its primary structure, led to the suggestion that it might contain an iron–sulfur (Fe/S) cluster (8). Indeed, recent characterization of the protein purified under anaerobic conditions showed that it harbors a [4Fe-4S] cluster that is absolutely required for activity (9, 10). Through bioinformatics analysis and characterization of cysteine variants, we and others have shown that three conserved cysteines, Cys113, Cys200, and Cys297, serve as ligands to a [4Fe-4S] cluster that is required for turnover, only one of which residue resides in the motif (1, 2). The remaining two cysteines, Cys291 and Cys294, exist in a CysXXCys motif. This sequence is conserved in the active site of thioredoxin–fold proteins, of which Trx is the
prototype family member (11). Trx catalyzes the reduction, formation and isomerization of disulfide bonds, and helps to maintain the reducing environment of the E. coli cytosol (12).

In recent studies of E. coli NadA (9, 10), the protein was maintained under anoxic and reducing conditions because of its reported lability in the presence of oxygen (8). This observed oxygen sensitivity was later reproduced; exposure of the protein to oxygen resulted in loss of the [4Fe-4S] cluster and its characteristic UV–visible signal, as well as loss of the enzyme’s ability to catalyze the reaction (10). Given that NadB uses dioxygen as a cosubstrate under aerobic conditions, catalyzing the formation of peroxide, another reactive oxygen species (ROS) that is known to have deleterious effects on Fe/S clusters (13), we re-investigated the oxygen sensitivity of NadA, and find that the activity of the protein is actually greater under oxic conditions than anoxic conditions. A single amino acid substitution of the two cysteines in the CysXXCys motif to either serine or alanine results in proteins that have the opposite effect. We demonstrate that this behavior is due to the formation of a redox–active disulfide bond between Cys291 and Cys294, which can regulated by thioredoxin. The midpoint reduction potential of the NadA disulfide bond is determined to be $-264 \pm 1.8$ mV.

3.3 Materials and Methods

Materials. All DNA modifying enzymes and reagents were purchased from New England Biolabs (Beverly, MA). PfuUltra High Fidelity DNA Polymerase and its associated $10\times$ reaction buffer were obtained from Stratagene (La Jolla, CA). Oligonucleotide primers for site-directed mutagenesis were obtained from Integrated DNA Technologies (Carlsbad, CA). Coomassie blue dye–binding reagent for protein concentration determination and the bovine serum albumin (BSA) standard (2 mg mL$^{-1}$) were obtained from Pierce (Rockford, IL). Whatman DE-52 resin was purchased through VWR International (West Chester, PA), while Sephacryl S-300 and Sephadex G-25 resins, as well as Nick pre-poured gel filtration columns were purchased from GE Bio-sciences (Piscataway, NJ). 2,3-Pyridinedicarboxylic acid (quinolinic acid) was obtained from Aldrich (St. Louis, MO). Dihydroxyacetone phosphate (dilithium salt), tris(2-
carboxyethyl) phosphine-HCl (TCEP), and oxaloacetate were obtained from Sigma (St. Louis, MO). Immobilized TCEP was obtained from Pierce (Rockford, IL). All other buffers and chemicals were of the highest grade available. The pTRX BL21(DE3) Trx expression vector was obtained from Dr. J. Martin Bollinger Jr. (Pennsylvania State University).

**General Procedures.** High performance liquid chromatography (HPLC) was conducted on a Beckman System Gold unit (Fullerton, CA), which was fitted with a 128 diode array detector and operated with the System Gold Nouveau software package. Sonic disruption of *E. coli* cells was carried out with a 550 sonic dismembrator from Fisher Scientific (Pittsburgh, PA), in combination with a horn containing a ½ in. tip. Procedures conducted under anoxic conditions were carried out in an anaerobic chamber from Coy Laboratory Products, Inc. (Grass Lake, MI) under an atmosphere of N<sub>2</sub> and H<sub>2</sub> (95%/5%), and with an O<sub>2</sub> concentration maintained below 1 ppm by the use of palladium catalysts.

**Spectroscopic Methods.** UV–visible spectra were recorded on a Cary 50 spectrometer (Varian; Walnut Creek, CA) using the associated WinUV software package. Low–temperature X-band EPR spectroscopy was carried out in perpendicular mode on a Bruker (Billerica, MA) ESP 300 spectrometer equipped with an ER 041 MR microwave bridge and an ST4102 X-band resonator (Bruker). The sample temperature was maintained with an ITC503S temperature controller and an ESR900 liquid helium cryostat (Oxford Instruments; Concord, MA).

**Expression and Purification of WT and Variant NadA Proteins.** All NadA proteins were overproduced and purified as described in Chapter 2 and as published (1). Steady–state kinetic analysis, disulfide–bond modification assays and cross–linking experiments were all performed with Cys→Ser variants of Cys291 and Cys294. The corresponding Cys→Ala variants were also constructed and used in EPR spectroscopic studies.

**Construction of the Cys35Ala Thioredoxin and Cys→Ala NadA Variants.** The Cys35Ala Trx variant and the Cys291Ala and Cys294Ala NadA variants were constructed using the QuickChange II Site–directed Mutagenesis Kit (Stratagene) according to the manufacturer’s
specifications, and as described previously \((14)\), in which the cycling protocol is adapted for the Stratagene Roboeyler thermocycler \((15)\). Plasmid pTRX was used as the template for the Cys35Ala Trx variant and pET28-NadA was used as the template for the Cys291Ala and Cys294Ala NadA variants, all in conjunction with the appropriate primers for each respective amino acid substitution (Table 3.1). All mutations were verified by DNA sequencing of the entire gene carried out at the Pennsylvania State University Genomics Core Facility (University Park, PA).

Table 3.1: Sequence of Primers used for Site-directed Mutagenesis

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</tr>
<tr>
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<td>CCTCACCTGC-3'</td>
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Bolded bases represent amino acid changes.

*Expression of Genes for E. coli Thioredoxin and the Cys35Ala Variant, and Purification of the Respective Gene Products.* A single colony was used to inoculate 100 mL Luria–Bertani (LB) media containing 100 µg/mL ampicillin and was cultured for ~7 h at 37 °C with shaking. A 60 mL portion of the culture was then evenly distributed into four 6 L Erlenmeyer flasks to inoculate 16 L of LB media containing 100 µg/mL ampicillin, which were cultured further at 37 °C with shaking (180 rpm). At an optical density (600 nm; OD\(_{600}\)) of 0.6, the cultures were cooled in an ice–water bath, and solid IPTG was added to each flask at a final concentration of 1
mM. The cultures were allowed to incubate further at 18 °C with shaking for 16 h. Cells were harvested at 10,000 × g for 10 min at 4 °C, frozen in liquid N₂, and stored at −80 °C until ready for use.

Trx was purified inside a cold room maintained at 4 °C. Frozen cells (40 g) were resuspended in 120 mL buffer A (50 mM Tris, pH 7.4, 3 mM 2-mercaptoethanol) at room temperature. Solid phenylmethylsulphonyl fluoride (PMSF) was dissolved in 95 % ethanol and added to a final concentration of 1 mM. Solid egg–white lysozyme was added to a final concentration of 1 mg/mL, and the mixture was stirred at room temperature for 30 min. After the solution was cooled in an ice–water bath to <8 °C, it was subjected to four 1 min bursts of sonic disruption (setting 7). Cellular debris was removed by centrifugation at 50,000 × g for 1 h. Streptomycin sulfate (10 % w/v in H₂O) was added to the supernatant to a final concentration of 2 % over 20 min while stirring. The solution was stirred for an additional 15 min and then subjected to centrifugation at 14,000 × g for 20 min. The supernatant was diluted to 400 mL in buffer A and loaded onto a DE-52 column (9 x 13 cm) equilibrated in buffer B (50 mM potassium phosphate, pH 7.3, 3 mM EDTA). The protein was eluted with a 1 × 1 L linear gradient from buffer B to buffer C (150 mM potassium phosphate, pH 7.3, 3 mM EDTA). Fractions were monitored by SDS-PAGE, and those containing Trx were pooled and concentrated in an Amicon stirred cell (Millipore, Billerica, MA) fitted with a YM-3 membrane (3,000 Da MW cutoff). The concentrated protein was loaded onto a Sephacryl S-300 size–exclusion column equilibrated in buffer D (50 mM Tris, pH 7.4, 3 mM EDTA). Fractions containing Trx were pooled and concentrated in an Amicon stirred cell prior to freezing in liquid N₂. The protein was stored in aliquots at −80°C until ready for use.

Preparation of Oxidized and Reduced Forms of Thioredoxin. Oxidized Trx (Trxₜₐₓ) was exchanged into buffer E (50 mM HEPES, pH 7.5, 0.1 M KCl and 20 % glycerol) by anaerobic gel filtration (Sephadex G-25) inside of a Coy anaerobic chamber. Reduced Trx (Trxₗₑₜ) was prepared
by incubation with 200 mM tris(2-carboxyethyl)phosphine (TCEP) for 1 h on ice. The protein was then subjected to anaerobic gel filtration into buffer E. The protein was aliquoted, frozen, and stored in liquid N\textsubscript{2} until ready for further use. The total thiol content was determined for both forms of Trx spectrophotometrically using 5,5'-dithiobis(nitrobenzoic acid) (DTNB) and its associated molar extinction coefficient \(\epsilon_{412} = 14,150 \text{ M}^{-1}\text{cm}^{-1}\) \(^{(16)}\).

*Determination of Protein Concentration.* Purification and reconstitution of *E. coli* NadA was carried out as described previously in Chapter 2 and as published \(^{(17)}\), as was the determination of its concentration. Trx concentration was determined using a molar extinction coefficient \(\epsilon_{280}\) of 13,700 M\(^{-1}\)cm\(^{-1}\) \(^{(18)}\).

*NadA Assays.* The activity of NadA proteins was determined by monitoring the formation of quinolinic acid at 37 °C during a 10 min incubation under anaerobic conditions or a 150 s incubation under aerobic conditions. The substrate IA was generated chemically by reacting OAA with an ammonia source \(^{(19)}\). The assay contained the following in a final volume of 1100 \(\mu\)L: 200 mM HEPES, pH 7.5, 1 mM DHAP, 0.1 M ammonium chloride, 5 mM OAA, 0.3 mM L-tryptophan (internal standard (IS)), and 5 \(\mu\)M reconstituted NadA. The reactions were initiated by addition of OAA after incubation of the other components at 37 °C for 3 min. At designated times, 200 \(\mu\)L aliquots of the assay mixture were removed and added to 40 \(\mu\)L of 2 M trichloroacetic acid (TCA) to quench the reaction. The precipitated protein was pelleted by centrifugation and the supernatant was analyzed by HPLC as described in Materials and Methods of Chapter 2 of this document, and as previously published \(^{(1)}\).

*Anaerobic and Aerobic Steady–state Kinetics Assays.* Initial rates of product formation for WT (anaerobic and aerobic) and variant (anaerobic only) NadA proteins were determined as described above, except that incubation times were extended to 20 min for anaerobic assays, and the concentration of DHAP was varied from 0.1 to 2.5 mM. The concentration of DTT in the assay mixtures was calculated to be 45 \(\mu\)M. The rates were then plotted as a function of DHAP
concentration and fitted to eq 1 by nonlinear regression to extract appropriate kinetic constants. In equation 1, \( v_0 \) is the observed initial rate at a given DHAP concentration \([S]\), \( V_{\text{max}} \) is the maximal velocity of the reaction, and \( K_m \) is the apparent Michaelis constant for DHAP.

\[
v_0 = \frac{V_{\text{max}} [S]}{K_m + [S]}
\]

Equation 1

All assays performed in these studies were in the presence of saturating concentrations of both substrates, conditions in which the rate equation for a bisubstrate system reduces to \( v = V_{\text{max}} \). Therefore turnover is expressed as this rate normalized to enzyme concentration, \( V_{\text{max}}/[E_{\text{T}}] \).

**Disulfide Bond Modification Assays.** Subsequent to purification, NadA is stored in gel–filtration buffer 1 (50 mM HEPES, pH 7.5, 0.1 M KCl, 20% glycerol, 10 mM DTT). Prior to assaying, the DTT was removed from the reduced NadA (NadA\(_{\text{red}}\)) by anaerobic gel filtration using a pre-poured Nick gel–filtration column equilibrated in gel–filtration buffer 2 (50 mM HEPES, pH 7.5, 0.1 M KCl, 20% glycerol). To prepare oxidized NadA (NadA\(_{\text{ox}}\)), the protein was oxidized by gel–filtration into gel–filtration buffer 2 prepared aerobically, and then exchanged into gel–filtration buffer 2 prepared anaerobically. To determine the activity in the presence of oxygen, NadA was assayed in gel–filtration buffer 2 prepared aerobically. For activity determinations in the presence of Trx, 166 \( \mu \)M of the protein (oxidized, reduced, or the Cys35Ala variant) was added to the assay. To assess the effect of DTT on the activity of NadA, NadA\(_{\text{ox}}\) was incubated on ice for 5 h in the presence or absence of 5 mM DTT before activity was determined. The effect of glutathione (reduced (GSH) and oxidized (GSSG)) was assessed by adding 5 mM of GSH or GSSG to the assay.

**Control Assays.** To ensure that there was not contaminating molecular oxygen or TCEP in the Trx, which would affect the activity of NadA, control assays were performed. To test for contaminating molecular oxygen, Trx\(_{\text{ox}}\) was incubated with 1.2 U of glucose oxidase, 14 mM D-glucose, and 1970 U of catalase for 1 h at RT to remove any contaminating molecular oxygen. To test for contaminating TCEP, Trx\(_{\text{ox}}\) was incubated with TCEP immobilized on agarose for 1 h at
RT. The resulting Trx\textsubscript{red} was then separated from the agarose by centrifugation. These Trx\textsubscript{ox} and Trx\textsubscript{red} control samples were then used in NadA assays to compare the rates with those from assays in which these precautions were not taken.

Formation of a NadA–Thioredoxin Mixed–disulfide Bond. A stable mixed–disulfide bond between NadA and Trx was generated by incubating 60 \(\mu\)M NadA\textsubscript{ox}, or the Cys291Ser or Cys294Ser variants, (in anaerobic gel–filtration buffer 2) with 416 \(\mu\)M Cys35Ala Trx in 50 mM Tris-HCl, pH 8.0 for 1 h at room temperature in the anaerobic chamber. The sample was then mixed with an equal volume of SDS-PAGE sample buffer that lacked reductant, and then removed from the anaerobic chamber and analyzed by SDS-PAGE (10%). Controls were conducted in which Cys35Ala Trx and each of the NadA proteins were incubated in the absence of the other protein.

Determination of the Midpoint Reduction Potential of NadA Disulfide Bond. To determine the midpoint reduction potential of the Cys291/Cys294 disulfide bond, NadA was exchanged into gel–filtration buffer 2, and then incubated (final concentration 6.8 \(\mu\)M) with varying ratios of Trx\textsubscript{ox} and Trx\textsubscript{red} (final concentration 166 \(\mu\)M) in 200 mM HEPES, pH 7.0 for 15 min at room temperature in an anaerobic chamber. The samples were subjected to activity determinations at 25°C for each given midpoint reduction potential, calculated from the ratio of Trx\textsubscript{ox} to Trx\textsubscript{red} using the Nernst equation and the known midpoint potential for Trx (−270 mV) (22). Samples were run in triplicate, and the resulting average observed rates with their associated standard deviations were determined and plotted versus the midpoint reduction potential of the assay. The resulting curve was fitted to eq 4, which is derived from the Nernst equation (see below), wherein \(k_1\) and \(k_2\) are the turnover numbers for NadA\textsubscript{red} and NadA\textsubscript{ox} respectively, \(n\) is the number of electrons transferred in the reaction (\(n = 2\)), \(F\) is Faraday’s constant (96,494 J/V*mol), \(R\) is the gas constant (8.314 J/mol), \(E\) is the actual measured (applied) potential, \(E_o\) is the standard potential of the electrode couple and [red] and [ox] are the concentrations of the reduced and oxidized species of the enzyme, respectively (23, 24).
Derivation of the equation for determining the midpoint reduction potential of NadA:

\[ E = E_0 - \frac{RT}{nF} \ln \left( \frac{[red]}{[ox]} \right) \]

Nernst Equation

\[ E = E_0 - 2.303 \left( \frac{RT}{nF} \right) \log \left( \frac{[red]}{[ox]} \right) \]

\[ E = E_0 - \left( \frac{0.059 V}{n} \right) \log \left( \frac{[red]}{[ox]} \right) \]

\[ \left( \frac{n(E - E_0)}{0.059 V} \right) = \log \left( \frac{[ox]}{[red]} \right) \]

\[ \frac{[ox]}{[red]} = 10^{\left( \frac{n(E - E_0)}{0.059 V} \right)} \]

\[ [red] = \frac{[ox]}{10^{\left( \frac{n(E - E_0)}{0.059 V} \right)}} \]

Equation 2

The sum of the oxidized and reduced species of the enzyme, \([ox] + [red]\), is equal to the total enzyme concentration, \([E_T]\):

\[ [ox] + [red] = [E_T] \]

Plug equation 2 in for \([red]\):
\[
[\text{ox}] = [E_T] - \frac{[\text{ox}]}{10^{\frac{n(E-E_0)}{0.059V}}}
\]

Rearrangement to obtain Equation 3:

\[
\frac{[\text{ox}]}{[E_T]} = \left(10^{\frac{n(E-E_0)}{0.059V}}\right) - \frac{[\text{ox}]}{[E_T]}
\]

Equation 3

The maximum rate of NadA \((V_{\text{max}})\) can be described by the following equation, where \(k_1\) is the rate of NadA_{ox}, \(k_2\) is the rate of NadA_{red}:

\[
V_{\text{max}} = k_1[\text{ox}] + k_2[\text{red}]
\]

\[
\frac{V_{\text{max}}}{[E_T]} = \frac{k_1[\text{ox}]}{[E_T]} + \frac{k_2[\text{red}]}{[E_T]}
\]

\[
\frac{V_{\text{max}}}{[E_T]} = \frac{k_1[\text{ox}]}{[E_T]} + \frac{k_2([E_T] - [\text{ox}])}{[E_T]}
\]

\[
\frac{V_{\text{max}}}{[E_T]} = k_1\left[\frac{[\text{ox}]}{[E_T]}\right] + k_2\left(1 - \frac{[\text{ox}]}{[E_T]}\right)
\]

Plug equation 3 in for \([\text{ox}]/[E_T]\):
\[
V_{\text{max}} / [E_T] = k_1 \left( \frac{10^{n(E-E_0)/0.059}}{1 + 10^{n(E-E_0)/0.059}} \right) + k_2 \left( 1 - \frac{10^{n(E-E_0)/0.059}}{1 + 10^{n(E-E_0)/0.059}} \right)
\]

Equation 4

Preparation of EPR Samples. For characterization by EPR, samples (250 µL) containing 300 µM NadA were treated with 2 mM sodium dithionite at room temperature inside the anaerobic chamber for 5 min, placed in EPR tubes (2 mm i.d.), and frozen in liquid N\(_2\). To oxidize the Fe/S cluster of NadA, the sodium dithionite was replaced with 2 mM potassium ferricyanide. For samples prepared in the presence of Trx\(_{\text{ox}}\), 700 µM NadA was treated with 2 mM sodium dithionite at room temperature for 5 min and then applied to a pre-poured Nick gel–filtration column to remove excess dithionite. The resulting protein (100 µM) was then incubated at room temperature for 1 h in the presence or absence of 500 µM Trx\(_{\text{ox}}\) prior to freezing.

3.4 Results

Expression and Purification of WT Thioredoxin and its Cys35Ala Variant. WT Trx and its Cys35Ala variant were overexpressed using the IPTG–inducible pTrx vector. The resulting cell paste was resuspended, lysed by sonication and centrifuged to separate cellular debris. The crude lysate supernatant (Figure 3.1, lane 2) was subjected to a 2 % streptomycin sulfate precipitation followed by centrifugation. The resulting supernatant (Figure 3.1, lane 4) was then subjected to anion exchange chromatography (DE-52 resin) followed by size–exclusion chromatography (S300) (Figure 3.1, lanes 7 and 8, respectively). A typical purification yielded approximately 1.4 g of protein that was ≈ 95 % pure from 40 g of cell paste.
Figure 3.1: SDS-PAGE analysis of the purification of *E. coli* thioredoxin. Lanes: 1, molecular mass markers; 2, crude lysate supernatant; 3, crude lysate pellet; 4, streptomycin sulfate supernatant; 5, streptomycin sulfate pellet; 6, DE-52 load eluate; 7, pooled DE-52 fractions; 8, pooled S300 fractions (pure).

For use in the disulfide bond modification assays, *Trx*<sub>ox</sub> was prepared by subjecting purified Trx (isolated aerobically in the absence of thiols) to gel–filtration chromatography into anaerobic buffer to remove oxygen. Alternatively, *Trx*<sub>red</sub> was prepared by incubating purified Trx with TCEP, followed by gel–filtration chromatography into anaerobic buffer. The oxidized and reduced states of the enzyme were confirmed by quantifying the free thiols using DTNB. No absorbance change was observed with *Trx*<sub>ox</sub> indicating that it contains no free thiols. When *Trx*<sub>red</sub> was treated with DTNB an absorbance change was observed that corresponded to 1.8 free thiols per protein as expected, corresponding to the two cysteine sulfhydryls present in the fully reduced state.

*Steady State Kinetic Analysis of WT NadA and NadA CysXXCys Motif Variants.* In Figure 3.2, a NadA–dependent activity determination under oxic and anoxic conditions is
displayed, in which L-aspartate and the NadB protein are replaced by addition of 100 mM ammonium chloride and 5 mM OAA, which affords a saturating concentration of IA. The use of chemically generated IA in place of NadB obviates complications associated with data interpretation deriving from to the different rates of IA formation by NadB when using oxygen as an electron acceptor as opposed to fumarate (5). Under both oxic and anoxic conditions, the NadA reaction displays clean Michealis–Menton behavior with respect to the varied substrate DHAP. Surprisingly, under oxic conditions, the $V_{\text{max}}/[E_{\text{T}}]$ of the reaction ($19.2 \pm 0.2 \text{ min}^{-1}$) is 12-fold greater than that observed under conditions that are both anoxic and reducing ($1.6 \pm 0.1 \text{ min}^{-1}$). Moreover, $V_{\text{max}}/[E_{\text{T}}] K_m$ for the reaction under oxic conditions ($80 \pm 0.1 \text{ min}^{-1} \text{ mM}^{-1}$) is 17-fold greater than that observed under anoxic and reducing conditions ($4.7 \pm 0.2 \text{ min}^{-1} \text{ mM}^{-1}$).

