RING ASYMMETRY IN THE BACTERIAL ENHANCER BINDING PROTEINS GUIDES INTERACTION WITH SIGMA-54 FORM OF RNA POLYMERASE

A Dissertation in
Biochemistry, Microbiology and Molecular Biology
by
Saikat Chowdhury

© 2012 Saikat Chowdhury

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2012
The dissertation of Saikat Chowdhury was reviewed and approved* by the following:

B. Tracy Nixon, Ph.D.
Professor of Biochemistry and Molecular Biology
Dissertation Advisor
Chair of Committee

Kathleen Postle, Ph.D.
Professor of Biochemistry and Molecular Biology

John H. Golbeck, Ph.D.
Professor of Biochemistry and Molecular Biology

David S. Gilmour, Ph.D.
Professor of Biochemistry and Molecular Biology

William O. Hancock, Ph.D.
Professor of Bioengineering

Craig E. Cameron, Ph.D.
Paul Berg Professor of Biochemistry and Molecular Biology
Associate Department Head for Research and Graduate Education

*Signatures are on file in the Graduate School
ABSTRACT

Bacteria can survive under different environmental conditions by regulating the expression of specific genes. Such targeted transcription requires the recruitment of the core RNA-polymerase (RNAP) to a specific promoter by a Sigma factor. Successful recruitment typically leads to a rapid isomerization from double-stranded (closed) form to a single stranded state (open complex) - from which transcription can begin. Contrary to this, a major alternative Sigma factor called σ54, can bind along with RNAP core to the promoter DNA and form stable closed complex. However, unlike all other Sigma factors they cannot spontaneously isomerize closed complex to open complex. For this transition, σ54 dependent transcription machinery needs to be remodeled by ATP-hydrolyzing activator proteins, also known as bacterial Enhancer Binding Proteins (bEBPs). These activators form a subset of the super-family of AAA+ ATPases (ATPases Associated with Various Cellular Activities).

These AAA+ ATPases are known to act as rings made up of chemically identical subunits. The exact mechanism by which the ring architecture or geometry assists them to perform mechanical work on targets is unknown. Prior structural studies have shown various changes in the ring conformation during different stages of nucleotide hydrolysis. These studies were done in saturating nucleotide concentrations and the oligomeric state of the ATPase was either hexamer (PspF, NtrC) or heptamer (NtrC1). Recent crystal structure (Sysoeva thesis and manuscript in preparation) of NtrC1 ATPase, from Aquifex aeolicus,
sub-saturating or stoichiometric amount of nucleotides have shown the formation of asymmetric gapped hexamer ring, with spiral staircase arrangement of the L1 or GAFTGA loops. The interface between the first and the sixth subunit had heterogeneous nucleotide occupancy. Striking similarity of this structure with distantly related AAA+ ATPases like E1 DNA translocase or Rho terminator protein (RNA translocase) suggests that allostery within the ring geometry of the ATPase leads to asymmetric functional states that exerts directional force to remodel asymmetric target macromolecules (here σ54-RNAP-promoter DNA closed complex).

To understand if this asymmetric geometry directs interaction of the bEBP ATPase with the σ54 transcription apparatus, structural studies were done using the NtrC1 ATPase in complex with σ54 and promoter DNA and also in presence of RNAP. Electron Microscopy (EM) reconstructions of the complex of NtrC1 ATPase with σ54 and promoter DNA revealed the persistence of an asymmetric gapped hexameric ATPase ring in the complex. An improved feed batch fermentation technique was developed for obtaining higher yields of deuterated proteins. This method was applied by a lab colleague to perform novel contrast matching neutron scattering experiments (SANS) showing that the ring geometry of the NtrC1 ATPase remained largely unchanged while interacting with its target σ54 (Sysoeva thesis and manuscript in preparation). This deuteration protocol can significantly reduce the cost of production of isotopically labeled proteins. Finally, an initial 3D EM reconstruction was made from images of negatively stained, full closed σ54 transcription apparatus complexed with NtrC1 ATPase.
This model together with class averages from preliminary cryo EM data of the complex provided additional evidence for the persistence of the asymmetric ATPase ring when bound to the transcription apparatus and promoter DNA.

This thesis presents low-resolution structural information that leads to the hypothesis that a bEBP ATPase acquires asymmetry in response to ATP occupancy that it uses to approach its asymmetric target, the σ54-closed transcription complex. This insight helps us understand σ54-dependent transcription and thus propels us toward harnessing bEBPs to block harmful bacterial activities and enhance beneficial ones. Viewed from a larger perspective, these observations suggest one way to understand how asymmetry can arise in the homomeric ring morphology of AAA+ ATPases to guide their interactions with different, frequently asymmetric, target macromolecules. In future, high-resolution information has to be obtained to understand and thus harness these important biological molecular motor proteins to alleviate a variety of human diseases, block virulence of bacterial and viral pathogens, and otherwise engineer biologically useful molecular motors.
# TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................... ix

LIST OF TABLES ............................................................................................................... xi

LIST OF ABBREVIATIONS ............................................................................................... xii

ACKNOWLEDGEMENTS .................................................................................................... xiv

Chapter 1  Introduction and Literature Review ................................................................. 1

General Overview ............................................................................................................. 1

Introduction to bacterial transcription machinery ......................................................... 2

σ54 dependent transcription .......................................................................................... 5

Bacterial Enhancer Binding proteins (bEBPs) .............................................................. 6

Prior studies on bEBP’s AAA+ ATPase domain ............................................................. 10

Transmission Electron Microscopy .............................................................................. 22

Theory and background of Electron microscopy ....................................................... 22

Transmission Electron Microscope ............................................................................ 23

Grids and sample preparations ...................................................................................... 25

Thin carbon film making by carbon evaporation ......................................................... 26

Preparation of thin carbon coated TEM grids ............................................................ 27

Sample preparation techniques ..................................................................................... 28

Theory of image formation in TEM ............................................................................ 31

Data collection strategy for single particle reconstructions ........................................ 32

Image processing and introduction to Single Particle Reconstructions ...................... 33

Solution Scattering techniques (X-ray and Neutron Scattering) ................................ 44

Overall Summary ........................................................................................................... 46

Chapter 2  Persistance of Asymmetry in NtrC1 AAA+ ATPase when in

Complex with σ54 and Promoter DNA ......................................................................... 49

Summary ......................................................................................................................... 49
# Introduction

Materials and methods

**Protein expression and purification**

Expression and purification of NtrC1 ATPase

Expression and purification of σ54

*nifH* promoter DNA

**Preparation of complex**

**Single particle reconstruction of the complex**

Preparation of grids for electron microscopy

Data collection and processing

Results

**Reference free class averages**

**Reconstructed asymmetric 3D volumes of the complex**

Discussion

Conclusions

---

Chapter 3  Novel Deuteration Protocol and Solution Scattering Approach for Studying Interaction of NtrC1 AAA+ ATPase with σ54

Summary

Introduction

Materials and methods

**Protein expression and purification**

Expression and purification of deuterated NtrC1<sup>C</sup>

**Contrast matching SANS studies of the complex of NtrC1<sup>C</sup> with σ54**

**Modeling solution X-ray scattering data of the complex of NtrC1<sup>C</sup> with σ54**

Results and Discussion

**Higher yield of deuterated proteins**

**NtrC1<sup>C</sup> ring structure does not change upon binding with σ54**

**Solution X-ray scattering models of complex of NtrC1<sup>C</sup> with σ54**
Conclusions .................................................................................................................. 99

Chapter 4  NtrC1 AAA+ ATPase interacts with Closed Complex with σ54-RNAP and Promoter DNA as an Asymmetric Split Ring ......................... 101

Summary ............................................................................................................... 101
Introduction ............................................................................................................ 102
Materials and methods ............................................................................................ 103
Protein expression and purification .............................................................. 103
Expression and purification of RNAP .............................................................. 103
Preparation of complex ..................................................................................... 105
Single particle reconstruction of the complex .............................................. 108
Preparation of grids for electron microscopy ............................................... 108
Data collection and processing .......................................................................... 109
Results ................................................................................................................. 112
Discussion ........................................................................................................... 113
Conclusions ......................................................................................................... 120

Chapter 5  Significance and Future Directions .................................................. 121

EM studies on complexes of NtrC1C with σ54, promoter DNA and also with RNAP .......................................................... 122
Deuteration of proteins for SANS experiments ............................................ 125
Crystallography of NtrC1C ............................................................................... 126

References .......................................................................................................... 130
LIST OF FIGURES

Figure 1.1: Comparison between σ70 and σ54 transcription initiation. ............... 17
Figure 1.2: Domain architecture of bEBPs. .................................................................... 18
Figure 1.3: SAS models of NtrC1 AAA+ ATPase in the presence of different ATP analogues and ADP ......................................................... 19
Figure 1.4: NtrC1 AAA+ ATPase domain..................................................................... 20
Figure 1.5: NtrC1 AAA+ ATPase in sub saturating or stoichiometric amounts of nucleotide ........................................................................... 21
Figure 1.6: Basic set up of Transmission Electron Microscope................................. 40
Figure 1.7: Sample preparation for single particle reconstructions by transmission Electron Microscopy........................................................................ 41
Figure 1.8: Image processing and Single Particle Reconstruction.............................. 42
Figure 1.9: Random Conical Tilt ................................................................................. 43
Figure 2.1: Purification of NtrC1C, σ54 and DNA by Gel-filtration.............................. 59
Figure 2.2: Tilt pair micrographs of the NtrC1C, σ54 and DNA complex............... 63
Figure 2.3: FSC curve for the final reconstructed volume of the complex............... 64
Figure 2.4: Class averages of the complex ................................................................. 67
Figure 2.5: Refined 3D reconstructions of the complex show an asymmetric NtrC1C ring structure ................................................................. 68
Figure 2.6: Fitting crystal structures of NtrC1 ATPase into the ring density of the EM reconstruction of the complex ............................................ 69
Figure 2.7: The gap in the NtrC1 ATPase hexamer structure matches with the gap in the ATPase ring density in the EM reconstructions and the density for σ54 and promoter DNA makes contact with the ATPase density opposite to the gap ............................................. 70
**Figure 2.8:** Asymmetric ATPase ring can be observed in PspF when in complex with σ54 or with σ54 and RNAP ......................................................... 71

**Figure 2.9:** Density for σ54-promoter DNA makes contact with subunits B, C and D of the NtrC1 ATPase at the opposite interface to where the gap is located ................................................................. 77

**Figure 3.1:** Relationship between percent deuteration of proteins expressed in *E. coli* and percent D$_2$O (v/v) in media and carbon source ...................... 89

**Figure 3.2:** Glycerol-based fermentations to produce deuterated NtrC1$^C$ ............... 95

**Figure 3.3:** Neutron scattering by hydrogenated σ54 and deuterated NtrC1$^C$ .......... 96

**Figure 3.4:** SAXS models of complex of NtrC1$^C$ with σ54 ................................ 98

**Figure 4.1:** Purification of NtrC1$^C$, σ54, RNAP and DNA complex by Gel-filtration ................................................................. 107

**Figure 4.2:** Negative stained micrograph of the NtrC1$^C$, σ54, RNAP and DNA complex ........................................................................... 114

**Figure 4.3:** Tilt pair micrographs of the NtrC1$^C$, σ54, RNAP and DNA complex .... 115

**Figure 4.4:** RCT reconstructions of class averages of the complex using ML2D/XMIPP ................................................................. 116

**Figure 4.5:** Classification of the un-tilted particles from the RCT data using ISAC/SPARX ................................................................. 117

**Figure 4.6:** 3D reconstruction of the complex of NtrC1$^C$, σ54, RNAP and DNA (negative stained untilted data set) and its comparison with the 3D reconstruction of PspF, σ54 and RNAP complex .............................................. 118

**Figure 4.7:** Initial Cryo EM data of NtrC1$^C$, σ54, RNAP and closed DNA complex ........................................................................... 119

**Figure 5.1:** Crystallography of NtrC1$^C$ ........................................................................... 128
LIST OF TABLES

Table 2.1: -11, -12 mismatch nifH promoter DNA ................................................. 57

Table 2.2 Media and buffers for protein expression and purification and complex formation ........................................................................................................... 58

Table 3.1: Media and buffers for protein expression and purification and complex formation ........................................................................................................... 88

