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EXPANDING THE VERSATILITY OF THE PS I—HydA NANOCONSTRUCT

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by
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In 2010, our group devised a photosystem I (PS I)—[FeFe] hydrogenase (CaHydA) nanoconstruct capable of light-driven H₂ evolution with a rate of 30.3 ± 0.8 μmol of H₂ (mg of Chl)⁻¹ h⁻¹. PS I from Synechococcus PCC 7002 and HydA from Clostridium acetobutylicum ATCC 824 were tethered by a molecular wire, 1,6-hexanedithiol. The molecular wire links PS I and CaHydA through the F₈ and Fe/S clusters in the enzymes, respectively, while eliminating diffusion as a factor for electron transfer between the two enzymes. Both clusters are [4Fe-4S] clusters missing one of the cysteine ligands generated by site-directed mutagenesis, leaving an open coordination site for the molecular wire to rescue the clusters chemically. Upon illumination, an electron is transferred along the electron transport chain (ETC) of PS I to CaHydA. Electrons ultimately reach the active site of CaHydA, the H-cluster, which catalyzes proton reduction to form molecular hydrogen, H₂(g).

This success has sparked interest in the application of PS I, especially the extraction of low-potential electrons from the F₈ cluster. Firstly, attempts were made to optimize the production of CaHydA in Shewanella oneidensis MR-1. Secondly, this study explored an alternative method serving to connect PS I with a redox-active protein reporter by using sulfhydryl crosslinkers. This protein reporter (i.e., N75) is the N-terminal domain of CaHydA containing a single prosthetic group, a [2Fe-2S] cluster. Therefore, N75 can demonstrate the possibility of directing an electron from PS I to an external protein redox cofactor that is not a [4Fe-4S] cluster. These studies expand the versatility of the nanoconstruct platform by extending its connectivity to allow studies of other protein cofactors of interest.
TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................... vi
LIST OF TABLES ............................................................................................................... vii
ACKNOWLEDGEMENTS ................................................................................................... viii

Chapter 1 Introduction ...................................................................................................... 1
1.1 The Photosystem I—[FeFe] hydrogenase (PS I—CaHydA) nanoconstruct ............... 1
1.2 The light-harvesting photochemical module—PS I .................................................... 1
1.3 The H₂-evolving catalytic module, hydrogenase (H₂ase) ...................................... 3
  1.3.1 Different types of H₂ase ................................................................................... 3
  1.3.2 The [NiFe]-H₂ase ........................................................................................... 3
  1.3.3 The [FeFe]-H₂ase ........................................................................................... 4
  1.3.4 The [FeFe]-H₂ase from Clostridium acetobutylicum—CaHydA ...................... 4
  1.3.5 The central molecular wire that links the modules—1,6-hexanediol .............. 5
1.4 Expanding the versatility of PS I—CaHydA nanoconstruct ................................... 6

Chapter 2 Overexpression of C. acetobutylicum [FeFe] Hydrogenase in S. oneidensis
MR-1 .................................................................................................................................. 13
2.1 Introduction ................................................................................................................... 13
2.2 Methods and Materials ............................................................................................... 15
  2.2.1 Strains and plasmids construction ................................................................. 15
  2.2.2 S. oneidensis MR-1 conjugation procedure ................................................. 15
  2.2.3 Recombinant CaHydA vectors expression level analysis ............................. 16
  2.2.4 H₂ formation assay on S. oneidensis MR-1 strains .................................. 17
  2.2.5 Heterologous expression, purification, and characterization of CaHydA ... 18
  2.2.6 Iron quantification assay .............................................................................. 19
  2.2.7 Purification of PS I, associated photosystem proteins, and preparation of
      photosystem F₅ core particles ........................................................................... 19
  2.2.8 Rebuilding of PS I with heterologously expressed PsaD and C13/33G
      PsaC and tethering with C97G HydA variant .................................................. 20
2.3 Results ......................................................................................................................... 21
  2.3.1 Transconjugation of S. oneidensis MR-1 hydA/hyaB double mutant ........... 21
  2.3.2 H₂ formation assay on S. oneidensis MR-1 strains .................................. 21
  2.3.3 H₂ formation assay on S. oneidensis MR-1 strains with different anaerobic
      respiratory substrates ....................................................................................... 22
  2.3.4 Heterologous expression, purification, and characterization of CaHydA .... 23
  2.3.5 H₂ evolution rate of purified CaHydA and PS I—CaHydA nanoconstruct ... 23
2.4 Discussion ..................................................................................................................... 24

Module Nanoconstruct ..................................................................................................... 42
3.1 Introduction ............................................................................................................................................. 42
3.2 Methods and Materials ......................................................................................................................... 44
  3.2.1 Strains and plasmids construction ................................................................................................. 44
  3.2.2 Heterologous expression, purification, and characterization of N75 ........................................ 45
  3.2.3 Guanidine denaturation of Fe/S cluster in N75 ............................................................................ 46
  3.2.4 Fe and S reconstitution, Fe and S quantification of N75 ................................................................. 46
  3.2.5 Spectroscopic measurements of N76 characterizing the [2Fe-2S] clusters .................................... 47
  3.2.6 Crosslinkers preparation and utilizations ...................................................................................... 47
  3.2.7 Construction of rebuilt PS I and PS I—N76 nanoconstruct ............................................................. 48
  3.2.8 Ultrafiltration of rebuilt PS I and N75 nanoconstruct assembly mixtures ..................................... 49
3.3 Results ................................................................................................................................................... 49
  3.3.1 Design of N75 based on structure of HN76 .................................................................................. 49
  3.3.2 Heterologous expression, purification, and characterization of N75 ............................................ 50
  3.3.3 [2Fe-2S] cluster sensitivity to oxygen and room temperature exposures ...................................... 51
  3.3.4 Synthesis and effectiveness of the crosslinkers, BIDBE and DTME ........................................... 51
  3.3.5 Deteriorating effects of crosslinkers to the [2Fe-2S] clusters ...................................................... 53
  3.3.6 Effects of crosslinkers on the environment of the [2Fe-2S] cluster .............................................. 54
  3.3.7 Crosslinker-facilitated interaction between rebuilt PS I and N75 ................................................. 55
  3.3.8 Flash-induced 820nm absorption transient spectroscopy and light-induced low-temp CW-EPR measurements of the PS I—N75 nanoconstruct ........................................ 56
3.4 Discussion ............................................................................................................................................. 58

References ..................................................................................................................................................... 83
LIST OF FIGURES

Figure 1-1: The structure of [NiFe]-H₂ase active sites ................................................................. 8
Figure 1-2: Active site structure of [FeFe]-H₂ases in the active state ............................................. 9
Figure 1-3: Structure of CpI and detailed schematics of the cofactors in its ETC ...................... 10
Figure 1-4: Proposed structure and detailed components of the PS I—CaHydA nanoconstruct...................................................................................................................... 11
Figure 1-5: Structure of 1-(3-thiopropyl)-10-(methyl)-4,4’-bipyridinium chloride ........... 12
Figure 2-1: Kovac’s reagent test on LB agar plates with E. coli / S. oneidensis ......................... 28
Figure 2-2: Immunoblot of CaHydA expression S. oneidensis strains........................................ 29
Figure 2-3: Immunoblot and SDS PAGE of various purification steps of CaHydA............ 31
Figure 2-4: UV-visible spectrum of purified WT CaHydA................................................................. 32
Figure 2-5: Mass spectrometry coverage map of CaHydA trypsin digested fragments....... 33
Figure 2-6: Rate of H₂ evolution of PS I—CaHydA nanoconstruct........................................ 35
Figure 2-7: Rare codon analysis of C. acetobutylicum hydA against codon usage frequencies of S. oneidensis MR-1................................................................. 36
Figure 2-8: The annotated ISC gene cluster in S. oneidensis MR-1 ............................................ 40
Figure 2-9: Sequence alignment of iscR in E. coli and S. oneidensis MR-1 ......................... 41
Figure 3-1: Distance between Cys39 and the [2Fe-2S] cluster in N-terminus of CpI .......... 62
Figure 3-2: Structure of the N-terminal domain of CpI .............................................................. 63
Figure 3-3: Amino acid sequence alignment between HN76 and N75................................. 64
Figure 3-4: SDS PAGE of N75 purification steps ...................................................................... 65
Figure 3-5: UV-visible spectrum of N75 .................................................................................... 67
Figure 3-6: Low temperature CW-EPR spectrum of reduced N75..................................... 68
Figure 3-7: Decrease of absorbance at OD₁₈₅nm observed from N75 exposed to aerobic and anaerobic environment in RT ......................................................... 69
Figure 3-8: Mass spectrometry spectra of BIDBE and DTME ................................. 70

Figure 3-9: SDS PAGE of N75 crosslinked with BIDBE and DTME ......................... 71

Figure 3-10: Decrease of absorbance at OD_{329 nm} and OD_{418 nm} observed from N75 crosslinked by different concentrations of BIDBE .............................................. 74

Figure 3-11: Decrease of absorbance at OD_{329 nm} and OD_{418 nm} observed from N75 crosslinked by different concentrations of DTME .............................................. 75

Figure 3-12: Low temperature CW-EPR spectra of N75, BIDBE-N75, and DTME-N75 reduced with 2 mM Na dithionite .............................................................................. 77

Figure 3-13: Midpoint potential titration curves of N75, BIDBE-N75, and DTME-N75 ...... 78

Figure 3-14: Silver stained SDS PAGE and immunoblot of various nanoconstruct assembly mixtures .................................................................................................. 79

Figure 3-15: 820 nm time-resolved optical spectroscopy of WT PS I, rebuilt PS I, rebuilt PS I mixed with N76, rebuilt PS I mixed with DTME-N76, and rebuilt PS I mixed with BIDBE-N76 ............................................... 80

Figure 3-16: Light induced low-temp CW-EPR measurements of rebuilt PS I, rebuilt PS I + N75, and rebuilt PS I + BIDBE—N75 nanoconstruct ................................. 82
LIST OF TABLES

Table 2-1: H₂ evolution rate of *S. oneidensis* MR-1 strains with different anaerobic respiratory substrates. ...............................................................30

Table 2-2: Specific activities and Fe quantification of WT and C97G HydA ..................34

Table 3-1: Average moles of Fe and S atoms per moles of reconstituted N75...............66

Table 3-2: The m/z of major peaks of crosslinked products in various BIDBE:N75 ratios ....72

Table 3-3: The m/z of major peaks of crosslinked products in various DTME:N75 ratios ....72

Table 3-4: Free sulfhydryl groups to protein ratios of N75 after various stages of crosslinking with BIDBE in different concentrations.................................73

Table 3-5: Free sulfhydryl groups to protein ratios of N75 after various stages of crosslinking with DTME in different concentrations. ......................................73

Table 3-6: The m/z of major peaks of crosslinked products after being reduced by DTT in various BIDBE:N75 ratios.................................................................76

Table 3-7: The m/z of major peaks of crosslinked products after being reduced by DTT in various DTME:N75 ratios.................................................................76
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Chapter 1

Introduction

1.1 The Photosystem I—[FeFe] hydrogenase (PS I—CaHydA) nanoconstruct

Ever since the onset of the energy crisis, researchers have been looking for an efficient and sustainable alternative energy source by exploiting components of photosynthesis (1-4). In previous works (5-7), Lubner et al. have devised a PS I—CaHydA nanoconstruct that harnesses solar energy to produce molecular hydrogen, H₂, which is a fuel source used in fuel cells. H₂ stores 143 kJ/g of energy that can be released in the presence of oxygen without giving off environmentally harmful greenhouse gases. This is a brilliant application of PS I because the energy stored in the produced H₂ by this method ultimately comes from sunlight, that faithfully shines on the earth abundantly with a solar constant of 1.37 kW/m². This nanoconstruct employs three critical components: 1) photosystem I from *Synechococcus* PCC 7002 (PS I); 2) the H₂-evolving hydrogenase from *Clostridium acetobutylicum* ATCC 824 (CaHydA); and 3) the central connecting molecular wire 1,6-hexanedithiol.

1.2 The light-harvesting photochemical module—PS I

PS I is the light-driven plastocyanin:ferredoxin oxidoreductase that reduces ferredoxin with an electron donated from the oxidation of plastocyanin (8). The electron from plastocyanin is obtained ultimately from the reaction of H₂O oxidation carried out by the manganese center in
PS II. The electrons obtained from H₂O are transferred after illumination from the special pair of chlorophylls in PS II (i.e., P680) and sequentially transferred for reduction of plastoquinone into plastoquinol. Plastoquinol carries these electrons to cytochrome b₆f complex where plastocyanin is reduced and plastoquinol is oxidized.