![Figure 3.2](image.png)

Figure 3.2: Steady state kinetic analysis of WT *E. coli* NadA. Activity determinations were performed using 5 mM OAA and 100 mM NH$_4$Cl under oxic (closed symbols) or anoxic (open symbols) conditions.
The observation of O2–dependent changes in NadA activity suggested redox regulation of the protein via oxidation of the Fe/S cluster or disulfide–bond formation, perhaps via Cys291 and Cys294. During incubation under oxidizing conditions (100 µM potassium ferricyanide), no evidence for a [4Fe-4S]3+ cluster was observed by electron paramagnetic resonance (EPR) spectroscopy; the protein remained largely diamagnetic with no signal present to indicate any [4Fe-4S]+ cluster, but displayed a small amount (<10%) of a [3Fe-4S]+ cluster (Figure 3.3), suggesting that cluster oxidation is not responsible for the increase in activity. This observation is also consistent with the previous finding that the [4Fe-4S]2+ cluster can be reduced to the +1 oxidation state (9, 10).

![Figure 3.3: EPR spectra of WT NadA in the presence of 2 mM sodium dithionite (black), and 2 mM potassium ferricyanide (blue).](image)

To assess the effect of the redox state of cysteines 291 and 294 on NadA activity, each was changed individually to serine, which blocks potential disulfide–bond formation between the two amino acids. Under conditions that are both anoxic and reducing, the Cys291Ser and
Cys294Ser variants displayed \( V_{\text{max}}/[E_T] \) values of \( 1.9 \pm 0.1 \) and \( 6.3 \pm 0.4 \) min\(^{-1}\), and \( V_{\text{max}}/[E_T] \ K_m \) values of \( 2.2 \pm 0.1 \) and \( 17 \pm 0.2 \) min\(^{-1}\) mM\(^{-1}\), respectively (Table 3.2). Both variants also display clean Michaelis–Menton behavior with respect to the varied substrate DHAP under anoxic conditions (Figure 3.4). Unlike the WT protein, the two variants were too unstable under oxic conditions for a complete steady–state analysis. To assess whether the observed rates for the Cys291Ser and Cys294Ser variants was due to an effect of the Cys→Ser substitution, the corresponding amino acids were also changed to Ala. The resulting Ala variants behaved similarly as the Ser variants with essentially the same \( V_{\text{max}}/[E_T] \) values.

Table 3.2: Steady state kinetic parameters for WT and variants under anaerobic conditions and WT under aerobic conditions.

<table>
<thead>
<tr>
<th></th>
<th>( V_{\text{max}}/[E_T] ) (min(^{-1}))</th>
<th>( K_m ) DHAP (mM)</th>
<th>( V_{\text{max}}/[E_T] \ K_m ) (min(^{-1}) mM(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT(_{\text{anaerobic}})</td>
<td>1.6 ± 0.1</td>
<td>0.34 ± 0.05</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>WT(_{\text{aerobic}})</td>
<td>19.3 ± 0.2</td>
<td>0.24 ± 0.01</td>
<td>80.4 ± 0.04</td>
</tr>
<tr>
<td>Cys291Ser</td>
<td>1.9 ± 0.1</td>
<td>0.88 ± 0.11</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Cys294Ser</td>
<td>6.3 ± 0.4</td>
<td>0.38 ± 0.09</td>
<td>16.7 ± 0.3</td>
</tr>
</tbody>
</table>
Figure 3.4: Steady state kinetic analysis of (A) Cys291Ser and (B) Cys294Ser *E. coli* NadA. Activity determinations were performed using 5 mM OAA and 100 mM NH₄Cl under anoxic conditions.


**Disulfide Bond Modification Assays.** In a separate series of experiments, activity determinations of WT NadA and the Cys291Ser and Cys294Ser variants were performed under various conditions (Table 3.3). When WT NadA was pre-exposed to oxygen to allow formation of the putative disulfide bond, the $V_{\text{max}}/[E_{\text{T}}]$ of the enzyme upon incubation in the presence of 5 mM DTT (1.6 min$^{-1}$) was 3.6-fold lower than that obtained in the absence of DTT (5.8 min$^{-1}$). By contrast, the Cys291Ser and Cys294Ser variants displayed opposite effects when treated similarly; their activities in the absence of DTT were lower, presumably because of their inability to form an intramolecular disulfide bond coupled with their increased instability (Table 3.3). Similar behavior was observed when NadA was prereduced by incubation with DTT and then subjected to gel–filtration under anaerobic conditions to remove the reductant. The activity of the protein was 2.3-fold greater when determined under oxic conditions (air) than when determined under anoxic conditions. Again, the Cys291Ser and Cys294Ser variants displayed opposite behavior; the activity of the Cys291Ser variant was slightly lower (3.3-fold) under oxic conditions as compared to anoxic conditions, while the activity of the Cys294Ser variant was significantly lower (13-fold).
Table 3.3: Activities of WT and variant *E. coli* NadA Proteins

<table>
<thead>
<tr>
<th>Condition</th>
<th><em>V</em>&lt;sub&gt;max&lt;/sub&gt;/<em>[E&lt;sub&gt;T&lt;/sub&gt;]</em> (min&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>NadA&lt;sub&gt;ox&lt;/sub&gt; plus DTT</td>
<td>1.6</td>
</tr>
<tr>
<td>NadA&lt;sub&gt;ox&lt;/sub&gt; minus DTT</td>
<td>5.8</td>
</tr>
<tr>
<td>NadA&lt;sub&gt;red&lt;/sub&gt;</td>
<td>2.3</td>
</tr>
<tr>
<td>NadA&lt;sub&gt;red&lt;/sub&gt; plus O&lt;sub&gt;2&lt;/sub&gt; (air)</td>
<td>5.3</td>
</tr>
<tr>
<td>NadA&lt;sub&gt;red&lt;/sub&gt; plus Trx&lt;sub&gt;ox&lt;/sub&gt;</td>
<td>12</td>
</tr>
<tr>
<td>NadA&lt;sub&gt;red&lt;/sub&gt; plus C35A Trx</td>
<td>3.5</td>
</tr>
<tr>
<td>NadA&lt;sub&gt;ox&lt;/sub&gt;</td>
<td>7.9</td>
</tr>
<tr>
<td>NadA&lt;sub&gt;ox&lt;/sub&gt; plus Trx&lt;sub&gt;red&lt;/sub&gt;</td>
<td>1.6</td>
</tr>
<tr>
<td>NadA&lt;sub&gt;ox&lt;/sub&gt; plus C35A Trx</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Because Trx is known to be involved in reversible disulfide bond reduction in the cytosol (25), its ability to regulate turnover of *E. coli* NadA was assessed (Table 3.3). When NadA<sub>red</sub> was added to an assay mixture containing excess Trx<sub>ox</sub>, the *V*<sub>max</sub>/*[E<sub>T</sub>]* of the enzyme was observed to increase 5.2-fold over that of prereduced enzyme treated similarly but added to an assay mixture lacking Trx<sub>ox</sub>. By contrast, the activities of the Cys291Ser and Cys294Ser variants remained relatively constant. Similarly, when WT NadA<sub>ox</sub> was added to an assay mixture containing Trx<sub>red</sub>, its activity decreased 4.9-fold. Again, the Cys291Ser and Cys294Ser variants responded differently; their activities increased slightly, presumably because of decreased lability of the cluster in the presence of a reductant. By contrast, the Cys35Ala Trx variant had little or no effect on the activity of WT NadA (Table 3.3), as did oxidized DTT, suggesting that the midpoint reduction potential of the Cys291/Cys294 disulfide bond is considerably more positive than −330 mV, the midpoint potential of DTT (26).
Control assays were performed to ensure that the observed rate increase in the presence of \( \text{Trx}_{\text{ox}} \) was not due to contaminating oxygen from the protein purification. In these controls, \( \text{Trx}_{\text{ox}} \) was incubated with the oxygen scrubbing system, consisting of glucose oxidase, D-glucose and catalase, and then reacted with \( \text{NadA} \) in an assay. Similarly, to show that the observed rate decrease in the presence of \( \text{Trx}_{\text{red}} \) was not due to contaminating TCEP, \( \text{Trx} \) was reduced with TCEP that is immobilized on agarose resin to allow for the separation of the \( \text{Trx} \) from TCEP–agarose by centrifugation. In both control assays, the observed \( \text{NadA} \) rate increase in the presence of \( \text{Trx}_{\text{ox}} \) and decrease in the presence of \( \text{Trx}_{\text{red}} \) was equal to those observed with \( \text{Trx}_{\text{ox}} \) or \( \text{Trx}_{\text{red}} \) in Table 3.3. The effect of glutathione—another common reagent used to reduce disulfide bonds—on \( \text{NadA} \) activity was also assessed. Because the oxidation–reduction potential of the GSH/GSSG redox couple \( (E_0 = 240 \text{ mV} \text{ (27)}) \) is more oxidizing than \( \text{Trx} \), we expected that incubation with GSSG would increase the activity of \( \text{NadA} \) in a similar manner. In the presence of GSSG, a 2.5-fold increase in the activity of \( \text{NadA} \) was observed, which is not as substantial as that observed in the presence of \( \text{Trx}_{\text{ox}} \) but comparable to that observed under oxic conditions. Surprisingly, activity also increased 3.4-fold in the presence of GSH as compared to that obtained in its absence. Neither GSH nor GSSG had any effect on the activity of the Cys291Ala or Cys294Ala variants. We have investigated possible interactions with the Fe/S cluster by EPR and Mössbauer spectroscopies and observed no changes to the spectra of WT \( \text{NadA} \) in the presence of GSH. The activity of \( \text{NadA} \) from \textit{Mycobacterium tuberculosis} is also redox regulated and evidence suggests it contains a disulfide bond with a more oxidizing midpoint reduction potential than the \textit{E. coli} enzyme (subject of Chapter 5 of this document). The addition of GSH to the \textit{M. tuberculosis} \( \text{NadA} \) has no effect on activity, while GSSG does increase activity approximately 2-fold. In addition, the \textit{Pyrococcus horikoshii} \( \text{NadA} \) lacks a disulfide bond and displays no activity change in the presence of GSH/GSSG. These results suggest that the effect of GSH is specific to \textit{E. coli} \( \text{NadA} \) disulfide–bond formation and is not due to changes to the [4Fe-4S] cluster, conserved in \textit{M. tuberculosis} and \textit{P. horikoshii} \( \text{NadA} \).
Crosslinking of the Cys35Ala Thioredoxin Variant with WT NadA. The reduction of disulfides by Trx takes place via a two-step process. The first step involves an intermolecular attack of Cys32, the more reactive cysteine, onto the disulfide bond, resulting in a mixed-disulfide intermediate. Cleavage of the mixed-disulfide occurs upon intramolecular attack of Cys35 onto Cys32 (28). The mixed-disulfide intermediate does not accumulate with E. coli Trx, because the second step of the reaction is significantly faster than the first. However, use of a Trx Cys35Ser or Cys35Ala variant allows the mixed-disulfide intermediate to be isolated in significant quantities (29). In Figure 3.5, a nonreducing SDS-PAGE analysis of the interaction of NadAox with WT and variant forms of E. coli Trx is displayed. NadAox migrates primarily as a monomer (molecular mass = 40 kDa), although a small amount (<10%) of dimer is observed (Figure 3.5, lane 2). Similar migratory properties are observed for the Cys291Ser and Cys294Ser variants (Figure 3.5, lanes 3 and 4). When WT NadAox is treated with the Trx Cys35Ala variant, a shift in the molecular mass of NadA is observed that is consistent with the addition of 12 kDa, the approximate molecular mass of E. coli Trx (Figure 3.5, lane 5). By contrast, intermolecular crosslinking is not observed with the NadA Cys291Ser and Cys294Ser variants (Figure 3.5, lanes 6 and 7). Figure 3.5 shows that the Trx dimer is generated under aerobic incubation via intermolecular disulfide formation, although most of the protein, which has exited the gel because of its small mass, is still monomeric. The appearance of small amounts of other bands at various molecular masses during aerobic incubation of NadAox with the Trx variant suggests that dimeric Trx may be cleaved by one or more of the nine Cys residues found in E. coli NadA.
Figure 3.5: Crosslinking of Trx Cys35Ala with NadA via disulfide–bond formation. Lanes: 1, molecular mass markers; 2, WT NadA; 3, NadA (C291S); 4, NadA (C294S); 5, WT NadA plus Trx (C35A); 6, NadA (C291S) plus Trx (C35A); 7, NadA (C294S) plus Trx (C35A); 8, Trx (C35A).

**NadA Midpoint Reduction Potential Determination.** To determine the midpoint potential of the Cys291/Cys294 disulfide–bond, the activity of WT NadA was assessed in the presence of varying ratios of oxidized to reduced Trx, using the known midpoint potential of Trx to calculate the midpoint reduction potential of the solution (Eₘ) at each [Trx]ox/[Trx]red combination (Figure 3.6). Observed rates versus Eₘ were plotted and fitted to a modified form of the Nernst equation (Eq 4), wherein k₁ and k₂ are kcat values for oxidized and reduced NadA, respectively; n is the number of electrons transferred (n = 2); E is the applied potential; E₀ is the standard potential of the redox couple; and Vmax[Eₗ] is the observed maximal velocity at a given applied potential (see
affording a midpoint potential ($E_0$) of $-264 \pm 1.8$ mV and rate constants $k_1$ and $k_2$ of $5.7 \pm 0.2$ and $0.9 \pm 0.2$, respectively.

$$V_{max} / [E_T] = k_1 (1 + 10^{\frac{n(E-E_0)}{0.059V}})^{-1} + k_2 (1 - (1 + 10^{\frac{n(E-E_0)}{0.059V}})^{-1})$$  \hspace{1cm} \text{Equation 4}

Figure 3.6: Midpoint potential determination of the disulfide bond in \textit{E. coli} NadA. The solid line is a fit to Eq 4, which assumes an obligate 2 e\textsuperscript{-} redox couple.
Electron Paramagnetic Resonance Spectroscopy Studies. The EPR spectra of the Cys291Ala and Cys294Ala variants, which are reduced in the presence of 2 mM sodium dithionite, indicates the presence of reduced $[4\text{Fe}-4\text{S}]^+$ cluster (Figure 3.7, blue and red spectra, respectively). The spectra, obtained at 13 K and 5 mW power, both show an axial signal with $g$-values of $g_1 = 2.05$ and $g_2 = 1.93$ for Cys291Ala and $g_1 = 2.06$ and $g_2 = 1.91$ for Cys294Ala. The observed $g$-values differ slightly from those of the WT ($g_1 = 2.05$ and $g_2 = 1.92$) (Figure 3.7, black spectra). The spectra also differ in intensity, with the spectrum of the Cys291Ala variant being the most intense, followed by those of WT NadA and the Cys294Ala variant in decreasing order.

Figure 3.7: EPR spectra of NadA in the presence of 2 mM sodium dithionite: WT (black), Cys291Ala (blue), Cys294Ala (red).
Experimental observations suggest that the observed increase in activity of WT NadA in the presence of Trx$_{\text{ox}}$ is due to oxidation of the NadA disulfide. The effect to the Fe/S cluster under these conditions was also investigated. WT NadA was first reduced to the [4Fe-4S]$^+$ cluster state in the presence of 2 mM sodium dithionite and then excess reductant was removed from the protein by gel–filtration. The NadA, pre-reduced with dithionite, was then incubated in the absence and presence of Trx$_{\text{ox}}$ prior to freezing the sample. The spectra, obtained at 13 K and 5 mW power, in the absence of Trx$_{\text{ox}}$ (Figure 3.8, black spectra) displays an axial signal with approximate $g$–values ($g_1 = 2.05$ and $g_2 = 1.93$) that are nearly identical to those previously observed for the [4Fe-4S]$^+$ cluster (1, 17). In addition to the [4Fe-4S]$^+$ cluster signal, another small feature is present ($g = 2.01$), which is stable at 80 K. This feature is most likely contaminating [2Fe-2S]$^+$ cluster, formed from the degradation of the [4Fe-4S]$^+$ cluster during the long incubation following reduction with sodium dithionite. In the presence of Trx$_{\text{ox}}$ the [2Fe-2S]$^+$ spectra disappears and a small increase in the intensity of the [4Fe-4S]$^+$ spectra is observed. This is possibly due to the protection of the [4Fe-4S]$^+$ cluster from degradation in the presence of the NadA disulfide bond.
3.5 Discussion

The biosynthesis and recycling of NAD$^+$ and its derivatives is a highly regulated process (3), and the intracellular redox state of *E. coli*, as reflected by the ratio of NADH to NAD$^+$, varies as a function of the environmental redox state of the organism (30). During shifts from aerobic to anaerobic growth in several facultative bacteria, the total intracellular NADH concentration has been shown to remain constant, while the intracellular concentration of NAD$^+$ decreases rapidly. It was suggested that the regulation of NAD$^+$ synthesis is directly or indirectly influenced by oxygen (31).
Under aerobic growth conditions, reactive oxygen species (ROS) are formed when electrons are transferred to oxygen, often from flavin enzymes in the respiratory chain (32). The exposure to ROS, in the form of superoxide anion (O$_2^{-}$•), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (HO••), can cause significant damage to proteins, nucleic acids and cell membranes. One potential target of these ROS is the [4Fe-4S] clusters of dehydratases, such as dihydroxyacid dehydratase, aconitase, and fumarase (33). In these enzymes, ROS destroy the Fe/S clusters, which results in loss of enzyme activity. Interestingly, one major source of H$_2$O$_2$ of $E. coli$ growing under oxic conditions is NadB (34). In the presence of ROS, cysteine residues are vulnerable to modification, either reversible or irreversible, which often results in altering of the protein function. These modifications include the oxidation to sulfenic acid (R-SOH), sulphinic (R-SO$_2$H), or sulphonic (R-SO$_3$H) acid, or the formation of S-thiolated proteins such as S-cysteinyalted or S-glutathionylated proteins (35). Protein sulfenic acids and S-thiolated proteins are reversible modifications and can be reduced back to the thiol form or further oxidized to form sulfenic and sulphinic acids.

Another reversible modification is the formation of disulfide bonds. Most sulphhydryl groups of cysteines have a pK$_a$ greater than 8 and therefore do not form disulfide bonds in the reducing environment of the cytoplasm. In contrast, cysteine residues of redox–sensitive proteins often have lower pK$_a$’s due to charge interactions with neighboring amino acids and exist instead as thiolate anions at neutral pH, which are more vulnerable to oxidation (36). The experiments described in these studies have demonstrated that following exposure to oxygen, $E. coli$ NadA forms a disulfide bond between two cysteine residues (Cys291 and Cys294) that reside in a CysXXCys motif. Disulfide–bond formation results in a change in NadA activity, which is up to 7.5-fold greater than that observed when Cys291 and Cys294 exist as sulfhydryls. When either cysteine is changed to serine or alanine, preventing disulfide bond formation, the variant protein is unstable in the presence of oxygen and enzyme activity is significantly decreased. This
behavior suggests that formation of the disulfide bond may also act to protect the Fe/S cluster from damaging oxidizing conditions.

The reducing cellular environment and thus the redox–state of protein cysteines are maintained by thiol reducing systems in the cytoplasm, such as Trxs, along with GSH/glutaredoxins (Grx) (37). The small tripeptide, GSH, is reduced by GSH oxidoreductase and NADPH, and is then able to reduce Grxs. While Grxs are capable of reducing protein disulfides, they are more efficient at reducing mixed disulfides of GSH. Trxs are part of the Trx system, in which electrons are transferred from NADPH to Trx reductase and subsequently Trx1. This system is best characterized in *E. coli*, in which the Trx1 has a midpoint potential of −270 mV (22), and is effective at maintaining protein disulfides in the reduced state in the *E. coli* cytosol (−280 mV) (38). Our studies show that the redox state of the *E. coli* NadA disulfide bond is regulated by Trx. Trx reduces protein disulfides through the oxidation of its own active site cysteines (Cys32 and Cys35), which reside in a CysXXCys motif. This reduction proceeds through a mixed disulfide intermediate between Cys32 of Trx and one cysteine of the target protein and is followed by cleavage of the mixed disulfide by intramolecular attack on Cys32 by Cys35 of Trx. By reacting a variant of the resolving cysteine (Cys35) of Trx with NadA<sub>ox</sub> we were able to observe this mixed disulfide intermediate by SDS-PAGE. The disulfide bond of *E. coli* NadA displays a midpoint potential (−264 mV) that is similar to that of *E. coli* Trx, which supports its physiological relevance in the regulation of NAD<sup>+</sup> biosynthesis and recycling.
3.6 References


Chapter 4

Active Site Modeling and Mutagenesis Studies of Quinolinate Synthase from *Pyrococcus horikoshii*

4.1 Abstract

Quinolinate synthase (NadA) catalyzes the condensation reaction between dihydroxyacetone phosphate (DHAP) and iminoaspartate (IA) to form quinolinic acid (QA), the central intermediate in the biosynthesis of nicotinamide adenine dinucleotide (NAD$^+$). Oxygen sensitivity of the enzyme and the presence of a CysXXCysXXCys motif in the primary structure led to the hypothesis that NadA contains an iron–sulfur (Fe/S) cluster (1, 2). Since then, the enzyme has been demonstrated to contain one [4Fe-4S] cluster that is required for activity (3, 4). A crystal structure was published in 2005 of NadA from *Pyrococcus horikoshii* that is lacking the essential Fe/S cluster (5). The protein displays pseudo 3-fold symmetry with the active site in the center. The three domains should be connected by loops, but these regions of the protein are missing electron density and therefore are not visible in the structure. Each of these loops contains or is adjacent to one of the conserved cysteines shown to ligate the Fe/S cluster of NadA from *Escherichia coli* (6, 7). We utilized this apo structure to model in the missing loops ligated to an Fe/S cluster in the active site along with a QA precursor in place of the malate bound in the structure. The active site of our model shows surprising similarities to the published structure of the enzyme IspH, involved in the isoprenoid biosynthesis pathway (8). This report describes our model and the identification of key conserved residues in the active site of NadA. Using site-directed mutagenesis, we made variants in *P. horikoshii* NadA of eight of the conserved residues in the active site positioned to interact with the QA precursor. Our results in conjunction with
those published for IspH allowed us to propose the role for conserved residues in the reaction to form QA.

4.2 Introduction

NAD$^+$ is an essential biological cofactor known primarily for its role in oxidation–reduction reactions, but is also involved in ADP ribosylation, adenylation and histone deacetylation (9). QA is the first common intermediate in the de novo biosynthesis pathway for formation of NAD$^+$ in all organisms. In most eukaryotes, QA is synthesized by five enzymes, three of which require molecular oxygen as a cosubstrate designating this the aerobic pathway (10). Most prokaryotes utilize an anaerobic pathway to form QA via the action of two enzymes that both function in the absence of oxygen. The first enzyme in this pathway, L-aspartate oxidase (NadB), catalyzes the oxidation of L-aspartate to IA. This is a two electron oxidation in which the electrons are transferred to the non-covalently bound flavin cofactor (11). IA is then condensed with DHAP by the second enzyme in the pathway, NadA, to form QA, inorganic phosphate (P$_i$) and 2 moles of water (12).