Table 3.2: Cross correlation NSD table between the Credo Models having σ54 placed on top of the NtrC1 C ring ............................................................................................................. 97

Table 4.1 Media and buffers for protein expression and purification and complex formation ........................................................................................................... 106
LIST OF ABBREVIATIONS

AAA+ ATPase: ATPases Associated with various cellular Activities
ADP: adenosine diphosphate
ADP-AIF$_x$ : aluminum fluoride
ADP-BeF$_x$ : beryllium fluoride
ATP: adenosine triphosphate
bEBPs: bacterial Enhancer Binding Proteins
CheY : chemotaxis regulator transmitting signal to flagellar motor component
ClpX: caseinolytic peptidase X
CtcC : chlamydial two-component system protein
cryo EM : cryo electron microscopy
CTF: contrast transfer function
D$_2$O: deuterium oxide
DBD: DNA binding domain
DctD: C4-dicarboxylic acid transport protein D
DNA: deoxy ribonucleic acid
EDTA: ethylene-diamine-tetra-acetate
EM: electron microscopy
FEG: field emission gun
FSC: fourier shell correlation
GAF: domain named after cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA
IPTG: isopropyl β-D-1-thiogalactopyranoside
NifA: nitrogen fixation regulatory protein A
NMR: nuclear magnetic resonance
NtrC: nitrogen regulatory protein C
NtrC1: homolog of NtrC in Aquifex aeolicus
PAS: periodic clock protein, aryl hydrocarbon receptor and single-minded protein
PPG: poly propylene glycol
Rg: radius of gyration
PspF, PspA: phage shock proteins F and A
PTS: phosphoenolpyruvate-dependent phosphotransferase system
RCT: random conical tilt
RNA: ribonucleic acid
RNAP: RNA polymerase core
SANS: small-angle neutron scattering
SAXS: small-angle X-ray scattering
TCEP: tris(2-carboxyethyl)phosphine
TEM: transmission electron microscopy
Tris: tris(hydroxymethyl)aminomethane
UAS: upstream activator sequences
WAXS: wide angle X-ray scattering
XylR: xylose operon regulatory protein
ACKNOWLEDGEMENTS

It would not have been possible for me to complete my Ph. D. all by myself had it not been for the support and help of several people. I take this opportunity to express my thanks and gratitude to all of them.

I would like to first thank my wonderful advisor Dr. B. Tracy Nixon. He has been a major source of inspiration for me and encouraged me to try and learn new techniques without being afraid of them. My undergraduate training was in Bioinformatics and I had no formal training in molecular biology and bench work when I joined his group. Without any reluctance he accepted me as a graduate student and taught me several techniques himself. It was because of him I was able to learn and use some advanced structural and biophysical techniques for my research. Dr. Nixon’s help went beyond the academic domain. I will never forget his help and support when I received the news of my father’s sudden demise in a road accident. My thanks to his family and especially to his wife, Mrs. Nixon for her warm affection and support she provided to me and my wife when we stayed at their home in Princeton during sabbatical.

I would like to thank Dr. Sacha De Carlo for introducing me to the field of electron microscopy and for providing financial support and resources to carry out the EM project. My special thanks to Dr. Ruben Diaz-Avalos for helping me with the electron microscopy project and providing me hands on training in electron microscopy. I would also like to thank the EM group at NYSBC, especially Drs. William Rice and David Stoke for allowing me to use the
resources and microscopes at NYSBC. It would have been impossible for me to process all my data without the help of High Performance Computing clusters at Penn State and especially Dr. Michael Fenn who was always very helpful with installation of EM data processing programs and troubleshooting and setting them up for me in the clusters.

I would like to thank Dr. Kevin Weiss for providing training on expressing deuterated proteins at Oak Ridge National Laboratory. I was able to learn this technique and improve upon it at Penn State Shared Fermentation Facility with the help of Mr. Mark Signs. Thanks to Mr. Signs, for helping me with all the fermentations for expressing various proteins for my work. I would also like to thank Dr. Tatiana Laremore for helping me with mass spectrometry to calculate the percentage deuteration of expressed deuterated proteins. I would like to thank Drs. Hemant and Neela Yennawar for helping me with x-ray crystallography and for allowing me to use the crystallography facility.

My special thanks to two former lab mates Drs. Bayou Chen and Tatyana Sysoeva. I was able to learn a lot from their experience and thanks for the help they provided during the initial years of my Ph. D. Thanks to Ms. Robin Rohwer, a former member of the lab for helping me with teaching responsibilities when I had to travel for interviews and conferences. My thanks to all my friends at State College because of whom my stay away from my home did not feel so bad during the last six years.

Finally nothing in my life so far would have been possible without the support and help from my family. My parents and wife have always been besides
me during bad and good times. Thanks to all of them for their wishes and support.
CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 General Overview

The purpose of this chapter is to provide a general overview, background information and description of techniques related to the understanding of this thesis.

To begin, I introduce bacterial transcription machinery and then describe the unique σ54-dependent transcription apparatus in bacteria. After that I provide background information and domain architectures of activators of σ54-dependent transcription, also known as bacterial Enhancer Binding Proteins (bEBPs). This background information is followed by reviewing prior structural studies done on the AAA+ ATPase (ATPase Associated with various Cellular Activities) domain of bEBPs, which sets the platform of my work by allowing me to frame the question: Is it possible, and important, for a homo-oligomeric ring of bEBP to use asymmetry amongst its protomers to guide interaction with its asymmetric target – the σ54 protein component of closed complex of promoter DNA and RNAP? My work answers this question positively, throwing light on how AAA+ ATPases couple nucleotide binding and hydrolysis to perform mechanical work on target macromolecules.
To aid the reader, I further introduce the major structural techniques I have used for my work, viz. Transmission Electron Microscopy (TEM) and solution scattering techniques (SAXS for X-rays; and SANS for neutrons). In this section I provide basic information on microscope handling, sample preparation for TEM, data collection strategy, processing and single particle reconstructions. For simplicity I do not describe any extensive mathematical details involved in these techniques.

Finally at the end of this chapter I provide the working hypothesis that embodies the question I address in this thesis. Here I also provide a basic overview of the subsequent chapters and explain how I address my questions in them.

1.2 Introduction to bacterial transcription machinery

Transcription is one of the fundamental processes that are necessary for the survival of all cells. It is the very first step in the “central dogma” of sequential information flow from genes to protein in the cell (Crick, 1970). Like many other organisms, in bacteria this process is carried out by DNA-dependent RNA polymerase. For survival and adaptation of bacteria it is crucial that this flow of information be highly regulated. One of the levels at which regulation is achieved is by controlling the RNA polymerase transcription machinery as it undertakes the process of transcription initiation. The bacterial RNA polymerase holoenzyme (RNAP) is a 400KDa hetero-hexameric complex (Murakami and Darst, 2003;
Helmann et al., 2009, Opalka et al., 2010). It can be further divided functionally and structurally into two basic parts – a catalytically active RNAP core, made up of two α subunits and single copies of subunits β, β’ and ω; and a second basic part, a dissociable σ subunit. The catalytic core is where the synthesis of new RNA takes place by polymerizing nucleotide tri-phosphate, complementary to the bases present in the template DNA strand. A “crab-claw” structure of the catalytic core is structurally conserved in all species (Murakami and Darst, 2003). Within a bacterial cell this RNAP core is ubiquitous and forms the common pool of catalytic transcription machinery. It is the σ factor that makes it possible for the core to transcribe specific genes, by assisting in promoter recognition and recruitment of the polymerase core to specific promoter DNA. This complex containing the catalytic core of RNAP, σ factor and the double-stranded promoter DNA is known as the “closed complex” (Browning and Busby, 2004). For all but the σ54-form of RNAP, the σ factor spontaneously facilitates the isomerization of closed complex to the “open complex” state or transcription bubble, by stabilizing the single stranded unwound DNA around -10 site (10 base pairs upstream of the transcription start site) (Browning and Busby, 2004). This eventually sets the pre-initiation stage of transcription, exposing the start site of the DNA template strand to the active site of the enzyme. It was believed that σ factor was a dissociable factor that got released after successful initiation (Travers et al., 1969; Hansen et al., 1980). However later studies have shown that in majority of early elongation complexes, the σ factor remains bound to it (Kapanidis et al., 2005; Mukhopadhyay et al., 2001). These facts suggest that σ factors not only play a
crucial role in transcription initiation, but also can be present at and regulate later stages of transcription.

Evolutionarily sigma factors are classified into two distinct families: σ70 and σ54. The founding member of the σ70 family, σ70 itself, forms the major σ factor and is also known as the “house keeping σ factor” as it mediates most of the transcription in rapidly growing cells (Sterberg et al., 2011; Paget and Helmann, 2003). In addition to σ70, many bacterial genomes code for different alternative σ factors like σE, σS, σF and σ32. These regulate genes under different growth or stress conditions (Seshasayee, 2011; Hellmann and Chamberlin, 1988). All these alternative sigma factors share significant sequence conservation with σ70 and together comprise the σ70 family of sigma factors. Its members are partitioned into separate sub-classes of sigma factors. As stated above, all members of the σ70 family spontaneously isomerize open complex formation from closed complexes. They accomplish this using several sub-domains that are organized identically within the larger protein. On the other hand, the σ54 family is made of only one type of sigma factor that is present in about 65% of sequenced bacterial genomes (Gruber and Gross, 2003; Pérez-Rueda et al., 2009). The σ54 proteins significantly differ from the σ70 family members. There is no sequence similarity between the two families and the members display different sub-domain architectures. Nonetheless, members of both families function as sigma factors together with the same core enzyme of RNAP.
Unlike members of the $\sigma_{70}$ family, $\sigma_{54}$ do not spontaneously isomerize closed complexes to open ones; rather, they form stable closed complexes with RNAP core and double stranded promoter DNA. Transition from closed to open complex state requires the help of certain activator proteins. It is this unique feature of $\sigma_{54}$-dependent transcription that I explore in this thesis (Figure: 1.1).

1.3 $\sigma_{54}$-dependent transcription

Due to historical influences, $\sigma_{54}$ is variously known in the literature as $\sigma_N$, RpoN, NtrA or GlnF. For $\sigma_{54}$-forms of RNAP to melt promoter DNA requires the help of activator ATPases, which bind to promoter-distal enhancer DNA sequences (sometimes these are called upstream activation sequences, or UAS; Buck et al., 2000; Popham et al., 1989; Wigneshweraraj et al., 2008). Historically $\sigma_{54}$-dependent transcription was first known to be associated with nitrogen fixation and metabolism (Hennecke et al., 1990; Michiels et al., 1998). Over time it has been found to be associated with several crucial functions like carbon source uptake and utilization (Wang et al., 1997), complex developmental processes where they are associated with expressing alternate sigma factors, RNA modification, phage shock response (Bordes et al., 2003), heat shock responses, motility and flagellar motion and bio-film formation (Wolfe et al., 2004). The most significant biomedical importance of $\sigma_{54}$-dependent transcription is that it is associated with bacterial pathogenesis. $\sigma_{54}$-dependent genes are responsible for vector host adaptations in the case of Borrelia burgdorferi, the spirochete
responsible for causing Lyme disease (Fisher et al., 2005). They are also known to be associated with pathogenesis and motility of *Vibrio cholera* that cause diarrheal epidemic in humans (Correa et al., 2000). Even if they are not the major σ factor, σ54 is almost ubiquitous from extreme hyper-thermophiles, spirochetes, enteric bacteria to photosynthetic bacteria (Buck et al., 2000; Studholme et al., 2000).

The mechanism of σ54-dependent transcription varies significantly from σ70-dependent transcription. For the σ70 form of transcription, regulation occurs, at least as a first limiting step, by the σ70-RNAP gaining access to the promoter (enhanced by activators or blocked by repressors). In stark contrast, σ54-RNAP remains constitutively pre-bound to its promoter with RNAP core in stable closed complex state (Jishage et al., 1996). Transition to open complex formation requires ATP hydrolysis by specific activators also known as bacterial Enhancer Binding Proteins (bEBPs) (Sasse-Dwight and Gralla, 1988; Merrick, 1993; Bose et al., 2008; Chen et al., 2007, 2008) that belong to AAA+ ATPase super-family (Figure: 1.1) (Erzberger and Berger, 2006).