Cyanobacterial PS I is a protein complex that consists of 12 subunits, 96 chlorophylls, 22 carotenoids, 4 lipids, 2 quinones, and 3 [4Fe-4S] clusters (9). At the core of PS I are 6 chlorophylls, 2 menaquinones, and 3 [4Fe-4S] clusters that compose the electron transport chain (ETC) of PS I (10). Starting from the lumenal side of the thylakoid membrane, two chlorophylls (Chl a and a') form the P700 special pair that acts as the primary electron donor of the ETC.

Previous studies (8, 11, 12) have shown that light energy absorbed through the antenna system of PS I is funneled to P700 in order to excite P700, which leads to the transfer of an electron along the ETC. Downstream of P700, the ETC is split into two branches (A and B) with each branch having sequentially 2 Chl a (A₋₁ and A₀) and 1 menaquinone (A₁). According to the literature, A₋₁ is involved in electron transfer either as part of the ETC between P700 and A₀ (13), or as the primary electron donor at which charge separation is initiated by light energy instead of P700 (14). The excited state of P700 first transfers an electron to the primary electron acceptor, A₀, either along branch A or B. This creates a charge-separation state, P700⁺A₀⁻, which has a lifetime of about 10 ns (15). Sequential transfer of that electron to the secondary electron acceptor, A₁, further prolongs the lifetime to about 10-100μs in the charge-separated state of P700⁺A₀A₁⁻. Branches A and B of the ETC converge onto one [4Fe-4S] cluster, Fₓ, to which the electron is transferred from A₁. The electron is eventually transferred sequentially to two [4Fe-4S] clusters associated with subunit PsaC, first to Fₐ and then to Fₜₜ.

The lifetime of the charge-separated state is prolonged with each transfer along the cofactors on the ETC by increasing the distance between the reduced and oxidized cofactors in PS I (16). When the electron reaches the terminal electron acceptor, Fₜₜ, the charge-separated state
has a lifetime of about 65 ms and must wait for the physiological redox partner (i.e., ferredoxin) to extract the low-potential electron. Because \( F_B \) has a midpoint potential of \(-580\) mV (17, 18), the electrons coming from \( F_B \) can supply sufficient reducing power for many reactions involved in phototrophic metabolism, such as carbon fixation. The abstraction of this electron from the \( F_B \) cluster is one key element of implementing the nanoconstruct design.

1.3 The \( \text{H}_2 \)-evolving catalytic module, hydrogenase (\( \text{H}_2 \text{ase} \))

1.3.1 Different types of \( \text{H}_2 \text{ases} \)

Hydrogenases (\( \text{H}_2 \text{ase} \)) are enzymes that catalyzes the reversible reaction of \( 2\text{H}^+ + 2e^- \rightarrow \text{H}_2 \), which has a standard midpoint potential of \(-420\) mV at pH 7.0 (19). The chemistry occurs deep inside the enzyme, where the active site is buried to avoid \( \text{O}_2 \), that is strongly inhibitory to most \( \text{H}_2 \text{ases} \). The active sites of \( \text{H}_2 \text{ases} \) consist primarily of transitional metal ions (i.e., Fe and Ni) that are abundant on Earth (19). Based upon the composition of their active sites, \( \text{H}_2 \text{ases} \) are categorized into 3 distinct groups: \([\text{NiFe}]\), \([\text{FeFe}]\), and \( \text{H}_2 \)-forming methylenetetrahydromethanopterin dehydrogenase (Hmd) (20, 21). The Hmd \( \text{H}_2 \text{ase} \) will not be discussed further here.

1.3.2 The \([\text{NiFe}]\)-\( \text{H}_2 \text{ase} \)

The \([\text{NiFe}]\) \( \text{H}_2 \text{ase} \) has an active site composed of a Ni atom and an Fe atom bridged by two cysteine residues (Fig. 1-1) (21). The Ni atom also has two other cysteine residues as ligands, and the iron atom has two cyanide (\( \text{CN}^- \)) and one carbon monoxide (\( \text{CO} \)) ligands (21, 22). These \( \text{H}_2 \text{ases} \) are usually heterodimeric if not multimeric (19). Besides the main subunit, these
complexes have accessory subunits containing Fe/S clusters that form part of the ETC for these enzymes. [NiFe]-H₂ases are sometimes referred to as uptake H₂ases because they often participate in the oxidation of H₂ as an energy source in the organisms in which they are discovered (19).

1.3.3 The [FeFe]-H₂ase

In contrast to [NiFe]-H₂ases, [FeFe]-H₂ases have active sites, named the H-cluster, composed of one [4Fe-4S] cluster and two Fe atoms, proximal and distal (Feₚ and Feₜ), named relative to their distance from the [4Fe-4S] subcluster (Fig. 1-2) (19). Feₚ is bridged to the [4Fe-4S] subcluster through a cysteine residue. In turn, the two Fe atoms are bridged by a CO and a dithiolmethylamine as ligands (19, 22). Both Fe atoms have one more CO ligand and one CN⁻ ligand to complete the composition of the H-cluster. These [FeFe]-H₂ases are usually H₂-producing H₂ases that help organisms dispose of excess electrons by reducing protons (21, 23).

1.3.4 The [FeFe]-H₂ase from Clostridium acetobutylicum—CaHydA

The H₂ase chosen for the nanoconstruct is the [FeFe]-H₂ase, CaHydA, from C. acetobutylicum ATCC 824 (5-7). C. acetobutylicum is the fastest reported microorganism for H₂ production from hexose, with a H₂ production rate of 2.4 L L⁻¹ h⁻¹ in a chemostat (24). CaHydA is very similar to the [FeFe]-H₂ase of C. pasteurianum (i.e., CpI). The crystal structure of CpI (Fig. 1-3) reveals an ETC composed of 3 [4Fe-4S] clusters leading from the surface of CpI to the interior where the active site, H-cluster, is buried (25). The three [4Fe-4S] clusters are named FS4A to FS4C starting from the one closest to the H-cluster. CpI also has a [2Fe-2S] cluster that is 17 Å away from FS4C and converges to the ETC at FS4B with a distance of 11 Å. The surface-located FS4C cluster in CpI has one solvent-exposed cysteine, Cys98, out of its four ligands. This
cysteine residue of Cpf corresponds to Cys97 in CaHydA. It is at this residue that a Cys97Gly site-directed mutation was introduced to leave an open coordination site for a thiol-containing molecular wire to access and rescue FS4C chemically (5, 6).

1.3.5 The central molecular wire that links the modules—1,6-hexanedithiol

The molecular wire is the most crucial as well as the most novel component of the nanoconstruct. With the employment of 1,6-hexanedithiol, the sulfhydryl groups on each end of the molecular wire are capable of chemically rescuing Fe/S clusters by replacing their missing cysteine ligand caused by site-directed mutagenesis (5, 6). In the PS I—CaHydA nanoconstruct, Cys13 of PsaC and Cys97 of CaHydA are converted into glycines, which leaves open coordination sites for the molecular wire to rescue the F_{B} and FS4C clusters, respectively. Cys33 of PsaC is also mutated into glycine, because it is in close proximity to the F_{B} cluster and might replace the missing ligand arising from the C13G mutation. It results in a nanoconstruct that links PS I and CaHydA through these two [4Fe-4S] clusters using the molecular wire. In other words, the molecular wire links the terminal electron acceptor site of PS I to the initial electron acceptor site of CaHydA.

A methyl viologen derivative, 1-(3-thiopropyl)-10-(methyl)-4,4'-bipyridinium chloride, has demonstrated the ability of PS I to transfer an electron forward to an external redox cofactor facilitated by the design of a molecular wire (26). The bipyridinium has a 3-carbon chain (i.e., the molecular wire) at one side that ends with a sulfhydryl functional group, which is used to rescue the F_{B} cluster (Fig 1-5). PS I rescued by this bipyridinium exhibits an absorbance increase at 600 nm when illuminated, which has a rate of 53.3 ± 5.1 µmol 4,4'-bipyridinium (mg Chl)\(^{-1}\) h\(^{-1}\) (26). The absorbance increase is due to the reduction of the bipyridinium by electrons transferred from
the F\textsubscript{B} cluster. The bipyridinium construct demonstrates how the F\textsubscript{B} cluster can reduce external redox cofactors when they are brought in close proximity by a molecular wire.

The principle behind the design and function of the molecular wire in the PS I—CaHydA nanoconstruct is the elimination of diffusion as a factor, which would otherwise limit the rate of electron transfer between PS I and CaHydA. Furthermore, the molecular wire has a length of ~10 Å (6), so it brings the ETCs of the two modules into close proximity without uncertainties concerning the distance between PS I and its redox partner, unlike other methods such as fusion proteins or protein scaffolding (27, 28). In essence, the molecular wire brings the two ETCs from two different enzymes to form a single circuitry for electron transfer.

1.4 Expanding the versatility of PS I—CaHydA nanoconstruct

In previous studies (5-7), the PS I–molecular wire–catalytic module nanoconstruct design was conceived as a platform that incorporated an efficient photosensitizer with a H\textsubscript{2}–evolving catalyst. Nevertheless, this platform can have a greater scientific impact if its application expands beyond uses for biofuel production, such as its use in the study of redox-capable factors in place of the catalytic module (26). The application would also benefit if the nanoconstruct assembly is not limited to only chemically rescuing [4Fe-4S] clusters with a molecular wire between Fe/S clusters in the two modules.

This idea was explored with a redox-active protein reporter, N75, cloned from the first 75AA in the N-terminal domain of CaHydA. N75 contains a [2Fe-2S] cluster and has a solvent-exposed cysteine (i.e., Cys39) 10.1 Å away from the cluster (25). It presents an opportunity for the study of using sulphydryl specific crosslinkers to bring an external redox cofactor to PS I without the use of molecular wires for chemical rescue. In addition, the [2Fe-2S] cluster is an external redox cofactor enveloped in a protein environment. This study essentially would explore
the usefulness of this platform not just applied in the field of bioenergy, but as a tool that could be applied in diverse research applications, including its use as a single-electron injection device that can be controlled by sequential illumination.
Figure 1-1: The structure of [NiFe]-H$_2$ase active sites, which possess inorganic CO and CN$^-$ ligands that are uncommon in biology. Catalysis occurs in the middle coordinated by both the Ni and Fe atoms.
Figure 1-2: Active site structure of [FeFe]-H₂ases in the active state. Catalysis at the H-cluster occurs at the Fe₄ atom.
Figure 1-3: Structure of Cpi (A) and detailed schematics of the cofactors in its ETC (B).
Figure 1-4: Proposed structure and detailed components of the PS I—CaHydA nanoconstruct.

The 1,6-hexanediol, PS I, and CaHydA are not drawn to scale.
Figure 1-5: Structure of 1-(3-thiopropyl)-10-(methyl)-4,4’-bipyridinium chloride that can chemically rescue an $F_B$ cluster of PsaC that has C13G/C33G mutations.
Chapter 2

Overexpression of *C. acetobutylicum* [FeFe] Hydrogenase in *S. oneidensis* MR-1

2.1 Introduction

Large quantities of CaHydA are needed for characterization and on-going implementation of the PS I—CaHydA nanoconstruct. Previous studies were performed with CaHydA overproduced from *E. coli* (5, 6). Although the yield of expression is great and typically ample amount of protein can be purified, the specific activity from these proteins are typically low, averaging about 75.6 µmol H₂ (mg of H₂ase)^⁻¹ min⁻¹ (6). This is not optimal compared with HydA expressed in other organisms that have specific activities averaging about 450 to 550 µmol H₂ (mg of H₂ase)^⁻¹ min⁻¹ (24, 29). The activity is even lower for the C97G variant of CaHydA that is required for the implementation of the nanoconstruct. Its specific activity, averaging 10.8 µmol H₂ (mg of H₂ase)^⁻¹ min⁻¹, is usually one order of magnitude lower than that of WT HydA (6). The most probable cause may be attributed to the incomplete maturation of the cofactors of the enzyme, such as the H-cluster. Many other studies have focused on overcoming this problem during the overexpression of various [FeFe]-H₂ases (23, 29, 30).