NadA from *E. coli* was initially proposed to contain an Fe/S cluster, due to its instability in the presence of molecular oxygen and the presence of a CysXXCysXXCys motif (1, 2), which commonly contains the cysteines that ligate a [4Fe-4S] cluster (13). It was later confirmed that this enzyme contains one [4Fe-4S] that is essential for catalysis (3, 4). Through further investigation of the cysteine variants of NadA, we have shown that the cluster is ligated by three cysteines that are conserved in all NadA proteins, only one of which lies in the CysXXCysXXCys motif (6). Interestingly, the other two cysteines in the CysXXCys motif form a redox–active disulfide bond, which regulates the rate of formation of QA (7, 14). Disulfide bond formation is regulated by thioredoxin (Trx) and the midpoint reduction potential of the NadA disulfide was determined to be $-264$ mV using the Trx redox couple (14). NadA is purported to be a member of the Fe/S–cluster dependent hydro–lyase class of enzymes (15). The prototype member of this class is aconitase, which contains one [4Fe-4S] cluster in which three irons are ligated by three
cysteine residues (16). The fourth iron is coordinated to water, or substrate/product via a bidentate coordination in the reaction. All the enzymes in the hydro-lyase class contain substrates with carboxyl and hydroxyl groups that are lost as water. In aconitase, both a carboxyl and hydroxyl bind to the unique iron of the Fe/S cluster. It is expected that NadA similarly utilizes its [4Fe-4S] cluster for direct involvement in the final dehydration step to form QA through coordination of the hydroxyl group to the Fe/S cluster (15). QA also contains two carboxyl groups, yet they are on the opposite side of the molecule from the hydroxyl group and therefore are not capable of binding simultaneously.

A crystal structure of NadA from *P. horikoshii* was solved in 2005 to a resolution of 2.0 Å (5). The 35-kDa enzyme exhibits a clover-leaf shaped structure with a pseudo 3-fold symmetry. Each of the three domains consists of a 4-stranded parallel β sheet with two α helices on each side. The structure lacks a Fe/S cluster, mostly likely because the enzyme was purified and crystallized in the presence of oxygen. There are three surface loops connecting each domain, corresponding to amino acids 79–90, 165–175, and 257–260, that were not visible in electron density maps. The authors also report significantly greater activity for the enzyme despite the fact that the Fe/S cluster was previously shown by two independent groups to be required for the *E. coli* enzyme (3, 4). A bioinformatic analysis of the amino acid sequence indicated that three cysteines are conserved in over 700 annotated NadA proteins (17). Two of the disordered loops contain two of the three conserved cysteines, Cys83 and Cys170. The third conserved cysteine, Cys256, is visible in the structure, but adjacent to one of the loops that is not visible.

Herein, we use the biochemical evidence of the requirement of the Fe/S cluster and the conserved cysteine residues ligating that cluster along with the proposed cluster-bound QA precursor to create a model of the active site of *P. horikoshii* NadA. This was carried out by overlaying the QA precursor onto the malate bound in the active site of the apo structure, positioning a [4Fe-4S] cluster within range to bind to that intermediate, and then building the missing loops containing the conserved cysteines that bind the Fe/S cluster. The modeled holo
structure shows striking similarity to the structure of IspH, a [4Fe-4S] cluster containing enzyme involved in isoprenoid biosynthesis (18, 19). The structure of the *Aquiflex aeolicus* IspH containing a [3Fe-4S] cluster but no bound substrate was solved in 2008 (20). More recently, in 2010, a structure of the *E. coli* enzyme containing an intact [4Fe-4S] cluster was solved with bound substrate, 1-hydroxy-2-methyl-(E)-butenyl-4-diphosphate (HMBPP) (8, 21). When NadA is overlaid with the latter IspH structure, the Fe/S cluster, two conserved histidine residues and one conserved glutamate residue align in the active site. Site-directed mutagenesis was used to create variants of these three residues, along with five other conserved residues in the NadA protein. Three variants, Tyr109Phe, Asn111Gln, and Glu198Gln, were found to be inactive for QA formation and release of inorganic phosphate. Additionally, we provide second evidence that glyceraldehyde-3-phosphate (G3P), a predicted intermediate in one proposed NadA mechanism (10), is a significantly poorer substrate than DHAP.

### 4.3 Materials and Methods

*Materials.* All DNA modifying enzymes and reagents, and Vent DNA Polymerase and its associated 10× ThermoPol reaction buffer were purchased from New England Biolabs (Beverly, MA). PfuUltra High Fidelity DNA Polymerase and its associated 10× reaction buffer were obtained from Stratagene (La Jolla, CA). Oligonucleotide primers for mutagenesis and cloning were obtained from Integrated DNA Technologies (Carlsbad, CA). *E. coli* strains BL21(DE3) and Rosetta 2(DE3) were obtained from Novagen (Madison, WI), as was vector pET-28a. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Biosynth International (Naperville, IL). Coomassie blue dye–binding reagent for protein concentration determination and the bovine serum albumin (BSA) standard (2 mg mL⁻¹) were obtained from Pierce (Rockford, IL). Nickel nitrilotriacetic acid (Ni-NTA) resin was purchased from Qiagen (Valencia, CA) while Talon metal affinity resin was purchased from Clontech (Mt. View, CA). Sephadex G-25 resin was purchased from GE Biosciences (Piscataway, NJ). 2,3-Pyridinedicarboxylic acid (quinolinic acid) was obtained from Aldrich (St. Louis, MO).
Dihydroxyacetone phosphate (dilithium salt), DL-glyceraldehyde 3-phosphate diethyl acetal barium salt, Dowex® 50× 4-200R resin, L-(+)-arabinose, ferric chloride, glyceraldehyde 3-phosphate dehydrogenase and α-glycerophosphate dehydrogenase were obtained from Sigma (St. Louis, MO). Malachite green oxalate was obtained from JT Baker. All other buffers and chemicals were of the highest grade available.

**General Procedures.** High performance liquid chromatography (HPLC) was conducted either on a Beckman System Gold unit (Fullerton, CA), which was fitted with a 128 diode–array detector and operated with the System Gold *Nouveau* software package, or on an Agilent (Foster City, CA) 1100 HPLC system, which included an autosampler and a variable wavelength detector. Data collection and analysis of samples run on the Agilent system were performed with the associated ChemStation software package. Iron and sulfide analyses were performed as previously described (22-24). Fast protein liquid chromatography (FPLC) was conducted on an ÄKTAFPLC™ system operated with the UNICORN™ software package (GE Biosciences). Sonic disruption of *E. coli* cells was carried out with a 550 sonic dismembrator from Fisher Scientific (Pittsburgh, PA) in combination with a horn containing a ½ in. tip. The horn was threaded through a port in an anaerobic chamber to allow the process to be conducted under anoxic conditions. The polymerase chain reaction (PCR) was performed with a Robocycler temperature cycler from Stratagene (La Jolla, CA). DNA sequencing was carried out at the Pennsylvania State University Genomics Core Facility (University Park, PA).

**Spectroscopic Methods.** UV–visible spectra were recorded on a Cary 50 spectrometer (Varian; Walnut Creek, CA) using the associated WinUV software package. Low–temperature X-band EPR spectroscopy was carried out in perpendicular mode on a Bruker (Billerica, MA) ESP 300 spectrometer equipped with an ER 041 MR microwave bridge and an ST4102 X-band resonator (Bruker). The sample temperature was maintained with an ITC503S temperature controller and an ESR900 liquid helium cryostat (Oxford Instruments; Concord, MA).
Structure Modeling of P. horikoshii NadA. Modeling of the active site was performed utilizing the published apo structure of P. horikoshii NadA (PDB ID: 1WZU) as a template (5). The QA precursor proposed to be bound to the Fe/S cluster in the last step of the reaction (Figure 4.1) was converted into PDB format using PRODRG (25). It was then aligned with the malate bound in the crystal structure, with both carboxylates overlayed and the nitrogen of the QA precursor overlayed with the hydroxyl of the malate. A [4Fe-4S] cluster was then modeled into the active site by positioning one iron atom within 2.3 Å from the hydroxyl of the QA precursor proposed to ligate to the Fe/S cluster. This distance was determined based on the distance measured between the iron and hydroxyl in the structure of aconitase, the model enzyme for NadA (26). The missing loops (amino acids 79–90, 165–175, and 257–260) were built into the structure using Swiss–PDB Viewer (27). The cysteine of each loop (Cys83, Cys170, and Cys256) was positioned approximately 2 Å from an iron atom of the Fe/S cluster. Using the cysteine and the amino acid immediately before or after the modeled loops as anchors, the Build Loop function was used to find the best loop conformation, determined by the least number of clashes (bad contacts or hydrogen bonds). Energy minimization of the entire structure including the Fe/S cluster and QA precursor was performed in Insight II (28). The resulting PDB file was viewed and figures were made using PyMOL (29).

Figure 4.1: Proposed binding of QA precursor to Fe/S cluster of NadA in the final dehydration reaction.
Construction of *P. horikoshii* NadA Variants. *P. horikoshii* NadA variants were constructed using the QuikChange II Site-directed Mutagenesis Kit (Stratagene) according to the manufacturer’s specifications, and as described previously (30), in which the cycling protocol is adapted for the Stratagene Robocycler thermocycler (31). Plasmid pET28-PhNadA was used as the template in conjunction with the appropriate primers for each respective amino acid substitution (Table 4.1). All mutations were verified by DNA-sequencing of the entire gene.
Table 4.1: Sequence of Primers used in Cloning and Mutagenesis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Purpose</th>
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<tbody>
<tr>
<td>PhNadAH21Q.for</td>
<td>5’- GCG ATA ATA CTG GCC CAA AAT TAT CAG CTT CCT GAA G-3’</td>
<td>Forward Primer for <em>P. horikoshii</em> H21Q NadA</td>
</tr>
<tr>
<td>PhNadAH21Q.rev</td>
<td>5’- C TTC AGG AAG CTG ATA ATT TTG GGC CAG TAT TAT CGC -3’</td>
<td>Reverse Primer for <em>P. horikoshii</em> H21Q NadA</td>
</tr>
<tr>
<td>PhNadAY109F.for</td>
<td>5’- GCT CCA GTT GTC TCT TTC GTG AAT AGT ACG GCT GAA GC -3’</td>
<td>Forward Primer for <em>P. horikoshii</em> Y109F NadA</td>
</tr>
<tr>
<td>PhNadAY109F.rev</td>
<td>5’- GC TTC AGC GTAT ACT ATT CAC GAA GAG AAC AAC TGG AGC -3’</td>
<td>Reversal Primer for <em>P. horikoshii</em> Y109F NadA</td>
</tr>
<tr>
<td>PhNadAN111Q.for</td>
<td>5’- GC TTC AGC GTT GTC TCT TAC GTG AAG AGC AGC GCT GAA GC -3’</td>
<td>Forward Primer for <em>P. horikoshii</em> N111Q NadA</td>
</tr>
<tr>
<td>PhNadAN111Q.rev</td>
<td>5’- GCA AAG CTA ATG ATC CAA CCT GAG TGC ATC CCA GAG G-3’</td>
<td>Reverse Primer for <em>P. horikoshii</em> N111Q NadA</td>
</tr>
<tr>
<td>PhNadAH196Q.for</td>
<td>5’- C TTC TGG GAT GCA CTC AGG TTG CAT TAG CTG TGC -3’</td>
<td>Forward Primer for <em>P. horikoshii</em> H196Q NadA</td>
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<tr>
<td>PhNadAH196Q.rev</td>
<td>5’- GCA AAG CTA ATG ATC CAT CCT CAG TGC ATC CCA GAG G-3’</td>
<td>Reversal Primer for <em>P. horikoshii</em> H196Q NadA</td>
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<tr>
<td>PhNadAE198Q.for</td>
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<td>PhNadAE198Q.rev</td>
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<td>EcNadAE228Q.for</td>
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<td>Forward Primer for <em>E. coli</em> E228Q NadA</td>
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<tr>
<td>EcNadAE228Q.rev</td>
<td>5’- GCC GAT TTT ATA AGG GAT GCC CTT GAA CTT GCA GAG AGG GC -3’</td>
<td>Reversal Primer for <em>E. coli</em> E228Q NadA</td>
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<td>PhNadAS38A.for</td>
<td>5’- GCC GAT TTT ATA AGG GAT GCC CTT GAA CTT GCA GAG AGG GC -3’</td>
<td>Forward Primer for <em>P. horikoshii</em> S38A NadA</td>
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<td>PhNadAY23F.for</td>
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<td>Forward Primer for <em>P. horikoshii</em> Y23F NadA</td>
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<tr>
<td>PhNadAY23F.rev</td>
<td>5’- GC CAT AAT ATG GCC CAT AAT TTT CAG CTT CCT GAA GTT CAA GAC ATT GC -3’</td>
<td>Reversal Primer for <em>P. horikoshii</em> Y23F NadA</td>
</tr>
<tr>
<td>PhNadAS126A.for</td>
<td>5’- GCC ACA GGC GTT CGT CGT CGG CGT AAC TGG AAC TGT AAC GTC AGC -3’</td>
<td>Forward Primer for <em>P. horikoshii</em> S126A NadA</td>
</tr>
<tr>
<td>PhNadAS126A.rev</td>
<td>5’- CG CAT AAT ATG GCC CAT AAT TTT CAG CTT CCT GAA GTT CAA GAC ATT GC -3’</td>
<td>Reversal Primer for <em>P. horikoshii</em> S126A NadA</td>
</tr>
<tr>
<td>PhNadB.Ndel.for</td>
<td>5’- CGG CCG CGT CTC CAT ATG ATG GAG ATG AGG GTT GGT ATC G G3’</td>
<td>Forward Primer for <em>P. horikoshii</em> NadB cloning</td>
</tr>
<tr>
<td>PhNadB.EcoRI.rev</td>
<td>5’- CGG CCG CGT CTC CAT ATG ATG GAG ATG AGG GTT GGT ATC G G3’</td>
<td>Reversal Primer for <em>P. horikoshii</em> NadB cloning</td>
</tr>
</tbody>
</table>

Bolded bases represent amino acid changes.

a Underlined bases represent *NdeI* restriction site.

b Underlined bases represent *EcoRI* restriction site.

Expression of the Genes Encoding Wild Type *P. horikoshii* NadA and Variants. Plasmid pET28-PhNadA, encoding wild type (WT) or variant NadA proteins, was cotransformed into *E. coli* Rosetta 2(DE3) with plasmid pDB1282 as described previously (32). A single colony was selected and used to inoculate 200 mL of Luria–Bertani (LB) media containing 50 µg/mL kanamycin, 100 µg/mL ampicillin, and 34 µg/mL chloramphenicol, and was cultured overnight at
37 °C with shaking (250 rpm). A 60 mL portion of the culture was then evenly distributed among four 6 L Erlenmeyer flasks to inoculate 16 L of LB media containing 50 µg/mL kanamycin, 100 µg/mL ampicillin, and 34 µg/mL chloramphenicol, and the bacteria were cultured further at 37 °C with shaking. At an optical density at 600 nm (OD$_{600}$) of 0.3, solid L-(+)-arabinose was added to each flask at a final concentration of 0.2 % (w/v). At an OD$_{600}$ of 0.6, solutions of IPTG and ferric chloride were added to each flask at final concentrations of 400 and 50 µM, respectively. The cultures were then allowed to incubate further at 37 °C with shaking for 4 h. Cells were harvested by centrifugation at 10,000 × g for 10 min at 4 °C, and the resulting cell paste was frozen in liquid N$_2$ and stored at − 80 °C until ready for use. Typical yields were 20-25 g of frozen cell paste per 16 L of culture.

**Purification of P. horikoshii NadA Proteins.** All steps of the purification were conducted inside of an anaerobic chamber from Coy Laboratory Products, Inc. (Grass Lake, MI) under an atmosphere of N$_2$ and H$_2$ (95%/5%) with an O$_2$ concentration maintained below 1 ppm by the use of palladium catalysts. Steps involving centrifugation and heat–treatment were performed outside of the anaerobic chamber in centrifuge tubes that were tightly sealed before removal from the chamber. All buffers were prepared using distilled and deionized water that was boiled for at least 1 h and then allowed to cool with stirring uncapped in the anaerobic chamber for 48 h. All plastic ware was autoclaved and brought into the chamber hot, and allowed to equilibrate overnight before use.

Protein purification was carried out by immobilized metal affinity chromatography (IMAC) using a nickel-nitrilotriacetic acid (Ni-NTA) matrix. In a typical purification, 20 g of frozen cells were resuspended in 80 mL of buffer A (50 mM HEPES, pH 7.5, 0.3 M KCl, 20 mM imidazole, and 10 mM 2-mercaptoethanol). Solid egg white lysozyme was added to a final concentration of 1 mg mL$^{-1}$ and the mixture was stirred at room temperature for 30 min before incubating further for 15 to 60 min at 37 °C. After the mixture was allowed to cool in an ice–
water bath to <8 °C, it was subjected to four 1 min bursts of sonic disruption (setting 7). Following sonication, a sodium sulfate solution was added to a final concentration of 0.2 M, and the suspension of cells was heated at 85 °C for 15 min. Cellular debris was removed by centrifugation at 50,000 × g for 1 h, and the resulting supernatant was loaded onto a Ni-NTA column (2.5 × 7 cm) equilibrated in buffer A. The column was washed with 100 mL of buffer B (50 mM HEPES, pH 7.5, 0.3 M KCl, 40 mM imidazole, 10 mM 2-mercaptoethanol, and 20% glycerol), and subsequently eluted with buffer B containing 250 mM imidazole. Fractions that were brown in color were pooled and concentrated in an Amicon stirred cell (Millipore, Billerica, MA) fitted with a YM-10 membrane (10,000 Da MW cutoff). The protein was exchanged into buffer C (50 mM HEPES, pH 7.5, 0.1 M KCl, 10 mM DTT and 20% glycerol) by anaerobic gel filtration (Sephadex G-25), concentrated, and stored in aliquots in a liquid N₂ dewar until ready for use. All buffers and purification steps were performed at ambient temperature.

**Cloning of P. horikoshii NadB.** The nadB gene was amplified from *P. horikoshii* (JCM 9974) genomic DNA by PCR technology using primers PhNadB.NdeI.for and PhNadB.EcoRI.rev (Table 4.1). Each amplification reaction contained the following in a volume of 50 µL: 0.4 µM of each primer, 0.25 mM of each deoxynucleoside triphosphate, 200 ng of *P. horikoshii* genomic DNA, 2 U of Vent DNA Polymerase, and 5 µL of 10× ThermoPol reaction buffer. The reaction mixture was overlaid with 40 µL of mineral oil. After a 9 min denaturation step at 95 °C, 35 cycles of the following program were initiated: 1 min at 95 °C, 1 min at 55 °C, 2.5 min at 72 °C. Following the cycling program, the reaction was incubated further for 10 min at 72 °C. The PCR product was digested with *Nde*I and *Eco*RI and then ligated into similarly digested pET-28a by standard methods. The correct construct was verified by DNA–sequencing and designated pET28-PhNadB.

**Expression and Purification of P. horikoshii NadB.** Plasmid pET28-NadB was transformed into *E. coli* Rosetta 2(DE3). A single colony was used to inoculate 100 mL of TB
media containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol, and was cultured overnight at 37 °C with shaking. A 60 mL portion of the culture was evenly distributed among four 6 L Erlenmeyer flasks to inoculate 16 L of TB media containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol, and was cultured further at 37 °C. At an OD₆₀₀ of 0.6, IPTG and riboflavin were added to each flask to final concentrations of 400 and 50 µM, respectively. The cultures were then incubated at 37 °C with shaking for 3 h. Cells were harvested at 10,000 × g for 10 min at 4 °C, and the resulting frozen cell paste was frozen in liquid N₂ and stored at −80 °C until ready for use. Typical yields of frozen cell paste were 20-25 g per 16 L growth.

In a typical purification, 25 g of frozen cells were resuspended in 80 mL of buffer D (50 mM HEPES, pH 7.5, 0.3 M NaCl, 20 mM imidazole, 10 mM succinate, pH 8, and 20 µM FAD). Solid egg white lysozyme and phenylmethanesulfonyl fluoride (PMSF) were added to a final concentrations of 1 mg mL⁻¹ and 1 mM, respectively, and the mixture was stirred at room temperature for 30 min, followed by a 15 min incubation at 37 °C. After the mixture was allowed to cool in an ice–water bath to <8 °C, it was subjected to four 1 min bursts of sonic disruption (setting 7). Following sonication, a sodium sulfate solution was added to a final concentration of 0.2 M, and the suspension of cells was heated at 85 °C for 60 min. Cellular debris was removed by centrifugation at 50,000 × g for 1 h, and the resulting supernatant was loaded onto a Talon cobalt affinity column (2.5 × 7 cm) equilibrated in buffer D. The column was washed with 200 ml of buffer D and subsequently eluted with buffer E (50 mM HEPES, pH 7.5, 0.3 M NaCl, 250 mM imidazole, 10 mM succinate, pH 8, 20 µM FAD, and 20% glycerol). Protein–containing fractions were pooled and concentrated in an Amicon stirred cell fitted with a YM-10 membrane (10,000 Da MW cutoff), and stored in aliquots in a liquid N₂ dewar until ready for use.

**Determination of Protein Concentrations.** Protein concentrations were determined by the Bradford dye staining procedure with BSA as the standard (33). Quantitative amino acid analysis on parallel samples of *P. horikoshii* NadA, conducted at the University of Iowa Molecular
Analysis Facility, determined that the Bradford method overestimates its concentration by a factor of 1.54.

**Activity Determinations.** The activity of WT and variant NadA proteins was determined by monitoring the formation of QA over a 20 min time period at 50 °C under anaerobic conditions. The substrate IA was generated enzymatically via the NadB reaction using fumarate as the electron acceptor, or chemically by inclusion of OAA and ammonium sulfate in assay mixtures \((34)\). Activity determinations conducted in the presence of NadB contained the following in a final volume of 1300 µL: 200 mM HEPES, pH 7.5, 25 mM L-aspartate/fumarate solution (titrated to pH 7), 25 µM FAD, 1 mM DHAP, 0.1 M KCl, 0.3 mM L-tryptophan (internal standard), and 50 µM NadA and 5 µM NadB. When IA was generated chemically, the aspartate/fumarate solution, FAD, and NadB were replaced with 30 mM OAA and 100 mM ammonium chloride. The reactions were initiated by addition of DHAP after incubation of the other components of the assay mixture at 50 °C for 3 min. At designated times, 200 µL aliquots of the assay mixture were removed and added to 40 µL of 2 M trichloroacetic acid (TCA) to quench the reaction. The precipitated protein was pelleted by centrifugation, and the supernatant was analyzed by HPLC with UV detection (268 nm) as previously published \((6)\) and as described in Materials and Methods section of Chapter 2 of this document.

**Assays with Glyceraldehyde-3-Phosphate.** To determine if G3P is an alternative substrate, assays were performed as described above except using 50 µM NadA, 5 µM NadB and DHAP was replaced with 1 mM G3P. The assay was performed at 37 °C, rather than 50 °C to ensure that G3P was not degrading at the increased temperature, and was quenched at designated time points and analyzed for QA.