### 1.4 Bacterial Enhancer Binding Proteins (bEBPs)

In response to certain stimuli or external cues bEBPs undergo conformational changes to an active form and then hydrolyze ATP to perform mechanical work on the target, closed σ54-RNAP-DNA complex. Like other AAA+ ATPases they are P-loop (phosphate binding loop) NTPases and convert
chemical energy of ATP binding and hydrolysis to perform mechanical work on target macromolecules (Patel and Latterich, 1998; Ogura and Wilkinson, 2001). bEBPs are typically made up of three distinct domains. They have an amino-terminal regulatory domain, a centrally located and highly conserved AAA+ ATPase domain, and a carboxy-terminal DNA binding domain (Figure: 1.2) (Studholme and Dixon, 2003; Kustu et al., 1991; Kustu et al., 1989). In response to stimuli the regulatory domain triggers conformational changes that activate bEBPs.

In Group-I bEBPs the regulation occurs by phosphorylation of the regulatory domain. The majority of this family of bEBPs is a part of a two-component signal transduction system (Nixon et al., 1986). The first component of this system is a donor Histidine kinase, which gets auto-phosphorylated in response to specific signal or stimuli. This labile phosphate group then gets transferred from the first component to the second component’s (bEBP) specific aspartate residue on the regulatory or receiver domain. This leads to activation of the bEBP (Scharf, 2010; Xu and Hoover, 2001). Some other members of this Group-I bEBPs like Levan utilization operon regulator (LevR) contains a phosphotransferase regulation domain (PRD), where phosphorylation of a conserved histidine residue leads to activation of this operon (van Tilbeurgh and Declerck, 2001). Group-II bEBPs are regulated by binding of small ligand molecules like Zinc in case of Zinc resistance-associated regulator (ZraR), Propionate in case of Propionate resistance-associated regulator (PrpR), Xylene in case of Xylene resistance-associated regulator (XylR) and Nitric Oxide in case
of Nitric oxide resistance-associated regulator (NorR) (Bush et al., 2011; Leonhartsberger et al., 2001; Shamanna and Sanderson, 1979, Studholme and Dixon, 2003). Group-III bEBPs are regulated by domain interactions between proteins like GAF domains (“GAF” expands to “cGMP-specific phosphodiesterases and adenylyl cyclase and FhIA”, with FhIA being adenylate cyclases formate hydrogen-lyase transcriptional activator) (Wigneshweraraj et al., 2005). Some bEBPs even lack any of the regulatory domains and are categorized into Group-IV, like PspF (Phase shock protein F) of *E coli*. In these proteins, separate regulatory proteins are presumed or shown to interact in trans to control the functional state of the ATPase.

The second domain in bEBPs is the AAA+ ATPase domain. It is a highly conserved region of about 200 amino acids and has an amino-terminal α/β sub-domain and a carboxy-terminal α-helical sub-domain. There are several conserved motifs in the AAA+ ATPase domain of bEBPs. These include the Walker A and Walker B motifs, involved in nucleotide binding and hydrolysis, respectively. From across the subunit interface, the Arginine-finger residue directly contacts the γ-phosphate of ATP. The nucleotide status of this active site is believed to be monitored by the Sensors I and II. Like other AAA+ ATPases the bEBPs work in higher order oligomers (pentamer, hexamer or heptamer). By bringing together subunits in this way, oligomerization creates the juxtaposition necessary for Arginine finger contact with bound ATP (Patel et al., 1998; Ogura et al., 2001). This arrangement has also been seen for the NtrC1 ATPase domain (Lee et al., 2003; Chen et al., 2008; Chen and Sysoeva et al., 2010).
The AAA+ ATPase domain of bEBPs has two special inserts that are unique to this subfamily. These inserts are Loop-1 that is inserted into α-helix 3 and Pre-sensor I loop or Loop2 in α-helix 4 of the AAA+ ATPase domain. Loop-1 has the highly conserved “GAFTGA” sequence that is the unique signature motif of bEBPs. These surface loops are arranged such that L2 loop supports presentation of the GAFTGA motif of L1 for contact with σ54-RNAP. This contact mediates mechanical work that remodels σ54-RNAP so that it can melt the promoter DNA. Mutation of the threonine residue in the GAFTGA loop prevents bEBPs from binding or interacting with σ54 (Rappas et al., 2007; Chen et al., 2010). Recent mutational studies have suggested that the Phenylalanine residue of the GAFTGA loop interacts with the promoter DNA and helps in sensing its state (Zhang et al., 2009). Some EBPs have just this central AAA+ ATPase domain and are known as Group-V bEBPs. The Chlamydial two-component system (CtcC) and the Flagellar gene regulator (FlgR) are such EBPs (Brahmachary et al., 2004; Collmer et al., 2000).

The third domain in bEBPs is the carboxy-terminal DNA binding domain containing the signature helix-turn-helix (HTH) motif (Buck et al., 1986; Chaney and Buck, 1999; Batchelor et al., 2009; Pelton et al., 1999). This domain recognizes Upstream Activator Sequences (UAS), usually about 100 base pairs upstream of the σ54 promoter region and thus ensures promoter specificity (Batchelor et al, 2008; Hong et al., 2009). Enhancer Binding Proteins having the DNA binding domain along with only the ATPase domain are categorized into Group-IV EBPs. The DNA binding domain helps to increase the local
concentration of bEBPs close to the σ54-closed complex and also interacts with the complex with a unique DNA looping mechanism (Wedel et al., 1990; Buck et al., 1986). In the absence of DNA binding domain bEBPs free in solution are even able to perform σ54 dependent transcription activation. This has been observed in Group-V bEBPs and also in several experimentally truncated bEBPs or those bearing mutations to create non-functional DNA binding domains (for example, see Ledebur et al., 1990 and North and Kustu, 1997).

Unlike σ70-based transcription, σ54-based transcription resembles many of the basic features of eukaryotic transcription by RNAP II, where along with ATP hydrolysis it also requires the action of transcriptional activators and enhancer binding proteins that act on the polymerase complex by a DNA looping mechanism (Lin et al., 2005). These similarities broaden the audience intrigued by σ54-dependent activation to include those focused on eukaryotic gene regulation.

1.5 Prior studies on bEBP’s AAA+ ATPase domain

AAA+ ATPases couple nucleotide hydrolysis to perform mechanical work on target macromolecules. Over time several studies have been carried out on the AAA+ ATPase domain of bEBPs to understand how they couple nucleotide hydrolysis to remodel the closed σ54 transcription complex. Lee et al. reported the very first high-resolution crystal structure (1NY6) of bEBP ATPase bound to nucleotide in 2003. This structure revealed a heptamer ring arrangement of the
NtrC1 ATPase subunits, when in complex with ADP and all the seven active sites were occupied with ADP (Lee et al., 2003). Buck and Zhang’s groups from 2005 to 2006 solved several crystal structures of another bEBP ATPase, PspF (2BJW, 2C96) (Rappas et al., 2005; Rappas et al., 2006). They were all derived from a single lattice of the apo form of the protein, some soaked with nucleotides in attempt to capture nucleotide-bound structural states. Prior to these crystal structures the same group obtained EM reconstruction of PspF in 2004 (Schumacher et al., 2004). The EM reconstruction showed a hexameric arrangement of PspF. The crystal structures showed helical arrangement of the PspF subunits. Upon soaking apo crystals in nucleotides, a minor side chain movement in key glutamate residue (Glu 108) was observed. This glutamate is in the Walker B motif and it could sense the presence of nucleotide and underwent rotameric change. Further this was sensed by a neighboring asparagine residue (Asn 64), that salt-bridged to the relocated glutamate. This was envisioned to eventually lead to the movement of L1 loop (GAFTGA Loop) that interacts with σ54. This phenomenon was known as the “glutamate switch” hypothesis. Rappas et al. did electron microscopic reconstruction of complex of PspF ATPase with σ54 in 2005. This showed that σ54 does interact with the ATPase on top of the ATPase ring (Rappas et al., 2005). The anticipation at that time was that the ring would be symmetric, and although inspecting their density map shows the sigma factor as bound off-center, they present and discuss it as being centered on the top of the ring. The results from my studies clearly place the sigma factor off center revealing the importance of the unexpected asymmetry of the ATPase
De Carlo and Nixon et al. in 2006 used a combination of EM and X-ray scattering techniques (SAXS/WAXS) to study another bEBP - NtrC from *Salmonella typhimurium*. These reconstructions showed that NtrC forms a hexameric ring upon mimicking phosphorylation of the receiver domain with Be-F$_3$/Mg$^{2+}$. Differences were observed in the structures when the protein was bound to ADP versus the transition state, non-hydrolysable ATP analog ADP-AlF$_3$/Mg$^{2+}$. In the presence of ADP-AlF$_3$/Mg$^{2+}$ density for the six DNA binding domains could be modeled as three dimers below the ATPase ring, while they were invisible in the presence of ADP (De Carlo et al., 2006).

Chen and Nixon et al., in 2007, showed by X-ray scattering (SAXS/WAXS) that NtrC1 AAA+ ATPase, from *Aquifex aeolicus*, undergoes large-scale conformational changes in the presence of various non-hydrolyzable ATP analogues in various stages of ATP hydrolysis. Upon comparing the SAXS reconstructions of apo, ATP ground state analogue (ADP-BeF$_3$) and transition state analogue (ADP-AlF$_3$) (Chaney et al., 2001) bound complexes, the central GAFTGA loop region was seen to move upward by about 10Å. In contrast to the small conformational changes being entertained based on the PspF studies, these solution studies of NtrC1 suggested large-scale changes occur in the bEBP AAA+ ATPase during various stages of nucleotide hydrolysis cycle (Figure: 1.3). Further, by studying various mutants of the NtrC1 ATPase they were able to demonstrate that those mutants that failed to undergo conformational changes in the presence of ATP or ATP analogues failed to interact with σ54 (Chen et al.,
In 2008 Bose and Zhang et al., solved an EM reconstruction of complex of σ54-RNAP with PspF AAA+ ATPase domain (Bose et al., 2008). This complex lacked the presence of promoter DNA. Based on this reconstruction and other biochemical studies they hypothesized that Region-I of σ54 blocked the active center of RNAP, thus preventing the access of the template promoter DNA to be loaded into the RNAP core and the location of the -12 region (bubble start site) was located far from this region. They concluded that ATP hydrolysis by the ATPase causes work to be done on σ54-RNAP, causing large-scale conformational changes and domain movement, leading to removal of this inhibition and also translocation of promoter DNA to bring the promoter into register with the catalytic core of RNAP. These mechanistic features remain possible, but detailed knowledge needed to describe them, or refute them, has not been forthcoming.

In 2010 Chen and Sysoeva et al., obtained the crystal structure (3MOE) of NtrC1 AAA+ ATPase with over saturating amount of ATP. Here a Walker mutant (E239A) was used, which could bind and respond to the presence of ATP but not hydrolyze it to release the γ phosphate of ATP. The crystal structure showed that NtrC1 AAA+ ATPase could exist as a heptamer ring with all seven active sites occupied with ATP. This crystal structure also revealed for the first time the interaction of the putative Arginine finger from neighboring subunit with the γ-phosphate of ATP. Upon superposition and comparing individual subunits from this crystal structure with the ADP bound crystal structure (1NY5) a rigid body roll
in the ATPase subunit was observed (Figure: 1.4(C)). This observation lead to the hypothesis that binding of ATP in the inter-subunit interface is detected by the R-finger of the apposing protomer, causing a rigid body roll motion in the R-finger subunit that poised the GAFTGA loop above the ring for interacting with σ54. This described an alternative to the glutamate switch hypothesis for how ATP-dependent atomic interactions can guide the GAFTGA loop to interact with σ54, and switched focus from changes in the ATP binding subunit to the neighboring one sensing the event via its Arginine finger (Chen et al., 2010).