The active site of CaHydA, the H-cluster, has been a major subject of research, especially concerning investigations of its maturation process (19, 21, 23, 25, 31-34). The maturation of the H-cluster requires at least three essential accessory proteins: HydE, HydF, and HydG. Both HydE (35) and HydG (36, 37) belong to the radical S-adenosylmethionine (AdoMet) enzyme family, while HydF is a GTPase (38-40). HydEFG are responsible for synthesizing the biologically rare CO and CN⁻ ligands for the composition of the H-cluster (34, 36). It has been demonstrated that
HydEFG synthesize the diiron subcluster of the H-cluster with HydF acting as the scaffold protein; HydE and HydG are responsible for synthesizing the CO and CN⁻ ligands for the cluster (34, 37, 40). These accessory proteins are unique for the maturation of [FeFe]-H₂ases, which *E. coli* does not possess since it only has genes encoding [NiFe]-H₂ases in its genome.

In order to give *E. coli* the ability to synthesize the H-cluster while *CaHydA* is being overexpressed, *hydA* from *C. acetobutylicum* was cotransformed with *hydEFG* in a duet plasmid system allowing all four proteins to be expressed simultaneously upon induction by IPTG (6, 23). However, this method does not seem to be an optimal method for maturation of all *CaHydA* overexpressed in *E. coli*, because many active sites remain empty without the H-cluster, thus lowering the specific activity. Perhaps there are some other necessary components for the synthesis of the H-cluster absent in *E. coli*, or the induction with IPTG simply does not allow enough time for the H-cluster maturation machinery to be readily available during the rapid overproduction of *CaHydA*. This calls for a solution that would allow overexpression of *CaHydA* in an environment that also has a robust and readily available H-cluster maturation machinery.

In 2008, Sybirna et al. used *Shewanella oneidensis* MR-1 for the overexpression of HydA cloned from *Chlamydomonas reinhardtii* (*CrHydA*) with high yield in protein amount and impressive specific activity in the range of ~700 µmol H₂ (mg of H₂ase)⁻¹ min⁻¹ (29). *S. oneidensis* MR-1 is a facultative anaerobe that is able to reduce Fe and a variety of organic solvents (41). It possesses [FeFe]-H₂ases and the *hydEFG* genes for the required accessory proteins (42). It also has a well-defined set of protocols for genetic manipulations and cloning (43). All these features make *S. oneidensis* MR-1 an ideal platform upon which to overexpress highly active *CaHydA* for the PS I—*CaHydA* nanoconstruct.
2.2 Methods and Materials

2.2.1 Strains and plasmids construction

The *S. oneidensis* MR-1 hydA'/hyaB' strain along with plasmids pJBC2 and pBBR-hydA1C were obtained from Dr. Alexander Beliaev from Pacific Northwest National Laboratory (PNNL). The hydA'/hyaB' double mutant strain was generated by double homologous recombination and lacked the large subunits of the [FeFe]- and the [NiFe]-H₂ase respectively; therefore, it lacks the ability to evolve H₂ (44). pBBR-hydA1C was a broad range-host plasmid containing hydA1 from *C. reinhardtii* with a Strep-tag®II at the C-terminus (29).

pJBC₂-hydA NS11 was constructed by subcloning the hydA gene from *C. acetobutylicum* from pETDuet-hydAE (23) using the existing NdeI and SalI restriction enzyme sites flanking hydA. pJBC₂-hydA NK16 was constructed by amplifying the Ca hydA gene from pETDuet-hydAE using hydAF1 5’-CTCcatagGGCAAAACAATAATC-3’ and hydAR1 5’-CTCggtaccGCCGAGCTCG-3’, then digested and cloned into pJBC₂ using NdeI and KpnI restriction enzyme sites. Both constructs leave an 8 amino acid long Strep-tag®II (WSHPQFEK) at the C-terminus of CaHydA. pAQ1Ex-hydA was constructed by subcloning the Ca hydA gene from pETDuet-hydAE into pAQ1Ex plasmid using the NdeI and BamHI restriction enzyme sites. The same was done for subcloning C97G hydA from pETDuet to pJBC₂.

2.2.2 *S. oneidensis* MR-1 conjugation procedure

The procedure for transforming *S. oneidensis* MR-1 through conjugation was accomplished using a modified procedure from reports (45). The donor strain was a diaminopimelic acid (DAP)-dependent auxotrophic *E. coli*, WM3064, provided by Dr. Beliaev at PNNL. *E. coli* WM3064 was first transformed with the desired vector through electroporation,
and the resulting strain was grown in LB medium overnight at 37°C with 100µg/mL DAP and appropriate antibiotics. The recipient strain was the \textit{S. oneidensis} MR-1 \textit{hydA'/hyaB'} double mutant strain grown overnight at 28°C. Cells (50 µL) of each culture were mixed with 4 mL of filter-steriled 10mM MgSO$_4$. The cell suspension was filtered through a 25 mm diameter Millipore type HA 0.45 microfilter after 15 min of MgSO$_4$ treatment. Afterwards, the filter was placed cell-side up on top of an LB agar plate supplemented with 100µg/mL DAP and incubated overnight (16-18 hr) at 30°C. Cells were resuspended from the filter by vortexing in LB, then 100 µL of this cell suspension was plated on LB agar medium supplemented with appropriate antibiotics. Several dilutions were needed for the plates to contain individually separate colonies that appeared red due to the abundance of \textit{c-type cytochromes} in \textit{S. oneidensis} MR-1 (43). Due to contamination with \textit{E. coli}, it was necessary to select and re-streak red colonies at least three times in order to have contaminant-free \textit{S. oneidensis} MR-1 stains. Kovac’s reagent was used to detect indole production from tryptophan indicating the presence of \textit{E. coli} contaminants.

### 2.2.3 Recombinant \textit{CaHydA} vectors expression level analysis

\textit{S. oneidensis} MR-1 \textit{hydA'/hyaB'} double mutant strains were grown to 0.6 OD$_{600}$ from an overnight inoculum, then 0.5 mM IPTG was added to initiate the expression at 28°C. After 3 h, cells were harvested by centrifugation at 6000 $\times$ g and resuspended with Bugbuster™ in a cell to Bugbuster™ 1:5 w/v ration. Cell suspensions were shaken for 20 min and SDS loading buffer was added prior to analysis by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE). Afterward, polypeptides were electrophoretically transferred to a nitrocellulose membrane using Bio-Rad Trans-Blot® SD semi-dry transfer cell at 15V for 20-25 min. The membrane was subsequently blocked with 5% w/v milk and 2% w/v BSA in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05 % (v/v) Tween 20) for 1 h at RT, and anti-\textit{CaHydA} rabbit antibodies
was added to the blocking TBST buffer to react with blot for another 1 h at RT or overnight at 4 °C. Subsequently, the membrane was washed 3 times with TBST buffer in RT. Then, the membrane was incubated with 2nd antibodies in TBST for 1 hr in RT, and washed with TBST 3 x before chromogenic or chemiluminescence development. The 2nd antibodies (anti-rabbit goat antibodies) were either conjugated to alkaline phosphatase or horseradish peroxidase for chromogenic or chemiluminescence detection, respectively.

2.2.4 H₂ formation assay on S. oneidensis MR-1 strains

_S. oneidensis_ MR-1 _hydA/hyaB_ double mutant strains harboring pJBC₂ and pBBR vectors were grown aerobically at 28 °C until OD₆₀₀ nm = 0.6, then 0.5 mM IPTG was added to induce synthesis of HydA. Upon induction, five different anaerobic respiration substrates, ferric citrate (10mM), fumarate (20mM), sodium thiosulfate (20mM), trimethylamine N-oxide (TMAO, 10mM), dimethylsulfoxide (DMSO, 10mM), were added with or without ferric chloride (20 mM) as Fe supplements. After being purged with Ar (g), cultures were grown overnight in 28 °C. All following steps were carried out in an anaerobic chamber. 1 mL of each culture was measured for OD₆₀₀ nm, and cells were then harvested by centrifugation and resuspended with 0.5 mL resuspension buffer (150 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). Samples were then mixed with 0.5 mL of reaction buffer (50 mM potassium phosphate pH 7.0, 10 mM methyl viologen, 20 mM Na-dithionite, 6 mM NaOH, 0.2% Triton X-100) and incubated in 37 °C to allow maximal hydrogenase activity (23). H₂ evolution was measured by taking 200 µL of headspace gas at different time points, which was analyzed by gas chromatography using a Shimadzu model GC-8A1T gas chromatograph.
2.2.5 Heterologous expression, purification, and characterization of CaHydA

Heterologous expression of CaHydA was done by IPTG induction when culture were grown aerobically to 0.6 OD<sub>600</sub> at 28 °C from an overnight inoculum. 10 mM TMAO was added to the culture as a substrate for anaerobic respiration. Subsequently, 0.1 mM IPTG was added to the culture, which was then sealed and purged with Ar (g) to begin induction. The IPTG-induced culture was allowed to grow overnight at 28 °C. After ~16h of incubation, cells were centrifuged at 6000 × g anaerobically in sealed 1 L centrifugation bottles added with 2 mM Na dithionite. All following steps were carried out in an anaerobic chamber. Cell pellets were resuspended with breaking buffer (150 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT, 1 mM Na-dithionite, 1 mM PMSF, 0.3 nM avidin, 5% (v/v) glycerol). The resuspension was sonicated in 10 intervals of 15 sec and 5 min sonication/resting cycles. Cell lysate was then centrifuged at 19000 × g for 30 min in sealed 30 mL centrifugation tubes in Beckman® L8-80M ultracentrifuge. Supernatant was saved as crude extract, while the pellet was discarded as cell debris. The crude extract was absorbed onto a Strep-Tactin® Superflow® high-capacity column that had been equilibrated with breaking buffer. After the crude extract was loaded, the column was washed with 5× column volume with breaking buffer, and bound proteins were eluted using breaking buffer supplemented with 2.5 mM desthiobiotin. Eluted fractions were analyzed first by SDS-PAGE. The fractions with CaHydA presence were diluted 100-fold with storage buffer (150 mM Tris-HCl, pH 8.0, 100mM NaCl, 1 mM dithiothreitol, 15% (v/v) glycerol) and concentrated by ultrafiltration with Amicon stirred cells fitted with a Millipore regenerated cellulose ultrafiltration membrane having 30000 NMWL.

The absorption spectrum of the concentrated protein solution was measured with a GENESYS™ 10 UV Scanning spectrophotometer. Activity of purified CaHydA was determined by an activity assay using 0.5 mL of concentrate and 0.5 mL reaction buffer (50 mM potassium
phosphate pH 7.0; 10 mM MV; 20mM Na-dithionite; 6 mM NaOH). The assay was incubated in 37 °C during which H₂ evolution was monitored by taking 200 µL of headspace gas in different time points that were analyzed by gas chromatography.

Protein identification by tryptic peptide mass fingerprinting was performed by the Proteomics and Mass Spectrometry Core Facility at The Pennsylvania State University at University Park. Samples were submitted as gel fragments containing a band ~65.5 kDa and subjected to in-gel protein digestion with trypsin. The resulting peptides were analyzed by tandem mass spectrometry (LC-MS/MS), and searched against an organism-specific (C. acetobutylicum ATCC 824) database using ProteinLynx Global SERVER.

### 2.2.6 Iron quantification assay

Purified WT/C97G CaHydA were assayed with a laboratory established protocol (46) in order to quantify the Fe to protein ratio. 300µL of protein was mixed with 300µL of reagent A (4.5% w/v SDS, 150 mM Na-acetate) and 300 µL of reagent B (274 mM ascorbic acid, 8.5mM Na-metabisulfate, 714 mM Na-acetate). The reaction was incubated at 30°C for 15min, and then 15µL of reagent C (1.8% w/v ferrene) was added. Finally, the absorbances of samples were measured at 593 nm.

### 2.2.7 Purification of PS I, associated photosystem proteins, and preparation of photosystem Fₓ core particles

The $psaD$ gene was amplified through PCR using $psaD5FN$-GGTcatatgTCTAACGAGCTTACTGG-3’ and $psaD3RH$-GTTaagettAGGCTTCGTAGGGG-3’. The PCR product was digested with NdeI and HindIII then ligated to pET-21a(+) as expression vector in BL21 (DE3).
The gene encoding \textit{C13/33G psaC} was amplified through PCR using psaC5FNdeI 5’-CACcatatgTCTCATAAGGTAAAATTTAC-3’ and psaC3RHindIII 5’-GGCaagcttAGTAGGCCAGACCC-3’. The PCR product was digested with NdeI and HindIII then ligated to pET-21a(+) as expression vector in BL21 (DE3) \textit{ΔiscR}.

The expression and purification of PsaD and C13/33G PsaC were modified from previous protocols (47). Both PsaD and C13/33G PsaC were expressed as inclusion bodies, and both had to be denatured with 9M urea prior to purification using gel filtration chromatography with Sephadex® G50 resin from Sigma-Aldrich®. C13/33G PsaC was reconstituted in an anoxic chamber according to previously described protocol (47). Purification of PS I and preparation of photosystem F\textsubscript{X} core particles were performed following previously described protocols (48).