**Detection of Triosephosphate Isomerase Contamination.** Both *P. horikoshii* NadA and NadB were analyzed for triosephosphate isomerase contamination by assaying for the conversion of G3P to DHAP utilizing α-glycerophosphate dehydrogenase. In this assay, the α-
glycerophosphate dehydrogenase catalyzes the reduction of DHAP to form α-glycerophosphate, which is coupled to NADH oxidation to NAD⁺ that is monitored by UV–visible spectroscopy. NadA and NadB were incubated under assay conditions in the presence of 100 mM HEPES, pH 7.5 and 1 mM G3P. The sample was then transferred to a cuvette to which 0.25 mM NADH was added and the absorbance at 340 nm was measured. α-Glycerophosphate dehydrogenase (5.6 U) was added to the cuvette, and the resulting decrease in absorbance at 340 nm (ε = 6440 M⁻¹ cm⁻¹) was recorded and then used to calculate the concentration of DHAP present.

**Malachite Green Assay for Inorganic Phosphate.** Activity determinations were also performed by quantifying the amount of inorganic phosphate formed as a function of time monitoring complex formation of malachite green with phosphomolybdate as previously described (35). To make the stock solution of malachite green, 60 mL of concentrated sulfuric acid was slowly added to 300 mL of water and stirred until cooled to RT. After cooling, 0.44 g of malachite green oxalate was added. On the day of the assay, fresh dye solution was mixed by adding 2.5 mL 7.5 % ammonium molybdate to 10 mL of the malachite green solution, followed by the addition of 0.2 mL 11 % Tween 20. Assay were performed as described above; however, 50 µL aliquots of the acid quenched assay mixture were added to cuvettes containing 200 µL of the dye solution in a 1 mL final volume. The samples were allowed to incubate for 10 min at RT and the absorbance at 630 nm was measured. The concentration of P₁ was determined by the molar extinction coefficient for the malachite green phosphomolybdate complex (ε₆₃₀ nm = 90,000 M⁻¹ cm⁻¹) (35).

**Preparation of EPR Samples.** Samples to be analyzed by EPR were prepared as follows. WT and variant *P. horikoshii* NadA samples (300 µM; 250 µL) were treated with 10 mM sodium dithionite at room temperature for 5 min, placed in EPR tubes (2 mm i.d.), and frozen in isopentane chilled in liquid N₂. Samples prepared under turnover conditions were reduced as described above and then mixed with 0.5 M ammonium chloride, 30 mM DHAP and 30 mM
OAA prior to freezing in EPR tubes. WT and Glu228Gln variant *E. coli* NadA samples were prepared similarly; however, they were reduced with 2 mM sodium dithionite and substrate concentrations were changed to 0.1 M ammonium chloride and 10 mM OAA. The buffer concentration in the *E. coli* samples was increased to 200 mM (final concentration) as compared to 50 mM in the *P. horikoshii* NadA samples, due to the instability of the *E. coli* NadA protein in the presence of high concentrations of OAA.

**Anaerobic Screening for Crystallography.** WT *P. horikoshii* NadA was prepared for crystal screening by reconstituting the Fe/S cluster following the reconstitution procedure as previously described (7), except the reconstitution was maintained at RT throughout the procedure. Following the concentration of the reconstituted (RCN) protein, it was loaded onto a prepacked HiPrep 16/60 Sephacryl S-200 HR column equilibrated in 50 mM HEPES, pH 7.5, 0.1 M KCl, 10 mM DTT and 10% glycerol, fitted on an FPLC system housed in an anaerobic glove box. The protein was chromatographed by size, and fractions containing NadA, determined by the absorbance monitored at 280 and 410 nm, were pooled and further concentrated. The protein was then treated with thrombin overnight at RT to cleave the N–terminal hexahistidine tag using the Novagen® Thrombin Cleavage Capture Kit. The protein was then further concentrated (to a final concentration of approximately 30 mg/mL) and diluted in water to a final concentration of 10 mg/mL in the presence of 10 mM malate (pH 5.5). The mixture was applied to a 0.22 µm sterile filter (Millipore Ultrafree®-MC centrifugal filter units) and incubated at RT in the anaerobic chamber overnight. The mixture was screened for suitable crystallization conditions using the Qiagen® Classics suite of protein crystallization conditions by hanging drop vapor diffusion method in the anaerobic chamber at RT. The optimal condition for crystallization of the *P. horikoshii* NadA protein was 0.2 M ammonium acetate, 0.1 M sodium acetate, pH 4.6, 30% PEG 4000.
4.4 Results

Structure Modeling of P. horikoshii NadA Active Site. Since the determination of the X-ray crystal structure of NadA from P. horikoshii in 2005 (5), there has been published evidence that conflicts with the findings of that paper. The publication reports significantly greater activity for the enzyme used for crystallography, despite the fact that the structure lacks the essential Fe/S cluster that has previously been characterized and determined to be required for activity in E. coli NadA (3, 4). To ensure that this difference was not attributable to enzymes from two different organisms, previous work in our laboratory showed that NadA from P. horikoshii does indeed have a [4Fe-4S]^{2+} cluster and that the cluster is required for QA formation (6). The published structure also lacks three surface loops that are disordered and not visible in the X-ray structure. Each of these loops contains or is directly adjacent to one of three cysteines conserved in all NadA sequences (Cys83, Cys170, and Cys256 in P. horikoshii NadA), which have been shown to ligate the essential Fe/S cluster in NadA from E. coli and B. subtilis (6, 7, 36). Because the evidence is clear that P. horikoshii NadA contains an Fe/S cluster that is ligated by the three conserved cysteines, the Fe/S cluster and the missing loops that ligate three of its iron atoms were modeled into the published apo structure (Figure 4.2). In place of the malate bound in the apo structure, the QA precursor postulated to form prior to the final dehydration step was positioned in the active site (Figure 4.1). Because the proposed role of the Fe/S cluster is to bind to the hydroxyl group of this QA precursor, the open iron coordination site of the Fe/S cluster was positioned 2.3 Å from the hydroxyl group. Energy minimization of the entire structure resulted in our model structure (Figure 4.2), allowing for identification of possible key conserved active site residues that may be involved in acid/base catalysis during the reaction (Figure 4.3). The eight variants identified and described in this study were His21Glu, Tyr23Phe, Ser38Ala, Tyr109Phe, Asn111Gln, Ser126Ala, His196Gln, and Glu198Gln.
Figure 4.2: Modeled Holo–NadA structure from *P. horikoshii* with modeled loops shown in magenta, conserved cysteine Fe/S cluster ligands in yellow, key conserved active site residues in green, [4Fe-4S] cluster in orange and yellow, and the QA precursor in cyan.
Recent studies on the enzyme IspH, the last enzyme in the nonmevalonate pathway for isoprenoid biosynthesis, along with the X-ray crystal structure of the enzyme with the [4Fe-4S] cluster bound, provide potential insight into the catalytic mechanism of NadA. The X-ray crystal structure of IspH displays a clover–like structure of three similar domains with a central essential [4Fe-4S] cluster (Figure 4.4 B) (8). The active site contains conserved residues His41, His124, and Glu126 in *E. coli*, which are in a similar sequence context to conserved residues (His21, His196, and Glu198) in *E. coli* NadA. An overlay of the two structures using these three residues as anchors shows that they and the Fe/S clusters of the two enzymes align almost perfectly (Figure 4.4 C). Substitution of these residues in IspH by site–directed mutagenesis results in very low activity for the Glu126Ala variant, ~ 2 % the activity of the WT, and lower activity than WT for the His42Ala and His124Ala variants, 20 and 4 % the activity of the WT, respectively (37).
Spectroscopic studies performed by the same group showed the formation of a reaction intermediate with the Glu126Ala variant that perturbs the EPR spectra of the Fe/S cluster (37). We observe similar behavior of the corresponding the variants of *P. horikoshii* NadA; the His21Gln and His196Gln variants have lower than WT activity, 4 and 13 % respectively, the Glu198Gln variant is completely inactive, and we observe a perturbation of the EPR spectra of the Glu198Gln variant in the presence of both substrates, possibly due to the formation of a reaction intermediate (see below). In addition to the striking similarities in the structures and conserved active site residues of these two enzymes also have similar function chemically. Both bind to substrates containing phosphate moieties, the substrate for IspH, HMBPP, contains pyrophosphate while one of the substrates for NadA, DHAP, contains a phosphate group. IspH catalyzes the $2 \text{H}^+/2\text{e}^-$ reduction of HMBPP to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which includes the removal of a hydroxyl group from HMBPP, which is positioned in the active site for protonation by Glu126 to form water. NadA also catalyzes two dehydration reactions in the formation of QA and also has the conserved Glu198 residue positioned in the active site to act to protonate the hydroxyl.
Figure 4.4: (A) Modeled *P. horikoshii* NadA structure, (B) solved IspH structure from *E. coli*, (C) overlay of active sites of NadA (green) and IspH (cyan), (D) NadA active site with QA precursor bound, and (E) IspH active site with substrate HMBPP bound.

Also in the active site of IspH are the conserved residues Ser225 and Asn227 in a Ser–X–Asn loop which has been shown to hydrogen bond to the diphosphate of the ligand and variants of either residue results in inactive protein (21). Our modeled structure of NadA contains a conserved Tyr109 and Asn111 in a similar sequence context and both residues are essential for catalysis (see below). These two regions of the enzymes do not overlap, but are instead on opposite sides of the active sites. Yet, based on the positioning of the QA precursor in our model,
we predict that the phosphate of DHAP would be positioned to interact with the tyrosine and asparagine residues.

**Characterization of WT and Variant P. horikoshii NadA Proteins.** WT and variant *P. horikoshii* NadA proteins were produced in the presence of the gene products from the plasmid pDB1282 as described previously (6, 38). Because the protein is from a thermophile, after performing cell lysis by sonication, the crude lysate was transferred to sealed centrifuge tubes, removed from the anaerobic glove box and subjected to a heat treatment for 15 min at 85 °C to denature native *E. coli* proteins. After centrifugation, the supernatant contains NadA that is ≥ 90% pure, and the majority of the *E. coli* proteins are present in the pellet (Figure 4.5, lanes 2 & 3, respectively). The supernatant is loaded onto Ni-NTA resin, which is then washed and eluted (Figure 4.5, lanes 4-6). All variants behaved similar to WT during the purification and were isolated in similar yields (~200 mg protein from ~15 g cell paste generated from 12 L media). The WT protein is isolated with 1.4 irons and 3.0 sulfides per protein, yet unlike NadA from *E. coli*, the cluster content and activity does not significantly increase after chemical reconstitution of *P. horikoshii* NadA at RT. Given that this organism is a hyperthermophile and has an optimal growth temperature of 98 °C (39), it is possible that the enzyme requires increased temperatures to chemically reconstitute the Fe/S cluster. The variants were isolated with varying amounts of iron and sulfide (Table 4.2).
Figure 4.5: Coomassie blue stained SDS-PAGE analysis of purification of the *P. horikoshii* NadA Tyr23Phe variant. Lanes: 1, molecular weight markers (kDa); 2, crude lysate supernatant; 3, crude lysate pellet; 4, Ni-NTA load eluate; 5, Ni-NTA wash eluate; 6, purified and concentrated protein.
Table 4.2: Properties of *P. horikoshii* Wild Type and Variant NadA Proteins

<table>
<thead>
<tr>
<th>sample</th>
<th>iron per polypeptide</th>
<th>sulfide per polypeptide</th>
<th>$V_{\text{max}}/[E_T]$ (min$^{-1}$)</th>
<th>$K_m$ DHAP (mM$^{-1}$)</th>
<th>$V_{\text{max}}/[E_T] K_m$ (mM$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.86 ± 0.28</td>
<td>2.95 ± 0.10</td>
<td>3.98</td>
<td>0.24</td>
<td>16.58</td>
</tr>
<tr>
<td>His21Gln</td>
<td>2.50 ± 0.20</td>
<td>3.69 ± 0.34</td>
<td>0.17</td>
<td>0.08</td>
<td>2.21</td>
</tr>
<tr>
<td>Tyr23Phe</td>
<td>2.59 ± 0.82</td>
<td>3.79 ± 0.47</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Ser38Ala</td>
<td>2.38 ± 0.26</td>
<td>4.06 ± 0.45</td>
<td>1.99</td>
<td>0.14</td>
<td>14.21</td>
</tr>
<tr>
<td>Tyr109Phe</td>
<td>2.26 ± 0.38</td>
<td>3.53 ± 0.36</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Asn111Gln</td>
<td>1.10 ± 0.05</td>
<td>3.01 ± 0.17</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ser126Ala</td>
<td>2.81 ± 0.52</td>
<td>4.35 ± 0.56</td>
<td>0.32</td>
<td>0.03</td>
<td>10.67</td>
</tr>
<tr>
<td>His196Gln</td>
<td>3.08 ± 0.07</td>
<td>3.80 ± 0.46</td>
<td>0.51</td>
<td>0.08</td>
<td>6.38</td>
</tr>
<tr>
<td>Glu198Gln</td>
<td>2.31 ± 0.05</td>
<td>3.40 ± 0.21</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$NA, not applicable, activity too low to determine values accurately.

$^b$ND, activity not detected. Reported errors reflect one standard deviation.

Cloning and Expression of the *P. horikoshii* NadB gene and Purification of Its Product.

The *nadB* gene from *P. horikoshii* was previously cloned and characterized (40); yet for ease of purification, the gene was cloned into a pET-28a expression vector, which allowed for production of a construct with an N–terminal hexahistidine–tag for rapid purification. The protein was purified similarly to NadA, except all steps were performed outside of the anaerobic glove box. Due to low levels of expression, yields of protein obtained during purification were significantly lower than those for NadA (~ 20 mg of protein from ~ 27 g of cells generated from 12 L media) (Figure 4.6). Also, all attempts to exchange the protein into anaerobic buffer by gel filtration chromatography caused precipitation of the protein. Therefore it was maintained in aerobic Ni-NTA elution buffer E. The UV–visible spectrum of the isolated protein displayed the typical absorbance of a flavin–bound enzyme, exhibiting maxima at 376 and 452 nm (Figure 4.7). The protein also was competent to form the substrate for NadA, IA, when incubated with L-aspartate.
Figure 4.6: Coomassie blue stained SDS-PAGE analysis of purification of *P. horikoshii* NadB.

Lanes: 1, molecular weight markers (kDa); 2, crude lysate supernatant; 3, crude lysate pellet; 4, Ni-NTA load eluate; 5, Ni-NTA wash eluate; 6, purified and concentrated protein.
Figure 4.7: UV–visible spectrum of purified *P. horikoshii* NadB

*Activity of WT and Variant *P. horikoshii* NadA Proteins.* To assess the ability of *P. horikoshii* NadA variants to catalyze turnover, the concentration of enzyme was increased to 50 µM. No activity was detected after 30 min for the Tyr109Phe, Asn111Gln, and Glu198Gln variants (limit of detection 5 µM QA). The two histidine variants, His21Gln and His196Gln, and the two serine variants, Ser38Ala and Ser126Ala, were capable of forming QA, and therefore assays were performed to determine $K_m$ values for DHAP (Table 4.2). In these assays, IA was formed chemically using 15 mM OAA in the presence of ammonia chloride, and DHAP concentrations were varied from 50 µM to 5 mM. The concentration of WT NadA and the Ser38Ala variant was 2.5 µM; yet, due to the lower activities of the other variants, their concentrations were increased to 15 µM (His21Gln and Ser126Ala) or 10 µM (His196Gln). Attempts were made to perform the same analysis with the Tyr23Phe variant, yet the activity was too low to obtain an accurate $K_m$ value for DHAP (turnover number 0.02 min$^{-1}$). The rate of
turnover for WT enzyme normalized to enzyme concentration, $V_{\text{max}}/[E_T]$, is 3.98 min$^{-1}$. Interestingly, the Ser38Ala variant displays a normalized rate that is only 2-fold lower than that of the WT enzyme despite the fact that Ser38 is an absolutely conserved residue. The other three variants all display rates that are at least 8-fold lower than WT (Table 4.2). The $K_m$ value for DHAP for the WT enzyme is 0.24 mM, while all variants display lower values. The $V_{\text{max}}/[E_T] \cdot K_m$ value for the two serine variants is similar to WT, while the two histidine variants display values that are 2.6 and 7.5 lower (His21Gln and His196Gln, respectively).

*Activity of WT P. horikoshii NadA with Glyceraldehyde-3-Phosphate.* Because the tautomerization of DHAP to G3P is the first step in one of the proposed mechanisms of NadA (10), we investigated whether G3P could replace DHAP as a substrate for NadA. Previous studies showed that G3P did act as a substrate for NadA, yet the reaction was inhibited by chloroacetol phosphate, a known inhibitor of triosephosphate isomerase (41). It was concluded that the activity observed was due to contaminating triosephosphate isomerase, which converted the G3P to DHAP. However, it is possible that G3P is a substrate for NadA, but that the NadA reaction is inhibited by chloroacetol phosphate. To probe deeper into substrate preference of NadA, assays were repeated in the presence of G3P but with NadA and NadB that were not contaminated with triosephosphate isomerase.

The purification of NadA from *P. horikoshii* includes heat treatment, normally 15 min at 85 °C, to allow for the precipitation of contaminating proteins from *E. coli* before loading the crude extract (~ 90 % pure as determined by SDS-PAGE, Figure 4.4) onto affinity resin. Because of the thermostability of NadA, the heat treatment was extended to 60 min, which thoroughly inactivates *E. coli* triosephosphate isomerase. In a typical assay, IA can either be produced chemically, using OAA and an ammonia source, or enzymatically, using NadB and L-aspartate. The chemically generated IA is formed by reaction of the ammonia nitrogen with the carbonyl of OAA via Schiff’s base. Assays were unable to be performed with G3P as the substrate using the chemically generated IA, since the ammonia can also react via Schiff’s base with the aldehyde of
G3P. Therefore, the IA must be generated enzymatically for these experiments, using the \textit{P. horikoshii} NadB which was heat treated as described for NadA to prevent triosephosphate isomerase contamination.

The QA formed in assays performed with G3P as the substrate was below the limit of detection (5 µM) until the 10 min time point and supported a turnover number that was 12-fold lower than that with DHAP as the substrate (Figure 4.8). There was no QA detected with G3P under normal assay conditions (5 µM NadA); activity was only observed with 50 µM NadA. In addition, when G3P was used as a substrate in an assay containing triosephosphate isomerase, the activity observed was equal to that with DHAP as the substrate. The small amount of QA formed may be due to remaining contaminating triosephosphate isomerase or slow turnover of G3P by NadA. Contaminating triosephosphate isomerase was tested for by reacting NadA, NadB and G3P and then detecting DHAP formed using the spectrophotometric assay with \textit{α}-glycerophosphate dehydrogenase and NADH. In these assays, 42 µM DHAP was detected in the presence of 50 µM NadA, 5 µM NadB and 1 mM G3P. Yet, 31 µM DHAP was detected in the presence of 50 µM NadA in the absence of G3P, suggesting that the DHAP may be bound to the enzyme and not a product of the reaction of triosephosphate isomerase with G3P. To test this, NadA was assayed for QA formation in the presence of the IA substrate and the absence of DHAP or G3P. It was observed that ~ 30% of the NadA enzyme isolated contains DHAP bound. This does not account for the 60 - 80 % DHAP observed in the spectrophotometric assays, yet these two experiments were performed with different enzyme preparations and therefore the amount of DHAP bound may vary.
Figure 4.8: Formation of QA by WT *P. horikoshii* NadA using DHAP (closed circles), G3P (open circles), and G3P + addition of triose phosphate isomerase to convert G3P to DHAP (open squares).

Quantification of Inorganic Phosphate Produced by WT and Variant *P. horikoshii* NadA Proteins. In addition to QA, P<sub>i</sub> is formed as a product of the reaction. Although Tyr109Phe, Asn111Glu, and Glu198Gln NadA variants were inactive when assayed for QA, depending on their role in the reaction, they may still catalyze release of P<sub>i</sub>. A spectrophotometric assay was therefore used to monitor release of P<sub>i</sub> upon its complexation with malachite green in the presence of molybdate, which absorbs at 630 nm. The linear range of the assay was 1 to 20 μM P<sub>i</sub>. Therefore enzyme concentration in the assay was increased to 50 μM to ensure detection of substoichiometric amounts.

DHAP is moderately unstable, and preparations of it contain small amounts of P<sub>i</sub>, which increase slightly with time during the assay. Therefore, control assays lacking NadA were
routinely performed to give a background rate of Pi release (Figure 4.9, open circles). The WT enzyme produced P_i, as expected, turning over almost all of the substrate DHAP (500 µM) in 20 min (Figure 4.9, closed circles). The two histidine variants that were capable of forming QA also produced P_i, yet at lower levels than the WT (Figure 4.9, His21Gln (closed squares), His196Gln (closed triangles)). With respect to P_i release, the His196Gln enzyme was more active than the His21Gln variant, which was also true with QA formation by these variants. These results show that our P_i assay is accurate, and that the rate of phosphate release correlates with the rate of QA formation. The three variants determined to be inactive for QA production, produced P_i at levels equal to that of the background control (Figure 4.9, Tyr109Phe (open squares), Asn111Gln (open triangles), Glu198Gln (open diamonds)). These assays were repeated with increased concentrations of the inactive variants (200 µM) to ensure that a single turnover would be detected and the same results were obtained. These data show that these three variants are unable to catalyze formation of QA or P_i. To confirm these results, a separate continuous enzymatic assay (EnzChek® Phosphate Assay Kit, Molecular Probes) was also used to monitor phosphate formation; the same rates were observed for the active variants and no P_i was detected for the inactive variants.
Our finding that the three variants inactive for QA formation, Tyr109Phe, Asn111Gln, and Glu198Gln, are also unable to produce \( P_i \) led us to investigate other active site residues in our model. When we compared the active site of NadA to that of aconitase, the model enzyme in the dehydratase class, it became apparent that like aconitase, NadA also had a conserved serine residue positioned to interact with the proton hypothesized to be abstracted during the dehydration step. In aconitase, Ser642 has been proposed to act as the general base in the reaction based on its positioning, along with the potential hydrogen bonding network from the nearby
Arg644, which would stabilize a serine alkoxide (26). In NadA, there are two serine residues, Ser38 and Ser126, on either side of the QA precursor in the active site, closest to the proton on the QA precursor that is removed in the proposed last step of the reaction (Figure 4.3). These two residues, especially Ser38 due to its closer proximity to the substrate (2.5 Å), were likely candidates to act as the general base in the reaction. Yet it was determined that both of the variants of these two residues are capable of forming QA. Both variants were assayed for P$_i$ formation with 200 µM enzyme and 1 mM DHAP and produced P$_i$ at similar rates to those for QA (Figure 4.10 A & C). It is possible that in the absence of one serine, the other may serve as the functional general base in the reaction. To test this, we plan on investigating the double variant in which both serines are changed to alanine.

Tyr23 is on the opposite side of the active site, closest to the nitrogen from IA (Figure 4.3). The QA precursor was modeled in the active site by aligning with malate bound in the apo structure by the two carboxylates of each molecule. Given the possibility that the chosen positioning could be incorrect and the QA precursor may instead bind a 180° flipped conformation, we investigated the Tyr23Phe variant as the possible general base in the reaction. This variant was capable of producing QA, albeit at a rate that was 200-fold lower than that of WT. Analysis for P$_i$ release showed that it also produces P$_i$ at the same rate as QA (Figure 4.10 B).
Figure 4.10: Time dependent formation of QA (closed circles) determined by HPLC and $P_i$ (open circles) determined spectrophotometrically by complex with malachite green for (A) Ser38Ala, (B) Tyr23Phe, and (C) Ser126Ala, each with 200 µM enzyme.