In order to study the NtrC1 ATPase in sub-saturating or stoichiometric concentrations of nucleotide Sysoeva performed SAXS nucleotide titration experiments with the ATPase (Sysoeva thesis and manuscript in preparation). While gradually increasing the nucleotide concentration in sub-stoichiometric proportions from the apo state, she observed that the ATPase oligomer undergoes two large-scale conformational transitions. The first transition was characterized by a decrease in the ring’s Rg (Radius of gyration) value so they proposed that it changes from heptameric to hexameric state with partial occupancy of ATP. This was followed upon further occupancy with ATP by the second transition, which has been attributed to restructuring of the GAFTGA loops. In order to verify this, Sysoeva was able to obtain new crystal structure with sub-saturating or stoichiometric amount of ATP analogue ADP-BeF₄/Mg²⁺. This structure showed, for the first time, the presence of a hexamer ring of NtrC1C. This ring is highly asymmetric and shows a spiral staircase arrangement of the GAFTGA loops. Further, the electron density between the first and sixth
subunit reveals less contact between two protomers, with a gap present at this interface. Heterogeneous nucleotide occupancy was also observed in this interface, as this site could be in apo, ADP-bound or ADP-BeF$_3$/Mg$^{2+}$-bound state (all three states were observed among the eight hexamer rings present in the two lattices that were examined; Figure: 1.5) (Sysoeva thesis and manuscript in preparation).

Similar to NtrC1, work from Wemmer’s group has shown the existence of another bEBP NtrC4 from *Aquifex aeolicus* in heptamer and hexamer oligomeric states. The isolated central AAA+ ATPase domain of NtrC4 exists as heptamer, but the full-length protein exists as hexamer. They have also found that constructs of NtrC4 lacking DNA binding domain, exists as heptamer in inactive state, but upon phosphorylation of the receiver domain exists as hexamer (Batchelor et al., 2008, 2009).

This asymmetry in the NtrC1 AAA+ ATPase was found to resemble two AAA+ ATPases. Similar asymmetry was observed in the E1 DNA translocase from Bovine papilloma virus and in Rho transcription terminator from *E coli* (Enemark and Joshua-Tor, 2006; Thomsen and Berger, 2008, 2009). In both the structures there was a helical arrangement of the AAA+ ATPase loops and a gap or less tight contact between the first and the last subunit. Furthermore this helical arrangement was suggestive of a sequential nucleotide hydrolysis mechanism in the ATPase, where the arrangement of the loops was a direct reflection of the nucleotide occupancy at that interface between the subunits and the gap is necessary for product release and new nucleotide uptake.
In spite of all these information about bEBP ATPase it is still unknown as to how the homomeric ring geometry of AAA+ ATPase is necessary for its function and how do rings of chemically identical subunits perform mechanical work on target macromolecules? It is essential to seek answers to these questions as AAA+ ATPase are molecular machines that remolds various target macromolecules in prokaryotic and eukaryotic cells and are crucial for their survival. Work done by Sysoeva on isolated NtrC1 AAA+ ATPase showed the existence of the ATPase in asymmetric hexamer ring. She proposed the hypothesis that in order to interact with asymmetric target, here σ54, the AAA+ ATPase ring made up of chemically identical subunit has to acquire asymmetric geometry. My work in this thesis confirms that hypothesis by showing the NtrC1 ATPase exists as asymmetric ring in the closed complex with σ54 and promoter DNA and also when RNAP is present. Further novel contrast matching experiments by Sysoeva showed that the bEBP ATPases acquire this asymmetric geometry prior to interacting with σ54.
Figure 1.1. Comparison between σ70 and σ54 transcription initiation. A. Spontaneous isomerization from closed to open complex takes place when σ70-RNAP binds to σ70 promoter. B. σ54 remains constitutively bound to closed double stranded σ54 promoter. Binding of RNAP to form this closed complex does not lead to spontaneous isomerization to single stranded open complex state. It requires mechanical work to be performed on σ54 by bEBP AAA+ ATPase. These bEBPs bind distal from the promoter at Upstream Activator Sequences (UAS), and come close to the transcription complex by DNA looping mechanism.
Figure 1.2. Domain architecture of bEBPs. In green is the amino terminal receiver or regulatory domain. The central orange domain is the highly conserved AAA+ ATPase domain in bEBPs. They contain the walker motifs for nucleotide binding and hydrolysis, the Arg-finger and the conserved signature GAFTGA loop (blue loop in the center). The carboxy terminal DNA binding domain is shown in yellow. They have the HTH motif for binding to UAS DNA. Different classes from Group I to Group V have been shown with candidate examples from each group (Joly et al., 2012).
Figure 1.3. SAS models of NtrC1 AAA+ ATPase in the presence of different ATP analogues and ADP. (From Chen et al., 2007) Various changes in the ATPase ring structure can be observed in the presence of different nucleotides. The blue envelopes are SAS based reconstructions or models. Each model has been superimposed with ADP-bound crystal structure of NtrC1 ATPase (1NY6; Lee et al., 2003). Note the density in the middle of the ring corresponding to the GAFTGA loop moved up upon binding to ADP-BeFx or ADP-AIFx and comes down in the presence of ADP or in the apo state.
Figure 1.4. NtrC1 AAA+ ATPase domain. A. Heptamer crystal structure of E239A mutant NtrC1 AAA+ ATPase with ATP (3MOE, Chen et al., 2010). The yellow circles represent the interface between subunits and this is where nucleotide binding and hydrolysis takes place. The central pore of this ring is made up of L1 and L2 loops. B. After superimposing the ADP (1NY6) and ATP (3MOE) bound structures, an enlarged view of the interface between two neighboring subunits is shown. An upward movement of the Arginine finger (red in presence of ATP and blue in ADP bound state) is observed in the presence of γ-phosphate. C. Rigid body roll motion between domains in the NtrC1 ATPase upon binding with ATP, leading to upward movement of the GAFTGA (L1) loops (Chen and Sysoeva et al., 2010).
Figure 1.5. NtrC1 AAA+ ATPase in sub saturating or stoichiometric amounts of nucleotide. (Sysoeva thesis and manuscript in preparation). A. The hexamer crystal structure with each of the 6 subunits (A-F) shown in different colors. The side view of the structure shows staircase like or cascading arrangement of the GAFTGA loops. B. Difference map showing nucleotide occupancy between each subunit. The interface between the first and fifth subunit may be apo, ADP-bound or ADP-BeF\textsubscript{6} bound. C. Density around the L1 and L2 loops in the center of the ring shows tight contact in this region between the first five subunits and a gap between the first and the sixth subunit. The arrows show the connectivity between the loop regions from neighboring subunits, which is missing between the first and sixth subunit.
1.7 Transmission Electron Microscopy

Theory and background of Electron Microscopy

A microscope is a device that helps in visualizing a small object by magnifying it. In the case of light microscopes, a series of lenses are used to focus light reflecting off of an object into a magnified image. The magnification of an optical system depends on the wavelength of the incident photons. Therefore in the case of light microscopes the resolution limit is around hundreds of microns. It is impossible to obtain higher resolution information of individual macromolecules with light microscopy. X-ray crystallography is a technique that is used for obtaining near atomic resolution structures of macromolecules, but the limitation of obtaining diffracting crystals makes it difficult to use this system for most large complexes. In addition to this limitation, the lattice of the diffracting crystal often restrains structures obtained by crystallography. This often traps a packable conformation and then restricts movement once the lattice is formed. In order to study flexible proteins or macromolecules in solutions one can make use of other techniques like solution scattering (X-ray and neutron) or electron microscopy. High voltage electrons have wavelengths in Angstroms. This property of electrons is utilized in electron microscopes to provide reasonably moderate to high-resolution information about macromolecules.

The two most common forms of electron microscopes are Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM). In the case of SEM, an electron beam is used to scan the surface of biological samples
coated with metal and the back-scattered electrons give rise to magnified images of the surface features of the molecule. Internal information about the sample cannot be obtained by SEM; therefore limiting the extent of high-resolution information that can be obtained. On the other hand in TEM the electron beam penetrates through the sample and a magnified image or projection is obtained by interference of the electrons passing through the complete thickness of the sample. Thus the projections obtained by TEM combine both the surface and internal details of the sample and provide higher-resolution information (Frank, 1996). Next I provide an overview of TEM and Single Particle Reconstruction, which I used to obtain the structures of NtrC1ATPase, σ54 and promoter DNA complex.

**Transmission Electron Microscope**

A Transmission Electron Microscope works similarly to a light microscope. It uses fast accelerating electrons instead of light and a set of electromagnetic lenses to focus the beam of electrons. By varying the current in the lenses their focal lengths can be changed and so the overall magnification can be altered. The entire setup is encased inside a high vacuum column to minimize the interaction of electrons with air. (Figure: 1.7)

The following are basic components of a TEM (Frank, 1996) (Figure: 1.7):

1. **Electron Source / Gun**: This is the source or origin of the electron beam. There are two types of electron guns based on how the electrons are generated. Electrons can be generated by thermionic emission by heating metal like
Tungsten or Lanthanum Hexaboride (LaB$_6$). Alternatively, they can emit from a Field Emission Gun (FEG) where application of high voltage to a pointed tip leads to the release of electrons. The electrons emitting from an FEG source are more coherent (all at same phase) than those from filament based electron guns, thus leading to fewer aberrations in the micrographs.

2. **Electromagnetic Lenses:** Like any optical system, the TEM has lenses; in this case, they are electromagnetic. There are four basic lenses that make up the TEM. The condenser lens is the topmost lens that helps in focusing the electron beam on the sample. The sample stage is below this lens. After passing through the sample, the electron beam is first magnified and focused by an objective lens. Intermediate lenses further magnify the image. Finally the projector lenses are used to focus the magnified image onto a phosphorescence screen, photographic film or CCD camera.

3. **Apertures:** The apertures are located close to different lenses and they control the size of the electron beam that passes through. The condenser aperture lies close to the condenser lens and regulates the spot size and coherence of the electron beam that is going to be projected on the sample. The objective aperture lies below the objective lens and by selectively blocking passage of diffracted electrons it enhances the contrast of the image.

4. **External Accessories:** All the above-mentioned parts are the basic components of the microscope. In addition to these there are other accessories that lie outside of the microscope. These include the computers and electronics that actually control the microscope and help collect images. There are switches
and knobs that help in adjusting beam brightness, beam size, magnification, alignment and focusing of the beam and movement of the goniometer. The sample is loaded on to the microscope on special holders, which can either function at room temperatures (for negative stained samples) or cryo holders for studying frozen, hydrated cryo samples. The cryo holders have a liquid nitrogen Dewar attached at one end to keep the sample in the frozen state. The microscope too has a cryo Dewar attached to it and it needs to be filled with liquid nitrogen in order to maintain the temperature inside the microscope, close to the sample’s cold temperature. This helps in keeping cryo samples frozen in vitreous ice and also functions as an anti-contaminator by condensing water vapors and other gases generated during beam exposure, and preventing them from condensing back on the specimen.

**Grids and sample preparations**

For TEM the samples are applied to special carbon coated copper grids (Ruiz and Radermacher, 2006). These copper grids act as support and being a good electrical conductor disperse any accumulated charges on the samples upon exposure to electrons. They vary in the number of mesh like 100, 200, 300 or 400. More mesh provides more support to the carbon, but limits the useful angle of tilt that can be used to obtain side views of the molecules. The carbon layer on top of the copper grid is where the samples are applied. There are basically two types of carbon layers that can be used:
1. **Continuous carbon:** In these grids a thin continuous carbon layer is deposited on top of the copper grid. The way of making the carbon layer is described later. Samples are applied on the carbon layer. These types of grids are more suitable for negatively stained samples, given their higher contrast than unstained cryo samples. In the latter case, the carbon film will obscure the view of the particles.

2. **Holey Carbon:** In these grids there are holes in the thin carbon layer. Depending on the type of holes they can be called lacey carbon with irregular shaped and sized holes or regular holey grids where, by lithographic techniques, regular fixed diameter circular holes are punched on the carbon film at fixed distances from each other. These holey grids are usually used for cryo samples where frozen hydrated samples are imaged in the ice layer in the holes. Sometimes a thin carbon film is deposited on the holes for samples that are charged and have high affinity for carbon. This allows for even spreading of sample on the holes and prevents them from being on the carbon edge of holes. The way the carbon films are prepared and deposited is described in the section below.

**Thin carbon film making by carbon evaporation:** In order to make the continuous carbon film for carbon coated EM grids the following procedure is used.

1. Clean Grade-V mica is first gently cleaved to expose fresh, smooth and clean surface for carbon deposition.

2. A flake is then placed inside an Edwards 360 vacuum evaporator with the fresh cleaved surface facing up.
3. The chamber with the mica sheet is then evacuated and current is applied between two graphite electrodes until it incandescses.