\textbf{2.2.8 Rebuilding of PS I with heterologously expressed PsaD and C13/33G PsaC and tethering with C97G HydA variant}

All steps were carried out in an anoxic chamber. PS I was rebuilt with photosystem F\textsubscript{X} core particles by incubating the complexes with heterologously expressed PsaD and C13/33G PsaC from \textit{E. coli} in a 1:10:20 ratio, respectively. Overnight incubation at RT was followed by 100-fold dilution with PS I buffer (50 mM Tris-HCl pH 8.3, 10 mM NaCl, 10 mM MgCl\textsubscript{2}, 0.05% v/v Triton X-100, 15% v/v glycerol), and the protein was subsequently concentrated by ultrafiltration with an Amicon stirred cell fitted with Millipore regenerated cellulose ultrafiltration membranes having 100000 NMWL. Rebuilt PS I was then tethered to C97G CaHydA by 1,6-hexanediol at a 1:20:30 ratio, respectively. The tethering reaction was incubated overnight at RT to allow nanoconstruct assembly. Eventually, the PS I—CaHydA nanoconstruct was tested for H\textsubscript{2} evolution under illumination. The nanoconstruct solution was aliquoted (250 µl) in sealed vials supplied with 100 mM ascorbate and 30 µM phenazine methosulphate (PMS) as sacrificial
electron donors and mediators. The headspace gas (200 µL) from these vials was analyzed at set time points by gas chromatography using a Shimadzu model GC-8AIT instrument.

2.3 Results

2.3.1 Transconjugation of *S. oneidensis* MR-1 hydA’/hyaB’ double mutant

Kovac’s reagent showed that some of the transconjugates were still contaminated with *E. coli*. Shown in Figure 2-1 are *E. coli* and *E. coli* contaminated plates indicated by the appearance of quinoidal red-violet compound confirming the presence of indole producing *E. coli*. On the other hand, *S. oneidensis* MR-1 double mutant strain and 3rd generation plates with transconjugants were yellow after addition of Kovac’s reagent, which indicated no formation of indole and hence absence of *E. coli*. This reassured the purity of the culture used to express CaHydA. The *E. coli* contaminant was able to grow in the selective medium by obtaining the expression plasmid pJBC2, which enabled *E. coli* to express CaHydA in the absence of HydEFG. These CaHydA expressed in *E. coli* would lack the H-cluster and contribute to the lowered specific activity of purified CaHydA from these cultures.

2.3.2 H₂ formation assay on *S. oneidensis* MR-1 strains

Five vectors, (pETDuet-hydA, pAQ1Ex-hydA, pBBR-hydA1C, pJBC2-hydA NS11 and NK16) were tested for expression level of HydA in *S. oneidensis* MR-1 hydA’/hyaB’ double mutant. The highest expression of HydA was detected with pJBC2-derived vectors. Shown in Figure 2-2, pJBC2-hydA NS11 and NK16 cultures expressed proteins detected in immunoblot at the 65.5-kDa region. Strains harboring other vectors had little expression of what could be HydA
around the same region. The plasmids pETDuet, pBBR, and pAQ1Ex corresponded to the lowest to highest expression levels, respectively.

This order of expression levels is logical since *S. oneidensis* MR-1 *hydA*/*hyaB* double mutant lacks a T7 RNA polymerase. Therefore, the T7 promoter on pETDuet will be ineffective in *S. oneidensis* MR-1. Both pJBC2 and pBBR use the lac promoter, and pJBC2 is lac-inducible due to the presence of the lac operator. In this manner, pJBC2 surpasses pBBR in terms of expression level and becomes the primary choice for increasing heterologous expression of CaHydA. While the cpcBA promoter on pAQ1Ex is a strong promoter in *E. coli* (49) its effectiveness in *S. oneidensis* MR-1 *hydA*/*hyaB* was unknown. Nevertheless, its expression level clearly was lower than the expression level from pJBC2-derived vectors.

### 2.3.3 H₂ formation assay on *S. oneidensis* MR-1 strains with different anaerobic respiratory substrates

The general trend was that anoxic respiration substrates alone were better than having Fe³⁺ as an iron supplement (Table 2-1). It is possible that Fe³⁺ plays a bigger role in participating in the anaerobic respiration of *S. oneidensis* MR-1 than to supplement iron levels to support Fe/S cluster biosynthesis. The differences between substrates somewhat mimicked the trend observed in a previous publication (29). Growth on DMSO was so low that cell densities from these cultures were minimal (data not shown). While other substrates yielded a H₂ evolution rate of 5-20 nmol H₂ mg⁻¹ min⁻¹, cultures grown with TMAO had an evolution rate of about 300-400 nmol H₂ mg⁻¹ min⁻¹. These rates were at least one order of magnitude higher than rates from all other substrates (Table 2-1). Therefore, TMAO alone was used as component for the expression medium in all subsequent studies on the expression of CaHydA.
2.3.4 Heterologous expression, purification, and characterization of CaHydA

Heterologous expression of CaHydA was carried out primarily with *S. oneidensis* MR-1 hydA’/hyaB’ transconjugated with pJBC2-hydA NS11 and NK16. Both strains and their C97G variants produced CaHydA with same amino acid sequence and were different only at which restriction enzymes (SalI or KpnI) was used for cloning at the 3’ end of the gene. These CaHydA had a C-terminal Strep-tag®II that was used for isolating CaHydA through the use of affinity chromatography. After cells were lysed, both WT CaHydA and C97G CaHydA were occurred in the soluble fraction of the cell lysate, and a one-step purification by affinity chromatography yielded a high purity solution of CaHydA (Fig. 2-3). UV-visible spectroscopy revealed spectrum of the purified protein having a slight shoulder at wavelengths 400-450 nm that suggested the presences of iron sulfur cluster(s) in the protein (Fig. 2-4). Mass spectrometric analysis of tryptic peptides from a polypeptide band at the 65-kDa on SDS-PAGE gels indicated that the purified protein was mostly CaHydA with over 50% coverage of the CaHydA amino acid sequence (Fig. 2-5).

2.3.5 H$_2$ evolution rate of purified CaHydA and PS I—CaHydA nanoconstrict

After the purified protein was confirmed to be CaHydA, the various proteins were assayed to determine their specific activities. Since the specific activities (Table 2-2) were low compared to the literatures (23, 29), Fe quantification was also done to determine the integrity of cofactors in CaHydA. As shown in Table 2-2, while the Fe to protein ratio in purified C97G CaHydA was close to the ideal 20:1 ratio, the ratio in purified WT CaHydA was significantly lower than the ideal ratio. Nevertheless, PS I—CaHydA nanoconstruct that employed C97G
CaHydA expressed from *S. oneidensis* MR-1 yielded a satisfactory rate of 24.0 µmol H₂ (mg Chl)⁻¹ h⁻¹ (Fig. 2-6) compared to results from a previous publication (6).

2.4 Discussion

The CaHydA expressed from *S. oneidensis* MR-1 had such low specific activities that it was evident certain elements in this expression system were incompatible. Sybirna and colleagues (29) searched the gene sequence of *C. reinhardtii hydA* against codon usage data of different organisms to determine which bacteria were most suited for the expression of CrHydA while possessing putative homologues of *C. reinhardtii* HydEFG. A similar analysis done with the codons in CahydA revealed a much different situation, because hydA contains many codons rarely used by *S. oneidensis* MR-1 (Fig. 2-7). Conversion of rare codons to more frequently used codons of *S. oneidensis* MR-1 would possibly improve this overexpression system with respect to the yield of CaHydA. However, because a substantial amount of protein was expressed (Fig. 2-3), translation inefficiencies of *Ca hydA* do not seem to be the major cause of low specific activities. Perhaps this codon incompatibility will become a larger issue when this expression system is in the optimization for production yield.

The main challenge in obtaining active CaHydA is not how to produce the protein, but how to produce the protein together with its cofactors, Fe/S clusters and the H-cluster active site. While the Fe/S clusters and Fe/S subcluster in the active site are synthesized by the ISC system (30), the di-Fe subcluster of the H-cluster has been extensively studied, and there is a clear consensus that three accessory proteins (i.e., HydEFG) are needed for the synthesis of the CO and CN⁻ ligands and the assembly and insertion of the H-cluster into HydA. Two different scenarios are considered to explain the incomplete cofactors in CaHydA. Either the materials consumed in
cluster synthesis (e.g., Fe and S) are limiting, or the cluster is not assembled as quickly as CaHydA is synthesized.

Perhaps this is best explained in the H$_2$ formation assays that led to optimization of the supplements used in the expression medium. It was demonstrated (Table 2-1) that additional Fe$^{3+}$ added as an Fe supplement did not positively affect H$_2$ evolution. Thus, Fe$^{3+}$ should not be the limiting factor in CaHydA expression and maturation. In addition, Fe quantification of purified WT CaHydA indicates incomplete cofactors with 11.12±1.22 Fe:protein ratio (Table 2-2). This is evidence that the timing between cluster maturation and protein expression is not synchronized. More specifically, the current strain creates an environment of slow cluster maturation and quicker CaHydA expression in contrast. It is noteworthy that while the Fe:protein ratio of WT CaHydA is half that of C97G CaHydA, their specific activities were nearly the same. This indicates that WT CaHydA would probably have a comparatively higher specific activity when WT and C97G CaHydA have the same Fe:protein ratio.

This was the rationale for experimenting with different anaerobic respiration substrates. Because many H$_2$ases in S. oneidensis MR-1 are used as part of its anaerobic respiration for dumping excess electrons (42), it is logical to reason that upregulation of HydA maturation proteins can be achieved by growing Shewanella spp. under conditions where the bacteria employs HydA as its sole, or at least as a major, part of its respiration. Substrates tested with the CaHydA expression strains are conventional anaerobic respiration substrates used for Shewanella spp. (50-52). While TMAO is probably best for inducing HydEFG expression in S. oneidensis MR-1 based on the H$_2$ formation assay (Table 2-1), the current protocol still has not reached the theoretically optimal conditions for increasing HydEFG expression compared to CaHydA specific activities reported in the literature (23, 29).

Potential obstacles may be due to inadequate nutrient or environmental signals provided by the current expression medium. Although the results were not satisfactory (data not shown),
different media (e.g., TB and SB) were tested to see if inadequate carbon source or electron-accepting substrates were contributing factors causing lack of energy for protein and cofactor synthesis under anoxic conditions. Nevertheless, other options are still available. Lactate and pyruvate are often used, either separately or together, as carbon sources in media for the study of *Shewanella* spp. under anoxic conditions. These could be the environmental signals that lead to increased expression of the accessory proteins for biogenesis of mature HydA. Furthermore, Sybirna and colleagues (29) used peptie soy broth to express highly active *CrHydA*. The casein digest peptone in the peptie soy broth might ease the intake of tyrosine, which was illustrated to be an important substrate for the synthesis of the CN/CO ligands in the H-cluster (53).

Another direction can be taken by enhancing Fe/S cluster biosynthesis. Manipulations can be performed on the genome to alter cellular regulation to produce FeS clusters constantly. This can be accomplished by knocking out the *iscR* gene of the ISC system. IscR is the repressor of the ISC gene cluster and controls FeS cluster biosynthesis attributed to the ISC system (30). Deleting *iscR* should render the ISC system unchecked by its repressor mechanism and the ISC Fe/S cluster synthesis system should then be expressed continuously. This has been done to overproduce *CaHydA* in *E. coli* with high activity (30). Perhaps the same can be done in *S. oneidensis* MR-1 by knocking out SO_2263 (Fig. 2-8 and 2-9), the annotated *iscR* gene in *S. oneidensis* MR-1 (44).

Besides modifying *S. oneidensis* MR-1 to allow more robust Fe/S cluster biosynthesis and H-cluster maturation machineries, it is possible that a slower rate of *CaHydA* expression is also required to synchronize the two processes. This implies that the use of *lac* promoter along with IPTG induction on pJBC₂ is expressing *CaHydA* so quickly that it outpaces the cofactor maturation process. In previous reports in the literature, pBBR has been used as the expression vector to yield active *CrHydA* (29). It has a *lac* promoter just as in pJBC₂, but is not *lac* inducible because of the absence of a *lac* operator. Therefore, using pBBR to express *CaHydA*
continuously at a low level might be better than expressing CaHydA at a high level using IPTG inducible pJBC2. By using these manipulations to slow CaHydA expression while speeding up cofactor maturation processes, there is a great possibility that the major challenges of synchronizing the two processes can be overcome to yield highly active CaHydA.