*Spectroscopic Analysis of WT and Variant P. horikoshii NadA Proteins.* The EPR spectrum of WT *P. horikoshii* NadA reduced in the presence of 10 mM sodium dithionite displays a weaker signal than observed with the enzyme from other organisms (Figure 4.11 A), yet with similar $g$–values of 2.04 and 1.94 in addition to a third $g$–value of 2.11 (6). The EPR
spectrum of the Asn111Gln variant signal is weak and has a different shape than that of WT (Figure 4.11 D). The other four variants all show more intense signals with slightly different features, with the two active histidine variants the most intense. The addition of both substrates to the samples causes their spectral intensities to decrease almost to baseline for all except the Glu198Gln variant. The Glu198Gln spectra under turnover conditions decreases in intensity, narrows significantly and loses one of the features observed without substrates present (g = 2.11) (Figure 4.12 A). Interestingly, the overlay of the Glu198Gln spectrum with substrates (Figure 4.12 B, dashed line) aligns very closely with the His196Gln spectrum without substrates (Figure 4.12 B, solid line), with different signal intensities. This suggests that the presence of substrates with this inactive variant, Glu198Gln, changes the Fe/S cluster environment to one that is more like that of the active variant, His196Gln; however, the Glu198Gln variant was unable to produce QA or P, as determined by our assays.
Figure 4.11: EPR spectroscopy of WT and variant *P. horikoshii* NadA proteins: (A) WT; (B) His21Gln; (C) Tyr109Phe; (D) Asn111Gln; (E) His196Gln; (F) Glu198Gln.
Figure 4.12: EPR spectra of (A) Glu198Gln only (solid line) and Glu198Gln in the presence of substrates (dashed line); (B) His196Gln only (solid line) and Glu198Gln in the presence of substrates (dashed line).
**Spectroscopic Analysis of E. coli Glu228Gln NadA Protein.** Because the Glu198Gln variant from *P. horikoshii* NadA displayed a spectral change under turnover conditions, we made the corresponding variant in *E. coli* (Glu228Gln) and performed similar studies. The EPR spectrum of the dithionite reduced variant displays a typical [4Fe-4S]^+ spectrum with a similar shape and $g$–values as the WT protein (Figure 4.13, black trace). The spectrum is rhombic; however the binding of IA sharpens the spectrum significantly (Figure 4.13, red trace). The same effect is observed in the presence of both substrates, DHAP and IA (Figure 4.13, green trace). The spectra in the presence and absence of DHAP are essentially identical suggesting only the binding of IA causes the conformational change to the protein that affects the Fe/S cluster or the *E. coli* enzyme may also purify with DHAP already bound as was observed with the *P. horikoshii* enzyme (Figure 4.13, blue trace). Similar results were observed with IA binding to both the Glu198Gln *P. horikoshii* NadA variant and WT *E. coli* NadA.

![Graph](image)

**Figure 4.13**: EPR spectra of *E. coli* NadA Glu228Gln in the absence of substrate (black), in the presence of DHAP (blue), IA (red), and both DHAP and IA (green).
Anaerobic Crystal Trials of NadA. In an attempt to obtain a crystal structure of NadA with the intact Fe/S cluster, NadA from *E. coli*, *M. tuberculosis*, and *P. horikoshii* were all screened for crystallization in the anaerobic chamber in the presence of the substrate mimic, malate. We identified conditions that allowed for the crystallization of protein from *P. horikoshii*, while we were unable to do so with the NadA from the other two organisms. Yet we were only able to obtain crystal with the protein treated with thrombin to cleave the N-terminal hexahistidine tag, which was not performed with NadA from the other two organisms. Attempts were made to crystallize the *P. horikoshii* NadA in the presence of either QA and DHAP were unsuccessful. Crystals obtained in the presence of malate are displayed in Figure 4.14. The crystals were sent to our collaborators (Dr. Amy C. Rosenzweig at Northwestern University) to collect X-ray diffraction data.
Figure 4.14: Protein crystals of *P. horikoshii* NadA in the presence of malate (top), single crystal rod, 0.30 mm (length) x 0.01 mm (width) (bottom).
4.5 Discussion

The cofactor requirement of NadA has been the subject of numerous studies in the literature (3, 4, 36, 42). Our laboratory, along with the Fontecave laboratory, first provided evidence that *E. coli* NadA contains one [4Fe-4S]\(^{2+}\) cluster per polypeptide that is required for activity (3, 4, 43). Following those studies, a report of an X-ray crystal structure of active *P. horikoshii* NadA lacking an Fe/S cluster surfaced (5). The protein was purified and crystallized in the presence of oxygen, conditions that are unfavorable for maintaining an intact Fe/S cluster. The resulting structure in complex with the substrate mimic malate was solved to 2.0 Å. The protein displays a pseudo 3-fold symmetry of three domains of αβα sandwich motifs. The domains are connected by three loops that lack electron density and are therefore not visible in the published structure. Each one of the loops contains, or is directly adjacent to one of the cysteine residues conserved in all NadA sequences and proposed to ligate the Fe/S. The active site is at the center of the three domains and is where malate is bound. The authors made attempts to soak diffraction quality crystals with DHAP, but the crystals quickly deteriorated. The observation that the DHAP disturbed the crystal quality in addition to the fact that the authors were unable to model both substrates into the active site due to limited space to accommodate the phosphate moiety of DHAP, suggests that there may be a significant conformation change to the enzyme upon DHAP binding. We have initiated our own crystallographic studies with this enzyme in the anaerobic chamber to obtain a structure with the intact Fe/S cluster. We have managed to grow small crystals in the presence of malate, but not in the presence of DHAP, supporting the observations by Sakuraba *et al* (5).

Since the publication of the apo NadA structure, the cluster–ligating cysteines have been identified and it has been demonstrated that mutagenesis of any one of those three cysteines results in inactive protein (6, 7). Our laboratory has also produced apo–protein by overproducing the protein in *E. coli* in the presence of the iron–chelating agent o-phenanthroline. The cluster–less NadA proteins from *E. coli* and *P. horikoshii* are unable to form QA (6). Other laboratories
have also characterized the Fe/S clusters of NadA from Arabidopsis thaliana (42) and Bacillus subtilis (36) and also identified the three conserved cysteines as ligands to the cluster in the B. subtilis enzyme.

In these studies, we modeled the [4Fe-4S] cluster in the active site of the published apo NadA structure using the malate bound in the crystal structure and the cysteines that ligate the Fe/S cluster. NadA is a purported member of the Fe/S cluster containing hydro–lyase class of enzymes, with the prototype member aconitase (15). NadA and aconitase both contain one [4Fe-4S]$^{2+}$ cluster required for activity and the cluster is ligated by three cysteines on the protein. It is expected that the role of the Fe/S cluster of NadA is similar to that of aconitase and is directly involved in the reaction. Based on this assumption, we modeled a QA precursor in the active site in place of the malate bound in the structure. The hydroxyl group of this precursor is proposed to bind to the Fe/S cluster in the last step of the reaction, a dehydration of the QA precursor to form QA. In our model, we positioned a [4Fe-4S] cluster within 2 Å of the hydroxyl group of the QA precursor and then built the missing loops from the apo structure by ligating the conserved cysteines to the Fe/S cluster. The resulting structure was energy minimized and then used to identify key conserved active-site residues that may be involved in the reaction, with special emphasis on the general acid/base involved in this last dehydration step.

Based on the modeled structure, eight conserved amino acids in the active site were identified and changed by site-directed mutagenesis, and the resulting variant proteins were characterized for their abilities to catalyze formation of QA and $P_i$. All the variants isolated displayed evidence for the presence of the essential Fe/S cluster: brown color, a distinct UV–visible feature at 400 nm, and iron and sulfide content. When assayed for QA formation, three of the variants (Tyr109Phe, Asn111Gln, and Glu198Gln) were inactive, while the other five (Tyr23Phe, His21Phe, Ser38Ala, Ser126Ala, and His196Glu) were capable of forming QA at varying rates.
There exist two proposed mechanisms in the literature and one unpublished by our laboratory (Figure 1.16, p. 33). In the first mechanism, proposed by Gholson, the first step of the reaction is the nucleophilic attack by C3 of the enamine form of IA on C3 of DHAP via an S\(_{N2}\) type reaction, resulting in P\(_i\) release. If the mechanism proceeds with P\(_i\) release in the first step, we expected to identify at least one active site variant that is able to form P\(_i\), but not QA. To test this, the three inactive variants were probed for P\(_i\) release using a colorimetric assay to quantify a phosphomolybdate complex formed from P\(_i\) and malachite green in the presence of molybdate (35). To our surprise, all of the variants that were inactive for QA formation were also inactive for P\(_i\) formation. We also quantified P\(_i\) formation catalyzed by the five active variants and found that it correlated almost exactly with QA formation.

The activity results suggest that the reaction may proceed by an alternate mechanism in which P\(_i\) is released in a latter step. A second mechanism, proposed by Begley and coworkers, is distinctly different and begins with isomerization of DHAP to G3P. The enamine form of IA then forms a Schiff–base with the C3 carbonyl of G3P, followed by the loss of P\(_i\). Early studies demonstrated that activity observed with G3P as the substrate in place of DHAP was due to contaminating triosephosphate isomerase in their enzyme preparations, which catalyzes the conversion of G3P to DHAP (41). This contamination with triosephosphate isomerase was shown by an observed decrease in activity in the presence of chloroacetol phosphate, an inhibitor of triosephosphate isomerase. We repeated these studies using NadA and NadB from the hyperthermophilic archaea \textit{P. horikoshii} to allow for the denaturing of native \textit{E. coli} enzymes by heat treatment. We observed very low NadA activity with G3P as the substrate, which may be due to remaining contaminating triosephosphate isomerase. Future studies using an inhibitor of triosephosphate isomerase, such as glycidol phosphate (44), may provide confirmation that this observed activity is due to such contamination. These studies demonstrate that DHAP is preferred over G3P, suggesting that the reaction mechanism either does not proceed through a G3P intermediate or the protonation state of the active site residues are not correct to facilitate
catalysis with G3P as the substrate. The third mechanism proposed by our laboratory also begins with the isomerization of DHAP to G3P followed by P_i release via an E1cb elimination, affording an α,β-unsaturated aldehyde Michael acceptor. Our data suggests that this mechanism is not likely, because it postulates a G3P intermediate and early release of P_i.

While reviewing the literature, we found striking similarities in the overall fold and active site of the solved X-ray crystal structure of IspH and our model structure of NadA, suggesting that these two enzymes are related by divergent evolution. IspH contains a [4Fe-4S] cluster that is bound to the substrate HMBPP. This enzyme catalyzes the reductive dehydration of HMBPP to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), two central intermediates in the biosynthesis of isoprenoids (18, 19). X-ray crystal structures of IspH from Aquifex aeolicus (20) and E. coli (8, 21) display three domains of pseudo-C_3 symmetry with conserved amino acids lining the active site at the center of the protein. The IspH structure from E. coli was solved with protein crystallized in the presence of the substrate, HMBPP, under anaerobic conditions to preserve the intact [4Fe-4S] cluster (8). When the structure of IspH and our model structure of NadA are overlaid, the Fe/S cluster, two histidine residues and one glutamate residue directly align in the active sites. Variants of the two His residues in IspH display a $V_{\text{max}}$ that is slightly lower than that for WT, while a variant of the Glu residue displays a $V_{\text{max}}$ that is significantly lower (37). Additionally, there is an observed change in the EPR spectrum of the Glu variant upon substrate binding. This Glu residue is proposed to be the general acid that donates a proton in the dehydration step of the reaction. Also in the active site of IspH, there is a conserved serine–X–asparagine loop, in which X is a single nonconserved amino acid, which has been shown to H–bond to the PP_i of the ligand. Variants of either residue are inactive.

Our studies of the corresponding NadA variants show that they display similar properties to their counterparts in IspH. This allows us to propose functions for some amino acids in the active site of our NadA model structure. The Glu198Gln variant of NadA is inactive, but displays significantly altered EPR spectra upon substrate binding. This residue is positioned 2.7 Å from
the hydroxyl group that is removed in the dehydration reaction, and may donate a proton to the departing hydroxyl group in the last dehydration step (Figure 4.15). The two histidine variants of NadA are active, but have a lower $V_{\text{max}}$ than the WT enzyme. In our model, they are positioned near the nitrogen on either side of the QA precursor and may play a role in Schiff–base formation. The other two essential residues, Tyr109 and Asn111, are positioned above the hydroxyl group of the QA precursor, and may form hydrogen bonds to the P$_i$ of the substrate DHAP. These Tyr–X–Asn residues are not spatially located near the Ser–X–Asn loop of IspH due to the positioning of the different substrates bound to the Fe/S cluster, but both are positioned to interact with the phosphate moieties of their respective substrates.
Figure 4.15: Modeled active site of \textit{P. horikoshii} NadA with QA precursor, Fe/S cluster, cysteine cluster ligands (blue), and conserved amino acids (red - variants result in inactive proteins, black - variants result in active protein).

The remaining three conserved residues described in this study, Tyr23, Ser38 and Ser126, do not have corresponding amino acids in IspH, but may provide important mechanistic information. Based on the close positioning to the proton that is removed in the second dehydration reaction, we expected that Ser38 may play a role as the general base in the final step of the reaction (Figure 4.15). Due to their high $pK_a$ value (~13), serines are typically not found as general bases in enzyme catalysis, yet serine is positioned similarly in the aconitase structure and is proposed to act as a general base in that reaction (45). We were surprised to find that the
variant of this residue displayed activity that was similar to that of WT enzyme and that the same rates were observed for P$_i$ and QA formation. The Ser126 is positioned on the opposite side of the active site from Ser38, more distant from the proton abstracted, but still investigated because of the possibility that the positioning of the QA precursor in the active site is not correct. The variant of this residue was also active, with a 12-fold lower rate than WT, and also displayed equal rates for P$_i$ and QA formation. Finally, the Tyr23 residue is positioned near the nitrogen of the QA precursor, on the opposite side of the active site from the proton that is abstracted. Because the intermediate was positioned in the active site by aligning the carboxylates of the two compounds and the nitrogen of the QA precursor to the hydroxyl group of malate, it is possible that the QA may actually bind 180 ° flipped configuration from our proposed model. In this arrangement, Tyr23 would be a likely candidate for the general base in the last step of the reaction. Interestingly, the variant of this residue had the lowest activity of all the variants, but produced P$_i$ and QA at similar rates.

We have characterized variants of eight conserved amino acids positioned to interact with the QA precursor in the active site of our NadA model. There are only four other conserved amino acids in the active site (Asp37, Thr126, Ser212 and Thr213) that we did not study, because they are positioned to interact with the carboxylates of the QA precursor (and malate in the apo structure). Of the eight that we did characterize, we were unable to identify a variant that is capable of forming P$_i$ but not QA, to allow for build–up of the modeled QA precursor or any other intermediate. Our studies have provided suggested roles for some of these residues for future studies. We propose that the Glu198 residue acts to protonate water in the final dehydration step, a similar function to the glutamate in the active site of IspH. The Glu198Gln is unable to form QA or P$_i$, possibly because this residue may be required in an early step of the mechanism, prior to P$_i$ release, or because all steps are readily reversible until the final dehydration reaction to give product. The other two residues found to be completely inactive when altered, Tyr109 and Asn111, we propose to interact with the P$_i$ of the substrate, similar to the serine and asparagine in
IspH. The changing of one of these residues may inhibit DHAP binding, or may be critical for phosphate release.

The mechanism of the NadA reaction is difficult to dissect because almost all intermediates in the proposed mechanisms are subject to hydrolysis until QA is formed. One method to observe these intermediates is by trapping with sodium borohydride and then analyzing the quenched samples by liquid chromatography combined with electrospray mass spectrometry (LC-MS). Using this technique, we hope to identify the intermediate from Gholson’s mechanism with a carbon–carbon bond and no phosphate, or that from Begley’s mechanism with a carbon–nitrogen bond and the phosphate group still present. We were unable to identify a variant that catalyzes release of P, but not formation of QA to probe for intermediates, yet we did find five variants displaying varying activities, all of which are less than WT, which can be probed for a burst of P release under pre-steady state conditions. In the event that the first step of the reaction is rate–limiting and no intermediates are observed, these variants may be critical for these studies.

Spectroscopic studies showed that there is a change to the Fe/S cluster environment upon binding of IA. This change is not observed in the presence of only DHAP. The proposed mechanism that our model is based on suggests that DHAP binds closest to the Fe/S cluster in the active site. The mode of substrate binding to the Fe/S cluster will be probed using electron double nuclear resonance (ENDOR) spectroscopy with specifically labeled substrates. Using this technique, we hope to observe an interaction between the nuclear spin of the labeled substrate and the electron spin of the reduced Fe/S cluster using both the WT and Glu228Gln E. coli NadA proteins.
4.6 References


28. Computational results obtained using software programs from Molecular Simulations Inc.-dynamics calculations were done with the Discover® program, using the CFF91 forcefield, ab initio calculations were done with the DMol program, and graphical displays were printed out from the Cerius² molecular modeling system.


Chapter 5

Redox Regulation of *Mycobacterium tuberculosis* NadA

5.1 Abstract

Quinolinic acid (QA) is the common intermediate in the biosynthesis of nicotinamide adenine dinucleotide (NAD⁺) in all organisms that synthesize the cofactor *de novo*. In most eukaryotes, QA is synthesized via the degradation of L-tryptophan by a series of enzymes, three of which require molecular oxygen as a cosubstrate (1). In contrast, the prokaryotic pathway for QA biosynthesis can function in the absence of oxygen by the action of two enzymes. The first enzyme in this pathway is L-aspartate oxidase (NadB), which converts L-aspartate to iminoaspartate (IA) using its flavin cofactor (2). Quinolinate synthase (NadA) then condenses IA with dihydroxyacetone phosphate (DHAP) affording QA, inorganic phosphate (Pᵢ) and two moles of water (3). Recent studies have focused mainly on NadA from *Escherichia coli*, characterization of the essential [4Fe-4S] cluster and identification of the protein ligands to the cluster (4-7). It was also discovered that the activity of this enzyme is regulated by a redox–active disulfide bond formed between two cysteines that exist in a CysXXCys motif (6, 8). The studies described herein have shifted focus on NadA from the lethal infectious bacterium, *Mycobacterium tuberculosis* (Mtβ), which has previously been shown by our laboratory also to contain an essential [4Fe-4S] cluster (7). We now demonstrate that like the *E. coli* enzyme, the activity of Mtβ NadA is also redox–regulated by a disulfide bond. This enzyme does not contain a CysXXCys motif in its primary structure, but instead has CysXCys and CysCys motifs, which have both been shown to form disulfide bonds in other enzymes. Our studies demonstrate that the two cysteines in the CysXCys motif, Cys230 and Cys232, are involved in forming the disulfide
bond responsible for increasing the activity of Mtb NadA under oxidizing conditions. We also hypothesize that the midpoint reduction potential of the Mtb NadA disulfide bond is more oxidizing than that of \textit{E. coli}, because it requires a more oxidizing disulfide bond isomerase, such as DsbA, to observe the activity increase.

5.2 Introduction

NAD$^+$ is an essential cosubstrate involved in numerous oxidation/reduction reactions in biology. NAD$^+$ is also a substrate in a number of other reactions, including ADP ribosylation, prokaryotic DNA ligation, and histone deacetylation (9, 10). In these reactions, NAD$^+$ is consumed, and pools need to be restored in the cell either through salvage or \textit{de novo} biosynthesis. The \textit{de novo} biosynthesis of NAD$^+$ differs in eukaryotes and prokaryotes, heightening the medicinal interest of the enzymes involved in these pathways, specifically that of the lethal infectious bacterium, Mtb. It was originally believed that Mtb had a nonfunctional recycling pathway, but was later shown to actually prefer to acquire nicotinamide exogenously from its host to produce NAD$^+$ utilizing a nicotinamidase (encoded by \textit{pncA}) (11). This nicotinamidase, which converts nicotinamide into nicotinic acid, has been implicated in the hydrolysis of pyrazinamide, a nicotinamide analog that is a component of the Mtb treatment (12). Mutations in \textit{pncA} cause the resistance of \textit{Mycobacterium bovis} to this drug, and therefore it is ideal to target both the \textit{de novo} and salvage pathways for NAD$^+$ biosynthesis in these bacteria (13).

The common intermediate in the prokaryotic and eukaryotic NAD$^+$ biosynthesis pathways is QA, which synthesized in most prokaryotes by the enzyme NadA. NadA from \textit{E. coli} has served as the prototype for studies of this enzyme, and numerous studies have focused on the characterization of the essential [4Fe-4S] cluster and its coordination to conserved cysteine residues (4-7). Our laboratory has also cloned genes encoding NadAs from Mtb and \textit{Pyrococcus horikoshii}, and isolated and characterized the corresponding enzymes, demonstrating that they also contain an essential [4Fe-4S] cluster (7). We have also demonstrated that \textit{E. coli} NadA
contains a redox–active disulfide bond that is regulated by the protein disulfide isomerase thioredoxin and has a midpoint potential of $-264 \pm 1.8$ mV (8). The two cysteines that form the disulfide bond reside in a CysXXCys motif, and an increase in formation of QA is observed when the protein is in the disulfide–bonded form.

NadA from Mtb contains ten total cysteines, but none are in a CysXXCys motif as is found in the E. coli enzyme. Instead this enzyme contains a CysXCys motif, which has been shown to form a disulfide bond in the YphP enzyme from Bacillus subtilis (14). There also exists two adjacent cysteine residues, a CC motif, which are capable of forming a disulfide bond in synthetic peptides and some enzymes (15). For both of these motifs, the typically observed midpoint reduction potentials for disulfide–bond formation are considerably higher than that observed for the CysXXCys motif in E. coli NadA. In the studies described herein, we characterized the activity of Mtb NadA under various oxidizing and reducing conditions. We found that Mtb NadA activity is also redox regulated by a disulfide bond, but it is not oxidized by thioredoxin ($E_o = -270$ mV (16)). However, the disulfide–bond could be reduced by reduced thioredoxin, suggested that its midpoint reduction potential is higher than that of Trx. DsbA, which catalyzes disulfide–bond formation in the E. coli periplasm and has an oxidizing midpoint reduction potential of $-120$ mV (17), could catalyze disulfide–bond formation on Mtb NadA. Moreover, similarly to that observed for E. coli NadA, disulfide–bond formation was accompanied by an increase (12-fold) in the activity of the enzyme. Five Cys→Ala variants of Mtb NadA were also characterized. Variants of Cys87, Cys155, and Cys314 all responded similarly to that of WT enzyme in the presence of DsbA with an observed increase in activity. By contrast, the activity of the variants of either residue in the CysXCys motif, Cys230 or Cys232, did not increase significantly upon incubation with DsbA, suggesting that these two cysteines are those involved in disulfide–bond formation.
Recent *in vivo* studies performed in the Fontecave laboratory have investigated the essentialness of specific cysteinyl residues of *E. coli* NadA by testing for the ability of corresponding variants to complement a ΔnadA *E. coli* strain (6). This strain is unable to synthesize NAD⁺ via the *de novo* pathway and therefore cannot grow on minimal media unless supplemented with nicotinic acid to form NAD⁺ via the salvage pathway. When the ΔnadA strain is transformed with a plasmid encoding WT *E. coli* NadA, QA biosynthesis is restored and the strain is able to grow on minimal media. The iron–sulfur (Fe/S) cluster of NadA is required for activity and substitution of any of the three conserved cysteines (Cys113, Cys200, Cys297) involved in ligating the cluster results in complete loss of *in vitro* activity (6, 7). Similarly, these variants are inactive *in vivo* and do not support the growth of the ΔnadA strain. Interestingly, the variants of the two cysteines that undergo reversible disulfide–bond formation (Cys291 and Cys294) are also unable to support growth of the ΔnadA strain, although the variants display turnover rates comparable or greater than WT *in vitro*. We repeated these studies and found that one variant (Cys294Ala) was able to complement the ΔnadA strain when the plasmid encoding the NadA variant was over–produced and the cells were grown in the absence of molecular oxygen. The other variant (Cys291Ala) did not grow under these conditions, most likely due to the lower observed activity of this enzyme *in vitro* compared to the Cys294Ala variant.