4. Carbon evaporates from the graphite rods and gets deposited on the mica. Depending on the time of exposure one can attain different thickness for the carbon film.

5. The carbon coated mica pieces are then carefully removed from the evaporator and placed in a 60°C oven for five to seven days for hardening. Freshly deposited carbon is highly brittle. The carbon can be left in the oven till further use. Once oven-hardened, the carbon is less brittle.

**Preparation of thin carbon coated TEM grids:** Specific mesh number grids are first washed three times with acetone in a sonicating water bath for 10 mins each time. After the acetone has been completely evaporated individual grids are carefully submerged in water and placed over a small rectangular piece of filter paper. This entire setup is in a small water tank with an outlet for water connected to a syringe at the bottom. The carbon-coated mica is then slowly and carefully lowered into the water at an angle, in such a way that the thin carbon film comes off of the mica and floats on the water surface directly above the grids. By slowly drawing out the water from the bottom of the tank, the carbon layer comes down with the water level and finally covers the grids. The carbon-coated grids are then taken out and left for drying until further use. The carbon layer on the grids are usually hydrophobic at this point of time and if used won’t
cause even spreading of samples. Right before use they are plasma cleaned in a
glow discharge instrument or plasma cleaner to make them hydrophilic.

Sample preparation techniques: Depending on the type and temperature at
which samples are visualized or imaged in TEM, they can be broadly classified
as follows:
1. Negative Stained samples: Biological samples usually have lower contrast
thus making them difficult to be visualized. One can enhance contrast by
preferentially staining the specimen or sample. In TEM molecules are stained
with salts of heavy metals like Uranium, Tungsten, Molybdenum or Vanadium.
For single particle techniques small volumes (3-5μl) of diluted sample are added
on plasma cleaned continuous carbon coated grids. After waiting for 30s excess
sample is blotted with Whatman-5 filter paper and immediately a 10μl drop of
stain is added on the sample. After 15s the excess stain is blotted and this
process is repeated twice. After the last drop of stain has been blotted the grid is
dried quickly by swaying the grid in air. These steps typically need to be
optimized, and there can be variations in this process. Optimizing these steps to
yield good images consumes a significant amount of time.

The interaction of the electron beam with the stained sample leads to
contrast. The stain forms a uniform layer around the sample and is excluded from
the volume occupied by the sample (Figure: 1.8 (A)). When the electron beam
passes through the stained specimen there is differential deflection of the beam.
The beam passing through the stain-excluded volume is deflected less than in
the stained region and the objective aperture below the grid blocks some of the deflected beam. Contrast in the sample arises from this preferential allowance of deflected beam along with their interference with less deflected beam. A smaller aperture will give better contrast, but can lower the resolution as the electrons deflected to wider angles are stopped.

Despite advantages of room temperature sample preparation and data collection, relatively easy sample handling, and strong contrast between particles and solution, there are some drawbacks to using negatively stained samples. The staining process can cause some artifacts like stain flattening and collapsing of proteins during the drying process. Uneven staining can also give rise to artifacts, and the maximum achievable resolution is limited to the grain size of the dried stain i.e. 20 - 25 Å or lower. Stain flattening can be overcome by adding 1-4% Trehalose to the sample (DeCarlo et al. 2011, Harris et al. 1995).

2. Cryo samples: In order to observe biological macromolecules in their native conditions or in frozen hydrated states, the technique of cryo transmission electron microscopy is used (Figure: 1.8 (B), (C)). Samples are applied on plasma cleaned holey carbon grids or with holey grids covered with a thin carbon film. Excess sample is then blotted away with Whatman-5 ash-less filter paper and the reduced volume is quickly plunge frozen by dropping the grid into a small cup of liquid ethane at around -178°C. The reason for plunge freezing into liquid ethane is that it has high specific heat capacity and can quickly freeze the sample into vitreous ice (amorphous ice) instead of forming hexagonal or cubic
ice crystals. Hexagonal or cubic ice form when water is frozen slowly and they readily absorb the electron beam and obscure the biological sample. The blotting process before freezing is carried out in a controlled environment of about 70-90% relative humidity. This prevents the sample from getting dried. Optimal ice thickness is necessary for cryo EM, as thicker ice can reduce the contrast and may even block the electron beam from passing through. If too much of the buffer gets blotted then the sample can get dehydrated. The composition of buffer does affect the quality of ice and contrast. Presence of glycerol or sugars should be avoided as they interfere with electrons and add to background noise, reducing contrast. High concentration of salt can affect the quality of ice and add significantly to background noise resulting in poor contrast. A problem often faced in cryo EM is non-uniform sample spreading and preferential attachment of sample to the surrounding carbon and not in the holes. In order to facilitate even spreading of macromolecules into the holes, a thin carbon film is deposited on the grid and samples are applied on this film. These types of grids are prepared in the same manner as described earlier. Sometimes addition of small amount of surfactants like detergents or bacitracin (Ruiz and Radermacher, 2006) can help in uniform spreading of particles on the grid. Sometimes this can be incompatible with the biological specimen and can cause complexes to fall apart. In comparison to negative stained samples, cryo samples have significantly lower contrast. Thus single particle reconstructions with cryo samples work better for particles larger than 400KDa in size (Frank, 1996). Large complexes like ribosome, virus particles and apoptosomes work best with this technique.
**Theory of image formation in TEM**

When an electron beam hits a sample, the following can happen to the electrons: The electrons can just bounce off of the sample without any loss of energy - this is known as elastic scattering. Alternatively, they can transfer some of their energy to the sample and bounce off in-elastically. The in-elastically scattered electrons contribute to noise in TEM micrographs. Many electrons get transmitted through the sample. Some of them are deflected; others are transmitted un-deflected through the sample. When these electrons coming at different angles interfere with each other contrast is created to define the image. As mentioned earlier, by blocking some of the electrons by the objective aperture the contrast can be enhanced. However the higher deflected electrons have higher resolution information, so a balance between contrast and resolution has to be sought in adjusting this aperture. The in-elastically scattered electrons have different wavelengths or energy and in modern microscopes they can be removed with the help of a device called an energy filter, which leads to lesser noise in the micrograph. Since in TEM the micrographs or projections are formed by interference of electrons that have passed through the samples, the images contain internal details of the sample. Thus, although the information is of relatively low resolution in comparison to X-ray crystallography or NMR spectroscopy, TEM images contain information about the 3D cloud of electrons possessed by macromolecules.
Data collection strategy for single particle reconstructions

(Ruiz and Radermacher, 2006; Frank, 1996) Biological samples are considered soft matter and require special delicate sample handling. In the cases of both negative stained and cryo samples, the “low dose technique” is used. In TEM high-energy electrons are used for imaging. They can cause radiation damage and can even destroy the ice or carbon layer on grids. Minimizing this damage is important for keeping the integrity of the sample intact. This is achieved by imaging at a low electron dosage per unit area, about 6 to 10 electrons / Å². One of the drawbacks of this technique is that lower electron dosage leads to a poorer signal to noise ratio. Because one needs to collect a certain number of scattered electrons, larger specimens are easier to study at low dose, especially by cryo EM.

The steps that are usually followed while collecting images after insertion of a suitable grid (sample containing grid) into the microscope are to focus and align the microscope for data collection. Images are collected at the eucentric focal height of the microscope, so that the area that is being imaged would not alter or be out of focus when the grid is tilted. First an area suitable for imaging is selected at a lower magnification. Following this, the beam is focused at an area nearby to the one that is to be imaged at a higher magnification (about 100K). This is done to avoid over exposure and radiation damage in the area to be imaged. After focusing it, the microscope is slightly defocused to about 1.5 to 4.5 Å under focus and the area to be imaged is exposed for a short period of time - about 1s. Taking images at a slight defocus improves the contrast. Usually
images are collected at different defocus values, giving rise to a defocus series of images that yield different resolution information (Penczek, 2010). Increase in defocus improves contrast, but reduces the high-resolution information in the micrograph. This is why images are collected at close to focus or smaller defocus values. An explanation for this has been provided in a later section. Other strategies of image collection by tilting the grid or “Random Conical tilt” are explained in later sections.

**Image processing and introduction to Single Particle Reconstructions**

In the previous sections I have described how samples are prepared and micrographs are collected on either CCD or photographic films. When film is used it needs to be developed and properly digitized. The very first processing that can be carried out on the micrographs is correcting for optical distortions.

This is a mathematical process and is known as “Contrast Transfer Function” correction (Radermacher et al., 1987; Penczek, 2010; Frank, 1996). Any image collected by an optical system undergoes certain distortions. A mathematical relation between the object and its image can be formed by a convolution term known as the “Point Spread Function”. The physical meaning of this function is that in reality there is no one to one correspondence between every point in an object to every point in its image. The mathematical function that describes this is known as the point spread function. A Fourier transform of this term is known as the Contrast transfer function (CTF) (Figure: 1.9). This is an
oscillating function and depends on defocus, spherical aberration of the microscope and the wavelength of electrons (this can be determined from the voltage of the microscope). The parameter that is varied while collecting images is the defocus. Depending on the defocus settings different features of the objects appear enhanced or suppressed in an image. Usually the CTF oscillates between positive and negative values and the point where it crosses zero is where no contrast is transferred and information is lost. The spatial frequency in inverse distance where the function comes down to zero depends on the defocus used. Higher defocus values cause the CTF to cross zero at lower spatial frequencies, thus losing higher resolution information. This is why as mentioned earlier micrographs are collected at different defocus values. Calculating the CTF parameters of a micrograph is crucial to make these corrections to obtain an undistorted reconstruction of the particles. The presence of noise in micrographs makes it difficult to have an accurate CTF estimation. This estimation is made possible by using several Fourier filters (mathematical removal of unwanted frequencies in Fourier space) to enhance the signal to noise ratio (Frank, 2006; Penczek et al., 1997).

After estimating the CTF parameters of each micrograph, individual particles are boxed out from the micrograph with the help of software packages like EMAN2 / Boxer (Ludtke et al., 2007), XMIPP (Scheres et al., 2008) or SPIDER / WEB (Wadsworth Center, Albany, NY). This can be done by automation in programs in EMAN2, but manual picking is better as one can avoid picking unwanted particles or artifact features from the micrographs. After the
individual particles are boxed they are usually converted into stacks of particles for a project. The particles at this stage are not perfectly centered. In order to center the particles an average of all the particles is calculated and individual particles are moved to align their center of mass with that of the average (Radermacher et al., 1987; Penczek et al., 1992; Frank, 1996). This process is repeated, until no significant shift becomes necessary. Centering can also be done with respect to some external reference (e.g. a prior model).

After centering the particles they have to be aligned, classified and averaged. During the alignment process the particles are placed in similar orientation with respect to each other. This is done by maximizing the cross correlation and auto correlation functions to learn how to rotate and shift for optimal initial alignment. This process is iterated until all the particles have similar orientations. Unlike this alignment procedure, which is reference free, alignment can also be done with a reference particle or projections from a reference structure and is known as reference based alignment (Radermacher, 1994).

Once the particles have been aligned, they are then grouped into distinct classes based on inter-correlations between the particles. Particles belonging to a single group are presumed to represent the same projection of the 3D object. Data collection continues until all experimentally available projections are present in the data set. Because particles tend to be in certain preferred orientations in the xy plane one’s ability to capture all projections is limited to certain angles in the bulk of a 360° range. Sometimes there are conformational heterogeneity between particles and might reflect either different conformations of the molecule.
or different subassemblies of a complex macromolecule. Various classification algorithms have been developed to group identical particles together in well-defined classes and separate them from other particles. Most of the EM single particle processing software use one of the following algorithms: Maximum Likelihood based classification is used in XMIPP suite (Scheres et al., 2008), SPIDER and IMAGIC uses Multivariate Statistical Analysis (Van Heel et al., 1980; 1981), Expectation Maximization and K-mean clustering is used in SPIDER and EMAN (Frank, 2006) and K-EQ mean clustering in ISAC / SPARX (Zhang et al., 2012). Details of each of these algorithms are beyond the scope of this thesis.