Although the current specific activity of CaHydA is low, the PS I—CaHydA nanoconstruct nevertheless produced a substantial rate of H₂ production. Because successful tethering of C97G CaHydA to PS I depends on the presence of an intact FS4C cluster, the Fe quantification of C97G CaHydA indicates an almost ideal Fe:protein ratio, which is encouraging evidence concerning the effectiveness of the nanoconstruct. Perhaps the expression of C97G CaHydA is more successful than expression of WT CaHydA under the currently employed conditions with S. oneidensis MR-1.
Figure 2-1: LB agar media plated with *E. coli* (A), *S. oneidensis* MR-1 *hydA'/hyaB'* double mutant (B), double mutant with pJBC₂-*hydA* NS11—1ˢᵗ generation (C), and double mutant with pJBC₂-*hydA* NS11—3ʳᵈ generation (D). Plates appearing red indicate the presence of a quinoidal red-violet compound that confirms the presence of indole-producing *E. coli*. 
Figure 2-2: Immunoblot of purified WT CaHydA (A), and cell lysates from *S. oneidensis* MR-1 double mutant transconjugated with pAQ1Ex-hydA (B), pJBC2-hydA NS11 (C), pJBC2-hydA NK16 (D), pBBR-hydA1C (E), pETDuet-hydA (F), along with protein ladder (Ld; masses in kDa). Red arrows indicate the major band (A) and the thin minor bands (B, C, D) at the 65-kDa region where expressed CaHydA should occur.
Table 2-I: H₂ evolution rate (nmol H₂ mg⁻¹ min⁻¹) of *S. oneidensis* MR-1 *hydA/hyaB* double mutant (MR-1), double mutant transconjugged with pBBR-*hydA*1C (pBBR), and with pJBC₂-*hydA* NS11 (pJBC₂) when undergoing HydA expression in different anaerobic respiration substrates with (A) or without (B) ferric chloride (20mM) as iron supplement.

<table>
<thead>
<tr>
<th></th>
<th>Ferric citrate (10mM)</th>
<th>Fumarate (20mM)</th>
<th>Na Thiosulfate (20mM)</th>
<th>TMAO (10mM)</th>
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<tr>
<td><strong>A</strong></td>
<td></td>
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<tr>
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Figure 2-3: SDS-PAGE visualized through Coomassie Brilliant Blue staining (A) and immunoblot (B) of fractions from the purification steps of WT CaHydA; SDS PAGE stained with Coomassie Brilliant Blue (C) for analyzing the purification steps of C97G CaHydA: protein ladder (Ld), cell lysate (CL), crude extract (CE), cell debris pellet (CP), affinity chromatography flowthrough (FT), wash (WH), elution (EL) fractions, and purified wild-type Ca HydA (WT).
Figure 2-4: UV-visible spectrum of purified WT CaHydA (6.7 µM) with slight shoulder at 400-450 nm.
Figure 2-5: Coverage map of CaHydA by tryptic peptides generated from purified WT CaHydA analyzed by mass spectrometry. Detected peptide fragments (bold font) covered 52.23% of CaHydA.
Table 2-2: Specific activities and Fe quantification of purified WT and C97G CaHydA. While specific activities were lower than literature reports, the Fe to protein ratio of C97G CaHydA was closer to the ideal ratio than that of WT CaHydA.

<table>
<thead>
<tr>
<th>CaHydA Specific Activity</th>
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<th>C97G CaHydA</th>
</tr>
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<tr>
<td>23.83 µmol H₂ mg⁻¹ min⁻¹</td>
<td>28.8 µmol H₂ mg⁻¹ min⁻¹</td>
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</tr>
<tr>
<td>Calculated Fe:protein ratio</td>
<td>11.12±1.22</td>
<td>19.43±1.12</td>
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Figure 2-6: Rate of H₂ evolution of PS I—CaHydA nanoconstruct using C97G CaHydA expressed from *S. oneidensis* MR-1. The calculated rate was 24.0 µmol H₂ (mg Chl)⁻¹ h⁻¹.
CodonTable: [Link to Codon Table]

Ordinate (y-axis): frequency
<20%  <10%

Sequence derived from Clostridium Acetobutylicum

[Graph showing codon frequencies]

created: 27.09.2010
Figure 2-7: Rare codon analysis of *C. acetobutylicum* hydA against codon usage frequencies of *S. oneidensis* MR-1. Codons colored in black are codons appear more than 20% frequency in the genome of *S. oneidensis* MR-1. Codons colored in gray and red appear less than 20% and 10% in the genome of *S. oneidensis* MR-1, respectively.
Figure 2-8: The annotated ISC gene cluster in *S. oneidensis* MR-1
Figure 2-9: Sequence alignment of *iscR* in *E. coli* and *S. oneidensis* MR-1 reveals a 61% identity between the two genes.
Chapter 3


3.1 Introduction

In order to expand upon the design of the PS I nanoconstruct to provide for greater versatility, the N-terminal domain fragment of CaHydA was used to demonstrate that the nanoconstruct platform concept could be applied to different research scenarios using methods other than tethering two [4Fe-4S] clusters together by a homo-bifunctional molecular wire (1,6-hexanedithiol). This study was facilitated by the vast amount of knowledge that already existed concerning CpI, which is very similar to CaHydA (25, 54-58). The first 76 amino acids (AA) from the N-terminal domain of CpI, termed HN76, was first studied by a group in France led by Jacques Meyer in 1998 (57). It contains a [2Fe-2S] cluster ligated by Cys34, Cys46, Cys49, and Cys62 and exhibits a ferredoxin-like folding. The [2Fe-2S] cluster has a midpoint potential of \(-400\text{mV}\) (vs. SHE), and is EPR active when reduced with one electron from the +2 to +1 state. It has also been well characterized spectroscopically by UV-visible spectroscopy and circular dichromism.

A crystal structure of CpI was published by John Peters and coworkers in 1998 (25), and has confirmed much of the structural information found by Meyer’s studies (57, 58). When the AA sequence of CpI is aligned with that of CaHydA, the corresponding cysteine residues that ligate the [2Fe-2S] cluster found to be Cys34, Cys45, Cys48, and Cys61 in CaHydA. There also exists a solvent exposed cysteine residue, Cys39, only 10.1 Å away from the [2Fe-2S] cluster in
CaHydA predicted from the structure of CpI (Fig. 3-1). These features on the N-terminal domain of CaHydA provide us with a unique opportunity to apply the nanoconstruct platform through a different method of implementation.

Firstly, although [2Fe-2S] clusters are in the same class of prosthetic group as [4Fe-4S] clusters, this study will be able to test a different kind of protein cofactor as an entry portal for the electron coming from PS I. Secondly, the solvent exposed Cys39 can be exploited using a hetero-bifunctional molecule that can substitute for the homo-bifunctional molecular wire, 1,6-hexanedithiol. This hetero-bifunctional molecule should crosslink to a sulphhydryl group (i.e., Cys39) at one end and will have a free sulphhydryl group at the other end to rescue $F_B$ with the C13G mutation chemically. Thirdly, the properties of the [2Fe-2S] cluster allow a greater variety of techniques available for the detection of electron transfer from PS I to this external protein redox factor, such as spectroscopic and biophysical techniques, in contrast with measurement of catalytic activities as for the PS I—CaHydA nanoconstruct.

In comparison to the PS I—CaHydA nanoconstruct, two components will be reengineered. Firstly, the catalytic model (CaHydA) will be replaced by a protein reporter that is the first 75AA of the N-terminal domain in CaHydA. This protein fragment, N75, contains one [2Fe-2S] cluster just as HN76. Secondly, a hetero-bifunctional molecule will replace the homo-bifunctional molecular wire. Two molecules, BIDBE and DTME, will be used to crosslink to Cys39 and rescue $F_B$. BIDBE and DTME crosslink sulphhydryl groups with iodoacetyl and maleimide groups, respectively, which will be located at both ends of the molecule. A disulphide bond in the middle of the molecule will mask the free sulphhydryl groups that will be employed in chemical rescue of the $F_B$ cluster. After crosslinking to Cys39, the disulphide bond in the middle of the crosslinkers will be reduced by DTT to expose the free sulphhydryl group available for $F_B$ chemical rescue. The successful implementation of this PS I/crosslinker/reporter module
nanoconstruct will expand the application of this platform with PS I as an efficient photosensitizer.

3.2 Methods and Materials

3.2.1 Strains and plasmids construction

The *C. acetobutylicum* *hydA* were previously cloned into the plasmid pJBC2. The N-terminal 225 bp of *hydA* was amplified by HydAN76F 5’-AGGAAACACatatgAAAAAACAATAATCTTAAATG-3’ and HydAN76R 5’-AGCgatccTGAATCGGATTCTG-3’. The PCR product was digested with NdeI and BamHI in order to be subcloned into pET26b-*HoxK*dCterS, which possessed a Strep-tag®II sequence downstream from the 3’ end of the restriction site of BamHI. The resulting pET26bN75 was then used to transform BL21 (DE3) ΔiscR pRARE.

BL21 (DE3) ΔiscR strain was generously provided by Dr. John W Peters from Montana State University. *iscR* was deleted from BL21 (DE3) via double homologous recombination with DNA introduced by bacteriophage P1. Its identity was confirmed by PCR using *iscRFw* 5’-CATTTTCTCGGCAACACGCGGGTCC-3’ and *iscRRev* 5’-ATCCGACAAACAGGTACAAACGCCACG-3’ that flanked the *iscR* gene. Subsequently, BL21 (DE3) ΔiscR pRARE was constructed in order to give the strain ability to translate rare codons more effectively. pRARE was purified through QIAGEN QIAprep® Miniprep, and its presence is confirmed by PCR product of the *cat* gene using CmRF1 5’-ACGGGGGCGAAGAAGTTGTC-3’ and CmRB1 5’-CGGGCGTATTTTTTGAGTTAGTTGTC-3’. The resulting transformant gains CmR due to the uptake of pRARE. Strain SY1 was constructed after BL21 (DE3) ΔiscR pRARE
was transformed with pET26bN76 and colonies were selected upon growth on LB medium supplemented with chloramphenicol and kanamycin.

3.2.2 Heterologous expression, purification, and characterization of N75

Strain SY1 was grown in LB at 37°C in 180 rpm until OD_{600 nm} reached 0.6 in oxic flasks. The agitation was decreased to 80 rpm and cultures were incubated overnight. Cells were pelleted by centrifugation at 6000 × g into a dark brown pellet, and were subsequently resuspended in breaking buffer (100 mM Tris pH 8.0, 10 mM DTT, 3 nM avidin). Afterward, cells were lysed either by passage through a French press or sonication, and the resulting lysate was clarified by centrifugation at 19,000 × g to remove cell debris in sealed 30 mL centrifugation tubes in a Beckman® L8-80M ultracentrifuge. From this point on, all following steps were carried out in an anoxic chamber. The resulting crude extract was loaded onto a DEAE anion-exchange chromatography column equilibrated with EB (100 mM Tris-HCl, pH 8.0). After absorbing the proteins, the column was washed with 3× column volumes of EB containing 50 mM NaCl, and proteins were eluted with EB containing 100 mM NaCl. The eluate was then loaded on a Strep-Tactin® Superflow® high-capacity resin column equilibrated with EB. After the eluate from the DEAE column was loaded, the Strep-Tactin® column was washed 5× column volumes of EB, and then bound proteins were eluted with EB supplemented with 2.5 mM desthiobiotin. Eluted fractions were analyzed first by SDS-PAGE on gels composed of 15% (w/v) acrylamide. The fractions containing N75 were then subjected to Fe/S reconstitution after the protein concentration was determined by Bradford assay according to a protocol provided by the manufacturer (Thermo Scientific).
3.2.3 Guanidine denaturation of Fe/S cluster in N75

The [2Fe-2S] cluster of N75 was denatured in 6 M guanidine (pH 7.0), 2.5 mM EDTA incubated in 37°C for ~1 h. Since N75 only had 1 aromatic residue (i.e., W81), an extinction coefficient at OD$_{280}$ nm was calculated from the UV-visible spectra of 67.5 µM tryptophan dissolved in 6 M guanidine denaturation buffer. Based on this extinction coefficient and spectra of denatured N75, a correction factor of 1.52 was determined by the ratio of protein concentrations calculated from OD$_{280}$ nm and Bradford assay.