### 5.3 Materials and Methods

**Materials.** All DNA modifying enzymes and reagents, and Vent DNA Polymerase and its associated 10× ThermoPol reaction buffer were purchased from New England Biolabs (Beverly, MA). PfuUltra High Fidelity DNA Polymerase and its associated 10× reaction buffer were obtained from Stratagene (La Jolla, CA). Oligonucleotide primers for mutagenesis and cloning were obtained from Integrated DNA Technologies (Carlsbad, CA). *Escherichia coli* strains BL21(DE3), vectors pET-26b, pET-28a, pACYC and pCOLA were obtained from Novagen (Madison, WI). *E. coli* genomic DNA (strain W3110) was obtained from Sigma Corp
(St. Louis, MO), and *Mycobacterium tuberculosis* genomic DNA was a gift of Dr. Peter Tonge (SUNY Stoney Brook). *E. coli* JW011 *nadA* deletion strain was obtained from the Keio collection (Japan), *E. coli* MG1655, P1vir phage, pCP20 and pTrc99a were gifts of Dr. Sarah Ades (Pennsylvania State University). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Biosynth International (Naperville, IL). Coomassie blue dye–binding reagent for protein concentration determination and the bovine serum albumin (BSA) standard (2 mg mL⁻¹) were obtained from Pierce (Rockford, IL). Nickel nitrilotriacetic acid (Ni-NTA) resin was purchased from Qiagen (Valencia, CA) Whatman DE-52 resin was purchased through VWR International (West Chester, PA), while Sephadex G-25 resin, HiPrep 16/60 Sephacryl S-200 HR (1 x 120 mL) pre-packed column and NAP pre-poured gel filtration columns were purchased from GE Biosciences (Piscataway, NJ). 2,3-Pyridinedicarboxylic acid (quinolinic acid) was obtained from Aldrich (St. Louis, MO). L-(+)-Arabinose, dihydroxyacetone phosphate (dilithium salt), ferric chloride, glutathione, and trans-4,5-dihydroxy-1,2-dithiane were obtained from Sigma (St. Louis, MO). All other buffers and chemicals were of the highest grade available.

**General Procedures.** High performance liquid chromatography (HPLC) was conducted on a Beckman System Gold unit (Fullerton, CA), which was fitted with a 128 diode array detector and operated with the System Gold *Nouveau* software package. Fast protein liquid chromatography (FPLC) was conducted on an ÄKTAfplc™ system operated with the UNICORN™ software package (GE Biosciences). Sonic disruption of *E. coli* cells was carried out with a 550 sonic dismembrator from Fisher Scientific (Pittsburgh, PA) in combination with a horn containing a ½ in. tip. The horn was threaded through a port in an anaerobic chamber to allow the process to be conducted under anoxic conditions. The polymerase chain reaction (PCR) was performed with a Robocycler temperature cycler from Stratagene (La Jolla, CA). DNA sequencing was carried out at the Pennsylvania State University Genomics Core Facility (University Park, PA).
Expression of the *M. tuberculosis* nadA Gene and Purification of the Gene Product. The
*Mtb* nadA gene was codon–optimized and synthesized by GeneArt (Regensberg, Germany), and
the product was digested with *Nde*I and *Eco*RI and ligated into similarly digested pET-28a by
standard methods (18). This plasmid, designated pET28a MtbNadAopt was cotransformed into *E.
coli* BL21(DE3) with plasmid pDB1282 as described previously (19). A single colony was
selected and used to inoculate 200 mL of Luria–Bertani (LB) media containing 50 µg/mL
kanamycin and 100 µg/mL ampicillin, and was cultured overnight at 37 °C with shaking (180
rpm). A 20 mL portion of this culture was then evenly distributed among four 6 L Erlenmeyer
flasks to inoculate 16L of M9 minimal media (18) containing 50 µg/mL nicotinic acid, pH 7.0, 50
µg/mL kanamycin and 100 µg/mL ampicillin and the bacteria were cultured further at 37 °C with
shaking. At an optical density at 600 nm (OD_{600}) of 0.3, solid L-(+)-arabinose was added to each
flask at a final concentration of 0.05% (w/v). At an OD_{600} of 0.6, the cultures were cooled in an
ice–water bath, and IPTG and ferric chloride were added to each flask at final concentrations of
200 and 50 µM, respectively. The cultures were then allowed to incubate further at 18 °C with
shaking for 16 h. Cells were harvested by centrifugation at 10,000 × g for 10 min at 4 °C, and the
resulting cell paste was frozen and stored in liquid N\(_2\) until ready for use. Typical yields were 40-
50 g of frozen cell paste per 16 L of culture. Purification was performed as previously described
(7).

Reconstitution of *M. tuberculosis* NadA. Reconstitution of Mtb NadA was performed as
previously described (7), with this one exception. After concentrating the reconstituted (RCN)
protein, it was loaded onto a prepacked HiPrep 16/60 Sephacryl S-200 HR column equilibrated in
50 mM HEPES, pH 7.5, 100 mM KCl, 10 mM dithithreitol (DTT) and 10% glycerol fitted on an
FPLC system housed in an anaerobic glove box. The protein was chromatographed by size, and
fractions containing NadA, determined by the absorbance monitored at 280 and 410 nm, were
pooled and further concentrated prior to freezing in liquid N\(_2\) for storage.
Construction of the *M. tuberculosis* NadA Variants. *M. tuberculosis* NadA variants were constructed using the QuikChange II Site-directed Mutagenesis Kit (Stratagene) according to the manufacturer’s specifications, and as described previously (20), in which the cycling protocol is adapted for the Stratagene Robocycler thermocycler (21). Plasmid pET28-MtbNadAopt was used as the template in conjunction with the appropriate primers for each respective amino acid substitution (Table 5.1). All mutations were verified by DNA-sequencing of the entire gene.

Expression and Purification of the *M. tuberculosis* NadA Variants. The expression and purification of the variants were as described above for the WT, except during overexpression, the concentration of IPTG was lowered to 50 µM (final concentration), and 100 µM cysteine (final concentration) was added along with IPTG.

Cloning of *E. coli* dsbA Gene. The *dsbA* gene was amplified from *E. coli* (strain W3110) genomic DNA by PCR technology using the primers EcDsbA.AHS.for and EcDsbA.AHS.rev (Table 5.1). Each amplification reaction contained the following in a volume of 50 µL: 0.4 µM of each primer, 0.25 mM of each deoxynucleoside triphosphate, 100 ng of *E. coli* genomic DNA, 2.5 U of Vent DNA Polymerase, and 5 µL of 10× reaction buffer. The reaction mixture was overlaid with 30 µL of mineral oil. After a 5 min denaturation step at 95 °C, 35 cycles of the following program were initiated: 1 min at 95 °C, 1 min at 55 °C, 2.5 min at 72 °C. Following the cycling program, the reaction was incubated further for 10 min at 72 °C. The PCR product was digested with *NdeI* and *EcoRI* and then ligated into similarly digested pET-26b by standard methods (18). The correct construct was verified by DNA sequencing and designated pET26b-DsbA.
<table>
<thead>
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<th>Primer</th>
<th>Sequence</th>
<th>Purpose</th>
<th>Underlined bases</th>
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<td>5' - CG CGG CGT CTC <strong>CAT ATG</strong> AAA AAG ATT TGG CTG GCG CTG GC -3'</td>
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<td>Reverse Primer for <em>M. tuberculosis</em> C155A NadA</td>
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<td>Forward Primer for <em>P. horikoshii</em> NadB cloning&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>PhNadB.KpnI.rev</td>
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<td>Reverse Primer for <em>P. horikoshii</em> NadB&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Underlined bases represent the *NdeI* restriction site.
<sup>b</sup>Underlined bases represent the *EcoRI* restriction site.
<sup>c</sup>Underlined bases represent the *KpnI* restriction site.
Bolded bases represent amino acid changes.
Expression of the dsbA Gene from E. coli and Purification of the Gene Product. A single colony was used to inoculate 100 mL of LB media containing 50 µg/mL kanamycin and was cultured overnight at 37 °C with shaking. A 60 mL portion of the culture was then evenly distributed into four 6 L Erlenmeyer flasks to inoculate 16 L of LB media containing 50 µg/mL kanamycin, and were cultured further at 37 °C with shaking (180 rpm). At an optical density (600 nm; OD600) of 0.6, the cultures were cooled in an ice–water bath, and solid IPTG was added to each flask at a final concentration of 200 µM. The cultures were allowed to incubate further at 18 °C with shaking for 16 h. Cells were harvested at 10,000 × g for 10 min at 4 °C, frozen in liquid N2 and stored at −80 °C until ready for use. Typical cell paste yield was 35-40 g from 16 L media.

Soluble DsbA was released from the periplasm by cold–osmotic shock (22). Frozen cells (37 g) were resuspended in 140 mL cold buffer A (100 mM Tris, pH 8.0, 0.5 mM EDTA, 0.5 mM sucrose, and 20 µg/mL PMSF (dissolved in ethanol)) and was incubated on ice for 5 min. The solution was centrifuged at 15,000 × g for 10 min at 4 °C. The pellet was warmed to RT and resuspended in 100 mL of ice–cold water. Following a 1 min incubation of ice, 5 mL of 20 mM MgCl2 was added and then the solution was centrifuged at 15,000 x g for 10 min at 4 °C. The following steps of the purification were performed inside of a cold room maintained at 4 °C. The supernatant containing the periplasmic proteins was loaded onto a DE-52 column (2.5 × 14.5 cm) equilibrated in buffer B (10 mM MOPS, pH 7.0) and subsequently washed with 350 mL of buffer B. The protein was eluted with at 250 × 250 mL linear gradient of buffer B containing 0-500 mM NaCl. Fractions were monitored by SDS-PAGE, and those containing DsbA were pooled and concentrated in an Amicon stirred cell (Millipore, Billericias, MA) fitted with a YM-10 membrane (10,000 Da MW cutoff). The concentrated protein was then brought into the anaerobic glove box and subjected to anaerobic gel filtration (Sephadex G-25), concentrated, and stored in aliquots in a liquid N2 dewar until ready for use. Protein concentration was determined using the
molar extinction coefficient for the unfolded, oxidized DsbA \( (\varepsilon_{280nm} = 21,740 \text{ M}^{-1}\text{cm}^{-1}) \), adjusted with the absorption coefficient of \( A_{280nm, 1\text{mg/mL}, 1\text{cm}} = 1.10 \) for the native, oxidized protein \((17)\).

**Assay of oxidized M. tuberculosis NadA in the presence and absence of DTT.** All assays were performed anaerobically at 37 °C over a 10 min time period and analyzed as previously described \((8)\). Prior to performing all of the assays described below, the DTT present in the gel filtration buffer for storage was removed by anaerobic gel filtration using a pre-poured NAP column equilibrated in gel–filtration buffer 2 (50 mM HEPES, pH 7.5, 0.1 M KCl, 20% glycerol) \((\text{NadA}_{\text{red}})\). To expose the protein to oxygen \((\text{NadA}_{\text{ox}})\), gel filtration was performed aerobically and then the protein was brought into the anaerobic glovebox to assay. To assess the effect of DTT on activity, 5 \( \mu \)M RCN NadA\(_{\text{ox}}\) was incubated on ice for 5 h in the presence of 5 mM DTT, then assayed for QA production. The assay contained in a final volume of 1300 \( \mu \)L: 200 mM HEPES, pH 7.5, 1 mM DHAP, 0.1 M ammonium chloride, 10 mM OAA, 0.3 mM L-tryptophan (internal standard) and 5 \( \mu \)M RCN NadA. The reactions were initiated by addition of OAA after incubation of the other components at 37 °C for 3 min. At designated times, 200 \( \mu \)L aliquots of the assay mixture were removed and added to 40 \( \mu \)L of 2 M trichloroacetic (TCA) to quench the reaction. The precipitated protein was pelleted by centrifugation and the supernatant was analyzed by HPLC as previously published \((7)\) and as described in the Materials and Methods section of Chapter 2 of this document.

**Assay of M. tuberculosis NadA in the Presence of Trx.** Our laboratory previously observed the optimum activity of *E. coli* NadA in the presence of the oxidized form of thioredoxin \((\text{Trx}_{\text{ox}})\) and utilized this activity differential to determine the midpoint reduction potential of NadA \((8)\). To assess the effect of Trx on activity of MtNadA, 5 \( \mu \)M RCN Mt NadA\(_{\text{ox}}\) was assayed in the presence of 166 \( \mu \)M Trx\(_{\text{red}}\) under the conditions described above. Also, the reverse experiment was performed; Mt NadA\(_{\text{red}}\) was assayed in the presence of 166 \( \mu \)M Trx\(_{\text{ox}}\) under the same conditions.
Assay of *M. tuberculosis* NadA in the Presence of Glutathione (GSH/GSSG). To assess the effect of glutathione on activity, 15 µM as–isolated (AI) Mtb NadA\textsubscript{red} and NadA\textsubscript{ox} were incubated with 5 mM reduced (GSH) or oxidized (GSSG) glutathione at RT for 1 h. The proteins were then assayed for the rate of QA production as described above, except in the presence of 5 mM OAA.

Assay of *M. tuberculosis* NadA in the Presence of DsbA. To assess the effect of DsbA on activity, 5 µM AI NadA\textsubscript{red} was incubated with 200 µM DsbA\textsubscript{ox} at RT for 1 h then assayed for QA production as described above.

Assay of *M. tuberculosis* NadA Variant Proteins in the Presence of DsbA. The DTT in the gel–filtration buffer was removed from the NadA variants by subjecting the proteins to anaerobic gel–filtration chromatography. AI NadA\textsubscript{red} (20 µM) was then incubated with 200 µM DsbA at RT for 1 h and assayed as described above.

Cloning for in vivo Complementation Studies. The pET28a plasmid containing *nadA* genes expressing WT & variant (Cys291Ala and Cys294Ala) *E. coli* NadA, WT Mtb NadA, and WT *P. horikoshii* NadA were digested with *Nco*\textsubscript{I} and *Not*\textsubscript{I} and then ligated into similarly digested pCOLA by standard methods (18). The pET26b plasmid containing the *nadB* gene from *E. coli* was digested with *Nde*I and *Xho*I and then ligated into similarly digested pACYC by the same methods.

The *nadB* gene from *M. tuberculosis* was amplified from *M. tuberculosis* (strain H37Rv) genomic DNA by PCR technology using primers MtNadB.for.AEG and MtNadB.rev.AEG (Table 5.1). Each amplification reaction contained the following in a volume of 50 µL: 0.8 µM of each primer, 0.25 mM of each deoxynucleoside triphosphate, 750 ng of Mtb genomic DNA, 2.5 U of Vent DNA Polymerase, and 5 µL of 10× reaction buffer. The reaction mixture was overlaid with 30 µL of mineral oil. After a 5 min denaturation step at 95 °C, 35 cycles of the following program were initiated: 1 min at 95 °C, 1 min at 55 °C, 2.5 min at 72 °C. Following the cycling program, the reaction was incubated further for 10 min at 72 °C. The PCR product was digested with *Nde*I.
and EcoRI and then ligated into similarly digested pET28a by standard methods (18). The pET28a plasmid containing the \textit{nadB} gene from Mtb was also digested with \textit{NcoI} and \textit{HindIII} and then ligated into similarly digested pACYC.

The \textit{nadB} gene from \textit{P. horikoshii} was amplified from pET28a PhNadB by PCR technology using primers PhNadB.NdeI.for and PhNadB.KpnI.rev (Table 5.1). Each amplification reaction contained the following in a volume of 50 µL: 0.4 µM of each primer, 0.25 mM of each deoxynucleoside triphosphate, 200 ng of pET28a PhNadA, 2.5 U of Vent DNA Polymerase, and 5 µL of 10× reaction buffer. The reaction mixture was overlaid with 30 µL of mineral oil. After a 9 min denaturation step at 95 °C, 35 cycles of the following program were initiated: 1 min at 95 °C, 1 min at 55 °C, 2.5 min at 72 °C. Following the cycling program, the reaction was incubated further for 10 min at 72 °C. The PCR product was digested with \textit{NdeI} and \textit{KpnI} and then ligated into similarly digested pACYC by standard methods (18). The correct constructs were all verified by DNA sequencing.

The pET28a plasmid containing \textit{nadA} genes expressing WT & variant (Cys113Ala, Cys200Ala, Cys291Ala, Cys294Ala, and Cys297Ala) \textit{E. coli} NadA, WT Mtb NadA, and WT \textit{P. horikoshii} NadA were digested with \textit{NcoI} and \textit{EcoRI} and then ligated into similarly digested pTre-99a by standard methods (18).

\textit{Complementation studies of Escherichia coli ΔnadA}. \textit{E. coli} JW0733nadA mutant strain was obtained from the Keio collection (23). For transformation with plasmids conferring kanamycin resistance, the \textit{kan} allele was removed with FLP recombinase by the method of Datsenko and Wanner (24). The procedure involved transformation of the ΔnadA strain with the heat-curable plasmid pCP20 encoding the FLP-recombinase and the ampicillin resistance gene, which was then grown overnight at 30 °C on a LB plate containing 100 µg/mL ampicillin. A single colony was then streaked on a LB plate containing no antibiotics and incubated overnight at 43 °C to heat cure the pCP20 plasmid. A single colony of the ΔnadA strain was tested for
growth on LB plates containing either kanamycin or ampicillin to confirm that the kanamycin
cassette was removed and the pCP20 plasmid was heat cured. The ΔnadA phenotype was tested
by the inability of the bacterium to grow on M9 minimal media without supplementation with
nicotinic acid. The ΔnadA JW0733 strain was then transformed with pCOLA containing a nadA
gene or pACYC containing a nadB gene or both. Each was tested for growth aerobically on M9
minimal media plates at 37 °C for 2 days.

For complementation studies in the presence of IPTG to induce expression of the nadA
genes in the pTre99a plasmid, the nadA::kan allele was moved from E. coli JW0733nadA by P1vir
transduction into the MG1655 acceptor strain according to standard methods (25). A P1 lysate of
the E. coli JW0733nadA donor strain was prepared as follows: 150 µL of an overnight culture
was inoculated into 15 mL of LB media and the culture was grown at 37 °C with shaking. When
the culture grew to an optical density at 600 nm (OD_{600nm}) of 0.2, CaCl$_2$ was added to a final
concentration of 10 mM along with 150 µL of the P1vir, a mutant phage that enters the lytic cycle
upon infection, and the culture was further grown for 3 h at 37 °C with shaking. A 15 mL portion
of the culture was then transferred to a conical tube, 500 µL of chloroform was added, and the
culture was vortexed lightly to lyse any remaining unlysed cells. The culture was then centrifuged
at 5,000 × g for 15 min, the supernatant was transferred to a glass tube, 500 µL of chloroform was
added, and the culture was stored at 4 °C while preparing the MG1655 acceptor strain. A 4 mL
overnight culture of the MG1655 WT stain was grown, 1.5 mL was removed and the cells were
pelleted by centrifugation. The cell pellet was resuspended in 500 µL of a 10 mM MgSO$_4$, 5 mM
CaCl$_2$ solution. A 100 µL volume of the ΔnadA P1 lysate was added to an equal volume of the
resuspended MG1655 cells and the mixture was incubated in a 30 °C water bath for 30 min. A 1
mL volume of LB media was added to the cells and they were further incubated at 37 °C for 1.5 h
and then plated on a LB kanamycin media to screen for MG1655nadA mutant (ΔnadA) colonies
containing the nadA::kan allele. The ΔnadA MG1655 strain was then transformed with each of
the pTrc99a plasmids containing the genes expressing the respective NadA, and each was tested for growth on M9 minimal media plates under various conditions. The plates were grown at 37 °C for 2 days in the presence and absence of IPTG (400 µM & 1 mM) to induce expression of the pTrc99a plasmids. Also, the bacteria were grown in the presence and absence of molecular oxygen, by incubating the plates for 1 week in a sealed anaerobic jar containing an anaerobic gas pack with palladium catalyst (BD Biosciences, Sparks, MD).

5.4 Results

*Expression and Purification of E. coli DsbA.* The gene encoding WT DsbA was cloned into an IPTG–inducible pET-26b vector for overexpression. To isolate the protein, the resulting cell paste was first converted into spheroplasts by treatment with EDTA and sucrose, followed by centrifugation to pellet the cells. A significant amount of the protein was lost in the supernatant in this step due to some cell lysis during cell resuspension (Figure 5.1, lane 2). The pellet was then subjected to osmotic shock by resuspending it in ice–cold water and then adding MgCl₂ to release the periplasmic proteins. Following centrifugation, the supernatant was loaded onto a DE-52 anion–exchange column equilibrated in 10 mM MOPS, pH 7.0, and the column was washed with column equilibration buffer and eluted with NaCl (0 to 500 mM gradient) (Figure 5.1, lanes 3-6). The resulting fractions containing DsbA (as determined by SDS-PAGE) were pooled, concentrated and subjected to gel filtration into anaerobic buffer in the glove box (Figure 5.1, lane 7). The purification yielded 100 mg of protein that was ≥95 % homogeneous as determined by SDS-PAGE.
Figure 5.1: Coomassie blue stained SDS-PAGE analysis of purification of the *E. coli* DsbA. Lanes: 1, molecular weight markers (kDa); 2, spheroplast supernatant; 3, supernatant after osmotic shock; 4, pellet; 5, DE-52 load eluate; 6, DE-52 wash eluate; 7, purified protein.