In order to overcome the preferred orientation problem one can obtain an additional view of the particle in azimuth (z-axis). This is done by collecting images or projections after tilting the grid (Figure: 1.9). This helps to separate out classes better and even helps to separate particles that vary in conformations. This technique is known as Random Conical Tilt (RCT) (Radermacher et al., 1987, 1988) (Figure: 1.9). In this technique two sets of images of the same area are collected. First, by tilting the grid at an angle of about 45° - 65° an image is collected and after that an un-tilted (0°) image of the same area is collected. Particles are picked in pairs from both the micrographs and only the un-tilted sets of particles are used for classification. After successful classification, particles in each class are averaged over each pixel to have better signal to noise ratio. Classification can be done in the absence or presence of reference projections.
The basic mathematical theory based on which 3D volume is reconstructed from 2D projections is known as the Projection Theorem or Central Section Theorem. This theorem states, “The 2D Fourier transform of a 3D density is a central section of the 3D Fourier transform of the density, perpendicular to the direction of the projection.” (Crowther, 1971) (Figure: 1.8).

Each particle from TEM micrographs is a projection of a 3D macromolecule that is lying in a unique random orientation on the grid or in the xy plane. The Fourier transform of each projection is a central section of the 3D Fourier volume of the macromolecule. The angle of orientation of a given central section is unknown – assigning Euler angles to each section is thus required for building a 3D reconstruction. Assigning Euler angles is done in variety of ways. The method of “Common Lines” uses the intersecting lines of two or more projections to organize them in 3D. This technique is also known as the Angular Reconstruction Technique (ART) (Van Heel et al., 1987). Methods of 3D reconstructions have been developed where cross-correlations have been used with Common Lines (Hall et al., 2007). A major disadvantage of this technique is that it works better for symmetrical particles and a loss of handedness in particles happens without implementing constraints based on apriori information. Angular assignments of projections can also be done on Sinograms built from Radon transforms (Radermacher, 1997). In this process the original projection is transformed into a series of equi-angular projections and the outcome is a 1D line, a Radon transform. Stacking of all Radon transforms generates a Sinogram from which the angular orientation can be estimated. Another method of obtaining initial
models is by Random Conical Tilt that was described above. The un-tilted projections are used for classification. The tilted view of each image of a given class provides a different angular view whose Euler angle is known relative to the other views within this class. Therefore a 3D reconstruction can be built for each class average. The presence of heterogeneity in the macromolecule preparation will be revealed in the 3D reconstructions of all the classes. Unlike all the other methods, RCT provides a way of assessing heterogeneity in a given dataset of images.

After generation of an initial 3D model, it is further refined to improve resolution. This is done by projection matching and angular refinement technique. At first equidistant projections from the initial model are generated at moderate angles like 10° or 15°. These projections are then cross-correlated with a larger experimental dataset of projections. The experimental projections that have the highest cross-correlation values with the reference projections are matched and the corresponding Euler angles of those projections are updated. After all the projections have been assigned new Euler angles a new refined 3D model is generated. This process is repeated with each new model and with finer or smaller angular projections over successive iterations. This iterative process continues until no more improvement in the re-constructed model occurs. Finally an angular distribution plot for the reconstruction is obtained which shows the angular coverage in 3D Euler space. If the dataset of images does not give adequate coverage of 3D Euler space, more images at different angles are required.
In Single Particle Reconstructions by TEM, the resolution of the reconstruction is reported in terms of the Fourier Shell Correlation (FSC) (Frank et al., 2010; Henderson et al., 2011; Van Heel, 1982). In order to do so the data set is randomly divided into two halves (sometimes into odd and even numbered particles) and independent reconstructions from each data set are carried out. Cross-correlations between 3D Fourier shells over various spatial frequencies are calculated and the frequency where the FSC value is 0.5 is reported as the resolution of reconstruction that is achievable from that data set. This is the most widely used criterion for reporting the resolution of EM reconstructions. Other criteria often used for reporting resolutions are the 3σ criterion, 0.5σ criterion or the 0.143 cut off. These are based on where the FSC value becomes more than the expected random correlation of background noise (Van Heel et al., 2005).

After a final 3D reconstruction of a complex or biological macromolecule is obtained, high-resolution structures of individual components can be superimposed or fit into the density. This can provide the organization of individual components in a biological complex or even by flexible fitting of crystal structures into EM reconstructions one can obtain the dynamic conformational changes in the molecule.
**Figure 1.6. Basic set up of a Transmission Electron Microscope.** To the left is a typical modern transmission electron microscope (Jeol 2100, NYSBC, New York). To the right is a diagrammatic representation of the various components of the microscope and the direction of the electron beam. The microscope acts as a black box, creating a distorted image of a given object by virtue of the microscope’s point spread function. By knowing this function, or its Fourier transform known as the CTF, one can reconstruct the object from a set of collected images that represent different views of the object.
Figure 1.7. Sample preparation for single particle reconstructions by Transmission Electron Microscopy. **A.** Diagram showing how a sample is covered with metal stain during negative staining. To the right of it is a micrograph of the stained sample where individual particles in random orientation can be seen in white and one particle boxed. **B.** A typical cryo plunge freezer for preparing cryo-grids. 1. Liquid nitrogen chamber, 2. Liquid ethane cup containing ethane at -180°C, 3. Humidity controlled chamber, 4. Plunger, 5. Blotter for blotting off excess sample before plunge freezing, 6. Holey grid attached to forceps. Samples are applied on this grid. The brown arrow indicates the direction of plunging. **C.** A typical holey carbon grid with a thin layer of ice containing particles. A cartoon representation of a magnified hole with a sheet of ice is shown, and below that is a cryo micrograph with a boxed particle. Notice the relatively lower contrast of the cryo micrograph than the negatively stained micrograph.
Figure 1.8. Image processing and Single Particle Reconstruction. The left hand side of the figure shows a typical micrograph with its power spectrum (Fourier transform of the pixel intensities of the image). The different bright concentric rings show the oscillating nature of the CTF, which is the intensity radiating away from the center of the image. The right side of this image shows a diagrammatic explanation of the “Projection Theorem”; the basic principle of reconstructing 3D volume from its 2D projections (Taken from Frank, 1996)
Figure 1.9. Random Conical Tilt. A. (Taken from Joachim Frank’s lecture at NYSBC, New York). 1. Random orientations are shown in the xy plane of particles (here hand) in their untitled projection. 2. When the same grid is tilted, the new tilted projection can distinguish between the various in-plane orientations of the hands. 3. By collecting several tilted projections and back projecting from those projections, one can reconstruct the particle. B. A tilt pair micrograph. The micrograph to the left is a 65° tilted one of the right (un-tilted). The red axis in the middle is the tilt axis.
1.8 Solution Scattering techniques (X-ray and Neutron Scattering)

This is another commonly used structural technique to study macromolecules in solution. Solution scattering includes both X-ray scattering (SAXS / WAXS) and neutron scattering techniques (SANS). From solution scattering data one can obtain information about shape, size and hydrodynamic radius (Rg or Radius of gyration) of macromolecules. These techniques yield reconstructions at nm range resolutions.

In X-ray scattering a highly collimated and focused X-ray beam is scattered by molecules in solution. The 1D scattering profile of intensity versus distance can be obtained from circular integration of a 2D projection of the scattered photons. This projection is detected by a flat 2D array of pixels. The molecules being studied in solution need to be in a concentration range of 1 – 10 mg/ml and has to be free of aggregates and inter particle interference (i.e., needs to be mono-dispersed). Aggregation and inter particle interference can severely effect the scattering and confound data analysis. X-rays are scattered by electrons in atoms and thus in a multi component macromolecular complex individual components cannot be distinguished from each other in the overall reconstruction. In order to get structural information from each component in a complex, their individual scattering length density needs to be altered. Alteration of X-ray scattering length density of biological macromolecules is not possible.

This is easily achievable in neutron scattering experiments. Neutrons are scattered by the nuclei of atoms and altering the isotope can alter the nuclear properties of the element. In the case of biological macromolecular complexes,
by selectively deuterating individual subunits one can alter the scattering property or contrast of the separate components of the complex. In a binary system if one of the components is deuterated and the other is protonated; by altering the deuterium content of the buffer one can match out the scattering from one of the components. The resulting scattering profile will have information from just the non-matched out component. This procedure is known as the contrast variation neutron scattering technique and data is collected in buffers containing different amounts of deuterium. Complexes containing DNA or RNA do not require deuteration as poly-nucleotides have different scattering length density than proteins and this natural contrast difference can be utilized in contrast variation SANS. So far the majority of successful SANS reconstructions have been for binary systems. Software suites like MulCh (Whitten et al., 2008) and ELLSTAT (Heller et al., 2006) can be used for processing and modeling of SANS data.

Efforts have been made to extend this approach to include a combination of X-ray and neutron scattering data to improve reconstructions of components in complexes. By using the MONSA software suite (Svergun, 1999) from EMBL one can do such processing and reconstructions. Based on calculations made by Volker Urban at the ORNL BioSANS group, it was hoped that these improvements would allow solving ternary complexes, and this was attempted in this thesis research. Unfortunately, the information content of the data has proven inadequate for unique solutions to be obtained. Fortunately, the methods I developed in pursuit of this project were used in collaboration with my former
lab colleague, Tatyana Sysoeva, to gather structural information for a binary complex.

1.9 Overall Summary

For a considerable period of time there has been an unanswered question of how chemically identical subunits containing the AAA+ ATPase fold does mechanical work on asymmetric target macromolecules. One such scenario is in the case of bEBPs that help in activating σ54 dependent transcription in bacteria. Evidence reviewed in the introduction show that the AAA+ ATPase domains of bEBPs assemble into homomeric rings that couple nucleotide hydrolysis to perform mechanical work on asymmetric σ54-RNAP-promoter DNA closed complex. Prior structural studies on the ATPase domains of the bEBPs PspF, NtrC and NtrC1 done in the presence of saturating nucleotides have shown the presence of symmetric hexamer or heptamer rings. Recent crystal structure of the NtrC1 ATPase with stoichiometric or sub-saturating amount of nucleotides has shown the formation of an asymmetric hexamer ring structure (Sysoeva thesis, and manuscript in preparation). In this structure the L1 or GAFTGA loop was observed to form a helical staircase-like arrangement and heterogeneous nucleotide occupancy was seen in the interface between the first and sixth subunit. This feature was strikingly similar to distant AAA+ ATPase like E1 and Rho nucleic acid translocase, where the gap is suggested to be responsible for nucleotide exchange. This suggested a similar mechanism in bEBPs, which
yielded the hypothesis that generation of this asymmetric state in the ATPase is essential for it to exert directional force on its asymmetric target. Significantly, these studies were done on isolated ATPase that was not interacting with its target. So the question that needs to be answered is what happens to the bEBP AAA+ ATPase in the context of the σ54 transcription activation complex – does the asymmetry persist, and does it guide interaction with the transcription machine’s components.

In order to address this I have studied the closed complex of NtrC1 ATPase with σ54 and promoter DNA. In the second chapter I have presented single particle EM reconstruction of this complex. This reconstruction showed the persistence of a gapped asymmetric ATPase ring in this complex and by superimposing NtrC1 crystal structures into the EM density, the gap in the asymmetric hexamer ring fits well with the gap in the EM density. The hypothesis is directly supported by these results.