3.2.4 Fe and S reconstitution, Fe and S quantification of N75

All steps were carried out in an anoxic chamber. The protein solution was made to have at most 100 µM N75 in 50 mM Tris-HCl, pH 8.3 and 4% β-ME. N75 was reduced in the solution for 20 min in RT with constant stirring. 20 mM Fe(NH$_4$)$_2$(SO$_4$)$_2$•6H$_2$O dissolved in 50 mM Tris-HCl, pH 8.3 was added to the protein solution drop-wise at a rate of 1 drop/sec. The protein solution was then incubated on ice for 30 min. Afterward, 20mM Na$_2$S dissolved in 50 mM Tris-HCl, pH 8.3 was added to the protein solution drop-wise at a rate of 1 drop per 4 sec. The resulting protein solution was then incubated overnight at 4 °C. The resulting dark brown solution of N75 was repeatedly diluted 6-fold and concentrated until the effluent was colorless in an Amicon stirred cell fitted with a Millipore, regenerated cellulose ultrafiltration membrane having 3000 NMWL. N75 was further concentrated routinely to about ~800 µM before being desalted on PD10 columns. The PD10 columns were equilibrated by either 100 mM Tris-HCl pH 8.0 or 100 mM Na-phosphate, pH 7.4 with 150 mM NaCl. The quantities of Fe and S in reconstituted N75 were determined according to previously described procedures (46, 59).
### 3.2.5 Spectroscopic measurements of N76 characterizing the [2Fe-2S] clusters

All UV-visible spectroscopy measurements were done with a Cary TM-14 spectrophotometer modernized for computerized data acquisition by OLIS, Inc. 9.5GHz X-band low temperature CW-EPR was carried out using a Bruker ELEXSYS spectrometer equipped with the ELEXSYS Super High Sensitivity Probehead. The probehead was equipped with an ESR900 continuous flow cryostat feed cooled with liquid helium through a LLT series liquid helium transfer tube. Light-induced CW-EPR spectra were taken under illumination from a Verdi™ V-5 Diode-Pumped Laser (Coherent Inc, Santa Clara, CA.) powered to 1.0 W. Midpoint potentials were determined by a previously described protocol (60).

### 3.2.6 Crosslinkers preparation and utilizations

*N,N*-Bis(α-iodoacetyl)-2,2’-dithiobis(ethylamine) (BIDBE) was synthesized according to a previously described protocol (61). The reagents needed for BIDBE were purchased from Sigma-Aldrich®. Dithiobismaleimidoethane (DTME) was purchased from Thermo Scientific. BIDBE and DTME were dissolved in DMSO at concentrations that allowed DMSO to be less than 5% (v/v) final concentration after addition to the protein solution. Both BIDBE and DTME were incubated at RT under dark with N75 solubilized in 100 mM Tris-HCl, pH 8.0 for ~3 h and 100 mM Na-phosphate, pH 7.4 with 150 mM NaCl for 1 h, respectively.

The ratio of crosslinker to N75 had to be optimized through studies done by UV-visible spectroscopy and mass spectrometry. The optimal ratio was found to be 1:1 crosslinker to N75. After incubation with the crosslinker, the solution was desalted on a PD10 column. The desalted solution was reduced with 10 mM DTT for 20 min at RT, which was followed by a 2nd desalting step using a PD10 column. The identities of the crosslinkers were confirmed by LC-MS/MS.
using Waters Synapt HDMS hybrid QTOF spectrometer coupled to a Waters Acquity UPLC system. The crosslinked N75 was also analyzed using an ABI Voyager DE-PRO Reflectron MALDI-TOF mass spectrometer. These samples were prepared in 70%:30% H₂O:acetonitrile with 0.2% TFA. All mass spectrometry measurements were done by Anne Stanley in the Proteins and Mass Spectrometry Core Facility at College of Medicine (Hershey), The Pennsylvania State University.

The ratio of free sulphydryl groups to N75 concentration was determined with Ellman’s reagent following a protocol of the manufacturer (Thermo Scientific). The concentration of sulphydryl groups determined from OD₄₁₂ nm was corrected by substracting the OD₄₁₂ nm of N75 measured prior to performing the assay.

### 3.2.7 Construction of rebuilt PS I and PS I—N76 nanoconstruct

PS I, PsaC, and PsaD were expressed and purified as previously described (47, 48). After the rebuilding PS I (47), the quality was confirmed with flash-induced 820 nm absorption transient spectroscopy.

Rebuilt PS I and various forms of N75 were incubated together in a ratio of 1:10 overnight to assemble the nanoconstruct. Flash-induced 820 nm absorption transient spectroscopy and light-induced low-temperature CW-EPR were used to observe photochemistry of the nanoconstruct as previously described (47). Nanoconstructs assayed with flash-induced 820 nm absorption transient spectroscopy were supplied with 10 mM Na ascorbate and 10 μM DCPIP as sacrificial donors, while nanoconstructs assayed by light-induced low-temperature CW-EPR were only supplied with 10 mM Na-ascorbate.
3.2.8 Ultrafiltration of rebuilt PS I and N75 nanoconstruct assembly mixtures

Nanoconstruct assemblies incorporating various crosslinked N75 were diluted 200-fold with 50 mM Tris, pH 8.3, 0.05% v/v Triton X-100, 15% v/v glycerol after overnight incubation. The assembly mixtures were diluted from 50 µL to 10 mL and were then concentrated to ~500 µL with an Amicon stirred ultrafiltration cell fitted with a Millipore regenerated cellulose ultrafiltration membrane having 100000 NMWL.

Concentrates were analyzed on SDS-PAGE and proteins were visualized through silver staining and immunoblotting. Immunoblot membranes were blocked with 5% w/v powdered milk and 2% w/v BSA in TBST for 1 h at RT, and StrepMAB-Classic HRP conjugate was added to the blocking TBST buffer to react with blot for another 1 h at RT or overnight at 4 °C. Subsequently, the membrane was developed for chemiluminescence detection.

3.3 Results

3.3.1 Design of N75 based on structure of HN76

As shown in Figure 3-2, N75 was designed on the basis of criteria from previous studies (57, 58). The N-terminal domain fragment of CaHydA was to include the four cysteine ligands of the [2Fe-2S] cluster at positions 34, 45, 48, and 61. According to the crystal structure of CpI, a hydrophilic loop separated two α-helices made up of residues 61-63 and 77-93. Atta and colleagues (57) chose to end the HN76 on Ser76 located at the end of that hydrophilic loop. In order to determine the C-terminus of N75, we assumed the tertiary structures of CpI and CaHydA were highly similar given the high degree of similarity in their primary structures (70% identical). In fact, HN76 and N75 have primary structures that are 50% identical (Fig. 3-3). Therefore, the
N-terminal fragment of CaHydA (i.e., N75) ending with Ser75 should behave like Ser76 in CpI by protruding from the surface of the HN76 globular structure. Ser75 would terminate N75 before the start of the second α-helix. The corresponding gene fragment, n75, was ligated to the 5’ end of a linker sequence and Strep-tag®II, which would become “DSGAWSHPQFEK” after translation.

3.3.2 Heterologous expression, purification, and characterization of N75

Dark brown cell pellets were harvested after overnight expression under micro-aerobic conditions, which usually yielded about 5.0-8.0 mg of N75 per L of cell culture. After cell lysis, N75 was purified through anion exchange and affinity chromatography. During the chromatographic purification, reddish brown proteins were isolated that bound loosely to the resin beds. Both SDS-PAGE (Fig. 3-4) and mass spectrometry supported the predicted molecular weight of N75 to be 9620 Da. Fe and S quantifications indicated that reconstituted N75 contained on average about 2 Fe atoms and 2 labile S atoms (Table 3-1).

UV-visible spectroscopy and low-temperature CW-EPR were consistent with the presence of an [2Fe-2S] cluster in reconstituted N75. Unlike HN76, N75 had absorption at 280 nm due to the presence of 1 aromatic residue, Trp81 which is part of the Strep-tag®II sequence. Additionally, the UV-visible absorption spectrum of N75 (Fig. 3-5) exhibited maxima at 329, 418, and 456 nm along with a slight shoulder at 540 nm, which can be attributed to a [2Fe2S]^{2+} cluster with all-cysteine ligation (57). Upon reduction with 2mM Na-dithionite, there was a significant absorbance decrease between 365-750 nm. This change of the chromophore is indicative of the change in oxidation state from [2Fe2S]^{2+} to [2Fe2S]^{+} (57).

Figure 3-6 shows the X band CW-EPR spectrum of N75 reduced with 2mM Na-dithionite taken at different temperatures (10 - 54 K). When the temperature was 20 K or higher,
the signal from reduced N75 exhibited a rhombic structure with \( g \)-values at 2.05, 1.95, and 1.93. The signal retained its structure while decreasing in intensity as temperature increased. The signal did not disappear even at \( T = 54 \text{K} \). Unlike typical [4Fe-4S] clusters, the spectral features of the signal were not prominent at 10 K (62, 63). These characteristics were consistent with the presence of a paramagnetic center with a low-spin state of \( S = \frac{1}{2} \), which should be associated with the \([2\text{Fe2S}^+\)]^1 cluster of N75.

### 3.3.3 [2Fe-2S] cluster sensitivity to oxygen and room temperature exposures

Understanding the stability of the [2Fe-2S] cluster in N75 under routine experimental conditions are critical, because the existence of the cluster makes it possible for N75 to become a protein reporter in the nanoconstruct. When N75 was in an oxic environment under RT, the characteristic absorbance features of the \([2\text{Fe2S}^+\)] cluster at 418 nm decreased 14% in the first hour (Fig. 3-7). In contrast, \( \text{OD}_{418 \text{ nm}} \) of N75 in an anoxic environment at RT decreased only 1.5% after the first hour. Even after prolonged exposure to RT, the \( \text{OD}_{418 \text{ nm}} \) of N75 in an anoxic environment decreased only 4% after 6 hours.

### 3.3.4 Synthesis and effectiveness of the crosslinkers, BIDBE and DTME

The identity of both BIDBE and DTME were confirmed by LC-MS/MS tandem mass spectrometry with major peaks found at \( m/z = 488 \) and 313, respectively (Fig. 3-8). The ability of these crosslinkers to crosslink N75 was demonstrated through SDS-PAGE and MALDI-TOF mass spectrometry. In SDS-PAGE analysis (Fig. 3-9), formation of N75 dimers through crosslinking was evident in the 20-kDa range. These dimers were detectable starting at a 1:1 ratio of crosslinker to N75 for both BIDBE and DTME. Beginning at crosslinker to N75 ratios from
2:1 and up, bands and smears appeared around 30 kDa and higher, which indicated that multimers were forming due to crosslinking.

When these crosslinked species were examined more closely using MALDI-TOF mass spectrometry, the data provided a clearer picture of how N75 reacted with the crosslinkers. Reactions with lower crosslinker to N75 ratios (i.e., from 1:10 to 1:2) did not increase the m/z ratio of the major peaks significantly, indicating that most of N75 as not crosslinked under these conditions. The m/z ratio of the major peaks increased by 233 and 312 when crosslinker to N75 ratio reached 1:1 for both BIDBE (Table 3-2) and DTME (Table 3-3), respectively. Because the crosslinkers were not cleaved by any reductant (e.g., DTT), the m/z increase by BIDBE and DTME should be 360 and 312 respectively. The m/z increase of 233 for BIDBE-modified N75 was 127 Da less than the predicted values, which could be attributed to the loss of an iodine atom from the unoccupied end of the crosslinker.

While confirmation of crosslinking to the cysteine residue was important, it was also important to seek evidence to insure the presence of a free sulphydryl group for chemical rescue of the F₈ cluster. Ellman’s reagent was used to determine the free sulphydryl group to protein ratio after each critical step of the N75 crosslinking protocol: 1) before crosslinking; 2) after crosslinking and 1st desalting; 3) after DTT reduction and 2nd desalting. At the same time, two different ratios of crosslinker to N75 (i.e., 1:1 and 2:1) were examined. Overall, the effect of both BIDBE (Table 3-4) and DTME (Table 3-5) seemed to be the same. When the crosslinker to N75 ratio was 1:1, the number of free sulphydryl relative to protein decreased by 1 after crosslinking and increased again by 1 after DTT reduction. On the other hand when the crosslinker to N75 ratio was 2:1, the number of free sulphydryls per protein decreased by 2 after crosslinking and increased again by 2 after DTT reduction.

Although the free sulphydryl:protein ratio was higher than 1:1 before crosslinking, which could be an indication of [2Fe-S] cluster deterioration, the disappearance and reappearance of 1
sulfhydryl group before and after crosslinking could be interpreted as masking of Cys39 by the crosslinkers and subsequently reexposure of a sulfhydryl group after reductive cleavage of the crosslinkers. The disappearance and reappearance of 2 sulfhydryl groups in reactions employing 2:1 crosslinker to N75 ratio suggested that there were two crosslinkers crosslinked to each N75.