*Expression and Purification of M. tuberculosis NadA.* The codon-optimized Mtb *nadA* gene showed a slightly higher level of expression than the non-optimized gene, yet the majority of the expressed protein was insoluble and present in the pellet following centrifugation after cell lysis. The purification yielded 79 mg of approximately 70% pure protein. Following chemical reconstitution with ferric chloride, sodium sulfate, and DTT as previously described (7), the protein was then subjected to gel filtration chromatography using an FPLC system, yielding 39 mg of pure (≥ 95%) protein. Iron and sulfide analyses indicated 3.75 ± 0.78 equiv of Fe and 2.92 ± 0.39 equiv of S²⁻ per polypeptide.
Expression and Purification of M. tuberculosis NadA Variant Proteins. We previously were unable to isolate variant proteins of the Mtb NadA due to solubility issues. Using the codon–optimized Mtb nadA gene and changing its conditions for expression (M9 minimal media, reducing temperature and increasing length of time of expression), we were able to produce and isolate a small amount of soluble variant proteins. The purification yielded 15 to 25 mg of approximately 60% pure protein. Due to stability issues, the protein was not subjected to chemical reconstitution or gel filtration using FPLC like the WT.

Assays of M. tuberculosis NadA under Oxidizing and Reducing conditions. We have previously determined that WT E. coli NadA_red displays a $V_{\text{max}}/[E_T]$ of 1.6 min$^{-1}$, and that this rate increases 7.5-fold under oxidizing conditions (12 min$^{-1}$ in the presence of Trx$^{\text{ox}}$) due to the formation of a disulfide bond between Cys291 and Cys294 (8). The NadA from Mtb does not contain this CysXXCys motif, yet surprisingly a similar rate increase is observed under aerobic conditions (Table 5.2). We typically observe approximately 3-fold lower activity with WT Mtb NadA_red ($V_{\text{max}}/[E_T]$ of 0.55 min$^{-1}$) than with WT E. coli NadA_red under similar conditions. The observed rates reported for the Mtb NadA under similar conditions varies between each experiment due to the use of protein from different purifications, with varying purity and Fe/S cluster content, resulting in varying activity. Following oxygen exposure, the activity increases 11-fold ($V_{\text{max}}/[E_T]$ of 6.1 min$^{-1}$), suggesting that Mtb NadA activity is also regulated by disulfide-bond formation. However, treatment of Mtb NadA_red with Trx$^{\text{ox}}$ has no effect on activity, and under reverse conditions, Mtb NadA_ox in the presence of Trx$^{\text{red}}$, activity is decreased, but only 2.2-fold and not to the basal activity level of Mtb NadA_red. Because the midpoint potential of E. coli Trx is $-270$ mV (16), the lack of its effect on the Mtb NadA suggested that the disulfide–bond may have a more positive, oxidizing midpoint reduction potential.

We hypothesized that the midpoint reduction potential of the Mtb NadA disulfide–bond is significantly more positive than that of E. coli Trx or NadA and tested that by determining the effect of a more oxidizing reagent, glutathione ($E_o = -240$ mV (26)) on the activity of Mtb
NadA<sub>red</sub> following incubation with the oxidized form of glutathione (GSSG). The $V_{\text{max}}/[E_T]$ increased 2.3-fold, but not close to the observed increase in the presence of oxygen (11-fold). The reduced form of glutathione (GSH) was able to reduce the activity of Mtb NadA<sub>ox</sub> 3.5-fold, but again not to the basal activity level of the fully reduced enzyme. Another chemical oxidant tested, trans-4,5-dihydroxy-1,2-dithiane (DTT<sub>ox</sub>), also had no effect on Mtb NadA<sub>red</sub> as expected due to its more reducing midpoint reduction potential (~ 330 mV (27)).

Finally effect of *E. coli* DsbA on Mtb NadA activity was tested. DsbA is one of the most oxidizing oxidoreductase in the thioredoxin class, with a midpoint potential of ~ 120 mV (17). The fact that the oxidized form of DsbA is less stable than the reduced form (28) and the more reactive cysteine has a very low pK<sub>a</sub> (~ 3) (29) compared to that of normal cysteine residues (~ 9) makes DsbA a powerful oxidant. This enzyme was overproduced in *E. coli* and subsequently isolated from the periplasm under aerobic conditions. It was then subjected to anaerobic gel filtration in the absence of reducing agents to remove molecular oxygen while retaining the enzyme in the oxidized form. Following incubation of Mtb NadA<sub>red</sub> with the DsbA<sub>ox</sub>, the $V_{\text{max}}/[E_T]$ increased 12-fold, a similar increase as observed in the presence of oxygen. This observation suggests that the rate of Mtb NadA-dependent QA formation is also regulated by a disulfide bond, yet the midpoint reduction potential is more oxidizing that that of the enzyme from *E. coli*.

The same set of experiments were also performed with NadA from *P. horikoshii* and unlike that of the *E. coli* and Mtb proteins, this enzyme was unstable in the presence of oxygen and lost all activity following oxygen exposure. Additionally, TrxA<sub>ox</sub> and DsbA had no effect on the *P. horikoshii* NadA activity, suggesting that there is no disulfide bond formed in this enzyme. Interestingly, when *E. coli* NadA was incubated with DsbA the activity decreased 2-fold. This is most likely due to the oxidizing nature of DsbA that often forms incorrect disulfide bonds with the *E. coli* NadA protein, which has nine total cysteines (30, 31). The activity of variants of
the cysteines involved in forming the disulfide bond in *E. coli* NadA also decreases, 1.3- and 3-fold for Cys291Ala and Cys294Ala, respectively.

Figure 5.2: Time–dependent formation of QA catalyzed by WT *M. tuberculosis* NadA in the reduced form (closed circles), oxidized by molecular oxygen (closed squares), and oxidized by DsbA (open circles).
Table 5.2: Activity of \textit{M. tuberculosis} NadA under various oxidizing and reducing conditions.

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<td>( \text{NadA}_\text{ox} )</td>
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<tr>
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<td>0.44</td>
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<tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>( \text{NadA}_\text{ox} ) with ( \text{GSH} )</td>
<td>1.0</td>
</tr>
<tr>
<td>( \text{NadA}_\text{ox} ) with ( \text{GSSG} )</td>
<td>2.2</td>
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</tbody>
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\textit{Assays of \textit{M. tuberculosis} NadA Variant Proteins.} We expected that the activity of the single variants of either of the disulfide–bond forming cysteines would not be affected by DsbA. To test this hypothesis, DTT was removed from the storage buffer of each variant by anaerobic gel–filtration chromatography, and then each protein was incubated in the presence and absence of DsbA. After incubation, the variants were assayed for QA formation to determine their
respective turnover rates, which are compiled in Table 5.3. The two variants tested that do not exist in any motif, Cys87Ala and Cys314Ala, both displayed activity slightly lower ($V_{\text{max}}/[E_T]$ of approximately 0.1 min$^{-1}$) than that of WT NadA in the absence of DsbA (reducing conditions). The activity in the presence of DsbA increased 9.5- and 7.7-fold for the Cys87Ala and Cys314Ala variants, respectively. This increase is similar to the 12-fold increase observed with WT NadA under the same conditions (Table 5.2). The next variant tested was Cys155Ala, which exists in a CysCys motif. This protein also responded similarly to WT NadA when exposed to DsbA. Finally, the variants of the two cysteines in the CysXCys motif, Cys230Ala and Cys232Ala, both displayed activity that was approximately 2-fold greater than WT NadA in the absence of DsbA. Additionally, the activity did not increase upon incubation with DsbA, but instead decreased slightly. These results show that Cys230 and Cys232 are involved in forming the disulfide bond in Mtb NadA.

Table 5.3: Activity of *M. tuberculosis* NadA variant proteins in the absence and presence of DsbA.

<table>
<thead>
<tr>
<th></th>
<th>$V_{\text{max}}/[E_T]$ (min$^{-1}$)</th>
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<tbody>
<tr>
<td></td>
<td>− DsbA</td>
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<tr>
<td>Cys87Ala</td>
<td>0.13</td>
</tr>
<tr>
<td>Cys155Ala</td>
<td>0.27</td>
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<tr>
<td>Cys230Ala</td>
<td>0.87</td>
</tr>
<tr>
<td>Cys232Ala</td>
<td>1.02</td>
</tr>
<tr>
<td>Cys314Ala</td>
<td>0.12</td>
</tr>
</tbody>
</table>
Complementation of JW0733 ΔnadA with pCOLA NadA and pACYC NadB Plasmids. An E. coli strain in which the nadA gene has been removed is only capable of growing when supplemented with nicotinic acid to allow for the production of NAD⁺ via the salvage pathway. We also observed that the E. coli JW0733nadA mutant strain also grows when supplemented with quinolinic acid to produce NAD⁺ via the de novo pathway. It was previously demonstrated that a plasmid encoding the WT E. coli nadA gene was able to complement the E. coli strain lacking the nadA gene when grown on minimal medium alone (6). These studies included the surprising findings that neither of the variants of the cysteines that form a disulfide bond in E. coli NadA (Cys291 and Cys294) were able to complement, although these variants are active in vitro. In fact, one variant (Cys294Ala) displayed activity that was greater than that of the WT enzyme. We obtained the E. coli JW0733nadA mutant strain from the Keio collection, repeated these studies and obtained the same results. In addition, we tested NadA from P. horikoshii and Mtb for complementation as well and found neither to be capable of complementing the JW0733nadA mutant strain. To investigate whether the inability to complement may be due to a requirement of greater concentrations of the unstable substrate, IA, to grow under these conditions, genes encoding each NadA were cloned into the pCOLA expression vector to allow for co-expression with the E. coli nadB gene cloned into pACYC. After removing the kan cassette from the E. coli JW0733nadA strain, each NadA–encoding plasmid, along with the NadB–encoding plasmid, was cotransformed into this strain and tested for their ability to grow on minimal media (Figure 5.3). The only growth observed was with the strain cotransformed with plasmids encoding WT E. coli NadA and NadB, while genes encoding NadA disulfide–bond variants (Cys291Ala and Cys294Ala), P. horikoshii NadA and Mtb NadA were all unable to complement the nadA knockout strain even in the presence of NadB.
Figure 5.3: Complementation studies of JW0733 ΔnadA cotransformed with pCOLA NadA and pACYC *E. coli* NadB plasmids on M9 minimal media plates, and grown in the presence of oxygen for 2 days at 37 °C.

The inability of the NadA proteins from *P. horikoshii* and Mtb to complement the *E. coli* JW0733nadA mutant strain, even in the presence of *E. coli* NadB, led us to investigate whether these enzymes may require that IA be supplied by a NadB from the same organism, which would suggest species specific protein–protein interactions that facilitate transfer of the unstable intermediate. Therefore, genes encoding NadB from Mtb and *P. horikoshii* were cloned into the pACYC vector, cotransformed with the pCOLA NadA from the same organism into the nadA knockout strain, and plated on minimal media (Figure 5.4). Again, the only growth observed was with the strain transformed with WT *E. coli* NadA.
Figure 5.4: Complementation studies of JW0733 ΔnadA cotransformed with pCOLA NadA and pACYC NadB plasmids from *P. horikoshii* (A) and *M. tuberculosis* (B) on M9 minimal media plates, grown in the presence of oxygen for 4 days at 37 °C. Also shown is pCOLA (empty vector) as the negative control and the WT EcNadA as the positive control.
Complementation of MG1655 ΔnadA with pTrc99a NadA Plasmids. To further investigate the mechanism of complementation, the studies described above were repeated using an MG1655 ΔnadA strain containing genes encoding WT or variant E. coli NadA, WT Mtb NadA, or WT P. horikoshii NadA cloned into an IPTG–inducible plasmid, pTrc99a. Each plasmid–containing strain was tested for growth on M9 minimal media in the absence and presence of 400 µM IPTG under normal aerobic conditions at 37 °C. In the absence of IPTG, the results were identical to those previously observed; only the ΔnadA strain containing the WT E. coli NadA plasmid was able to grow (Figure 5.5 A). When the same strains were grown in the presence of IPTG, growth was observed with the ΔnadA strain containing the WT Mtb NadA (Figure 5.5 B). Further studies showed that the ΔnadA strain containing WT E. coli NadA is also able to grow in the presence of IPTG, but at a slower rate.

Figure 5.5: Complementation studies of MG1655 ΔnadA with pTrc99a NadA plasmids on M9 minimal media plates in the absence (A) and presence (B) of 400 µM IPTG, grown in the presence of oxygen for 2 days at 37 °C. MG1655 is the WT strain as a positive control and pTrc is the MG1655 ΔnadA strain with the empty vector as a negative control.
We previously observed that variants of the cysteine residues involved in forming the disulfide bond (Cys291 and Cys294) were unstable in the presence of oxygen, resulting in significantly decreased activity (8). This led us to propose that exposure to oxygen under aerobic growth conditions prevented variants of these two residues from complementing the $\Delta$nadA strain. Plates were therefore placed in a sealed jar with a gas pack and palladium catalyst to create an anaerobic environment before incubating at 37 °C. After 2 days, growth is observed with the $\Delta$nadA strain containing the Cys294Ala E. coli NadA variant in the presence of IPTG (Figure 5.6 B). Growth is also observed with the $\Delta$nadA strain containing WT E. coli NadA in the presence of IPTG under anaerobic conditions. Growth is still not observed with the Cys291Ala E. coli NadA variant or either Mtb or P. horikoshii NadAs.

Figure 5.6: Complementation studies of MG1655 $\Delta$nadA with pTrc99a NadA plasmids on M9 minimal media plates in presence of 1 mM IPTG, grown in the presence (A) and absence (B) of oxygen at 37 °C. Due to the decreased growth rate under anaerobic conditions, the aerobic plates were incubated for 2 days and the anaerobic plates for 1 week.
5.5 Discussion

NadA from *E. coli* has been characterized to contain one [4Fe-4S] cluster that is required for activity (4, 5). The Fe/S cluster was originally proposed to be ligated by three cysteine residues on the protein that reside in a CysXXCysXXCys motif (32). Studies in our laboratory, along with the Fontecave laboratory, have demonstrated that the Fe/S cluster is instead ligated by the three conserved cysteines on the protein (Cys113, Cys200, and Cys297) and any single amino acid substitution at those residues results in inactive protein (6, 7). Only one cysteine in the purported CysXXCysXXCys motif is actually involved in binding the Fe/S cluster. The other two cysteines, existing in a CysXXCys motif, have been shown to form a redox–active disulfide bond that is regulated by thioredoxin (6, 8). In addition to characterizing the Fe/S cluster ligands *in vitro*, Rousset *et al.* performed studies with an *E. coli* nadA deletion strain (ΔnadA). This strain exhibits NAD⁺ auxotrophy unless supplemented with nicotinic acid or complemented by transforming with a plasmid encoding the WT *E. coli* nadA gene (6). Given the results of the *in vitro* studies, it is not surprising that variants of any of the three cluster ligating cysteines were not capable of complementing ΔnadA. The authors also found that plasmids containing variants of either cysteine involved in disulfide bond formation (Cys291 or Cys294) also do not complement, despite their ability to form QA at comparable rates to that of WT *in vitro* (7). More studies performed in our laboratory showed that these variants display significantly decreased activity after oxygen exposure (8), suggesting that the disulfide bond may play a role in protecting the Fe/S cluster from oxidative stress.

We have repeated these growth studies in an MG1655 *E. coli* ΔnadA strain and tested for complementation using WT and variant NadAs in an IPTG–inducible plasmid (pTrc99a). We found that one of the two variants of the disulfide–bond forming cysteines, Cys294Ala, is capable of complementing the ΔnadA strain. Complementation was only observed in the presence of IPTG, to induce overexpression of the protein, and in the absence of oxygen. These results
corroborate our hypothesis that the function of the *E. coli* NadA disulfide bond is to protect the enzyme, most likely the Fe/S cluster, from oxidative stress. The variant of the second cysteine involved in forming the disulfide bond, Cys291Ala, does not complement under these conditions. This variant has a 3.3-fold lower $V_{\text{max}}/[E_T]$ that Cys294Ala *in vitro* (8), therefore the lack of growth may be due to a lower activity *in vivo*.

NadA from *Mtb* has previously been characterized by our laboratory and shown, similarly to *E. coli* NadA, to contain an essential [4Fe-4S] cluster similar to *E. coli* NadA (7). We have found that this enzyme is also redox–regulated, but does not contain the CysXXCys motif involved in forming the disulfide bond in *E. coli*. *Mtb* NadA does contain a CysXCys motif (Cys230 and Cys232) and two adjacent cysteines (Cys154 and Cys155) that are both moderately conserved in actinomycetes. Disulfide bonds formed from cysteines in CysXCys motifs are rare in nature due to their instability (33), yet this sequence has been found to be conserved in the DUF1094 family of proteins that display isomerase activity (14). The recent crystal structure of YphP from *Bacillus subtilis* was solved and it was demonstrated that the cysteines in the CysXCys motif form a disulfide bond with a reduction potential of $-130 \pm 5 \text{ mV}$ (14). This protein has not been assigned a function, but has been proposed to be a member of a family of oxidoreductases. The structure of YphP is similar to that of Trx, with the CysXCys motif of YphP in place of the CysXXCys motif of Trx in the active site. Also, the deletion of Pro34 of thioredoxin (one amino acid in the X of CysXXCys) to create a CysXCys motif resulted in a 70 mV increase in midpoint reduction potential from that of the WT enzyme (34). This behavior suggests that this motif may indicate a disulfide bond with a higher midpoint reduction potential than that of the CysXXCys motif. Our results with *Mtb* NadA are consistent with these findings, given that the disulfide is not oxidized by thioredoxin, but instead requires a protein disulfide isomerase, DsbA, with a much higher midpoint reduction potential. Adjacent cysteine residues have also been studied in synthetic peptides (15, 34) and shown to form disulfide bonds in nature,
which serves a regulatory role in the acetylcholine receptor (35). Therefore it is also possible that the disulfide bond in Mtb NadA is formed between the adjacent cysteines (Cys154 and Cys155).

To determine which cysteines are involved in forming the disulfide bond in Mtb NadA, we changed five of the cysteines to alanine individually and characterized the activity of each variant under reducing and oxidizing conditions. These studies included the two cysteines in the CysXCys motif (Cys230 and Cys232), one cysteine in the CysCys motif (Cys155), and two cysteines that do not exist in any motif (Cys87 and Cys314). We found that the activity of the Cys230Ala and Cys232Ala variants did not increase in the presence of DsbA, while the other three variants responded similarly to WT. These variant studies suggest that a disulfide bond is formed between the two cysteines in the CysXCys motif, which also corroborates our observation that the Mtb NadA requires a stronger oxidant to form this disulfide. Mtb NadA contains ten cysteines, three of which are conserved (Cys114, Cys201, and Cys300). We and others have shown that the corresponding cysteines in the *E. coli* enzyme ligate the Fe/S cluster (7), leaving seven other cysteines available to form a disulfide bond. There are two other cysteines that we have not yet investigated (Cys154 and Cys176), leaving the possibility that more than one disulfide bond is present in this enzyme. We have also proposed that the MtbNadA disulfide bond is more oxidizing than that of the *E. coli* enzyme (*E_o = −264 mV*) (8), but have not yet attempted to determine the midpoint reduction potential.

In growth studies with an MG1655 *E. coli ΔnadA* strain, we found that only one residue, Cys294, involved in disulfide bond formation in *E. coli* NadA is able to complement under conditions in which the protein is over–produced in the absence of oxygen. Given these results, we wanted to investigate whether Mtb NadA is able to complement this strain. Unlike *E. coli* NadA, Mtb NadA was not able to complement the *ΔnadA* strain by leaky expression, but instead required the addition of IPTG to induce overproduction of the protein. Interestingly, we observed less growth with WT *E. coli* NadA under these same conditions. The same experiments were performed with NadA from a third organism, *P. horikoshii*, hyperthermophilic archaean organism,
which is an obligate anaerobe. The *P. horikoshii* NadA was unable to complement under any of the conditions tested. This enzyme is very unstable in the presence of oxygen and exhibits no activity after oxygen exposure *in vitro*. Also, when tested for activity in the presence of Trx or DsbA, oxidized or reduced, there was no effect on NadA from *P. horikoshii* suggesting that it is not redox–regulated by disulfide bond formation. We also observe low expression of the *P. horikoshii* nadA gene in BL21(DE3) *E. coli* strain when over–producing for purification and instead use the Rosetta2(DE3) *E. coli* strain to provide tRNA for rare codons not commonly found in *E. coli*. Therefore, the lack of complementation with the *P. horikoshii* NadA may be due to low expression levels.

Our studies suggest that the disulfide bond in both *E. coli* and Mtb NadA plays a role in regulating the activity of these enzymes in the presence oxygen. This mechanism is not present in NadA from *P. horikoshii*, which is an obligate anaerobe that is not exposed to oxygen. Phylogenetic analysis of a number of prokaryotes by the similarity in the amino acid sequences is displayed in Figure 5.7. Each branch of the tree indicates the value for the number of amino acid substitutions per site, therefore a greater number indicates an increased difference between two sequences. In the tree, *E. coli* is at the top along with other organisms in the Enterobacter family (*Shigella boydii, Salmonella typhimurium, and Yersinia pestis*), of which all are gram negative, facultative anaerobes, and contain the CysXXCys motif in the NadA amino acid sequence. The next group below contains two representatives of Actinobacteria, *Rhodococcus jostii* and *Mycobacterium tuberculosis*, of which both are gram positive, aerobes, and contain the CysXCys motif in the NadA amino acid sequence. These are followed by a grouping of spore–forming gram positive bacteria, *Bacillus subtilis* and *Streptomyces coelicolor*, which are obligate aerobes, with the exception of *B. subtilis* which has been demonstrated to also grow under anaerobic conditions (36). The amino acid sequence of NadA from these organisms does not contain any common disulfide bond forming motif, yet they do contain a CysXCys motif in which the later cysteine acts as a ligand to the Fe/S cluster while the other has been demonstrated to be required
for growth under aerobic conditions in studies in a \textit{B. subtilis} nadA knockout strain \cite{37}. The two hyperthermophilic archaea, \textit{Pyrococcus horikoshii} and \textit{Thermotoga maritima}, are both obligate anaerobes, with no disulfide bond forming motif, and no evidence of disulfide bond formation in our studies of the \textit{P. horikoshii} NadA. Lastly, the aerobic thermophilic archa, \textit{Sulfolobus tokodaiii}, also does not contain cysteines in a disulfide bond forming motif, yet it does contain a CysXXCys motif in which the later is a ligand to the Fe/S cluster. The more N–terminal cysteine may be required for growth as was observed with the \textit{B. subtilis} NadA \cite{37}.

Figure 5.7: Phylogenetic tree of quinolinate synthases from various prokaryotes. The values on each branch provide the number of amino acid substitutions per site between the connected organisms.
Interestingly, the cellular concentrations of NAD\(^+\) in many of these organisms have been investigated and fall into three distinct classes. In these studies it was found that strict anaerobes had the highest concentrations of NAD\(^+\), followed by facultative anaerobes, and finally strict aerobes with the lowest concentrations of NAD\(^+\) (38). Since the rate of NAD\(^+\) turnover is greater under aerobic conditions (39), it is possible that the role of the disulfide bond in NadA is to provide more QA for the biosynthesis of NAD\(^+\) when it is needed under aerobic conditions. Formation of the disulfide bond increases the activity of NadA, which results in the production of more QA to feed into the biosynthesis of NAD\(^+\). Future studies in our laboratory will focus on investigating the redox status of NadA \textit{in vivo} under normal growth conditions and following exposure to oxidants. If it is found that the disulfide bond is formed under oxidizing conditions \textit{in vivo}, the concentrations of NAD\(^+\) and NADH will be quantified to determine if there is a correlation between the disulfide bond and NAD\(^+\) concentrations.