Further in chapter three by using contrast matching SANS experiments on complexes of NtrC1 ATPase with σ54, it has been observed that the asymmetric hexamer ring geometry of the ATPase does not significantly change when it interacts with its target σ54 (Sysoeva unpublished work). One of the crucial raw materials needed for doing such experiments is large quantity of deuterated proteins. I have described an improved feed batch method of expressing deuterated proteins. This is also of value for techniques that require labeled proteins like NMR.
Finally, to take the structural studies from a partial to a full transcription apparatus, in chapter four I have presented the negative stained EM reconstructions of the full σ54 transcription complex (NtrC1 ATPase in complex with σ54-RNAP-closed promoter DNA). While the cryo study is not yet completed, I have presented class averages from an initial cryo EM data set for this complex. Persistence of asymmetric, gapped ATPase ring was observed in these class averages. These results strongly support the working hypothesis that the bEBP AAA+ ATPase motor is made of chemically identical subunits that assemble into an asymmetric structure to perform mechanical work on an asymmetric target, the σ54-form of RNA polymerase bound to promoter DNA.
CHAPTER 2

PERSISTANCE OF ASYMMETRY IN NTRC1 AAA+ ATPASE WHEN IN COMPLEX WITH σ54 AND PROMOTER DNA

Summary

The fundamental difference between transcription initiation dependent upon σ70 and that dependent upon σ54 is that the later requires a bEBP ATPase to isomerize the closed transcription complex to the opened state. These ATPases play a crucial role in this process and it is necessary to understand how they couple ATP hydrolysis to perform mechanical work on their targets. NtrC1 from the extreme hyper-thermophile *Aquifex aeolicus* is one such bEBP who's central AAA+ ATPase domain (NtrC1<sub>C</sub>) is being extensively studied in the lab. Prior solution X-ray scattering experiments (SAXS/WAXS) have shown large-scale conformational changes in NtrC1<sub>C</sub> between its ADP-bound and ATP-bound states (Figure: 1.3) (Chen et al., 2007). Comparison between single subunits of the heptamer crystal structures of NtrC1<sub>C</sub> bound to ADP (1NY6) with the ATP bound structure (3MOE) has revealed a rigid body roll motion, with upward movement of the L1 or GAFTGA loop in presence of ATP (Figure: 1.4) (Chen et al., 2010). Recent SAXS experiments and crystal structures of NtrC1<sub>C</sub> in sub-saturating or stoichiometric amount of ATP have revealed the transition of the NtrC1<sub>C</sub> ring from symmetric heptamer to an asymmetric hexamer structure (Sysoeva thesis and manuscript in preparation). This leads to the hypothesis that
allosteric response to ATP binding by otherwise chemically identical subunits guides the ring to adopt an asymmetric structure. This asymmetric ring provides the scaffold for delivering directional force required for the bEBP subfamily of AAA+ ATPases to perform mechanical work on the asymmetric target molecule, σ54-RNAP-DNA closed complex.

So far all these observations were made in isolated NtrC1C ATPase. In order to understand what happens to the ATPase when it is interacting with its target, the σ54-promoter DNA closed complex, structural studies were carried out on this complex. This chapter presents the Electron Microscopic (EM) reconstruction of negatively stained closed complex of NtrC1C with Klebsiella σ54 and nifH promoter DNA from Rhizobium sp. This reconstruction of optimally interacting components shows the persistence of an asymmetric hexamer ring of the NtrC1C AAA+ ATPase in the complex. Re-evaluation of published observations of the PspF ATPase structure when in complex with σ54 and RNAP revealed a similar asymmetry. These observations strongly support the hypothesis that bEBP ATPases function as asymmetric hexamer rings via a combination of sequential nucleotide hydrolysis with rigid body roll in subunits providing the necessary mechanical force for them to remodel σ54-closed transcription complex.
2.1 Introduction

It is known that σ54 can recruit RNAP to specific promoters and form stable closed complexes. Unlike σ70 transcription initiation, the σ54-RNAP-closed promoter complex cannot spontaneously isomerize to open complex form. The σ54-RNAP closed complexes require mechanical work to be performed on them by bEBP ATPase before they can melt the promoter. These bEBPs belong to AAA+ ATPase super-family and function as oligomeric rings, with the interface between each pair of subunits acting as the active site for nucleotide binding and hydrolysis. NtrC1 is one such bEBP from *Aquifex aeolicus*. Prior studies on NtrC1 ATPase domain have shown that ATP binding causes large scale conformational changes and rigid body roll in the ATPase subunit (Chen et al., 2007, 2010). This leads to an ATP-driven, upward movement of the L1 loops or GAFTGA loops, which are highly conserved in bEBPs and are responsible for interacting with σ54. A power stroke presumably follows hydrolysis of ATP.

A fundamental question that remains to be answered is how do these molecular motors made up of chemically identical subunits interact with highly asymmetric targets? It is crucial to seek an answer to this question as AAA+ ATPases are ubiquitous in all kingdoms of life and they work on variety of macromolecules in the cell. Recent SAXS experiments and crystal structures of NtrC1 with sub-saturating or stoichiometric amounts of ATP has shown the reorganization of the ATPase from heptamer to a hexamer structure (Figure: 1.5) (Sysoeva thesis and manuscript in preparation). The NtrC1 ring appears to be
asymmetric with a quasi split between the first and sixth subunit. This interface also has heterogeneous nucleotide occupancy. It can be in apo, ADP-bound or ATP bound states. Along with this heterogeneous nucleotide occupancy the L1 and L2 loops were in a staircase like arrangement above the plane of the ring. This lead to the theory that the process of transcription activation by bEBP AAA+ ATPase happens by a sequential ATP hydrolysis mechanism, whereby varied functional and structural identity of the subunits is due to allosteric distribution of ATP and ADP. Further similar asymmetric ring structures in AAA+ ATPase were observed in distantly related members like Rho and E1 helicases (Enemark and Joshua-Torr, 2006; Erzberger and Berger, 2006), where the gap in the ring is proposed to mediate ADP-ATP exchange.

For bEBPs, all these studies were done on isolated ATPase ring, but the question that needs to be answered next is what happens to the ATPase when it is interacting with its target or in context of the full functional complex? The only two complexes of bEBP ATPase whose EM reconstructions are available are PspF ATPase with σ54 (EMD-1109) (Rappas et al., 2005) and the complex of PspF ATPase with σ54 and RNAP (EMD-1566) (Bose et al., 2008). Both these complexes lack promoter DNA and upon careful observation of the PspF ATPase ring density, it appears in hindsight to be asymmetric and have a gap or split. In σ54-PspF complex the σ54 density is on top of the ATPase, but positioned off center. This off centered positioning of the σ54-polymerase and the split ring architecture of the bEBP ATPase has not been noted or discussed by the authors.
In this chapter I present the electron microscopic studies on the negative stained closed complex of σ54-promoter DNA with NtrC1C in presence of non-hydrolysable ATP-transition state analogue, 1mM ADP-AlF₆/Mg²⁺. The analysis of class averages shows the persistence of a gapped asymmetric ring in this complex. Both the heptamer (3MOE) and asymmetric hexamer crystal structure of NtrC1C fits equally well into the 23Å RCT reconstructions of this complex, but the gap in the hexamer crystal structure matches with that in the ATPase ring the EM reconstruction. Further the σ54 and promoter DNA density makes maximum contact with the ATPase at the diametrically opposite interface of this gap. This asymmetry suggests a possible directional functional state of individual subunits in the ATPase depending on the nucleotide state at the interface. It provides significant restraints for all possible mechanisms of interaction between the bEBP ATPase with its asymmetric target.

2.2 Materials and methods

**Protein expression and purification**

(Composition of media and buffers are provided in Table: 2.2)

**Expression and purification of NtrC1 ATPase**

Plasmid containing the NtrC1 ATPase (NtrC1C) gene from *Aquifex aeolicus* was transformed into *E.coli* RosettaR(DE3) ultra-competent cells
(Novagen, USA), as described (Inoue et al., 1990). These cells were grown in 16 L of rich LB media (see Table 2.2) containing 50 μg/ml of ampicillin and 50 μg/ml chloramphenicol in a 20L New Brunswick Fermentor (at Penn State Shared Fermentation Facility) maintained at 37°C with constant agitation of 700 r.p.m. When the cultures reached a cell density (OD$_{600nm}$) of 4, the temperature was reduced to 15°C and expression induced with 0.5mM IPTG. After 12-16 hours of expression, cells were harvested by centrifugation and stored as cell paste at -80°C.

Twenty four grams of the frozen cell paste were suspended in 50ml of Lysis buffer containing one tablet of EDTA free protease inhibitor (Roche) in 50ml Falcon tube and the cells were disrupted by ultra-sonication over ice water for 10 cycles of 30s on plus 30s off. The cell lysate was then subjected to centrifugation at 100,000 x g, in Ti-70 rotor in Beckman Ultra-centrifuge, for 45mins, to remove cell debris. To the supernatant 5mM of TCEP was added before heating and holding the extract at 70°C for 30mins to denature most of the E.coli proteins. This was then centrifuged at 100,000 x g for 45mins at 4°C to remove the heat denatured, insoluble proteins. NtrC1$^C$ was precipitated with Ammonium sulfate at 80% saturation at 4°C. This was followed by centrifugation at 33,000 x g for 30mins at 4°C and the pellet was then re dissolved in 50ml buffer containing 20mM Tris-HCl, pH 8.0 and 5% (w/v) glycerol (Buffer A). The re-dissolved protein was then subjected to dialysis for 16hrs at 4°C in 4L of the same Buffer A. The dialyzed protein was purified by cation exchange chromatography using a HiTrap S column (GE Healthcare) at room temperature. NtrC1$^C$ was eluted from the
column with a linear gradient of loading buffer (Buffer B) to the same containing 1M KCl. Thirteen mg/ml concentration of purified NtrC1\textsuperscript{C} protein was obtained and 5mM TCEP was added to it before storing aliquots at -80°C until further use. Protein aliquots were thawed only one time just before use; excess was discarded.

**Expression and purification of σ54**

Plasmid encoding an N-terminal His6-tag on the σ54 gene from *Klebsiella pneumoniae* was transformed into *E. coli* BL21(DE3) ultra-competent cells and expressed in a similar way as described above and the cell paste was stored at -80°C until further use. Twenty-five grams of the frozen cell paste were suspended in 50 ml of lysis buffer containing one tablet of EDTA free protease inhibitor in 50ml Falcon tube and the cells were disrupted by ultra-sonication over ice water. The cell lysate was the subjected to centrifugation at 100,000 x g at 4°C for 45 min and the supernatant was subjected to affinity purification by loading on 5ml Ni-NTA agarose column (GE Healthcare) at 4°C and eluted by step elution with 0.4M Imidazole containing buffer (Buffer C). After adding 5mM TCEP to the eluted protein, it was subjected to overnight dialysis at 4°C in 4L of loading buffer (Buffer D) for binding to a heparin column. Following dialysis σ54 was further adsorbed to a 5ml Heparin Column (GE Healthcare) and eluted by gradient elution from 50mM NaCl to 1M NaCl (Buffer E). The eluted protein was then dialyzed overnight in 4L of loading buffer (Buffer F) for a Q-column at 4°C
and then subjected to further purification by anion exchange chromatography with a 5ml HP-Q column (GE Healthcare). Pure σ54 was then eluted from the column by gradient with 1M NaCl containing buffer (Buffer G). The peak for σ54 eluted between 0.2 and 0.3 M NaCl. To this 5mM TCEP and 30% (w/v) of Glycerol was added and the final concentration of protein was 3.23mg/ml. This was stored at -80°C, until further use. Aliquots of 1 ml protein were slowly thawed on ice to avoid precipitation that occurred with more rapid thawing.