In summary, these data showed that, at low ratios of cross-linker to N75, each N75 monomer was modified by only one crosslinker molecule. Both BIDBE and DTME were capable of crosslinking N75 into dimers and multimers of higher order according to the SDS-PAGE analysis. It is likely that the crosslinkers damage the [2Fe-2S] clusters in N75 when the crosslinker concentration is high.

3.3.5 **Deteriorating effects of crosslinkers to the [2Fe-2S] clusters**

Because there is only one solvent-exposed cysteine on N75, the fact that high concentration of crosslinkers could promote multimer formation with N75 could indicate the crosslinkers were crosslinking unspecific residues or the cysteine ligands of the [2Fe-2S] clusters. Unspecific crosslinking happens usually when conditions are non-suitable to the crosslinking functional groups. However, because the crosslinking reactions were done in appropriate conditions, it was more likely that the crosslinkers had competed for the cysteine residues ligating the [2Fe-2S] clusters. These reactions would cause deterioration of the [2Fe-2S] clusters that are detectable by spectroscopic methods. Two particular crosslinker to N75 ratios, 1:1 and 2:1, were selected for closer study through UV-visible spectroscopy and MALDI-TOF mass spectroscopy in order to observe the effects of the crosslinkers on the [2Fe-2S] cluster.

When studied by UV-visible spectroscopy, the absorbance at 329 and 418 nm of N75 could reflect the integrity of the [2Fe-2S] cluster after each step of the crosslinking process. These spectroscopic features did not change significantly after N75 was modified by a 1:1 ratio of
crosslinker to N75 with both BIDBE (Fig. 3-10) and DTME (Fig. 3-11). On the other hand, N75 modified by a 2:1 crosslinker to N75 ratio showed a 15-20% decrease in absorbance at 329 and 418 nm.

Similarly, the m/z increases of crosslinked products were different between reactions with 1:1 and 2:1 crosslinker to N75 ratios. According to the molecular structure and anticipated reaction mechanisms of the crosslinkers, BIDBE and DTME should increase the m/z of N75 by 117 Da and 157 Da, respectively, after the crosslinked N75 samples were reduced with DTT. These increases were easily detectable by mass spectrometry. N75 reacted with crosslinkers in 1:1 ratio had an increase of m/z indicating 1 crosslinked BIDBE (Table 3-6) and DTME (Table 3-7) per N75. N75 reacted with 2:1 crosslinker to N75 in concentrations had an increase of m/z indicating the presence of 2 molecules of crosslinker on each molecule of N75. Both spectroscopic and mass spectrometric data illustrated the deteriorating effects that high concentration of crosslinkers had on the [2Fe-2S] clusters.

### 3.3.6 Effects of crosslinkers on the environment of the [2Fe-2S] cluster

Based upon the results described above, a 1:1 ratio of crosslinker to N75 was selected as the optimal condition for crosslinking N75 to be used in the assembly of the PS I—N75 nanoconstruct once the effects of the crosslinkers on the [2Fe-2S] clusters had been thoroughly investigated. Because high concentrations of crosslinker would damage the [2Fe-2S] clusters, it was logical to assume that the crosslinkers could have a conformational effect on N75. Therefore, low-temperature CW-EPR and midpoint potential analysis were carried out to characterize the [2Fe-2S] cluster in properly modified N75.

Figure 3-12 shows the X-band CW-EPR spectra of reduced crosslinker-treated N75 taken at 20 K. The signals of all 3 differently manipulated N75 samples exhibited similar rhombic
structure with g-values at 2.05, 1.95, and 1.93. Whether N75 had been modified by crosslinker or not, the signal at g-values = 2.05 remained the same in terms of amplitude and line width. However, signals at g-values = 1.95 and 1.93 were altered in different manner, depending on which crosslinker was used.

For N75 modified with BIDBE, both signals were altered compared to their counterparts from non-modified N75. At g-value = 1.95, there was both a decrease in its amplitude and line width of the signal. At g-value = 1.95, the signal had a slight increase in amplitude and broadening on the line width. On the other hand, DTME-modified N75 only had a slightly broadened line width and decreased amplitude of its signal at g-value = 1.95. These alterations in the X-band CW-EPR signals could indicate the protein environments with which the [2Fe-2S] clusters interacted were affected differently by each crosslinker.

Electrochemical midpoint potential determinations of crosslinker-treated N75 also showed differences for the differently modified proteins (Fig. 3-13). The midpoint potential of the [2Fe-2S] cluster in unmodified N75 was -427 mV, like that of HN76 reported in the literature (57). On the other hand, the midpoint potentials of BIDBE and DTME modified N75 were -435 mV and -420 mV, respectively. This alteration of midpoint potential upon crosslinked N75 indicated slight modification to the protein environment surrounding the [2Fe-2S] cluster. It is not surprising that a chemical modification close to the [2Fe-2S] cluster could modify the local conformation and redox properties of this cofactor.

3.3.7 Crosslinker-facilitated interaction between rebuilt PS I and N75

Figure 3-14 illustrates that the nanoconstruct assembly solution employing BIDBE-crosslinked N75 contained a higher amount of N75 compared to solutions composed of unmodified N75 or DTME-crosslinked N75. After dilution and ultrafiltration, a solution
containing rebuilt PS I and unmodified N75 lost most of its N75 (Lane I). This is consistent with the idea that covalent attachment of N75 to rebuilt PS I cores requires the crosslinker. When DTME-modified N75 was incubated with rebuilt PS I and then subjected to ultrafiltration, less N75 was retained (lanes IV) than when BIDBE-modified N75 was used (Lane II). These data suggest that BIDBE was more effective in producing complete, covalently linked nanoconstructs than DTME.

3.3.8 Flash-induced 820nm absorption transient spectroscopy and light-induced low-temp CW-EPR measurements of the PS I—N75 nanoconstruct

Figure 3-14 (A) shows the flash-induced absorbance decay at 820 nm of WT PS I that was used to rebuild the PS I employed in nanoconstruct assembly. The kinetic trace was fitted with 3 separate phases. The major phase of the fit, consisted of 44.3% of the signal and had a lifetime of 62.9 ms. This is the typical back-reaction lifetime of the charge-recombination event between P700⁺ and F₆H⁻. The 2nd minor phase (33.4%) with a decay lifetime of 74.6µs could be attributed to charge-recombination event between P700⁺ and the A₁ menaquinones. This signal arises from damaged PS I that can not transfer electrons beyond the A₁ site. The 3rd phase (22.3%) with the prolonged lifetime of 876 ms was characteristic of forward electron transfer from ascorbate/DCPIP to P700⁺. The PS I complexes reflected in this signal had had their electrons removed by O₂, which diffused away and rendered the back-reaction impossible. This population of PS I could either be intact or damaged.

In contrast, the flash-induced absorbance decay at 820 nm of rebuilt PS I showed a much different picture than WT PS I (Fig. 3-14, B). The fitting of its trace was composed of 2 phases. The minor phase (34.3%) with a lifetime of 84.7 µs can be associated with damaged PS I that could not transfer an electron beyond the A₁ menaquinones. The major phase (65.7%) had a
lifetime of 129 ms. Because it was known that the sample only contained factors necessary for rebuilding PS I, this long-lived phase should be associated with charge-recombination event between P700$^+$ and F$_B^-$. However, the lifetime was prolonged much longer than the typical lifetime of P700$^+$ F$_B^-$ charge-separation state, most likely due to the fact that properties of the F$_B$ cluster in rebuilt PS I could be affected by the C13/33G mutation on PsaC. It is noteworthy that there was not a 3$^{rd}$ minor phase with a very long-lived phase with a lifetime in the ms-scale. This is most probably a sign indicating the absence of O$_2$, which can be easily explained because all steps involved in the rebuilding of PS I were performed under anoxic conditions.

After N75 had been modified with various crosslinkers (i.e., BIDBE and DTME), crosslinked N75 and rebuilt PS I were incubated overnight in a 10:1 ratio together to form a light-sensitive mixture. The behavior of electrons within these mixtures could be observed after being excited through the absorption of single flashes of photons. This can be done also by observing the lifetime of P700$^+$ absorbance decay through the use of the flash-induced 820 nm absorption transient spectroscopy. The effects of N75 on rebuilt PS I were different based on how N75 was modified and whether it was crosslinked or not.

The flash-induced absorbance decay at 820 nm of rebuilt PS I mixed with unmodified N75 was fitted with 4 phases (Fig. 3-14, C). The major phase (38.4%) with a lifetime of 152 ms could be associated with the back-reaction between P700$^+$ and F$_B^-$ of rebuilt PS I just as shown in Figure 3-14 (B). The 2$^{nd}$ major phase (33.4%) with a very long-lived lifetime of 839 ms could be associated with forward electron transfer from ascorbate/DCPIP to P700$. This is indicative of O$_2$ being introduced into the mixture from components (e.g., rebuilt PS I or N75) exposed to O$_2$ during storage. The other 2 minor phases with lifetimes of 143 µs (9.65%) and 3.14 ms (18.6%) were most likely characteristic of the back-reaction from A$_1$ and F$_A$ to P700$, respectively. These minor phases reflected the amount of damaged PS I, which could not undergo forward electron transfer to F$_A$/F$_B$, inherited from previous PS I manipulations. The flash-induced absorbance
decay at 820 nm of rebuilt PS I mixed with DTME-crosslinked N75 (Fig. 3-14, D) exhibited almost identical characteristics.

On the other hand, the flash-induced absorbance decay at 820nm of rebuilt PS I mixed with BIDBE-modified N75 was fitted with 3 phases. The 2 major phases with much prolonged lifetime of 1.97 s (50.7%) and 449 ms (23.4%) were much longer than the back-reaction lifetime between P700⁺ and F_B⁻ in rebuilt PS I. This clearly indicated that electron transfer had proceeded beyond the F_B cluster of rebuilt PS I, and that the electron had most likely reached the [2Fe-2S] cluster in N75. This signified the successful assembly of a PS I—BIDBE—N75 nanoconstruct.

The PS I—BIDBE—N75 nanoconstruct was further tested with light-induced low-temperature CW-EPR along with rebuilt PS I and mixture of rebuilt PS I with unmodified N75 (Fig. 3-15). However, the light-induced EPR spectrum of the nanoconstruct was not different from the light-induced signal of rebuilt PS I with or without unmodified N75. The complex signals for all 3 spectra, which spanned from g-values of 1.86 to g-values of 2.05, were characteristics of P700⁺F_A/F_B⁻. Because no signals could be attributed to other paramagnetic centers, it was assumed that there was no electron transfer beyond the F_B clusters of rebuilt PS I under the conditions of this experiment.

3.4 Discussion

Experimentation to expand applications of the nanoconstruct platform concept, which exploits the principle of eliminating diffusion limitations of intermolecular electron transfer, was somewhat fruitful. A nanoconstruct was assembled from a rebuilt PS I complex and BIDBE-modified N75. Compared to the original light-driven H₂-evolving nanoconstruct, this platform replaces the catalytic module, CaHydA, with a reporter module, N75. The interaction of the two modules was facilitated by using a hetero-bifunctional molecule (i.e., BIDBE). While one end of
the molecule was crosslinked to a specific residue on the protein structure of the reporter module, the other end can link the two modules by rescuing the F\textsubscript{B} cluster in PS I chemically.

Along with the traditional tethering of the two modules through chemical rescue of two [4Fe-4S] clusters at both ends of a molecular wire, this new method of nanoconstruct assembly will perhaps increase the versatility of this platform. Both methods have merits, because they can both direct the electron entry portal (e.g., [4Fe-4S] or [2Fe-2S] cluster) of the electron-accepting module (e.g., CaHydA or N75) to the light-harvesting module specifically within close proximity. However, the protein-crosslinking method allows artificial introduction of a coordination site on the electron-accepting module. For example, this could be accomplished by the introduction of a solvent-exposed cysteine residue adjacent to the electron entry portal by site-specific mutagenesis. This can be a very advantageous technique, because it may not always be possible to create a mutant that lacks a cofactor ligand. Furthermore, this method of nanoconstruct assembly may also allow different types of prosthetic groups, other than FeS clusters, to become electron entry portals as long as they are parts of an ETC. This would significantly expand the versatility of the nanoconstruct platform not only upon the applicable candidates for the electron-accepting module, but also different purposes for which the nanoconstruct platform can be employed. The ultimate goal would then be to demonstrate the possibility of using PS I as a light-driven sequential single-electron photosensitizer.