Interestingly, one major source of oxidative stress in \textit{E. coli} is from L-aspartate oxidase (NadB), the first enzyme in the QA biosynthesis pathway (41). NadB catalyzes the 2-electron oxidation of L-aspartate to IA utilizing the non–covalently bound flavin cofactor (2). In order to perform subsequent rounds of turnover, FADH\(_2\) is oxidized by molecular oxygen affording hydrogen peroxide. Under anaerobic conditions, fumarate acts as the electron acceptor, which is reduced to succinate (42). In recent studies performed by Kurshunov and Imlay, hydrogen peroxide production was suppressed by 25-30\% when the \textit{de novo} NadB/NadA pathway was circumvented by supplementing the media with nicotinic acid (41). This observation further supports our hypothesis that organisms that are exposed to oxidative stress utilize a disulfide bond on NadA to increase the production of QA, which feeds into the \textit{de novo} synthesis of NAD\(^+\). The disulfide bond may also protect the oxygen–sensitive Fe/S cluster that is essential for NadA activity.
5.6 References


Appendix

Steady State Kinetic Analysis of *Escherichia coli* NadA

A.1 Abstract

Quinolinate synthase (NadA) catalyzes the condensation of dihydroxyacetone phosphate (DHAP) with iminoaspartate (IA) to form quinolinic acid (QA). *In vivo*, IA is produced from L-aspartate by the flavin–dependent enzyme L-aspartate oxidase (NadB). It can also be generated chemically by Schiff-base formation between oxaloacetate (OAA) and ammonia (1). In these studies, we have investigated the steady state kinetic parameters for *Escherichia coli* NadA using both sources of IA. We have previously shown that the activity of NadA from *E. coli* is redox–regulated via a disulfide bond that forms under oxidizing conditions (2). We probe for any effect to the kinetic parameters under aerobic conditions when this disulfide bond is formed. Initial velocity and product inhibition studies provide some insight into the kinetic mechanism for the NadA reaction.

A.2 Introduction

QA is the first common precursor to nicotinamide adenine dinucleotide (NAD\(^{+}\)) in all organisms that synthesize NAD\(^{+}\) *de novo* (3). In most prokaryotes, NadA catalyzes the condensation of DHAP and IA, affording QA, inorganic phosphate (P\(_i\)) and two moles of water (4). The substrate, IA, is produced *in vivo* by the flavin–dependent enzyme NadB (5). NadB catalyzes the two electron oxidation of L-aspartate and transfers the electrons to non-covalently bound FAD, forming the reduced form of the cofactor, FADH\(_2\) (6). Under aerobic conditions, the cofactor is oxidized by molecular oxygen forming hydrogen peroxide. NadB also functions in the absence of oxygen, utilizing fumarate as the electron acceptor, forming succinate (7). Therefore,
this pathway has been designated the anaerobic pathway, in contrast to the aerobic pathway for QA biosynthesis utilized by most eukaryotes in which three of the five enzymes require molecular oxygen as a cosubstrate (3).

NadA from *E. coli* had been characterized and was demonstrated to contain one essential [4Fe-4S]^{2+} cluster that is ligated by three strictly conserved cysteines on the protein (8-11). The activity of the *E. coli* enzyme is redox–regulated by formation of a disulfide bond between cysteines that exist in a CysXXCys motif in the amino acid sequence (2, 11). Under aerobic conditions, or in the presence of oxidized thioredoxin, the rate of formation of QA by *E. coli* NadA is increased up to 7.5-fold compared to anaerobic, reducing conditions (2).

The mechanism by which the condensation reaction takes place is unknown, but two possible mechanisms have been proposed in the literature. The first proposed mechanism begins with the nucleophilic attack by C3 of the enamine form of IA on C3 of DHAP via an S_{N}2 type reaction, resulting in release of P_{i} (5). Subsequent keto–enol tautomerizations lead to Schiff–base formation, which closes the ring and removes the first water molecule. Proton abstraction and the second dehydration, hypothesized to be facilitated by the Fe/S cluster, allows for formation of the product QA. A second, distinctly different proposed mechanism begins with isomerization of DHAP to glyceraldehyde 3-phosphate (G3P), which is followed by Schiff–base formation prior to the loss of P_{i}. Subsequent electrocyclic ring closure, keto–enol/enamine tautomerizations, and dehydration result in formation of QA (12).

The only published crystal structure of NadA is from *Pyrococcus horikoshii* and is lacking the essential iron–sulfur (Fe/S) cluster (13). The enzyme used for crystallography was isolated and crystallized in the presence of molecular oxygen, conditions that we observe cause degradation of the Fe/S cluster and subsequently complete loss of activity (8). The authors attempted to model both substrates, DHAP and IA, into the active site based on the position of the substrate mimic, malate, in the structure. Even in the absence of the Fe/S cluster, they state that there was not enough space to accommodate the phosphate moiety of the DHAP. We have modeled
the cluster into the active site and used this model to characterize variant proteins containing key conserved substitutions at active-site residues (Chapter 4). Of the eight variants characterized in these studies, we identified three amino acids that are absolutely required for formation of both QA and P$_i$. The remaining five variants displayed varying activities that were all lower than that of the wild-type (WT) protein, and all variants that catalyzed production of P$_i$ also catalyzed production of QA. In our own modeling studies, we also had difficulty fitting both substrates into the active site and instead used an intermediate in the reaction that lacks the P$_i$ substituent.

The results from the modeling studies suggests that there is not enough space for the P$_i$ from DHAP to be present simultaneously with IA in the active site. This observation might suggest that the P$_i$ release step of the NadA mechanism may occur early in the reaction and possibly prior to IA binding. Yet, our studies of conserved active site variants did not identify an amino acid substitution that resulted in enzyme that released P$_i$ without catalyzing formation of QA as would be expected if the P$_i$ were released early in the reaction.

In the studies discussed herein, we investigate the steady state kinetic mechanism of NadA under anerobic and aerobic conditions. These studies were performed with both methods for formation of IA, enzymatically by reacting L-aspartate with NadB and chemically by reacting OAA with an ammonia source (1). Initial velocity studies performed by varying DHAP concentrations at fixed concentrations of IA indicate a sequential mechanism for substrate binding and product release. To gain further insight into the order of events, product inhibition studies were performed in the presence of P$_i$, which we show is a noncompetetive inhibitor of QA.

A.3 Materials and Methods

Materials. 2,3-Pyridinedicarboxylic acid (quinolinic acid) was obtained from Aldrich (St. Louis, MO). Dihydroxyacetone phosphate (dilithium salt) and oxaloacetic acid were obtained from Sigma (St. Louis, MO). All other buffers and chemicals were of the highest grade available.
General Procedures. High performance liquid chromatography (HPLC) was conducted on a Beckman System Gold unit (Fullerton, CA), which was fitted with a 128 diode array detector and operated with the System Gold *Nouveau* software package. Procedures conducted under anoxic conditions were carried out in an anaerobic chamber from Coy Laboratory Products, Inc. (Grass lake, MI) under an atmosphere of N$_2$ and H$_2$ (95%/5%), and with an O$_2$ concentration maintained below 1 ppm by the use of palladium catalysts.

Activity Determinations. The activity of WT *E. coli* NadA was determined by monitoring the formation of QA at 37 °C over a 20 min time period under anaerobic conditions or a 2.5 min time period under aerobic conditions. The substrate IA was generated enzymatically via the NadB reaction using fumarate as the electron acceptor, or chemically by inclusion of OAA and ammonium sulfate in assay mixtures (1). Activity determinations conducted in the presence of NadB contained the following in a final volume of 1300 µL: 200 mM HEPES, pH 7.5, 25 mM L-aspartate/fumarate solution (titrated to pH 7), 25 µM FAD, varying DHAP concentrations (0.1 to 5 mM), 0.1 M KCl, 0.3 mM L-tryptophan (internal standard), and 5 µM reconstituted (RCN) NadA and 5 µM NadB. When IA was generated chemically, the aspartate/fumarate solution, FAD, and NadB were replaced with 30 mM OAA and 100 mM ammonium chloride (NH$_4$Cl). The reactions were initiated by addition of either NadB or OAA after incubation of the other components of the assay mixture at 37 °C for 5 min. At designated times, 200 µL aliquots of the assay mixture were removed and added to 40 µL of 2 M trichloroacetic acid (TCA) to quench the reaction. The precipitated protein was pelleted by centrifugation and the supernatant was analyzed by HPLC as previously published (8) and as described in the Materials and Methods section of Chapter 2 of this document.

$K_m$ Studies. The first studies performed were to determine the $K_m$ for each substrate under various assay conditions. Assays were performed only with IA at constant and saturating concentrations (5 mM OAA and 100 mM NH$_4$Cl) while varying the concentration of DHAP,
because of the inability to determine IA concentrations accurately. All assays were performed with 5 μM RCN *E. coli* NadA. The observed rates were plotted versus the concentration of DHAP, and displayed clean Michaelis–Menton behavior. The resulting curve was fit to eq 1

\[ v = \frac{V_{\text{max}}[S]}{K_m + [S]} \]  

(Eq 1)

using Igor Pro where \( v \) is the initial reaction rate, \( V_{\text{max}} \) is the maximum rate, \( S \) is the varied substrate concentration, and \( K_m \) is the Michaelis constant for substrate \( S \). All assays performed in these studies were in the presence of saturating concentrations of both substrates, conditions in which the rate equation for a bisubstrate system reduces to \( v = V_{\text{max}} \). Therefore rates are expressed as this rate, normalized to enzyme concentration, \( V_{\text{max}}/[E_T] \) or Rate (min\(^{-1}\)).

**A.4 Results**

*Determination of the \( K_m \) for DHAP.* NadA undergoes reversible disulfide–bond that regulates the activity of the enzyme; therefore, assays were performed under both anaerobic and aerobic conditions (Figure A.1, A & B, respectively). Because NadA is more active under oxidizing conditions, assays were performed over 20 min under anaerobic conditions and 2.5 min under aerobic conditions, each with a total of six time points over each time span. The increased activity observed with the disulfide–bonded form of the enzyme is reflected in the 7-fold increase in \( V_{\text{max}}/[E_T] \) under aerobic conditions versus anaerobic conditions (Table A.1). Also, the \( K_m \) for DHAP is 2-fold greater under anaerobic conditions.
Figure A.1: Steady state kinetic analysis of WT *E. coli* NadA. Activity determinations were performed using 5 µM RCN NadA, 5 mM OAA and 100 mM NH₄Cl to make IA, and varying concentrations of DHAP under anaerobic conditions (A) and aerobic conditions (B).
Similar assays were performed with enzymatically generated IA by replacing the OAA and NH₄Cl with 5 µM NadB, 25 µM FAD (the cofactor for NadB) and 25 mM L-aspartate in the assay. NadB catalyzes the two electron oxidation of L-aspartate to IA with concomitant reduction of the noncovalently bound FAD to FADH₂ (5). In order to perform subsequent rounds of turnover, the flavin cofactor requires an electron acceptor to re-oxidize the flavin, which is satisfied by molecular oxygen under aerobic conditions, affording production of hydrogen peroxide. Under anaerobic conditions, it has been demonstrated that fumarate acts as the electron acceptor, which gets converted to succinate (7). Therefore 25 mM fumarate was included in anaerobic assays. The observed rates under anaerobic conditions were plotted versus DHAP concentration, and followed typical Michaelis-Menten behavior (Figure A.2 A). The data were fitted to equation 1, affording the same $V_{\text{max}}/[E_T]$ and $K_m$ within error as that obtained using OAA & NH₄Cl (Table A.1). Rates observed under aerobic conditions decrease at high concentrations of DHAP (Figure A.2), typical of substrate inhibition and necessitating that the kinetic data be fitted to equation 2

$$v = \frac{V_{\text{max}}[S]}{K_m + [S](1 + \frac{[S]}{K_i})} \quad \text{(Eq 2)}$$

using Igor Pro, where $K_i$ is the dissociation constant for the inhibiting substrate.
Figure A.2: Steady state kinetic analysis of WT *E. coli* NadA. Activity determinations were performed using 5 μM RCN NadA, 5 μM NadB and 25 mM L-aspartate to form IA, with varying concentrations of DHAP under anaerobic conditions with 25 mM fumarate as the electron acceptor (A) or aerobic conditions with O₂ as the electron acceptor (B).
Determination of the $K_m$ for DHAP. The exact concentration of IA formed by the reaction of OAA and NH$_4$Cl is unknown due to its instability; it hydrolyzes to ammonia and OAA at 37°C and pH 8.0 with a half-life of 144 s (5) and is also susceptible to decarboxylation yielding iminopyruvate and CO$_2$. Yet, an upper limit for the $K_m$ of IA can be determined by assaying for QA production at a constant DHAP (500 µM) and NH$_4$Cl concentration (100 mM) while varying the OAA concentration (Figure A.3). The DHAP concentration was chosen based on the observation of substrate inhibition at higher concentrations under aerobic conditions (Figure A.2). The resulting data were fitted to equation 1 for Michaelis–Menten kinetics, which afforded a $V_{max}/[E_T]$ that is similar to those obtained from studies wherein DHAP was varied under the same conditions, and a $K_m$ for IA was estimated to be 0.71 mM (Table A.1). The data for the assays under anaerobic conditions were not sufficiently accurate to be fitted to equation 1, most likely due to the longer time span of the anaerobic assay (20 min) as compared to the aerobic assay (2.5 min), which allows for more IA to degrade.
Figure A.3: Steady state kinetic analysis of *E. coli* NadA. Activity determinations were performed using 5 μM RCN NadA, 500 μM DHAP, 100 mM NH₄Cl and varying concentrations of OAA to form IA under aerobic conditions.
Table A.1: WT E. coli NadA Steady State Kinetic Analysis.

<table>
<thead>
<tr>
<th>Varied substrate &amp; conditions</th>
<th>$V_{max}/[E_T]$ (min$^{-1}$)</th>
<th>$K_m$ varied substrate (mM)</th>
<th>$K_i$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHAP, Anaerobic, 5 mM OAA</td>
<td>1.84 ± 0.28</td>
<td>0.47 ±0.23</td>
<td></td>
</tr>
<tr>
<td>DHAP, Aerobic, 5 mM OAA</td>
<td>13.69 ± 1.19</td>
<td>0.19± 0.064</td>
<td></td>
</tr>
<tr>
<td>DHAP, Anaerobic, NadB/Asp</td>
<td>1.91 ± 0.43</td>
<td>1.05 ± 0.65</td>
<td></td>
</tr>
<tr>
<td>DHAP, Aerobic, NadB/Asp*</td>
<td>14.88 ± 5.50</td>
<td>0.20 ± 0.16</td>
<td>5.59 ± 5.89</td>
</tr>
<tr>
<td>IA, Aerobic, 500 µM DHAP</td>
<td>10.87 ± 0.85</td>
<td>0.71 ± 0.17</td>
<td></td>
</tr>
</tbody>
</table>

Results are from fitting data to equation 1 or equation 2*

*Initial Velocity Plots. To determine whether the NadA reaction follows a sequential or ping–pong mechanism, assays were performed with varying DHAP concentrations at a constant OAA/ammonia concentration. The rate of QA formation was monitored over 20 min at 37°C under anaerobic conditions at various DHAP concentrations (0.1, 0.25, 0.5, 1, 2, and 3 mM). The OAA concentration was fixed at 5, 10, 15, 20, and 30 mM for each assay, while the ammonia was constant at 100 mM. These rates were then plotted as a function of DHAP concentration for each OAA concentration (Figure A.4 A). The resulting curves were fitted to equation 3

$$v = \frac{V_{max}[DHAP][IA]}{K'_{DHAP}K_{IA} + K_{IA}[DHAP] + K'_{DHAP}[IA] + [DHAP][IA]} \quad \text{(Eq 3)}$$

using GraFit 5 and produced the following values:

$V_{max} = 7.41 \pm 0.85$ min$^{-1}$

$K_{DHAP} = 0.68 \pm 0.21$ mM

$K_{IA} = 15.26 \pm 3.68$ mM

$K'_{DHAP} = 0.62 \pm 0.24$ mM

The inverse rate plotted versus the inverse DHAP concentration produces the linearized Lineweaver–burk plot (Figure A.4 B). The best fit was to the sequential mechanism, as indicated
by the set of intersection lines, rather than a ping-pong mechanism which should result in parallel lines.
Figure A.4: (A) Initial velocity plot for DHAP performed with 5 µM RCN NadA, and 100 mM NH₄Cl at varying concentrations of OAA; 5 mM (open circles), 10 mM (closed circles), 15 mM (open squares), 20 mM (closed squares), & 30 mM (open triangles), (B) Lineweaver–Burk plot of the data.
**Product Inhibition Studies with Inorganic Phosphate.** To determine the order of substrate binding and product release, product inhibition studies must be performed. These were first conducted by looking at the inhibition of $P_i$ on DHAP. Assays were performed as described above, with the exception that incubation was carried out over a 10 min time span. The concentration of OAA was fixed at 5 mM, while DHAP concentrations (0.5, 1, 2, 3, and 5 mM) and $P_i$ ($Na_2HPO_4$, pH 7) concentrations (1, 5, and 10 mM) were varied. These rates were then plotted as a function of DHAP concentration for each $P_i$ concentration (Figure A.5 A). The resulting curves were fitted to equation 4

$$v = \frac{V_{\text{max}} [\text{DHAP}]}{K_m + \frac{1}{1 + \frac{[P_i]}{K_i}}} \quad \text{(Eq 4)}$$

using GraFit 5 which produced the following values:

$V_{\text{max}} = 4.92 \pm 0.29 \text{ min}^{-1}$

$K_m = 1.63 \pm 0.22 \text{ mM}$

$K_i = 17.13 \pm 0.25 \text{ mM}$

The inverse rate plotted versus the inverse DHAP concentration produces the linearized Lineweaver burk plot. The best fit was to a noncompetitive inhibition mechanism, producing a set of lines that intersect to the left of the y-axis (Figure A.5 B), indicating that $P_i$ combines with a different enzyme form than that to which DHAP combines. If $P_i$ and DHAP both combined to the same enzyme form, then the inhibition would be competitive and we would expect to observe lines that intersect on the y-axis.
Figure A5: (A) Phosphate inhibition with respect to the substrate DHAP performed with 5 μM RCN NadA, and 100 mM NH₄Cl and 3 mM OAA to form IA at varying concentrations of P; 1 mM (open circles), 5 mM (closed circles), 10 mM (open squares), (B) Lineweaver–burk plot of the data.
A.5 Discussion

Studies of the mechanism of NadA have been hindered due to the instability of the Fe/S cluster in the presence of molecular oxygen, along with the instability of the imine intermediates of the reaction prior to QA formation (17). We have found that NadA is stable after brief exposures to oxygen (over the time course of an assay) due to the formation of a disulfide bond between cysteines 291 and 294 (2). This allowed us to investigate the steady state kinetics of NadA under both anaerobic, reducing conditions and aerobic conditions, wherein a disulfide bond is present. The one substrate for NadA, IA, can be produced either by reacting L-aspartate and NadB together in the assay or by reacting OAA with an ammonia source (1). Due to the instability of IA, we are unable to quantify the concentrations in the assay. Therefore only the second substrate, DHAP, was able to be varied to obtain accurate $K_m$ values with the different IA sources under oxidizing and reducing conditions.

Under oxidizing conditions with either source of IA, the activity of NadA is significantly greater, with an observed 7-fold increase in $V_{max}/[E_T]$ while the $K_m$ value for DHAP remains the same. When using NadB and L-aspartate to form IA in the assay under aerobic conditions, we observe substrate inhibition at DHAP concentrations above 1 mM. Although we are unable to determine the exact concentration of IA in the assays, we are able to estimate this concentration by assuming that all of the OAA added to the assay reacts with ammonia to form IA. By varying the concentration of OAA at a constant DHAP concentration we were able to obtain a Michaelis–Menten plot and estimate the upper limit for the $K_m$ of IA to be 0.71 mM.

The order of substrate binding and product release was of interest to us to provide insight into the chemical mechanism of NadA. We first investigated whether the reaction proceeded via a sequential or ping–pong mechanism, wherein we varied concentrations of DHAP at fixed concentrations of IA, generated chemically with OAA and ammonia. The resulting Michaelis–Menten plots were linearized and replotted in Lineweaver–burk format. The patterns of these lines were intersecting, indicating a sequential kinetic mechanism, such that both substrates bind prior
to the occurrence of chemical steps and then both products are released. This mechanism is in contrast to a ping–pong mechanism, indicated if the lines were parallel, in which one substrate binds and reacts to release one product, followed by binding of the second substrate and reaction to form the second product. The only possible way the NadA reaction could occur via a ping–pong mechanism is if DHAP were to bind and phosphate were to be released prior to IA binding.

To investigate the order of substrate binding and product release, product inhibition studies were performed. We observed inhibition by $P_i$ on the reaction and looked at the affect of increasing the concentration of that inhibitor on the Michaelis–plots in which we varied DHAP at a fixed IA concentration. The resulting plots were linearized and replotted in Lineweaver–burk format, giving rise to lines that intersected to the left of the y-axis, thus indicating that $P_i$ is a noncompetitive inhibitor with respect to DHAP.

These steady state kinetic studies of the NadA reaction have provided some information into the reaction mechanism. We know that substrate binding and product release is ordered and does not proceed via a ping–pong mechanism. Also, the product, $P_i$, and substrate, DHAP, are noncompetitive inhibitors and do not bind to the same enzyme form. Since the expected pattern of inhibition for an ordered mechanism predicts noncompetitive inhibition for the first product released ($P$) with respect to both substrates ($A$ & $B$), and the second product released ($Q$) with respect to the second substrate that binds ($B$), this study does not provide information on the order of substrate binding or product release (Figure A.6). To obtain that information, we need to observe a competitive product inhibition pattern, which should occur with the last product released ($Q$) and the first substrate to bind ($A$), because they bind to the same form of the enzyme ($E$). Future inhibition studies with QA may provide further information into this mechanism.
Figure A.6: Reaction scheme for ordered substrate binding (A & B) and product release (P & Q) from enzyme (E).

Unfortunately, due to the instability of the IA substrate, we are unable to quantify the amount formed in the assay via the OAA and ammonia reaction. This prevents us from accurately varying this substrate to determine the type of inhibition of P_i with respect to IA. Attempts were made to determine the inhibition with QA with respect to DHAP, yet we were unable to increase the QA concentration high enough to observe inhibition (above 500 µM) using our HPLC assay to detect QA formed in the assay. Since then, a spectrophotometric assay to detect P_i (complex with malachite green phosphomolybdate) has been developed. This assay may be useful to observe the inhibition with higher concentrations of QA because we are monitoring the other product of the reaction, P_i. We are also in the process of developing a method to assay NadA by liquid chromatography – electrospray mass spectrometry (LC–MS). If we use a labeled substrate in the NadA reaction, such as ^15_N-labeled ammonium chloride, using MS, we should be able to discern the ^15_N-labeled QA produced in the reaction from the unlabeled QA added as the inhibitor.
A.6 References


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