Some limitations still persist for the design tested here. The assembly of nanoconstructs still needs to occur in an anoxic environment, not only because the [2Fe-2S] cluster is sensitive to oxygen. A free sulphhydryl group on the crosslinker, just as was the case for the original molecular wires (REFS), is required for chemical rescue of the F\textsubscript{B} cluster in PS I. This free sulphhydryl group has to be maintained in an anoxic environment until assembly of nanoconstruct is completed. The distance between the F\textsubscript{B} cluster and the electron entry portal is also crucial to the success of facilitating two ETCs from the two modules to essentially become one ETC.
In this study, the distance between the coordination site (Cys39) and the electron entry portal ([2Fe-2S] cluster) is 10.1 Å. This amounts to a total distance of 18.6 Å and 16.7 Å between the solvent exposed free sulphhydryl group and the entry portal after BIDBE and DTME are crosslinked to Cys39, respectively. Although both distances are within the theoretical limits allowed for electron transfer between two cofactors, the results indicated that only BIDBE-crosslinked N75 was able to facilitate forward electron transfer beyond the F_B cluster with a prolonged charge recombination lifetime of >449 ms. On the other hand, DTME-crosslinked N75 was unable to affect significantly the charge recombination lifetime of 129 ms, indicating that it was still reflecting the back reaction between P700^+ and F_B^- . The inability of DTME-crosslinked N75 to reduce PS I could be caused by steric hindrance, either by the bulky maleimide ring or the short length of the crosslinker. Either of these effects might prevent DTME from connecting the two modules as required.

The number of conditions for which the nanoconstruct concept can be used may be limited. Low temperature (<20K) conditions seem to inhibit forward electron transfer beyond the F_B cluster, most likely because there may not be sufficient free energy for electron tunneling to occur between F_B and the entry portal. Another reason could be attributed to the [2Fe-2S] cluster signal at the g = 2 region being masked by the strong axial radical signal of P700^+. Nevertheless, the nanoconstruct was capable of demonstrating forward electron transfer beyond the F_B cluster of the platform at RT. This condition can be a great tool to continue develop and fine tune various parameters involved in the design of the nanoconstruct platform, such as, pH, temperature, ionic strength, types of prosthetic groups as entry portals, and distance between entry portals and the F_B.

However, there are some constraints in terms of creating a flash-inducible “freeze-quench” apparatus based on the nanoconstruct platform. First of all, the nanoconstruct must be designed such that forward electron transfer is possible while competing backreactions are
sufficiently slowed so that the electron can be trapped at the terminal electron acceptor. If more than one electron is desired to be transferred beyond F_{B}, the experimental condition will also have to account for the ability of sacrificial electron donors (e.g., ascorbate and DCPIP) to donate an electron to P700\(^+\) before the backreaction occurs. Perhaps this constraint could be overlooked when only two electrons are desired for forward transfer by employing cytochrome c_{6}-crosslinked, rebuilt PS I in the nanoconstruct. In any event, the optimal conditions for this implementation will most likely involve subjecting the nanoconstruct to cryogenic temperatures. Because forward electron transfer below 20K could not be demonstrated for the PS I—BIDBE—N75 nanoconstruct, the successful development of this application will require further testing of different entry portals with different crosslinkers/molecular wires under different reaction conditions. Nevertheless, if these parameters and components can be optimized, the possibilities are endless regarding the applications of the nanoconstruct platform, whether it can be used for biofuel generation or characterization of prosthetic groups and redox enzymes for biophysical research.
Figure 3-1: Distance between Cys39 and the [2Fe-2S] cluster in the N-terminal domain of CpI is \(~10.1\ \text{Å}\). All four ligands of the [2Fe-2S] cluster (i.e., Cys34, Cys46, Cys49, and Cys62) and Cys39 are shown as stick models.
Figure 3-2: Structure of the N-terminal domain of Cpl, including two α-helices made up of residues 61-63 (shown in blue) and 77-93 (shown in cyan). Between them exists the hydrophilic loop (shown in pink) ended with Ser76 (shown in yellow). The solvent exposed Cys39 (shown by the red arrow) is represented as a stick model. The [2Fe-2S] cluster is shown as space-filling Fe and S atoms in orange and yellow, respectively.
Figure 3-3: Amino acid sequence alignment between HN76 and N75 reveals 50% overall identity.
Figure 3-4: SDS-PAGE of N75 purification steps from DEAE anion exchange chromatography (A) and Strep-Tactin® affinity chromatography (B). Proteins were stained with Coomassie Brilliant Blue. Samples: protein ladder (Ld), chromatography flowthrough (FT), wash (WH), and elution (EL) fractions. N75 has an apparent mass of less than 10kDa and elutes from the DEAE column starting at 100 mM NaCl (EL1).
Table 3-1: Average moles of Fe and S atoms per moles of reconstituted N75

<table>
<thead>
<tr>
<th>Fe : N75 Ratio</th>
<th>S : N75 Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.44 ± 0.02</td>
<td>1.88 ± 0.07</td>
</tr>
</tbody>
</table>
Figure 3-5: UV-visible spectrum of N75 (100 µM): as purified (solid line) and reduced with 2 mM Na-dithionite (dashed line).
Figure 3-6: Low temperature CW-EPR spectra of N75 reduced with 2 mM Na-dithionite exhibits a rhombic spectrum with $g$-values = 2.05, 1.95, and 1.93. Experimental conditions: $T = 10$ K (dotted line), 20 K (solid line), 48 K (dashed line), 54 K (dashed and dotted line); microwave power = 1 mW; microwave frequency = 9.38 GHz; mod. amplitude = 10 G; mod. frequency = 100 kHz; receiver gain = 60 dB; conversion time = 50 ms.
Figure 3-7: Decrease of absorbance at OD$_{418}$ nm observed from N75 exposed to oxic conditions (shown in squares) and anoxic conditions (shown in triangles) at RT.
Figure 3-8: Mass spectrometry spectra of BIDBE (A) and DTME (B) measuring the molecules’ m/z ratio in isotope mode.
Figure 3-9: SDS-PAGE of N75 crosslinked with BIDBE (A) and DTME (B) stained with Coomassie Brilliant Blue. The BIDBE:N75 ratios are 0 (I, A); 1:10 (II, A); 1:4 (III, A); 1:2 (IV, A); 1:1 (V, A); 2:1 (VI, A); 10:1 (VII, A); 50:1 (VIII, A). The DTME:N75 ratios are 0 (I, B); 1:10 (II, B); 1:4 (III, B); 1:2 (IV, B); 1:1 (V, B); 2:1 (VI, B); 3:1 (VII, B); 5:1 (VIII, B).
Table 3-2: The $m/z$ of major peaks of crosslinked products in various BIDBE:N75 ratios

<table>
<thead>
<tr>
<th>BIDBE : N75 Ratios</th>
<th>Major Peak (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9644.07</td>
</tr>
<tr>
<td>1 : 10</td>
<td>9643.83</td>
</tr>
<tr>
<td>1 : 4</td>
<td>9640.61</td>
</tr>
<tr>
<td>1 : 2</td>
<td>9638.19</td>
</tr>
<tr>
<td>1 : 1</td>
<td>9877.64</td>
</tr>
<tr>
<td>2 : 1</td>
<td>9878.96</td>
</tr>
<tr>
<td>10 : 1</td>
<td>10343.20</td>
</tr>
<tr>
<td>50 : 1</td>
<td>9905.32</td>
</tr>
</tbody>
</table>

Table 3-3: The $m/z$ of major peaks of crosslinked products in various DTME:N75 ratios

<table>
<thead>
<tr>
<th>DTME : N75 Ratios</th>
<th>Major Peak (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9639.04</td>
</tr>
<tr>
<td>1 : 10</td>
<td>9641.89</td>
</tr>
<tr>
<td>1 : 4</td>
<td>9652.46</td>
</tr>
<tr>
<td>1 : 2</td>
<td>9644.98</td>
</tr>
<tr>
<td>1 : 1</td>
<td>9959.77</td>
</tr>
<tr>
<td>2 : 1</td>
<td>9967.45</td>
</tr>
<tr>
<td>3 : 1</td>
<td>9985.47</td>
</tr>
<tr>
<td>5 : 1</td>
<td>10201.66</td>
</tr>
</tbody>
</table>
Table 3-4: Free sulphydryl groups to protein ratios of N75 after various stages of crosslinking with BIDBE in different concentrations.

<table>
<thead>
<tr>
<th></th>
<th>1BIDBE:1N75</th>
<th>2BIDBE:1N75</th>
</tr>
</thead>
<tbody>
<tr>
<td>N75</td>
<td>3.73</td>
<td>3.73</td>
</tr>
<tr>
<td>+ BIDBE (desalted)</td>
<td>2.40</td>
<td>1.89</td>
</tr>
<tr>
<td>+ DTT (desalted)</td>
<td>3.73</td>
<td>4.11</td>
</tr>
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</table>

Table 3-5: Free sulphydryl groups to protein ratios of N75 after various stages of crosslinking with DTME in different concentrations.

<table>
<thead>
<tr>
<th></th>
<th>1DTME:1N75</th>
<th>2DTME:1N75</th>
</tr>
</thead>
<tbody>
<tr>
<td>N75</td>
<td>2.75</td>
<td>2.75</td>
</tr>
<tr>
<td>+ DTME (desalted)</td>
<td>1.31</td>
<td>0.85</td>
</tr>
<tr>
<td>+ DTT (desalted)</td>
<td>2.65</td>
<td>2.49</td>
</tr>
</tbody>
</table>
Figure 3-10: Decrease of absorbance at $\text{OD}_{329\,\text{nm}}$ (A) and $\text{OD}_{418\,\text{nm}}$ (B) observed from N75 crosslinked by different concentrations of BIDBE.
Figure 3-11: Decrease of absorbance at OD$_{329}$ nm (A) and OD$_{418}$ nm (B) observed from N75 crosslinked by different concentrations of DTME.
Table 3-6: The m/z of major peaks of crosslinked products after being reduced by DTT in various BIDBE:N75 ratios

<table>
<thead>
<tr>
<th>BIDBE : N75 Ratios</th>
<th>Major Peak (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9625.90</td>
</tr>
<tr>
<td>1 : 1</td>
<td>9714.66</td>
</tr>
<tr>
<td>2 : 1</td>
<td>9862.88</td>
</tr>
</tbody>
</table>

Table 3-7: The m/z of major peaks of crosslinked products after being reduced by DTT in various DTME:N75 ratios

<table>
<thead>
<tr>
<th>DTME : N75 Ratios</th>
<th>Major Peak (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9604.63</td>
</tr>
<tr>
<td>1 : 1</td>
<td>9758.18</td>
</tr>
<tr>
<td>2 : 1</td>
<td>9912.74</td>
</tr>
</tbody>
</table>
Figure 3-12: Low temperature CW-EPR spectra of N75 (solid line), BIDBE-N75 (dotted line), and DTME-N75 (dash line) reduced with 2 mM Na-dithionite exhibits a rhombic structure with three g-values = 2.05, 1.95, and 1.93. Experimental conditions: T = 20K; microwave power = 1mW; microwave frequency = 9.38 GHz; mod. amplitude = 10 G; mod. frequency = 100 kHz; receiver gain = 60 dB; conversion time = 50 ms.
Figure 3-13: Midpoint potential titration curves of N75 (A), BIDBE-N75 (B), and DTME-N75 (C).
Figure 3-14: SDS-PAGE developed with silver staining (A) and immunoblot (B) confirming the presence of N75 from various ultrafiltered nanoconstruct solutions containing rebuilt PS I incubated overnight with unmodified N75 (I); BIDBE-crosslinked N75 (II); and DTME-crosslinked N75 (IV). Unfiltered rebuilt PS I mixed with N75 in 1:10 ratio (III) was used as the control. All lanes were loaded with samples containing 2 µg Chl.
Figure 3-15: 820 nm time-resolved optical spectroscopy of WT PS I (A), rebuilt PS I (B), rebuilt PS I mixed with N76 (C), rebuilt PS I mixed with DTME-N76 (D), and rebuilt PS I mixed with BIDBE-N76 (E).
Figure 3-16: Light induced low-temp CW-EPR measurements of rebuilt PS I (dotted), rebuilt PS I + N75 (solid), and rebuilt PS I + BIDBE—N75 nanoconstruct (dashed). Experimental conditions: T = 10K; microwave power = 31.7mW; microwave frequency = 9.38 GHz; mod. amplitude = 10 G; mod. frequency = 100 kHz; receiver gain = 60 dB; conversion time = 50 ms.
References


the dissimilatory metal ion–reducing bacterium *Shewanella oneidensis*. Nat. Biotech. 20, 1118–1123