*nifH* Promoter DNA

Individual strands of early melt (-11, -12 mismatch) *nifH* containing promoter DNA was synthesized by Integrated DNA Technology (Table: 2.1). Prior gel shift assays by Cannon from Buck’s group (Cannon et al., 2000) showed that the -11, -12 pre melt *nifH* promoter forms the most stable complex with σ54. These components were also shown to activate transcription *in vitro* (Chen thesis; Chen et al., 2010). Pellets of individual strands were re-suspended in buffer containing 10mM Tris-HCl, pH 8.0, 50mM NaCl and 1mM EDTA. Equimolar amounts of each strand were mixed together in 15ml Falcon tube and put in a boiling water bath for 5mins and then left to cool to room temperature for 6hrs for complete annealing. The final concentration of double stranded DNA was 6.28mg/ml. It was stored in 1.5 ml aliquots at -80°C, until further use.
Table 2.1. -11, -12 mismatch \textit{nifH} promoter DNA.

\begin{tabular}{|c|c|}
\hline
5' - CAGACGGCTGGCACGACTTTTGCAGATCAGCCTG - 3' & 11022.2 Da \\
3' - GTCTGCCACCAGCTGCTGAAAACGTCTAGTCGGGAC - 5' & 11102.2 Da \\
\hline
\end{tabular}

Preparation of complex

NtrC\textsubscript{1C} (5.5nmols), \(\sigma\textsubscript{54} \) (8.3nmols) and promoter DNA (10.9nmols) were added to 10ml of 1mM ADP-AlF\(_x\)/Mg\(^{2+}\) containing buffer (Buffer GF1) and concentrated to 150\(\mu\)l using 10KDa Amicon Concentrator (Millipore). The concentrated protein was then subjected to size exclusion chromatography in a 24 ml Superdex-200 column (GE Healthcare), which was pre-equilibrated with buffer GF1. The NtrC\textsubscript{1C}, \(\sigma\textsubscript{54}\) and DNA complex elution peak was around 11.5ml (Figure: 2.1) and the concentration of the complex was estimated to be 0.22mg/ml using the Bradford assay (Bradford, 1976).
Table 2.2. Media and buffers for protein expression and purification and complex formation.

| **Fermentation Media (10 L):** | 200 g peptone; 400 g yeast extract; 300 g glycerol; 80 g NaH$_2$PO$_4$; 70 g K$_2$HPO$_4$; 0.6ml Poly Propylene Glycol-200 (antifoaming agent; more may be added later); Antibiotics (0.5 g kanamycin, 0.5 g ampicillin or 0.34 g chloramphenicol) were added after autoclaving. |
| **Buffers for the NtrC1$^c$ purification:** | **Lysis buffer:** 500 mM NaCl; 5 mM EDTA; 20 mM TrisHCl pH 8.0; 5%(w/v) glycerol. |
|  | **Buffer A:** 20 mM TrisHCl, pH 8.0; 5%(w/v) glycerol. |
|  | **Buffer B:** 20 mM TrisHCl, pH 8.0; 1 M KCl; 5%(w/v) glycerol. |
| **Buffers for σ54 purification:** | **Lysis buffer:** 25 mM Na$_2$HPO$_4$.H$_2$O, pH8.0; 0.5M NaCl; 5%(w/v) glycerol. |
|  | **Buffer C:** 25 mM Na$_2$HPO$_4$.H$_2$O, pH8.0; 0.5 M NaCl; 5%(w/v) glycerol; 0.4 M imidazol. |
|  | **Buffer D:** 10 mM TrisHCl, pH8.0; 50 mM NaCl; 10 mM MgCl$_2$; 0.1 mM EDTA; 1 mM DTT; 5%(w/v) glycerol. |
|  | **Buffer E:** 10 mM TrisHCl, pH8.0; 1 M NaCl; 10 mM MgCl$_2$; 0.1mM EDTA; 1mM DTT; 5%(w/v) glycerol. |
|  | **Buffer F:** 50 mM TrisHCl, pH8.0; 5%(w/v) glycerol. |
|  | **Buffer G:** 50 mM TrisHCl, pH8.0; 5%(w/v) glycerol; 1 M KCl. |
| **Buffers for Gel-filtration:** | The nucleotide buffer was prepared by mixing components in the following proportions: 1 [ADP]:1 [AlCl$_3$]:8 [NaF]:1 [MgCl$_2$]. The buffer after preparation was filtered with 0.45μ filter and re-filtered again right before Gel-filtration. |
|  | **Buffer GF1:** 20 mM TrisHCl, pH 7.8; 200 mM KCl; 1%(w/v) trehalose; 1mM TCEP; 1mM ADP; 1mM AlCl$_3$; 8mM NaF; 1mM MgCl$_2$. |
Figure 2.1. Purification of NtrC1<sup>C</sup>, σ54 and DNA by Gel-filtration. Gel-filtration over Superdex-200 for purification of NtrC1<sup>C</sup>, σ54 and promoter DNA closed complex in 1mM ADP-AlF<sub>6</sub>/Mg<sup>2+</sup> buffer. The elution peak of the complex is around 11.5ml and shown by an arrow. Elution profiles of the individual components and smaller sub-complexes have been shown to illustrate separation of the complex peak from all others. The SDS-PAGE of the complex is shown inset, with the dashed box highlighting the complex lane. Lane labels are as follows: 1 – molecular weight markers; 2 – σ54; 3 – NtrC1<sup>C</sup>; 4 – promoter DNA (runs at dye front; stained with ethidium bromide); 6 – complex. This lane is marked with dotted box.
Single particle reconstruction of the complex

Preparation of grids for electron microscopy

The purified complex was further diluted to sixty, seventy and eighty fold with Buffer GF1. Two sets of grids were prepared for two different procedures of data collection. In order to collect large un-tilted data for refinement during single particle reconstructions thin carbon coated holey grids (Ted Pella) were used. Random conical tilt data was collected using home made thin carbon coated 300 mesh grids. These grids were prepared as described in Chapter 1, Section 1.7. Both these types of grids were plasma cleaned for 30s with hydrogen and oxygen in a Solarus plasma cleaner. Five micro liters of protein sample were absorbed on the grid and stained with 0.75% (w/v) Uranyl Formate and air-dried. The detailed procedure for negative staining is described in Chapter1, Section 1.7.

Data collection and processing

After screening through various diluted samples the eighty fold diluted sample containing grid showed well separated and uniformly distributed particles. An initial set of 204 micrographs were collected at 0° in a JEOL2100 TEM, operated at 200KV at a nominal magnification of 50,000X, at New York Structural Biology Center (NYSBC), New York. These micrographs were collected over a
defocus range from 1.5μ to 2.5μ on a 2K X 2K CCD Tietz camera with a 24μ pixel size. The actual calibrated magnification on CCD was 80,000X (calibrated with TMV (Tobacco Mosaic Virus)). These micrographs were then preprocessed using XMIPP suite (Marabini et al., 1996) and were down sampled by two pixels and background normalized. CTF parameters were then estimated for each micrograph and bad micrographs seen by manual inspection to contain astigmatism were then discarded. The binned micrographs had pixel size of 6.016 Å/pixel, and in total yielded 17,745 particles by manual picking with a box size of 46X46 pixels. The picked CTF-corrected particles were centered and stacked into a single file using XMIPP boxing package. Maximum likelihood based reference free classification was done using the ML2D program of XMIPP.

One hundred and seventy eight tilted-untilted micrograph pairs were then collected from the eighty-fold diluted sample applied on homemade thin carbon coated grids (Figure: 2.2). Tilted data were collected by tilting the grid at 45° to 65° angles. This data was then preprocessed and CTF parameters were estimated for each micrograph. A total of 8,312 tilt pair particles were then manually picked using XMIPP and boxed out in 46X46 pixel boxes. The tilted and the untilted particles were saved as separate image stacks. The untilted particles were then aligned and classified using the ML2D program of XMIPP. This classification was repeated over several cycles with different numbers of classes. 3D reconstructions of the class averages were carried out using the tilted data set. This was done using XMIPP's RCT module. The basic principle of RCT reconstruction has been explained in Chapter 1, Section 1.7. The volume of the
complete complex had split ring architecture of the ATPase and density on top and towards the edge for σ54 and promoter DNA. This model was further refined against the larger untilted data set by angular refinement and projection matching, using the projection matching refinement module of XMIPP. This was further low pass filtered with a 20Å cut-off to remove noise. The final reconstruction had a resolution of ~23Å based on 0.5 FSC (Figure: 2.3).

Both the tilt pair data set and untilted data set were re-analyzed using a recent Iterative Stable Alignment and Classification (ISAC) program of SPARX/EMAN2 (Yang et al., 2012). The XMIPP picked particle stacks were first converted to a format compatible with SPARX. The tilt pair data set was first processed with ISAC. All the untilted particles were first aligned by reference free alignment in SPARX. After aligning and centering the particles in SPARX, ISAC runs were carried out for stable classification. The class averages generated by ISAC showed several different orientations of the particles and also the presence of split ATPase ring. These averages were then sorted based on similarity and the most relevant and similar averages were joined by combining all the particles belonging to those classes into one class. This new group of particles was further checked for homogeneity by k-mean clustering using SPARX. The corresponding tilted particles were then selected and by back projection the initial 3D RCT volume of the complex was reconstructed. This volume was then further refined by angular refinement and projection matching against a larger data set comprising of the large untilted stack of particles and the composite tilted and
untilted particles from the tilt pairs. The final volume was then low pass filtered with a cut-off of 20Å to remove noise.

Figure 2.2. Tilt pair micrographs of the NtrC1\textsuperscript{C}, σ54 and DNA complex. On the left hand side is a tilted micrograph (tilt angle 65°) and on the right is the corresponding untilted micrograph (tilt angle 0°) of the same area. This is exemplary of the remaining tilt pair micrographs that were used to generate the complete RCT data set.
Figure 2.3. FSC curve for the final reconstructed volume of the complex. The resolution of the final reconstructed volume based on 0.5 FSC is ~23Å.

2.3 Results

Reference free class averages

Reference free classification was carried out using two different approaches with two programs: maximum likelihood classification of XMIPP and
EQ-K mean classification of ISAC. In both cases the candidate class averages clearly showed the density of the NtrC1\textsuperscript{C} ring. The ATPase ring appears to be split from one of the sides. Diametrically opposite to this side and on top of the ring some asymmetric density can be observed. The XMIPP class averages provided limited orientations of the particles. In comparison the ISAC class averages that were generated had more orientations and views of the particles (Figure: 2.4).

**Reconstructed asymmetric 3D volumes of the complex**

The refined 3D volumes reconstructed by XMIPP and ISAC/SPARX clearly showed a split ring structure. Reducing the iso-surface threshold value using UCSF Chimera (Pettersen et al., 2004) revealed the gap prominently. The heptamer ATP bound NtrC1\textsuperscript{C} crystal structure (3MOE) and the recent hexamer structure of the ATPase, upon super-positioning into the ATPase density in the EM reconstruction fits equally well (Figure: 2.6). The correlation of fitting into the XMIPP generated density for the heptamer crystal structure was 0.9184 and the hexamer structure was 0.9202; and into the ISAC/SPARX generated density for the heptamer crystal structure was 0.8735 and the hexamer structure was 0.8966. However the gap in the asymmetric hexamer ring structure overlaps with the gap in the EM density (Figure: 2.7), suggesting that the ATPase in the complex has similar ring geometry and is most likely to be a gapped hexamer. For better comparison the hexamer NtrC1\textsuperscript{C} crystal structure was transformed into
an electron density map at the same resolution as the EM reconstruction (23Å), using Sculptor (Birmanns et al., 2010). Lowering of the iso-surface threshold of this map revealed a similar gap region as is observed in the EM reconstruction (Figure: 2.5 (B), (C)). In both XMIPP and ISAC/SPARX reconstructions the density for σ54 and DNA was not so well defined perhaps because of flexibility in σ54 and DNA and also due to their smaller size.

The asymmetric, off-centered blob, on top and to the edge of the ATPase's EM density, is that of σ54 and promoter DNA as the ring shaped density can be attributed to NtrC1 ATPase. The maximum contact of σ54 and DNA with the ATPase ring seems to happen at an interface diametrically opposite to that of the gap region (Figures: 2.5; 2.7). Lowering the iso-surface threshold of the EM density of the complex of PspF ATPase with σ54 (EMD-1109) (Rappas et al., 2005) shows a gap in the PspF ring. Gradual increase in the threshold value of the density reveals that the first contact the σ54 density makes with the ATPase ring occurs at the opposite interface of the gap in the PspF ATPase (Figure: 2.8 (A),(B)). At this resolution of reconstruction detailed features of individual GAFTGA loops are not prominent.
Figure 2.4. Class averages of the complex. Class averages from the untilted micrographs. 
A. Class averages generated from XMIPP. Particles have been classified into fewer orientations or populations. In averages like Nos. 13, 14, 19 one can get side views of the complex and can clearly see the split ring and densities of σ54 and promoter DNA on top. B. Class averages from ISAC/SPARX. Class averages showing several random orientations of the complex and different variants can be seen.
Figure 2.5. Refined 3D reconstructions of the complex show an asymmetric NtrC1C ring structure. A. Comparison between reconstructions obtained by using XMIPP (Left and in blue) and by using ISAC/SPARX (right and pink). Different views of the volumes show overall similarity between the two reconstructions. B. Upon reduction of iso-surface threshold the gap in the ATPase ring becomes prominent. Both reconstructions are shown from top and from bottom. C. Blurred density of the asymmetric, hexamer crystal structure of NtrC1C at 23Å resolution (left). The gap in the ATPase density becomes prominent (right), when the iso-surface threshold is reduced. Note the similarity in the gap in these models with the EM reconstructions of the complex.
Figure 2.6. Fitting crystal structures of NtrC1 ATPase into the ring density of the EM reconstruction of the complex. A. Heptamer crystal structure of NtrC1\(^C\) (3MOE) (shown in green) has been fitted in the ATPase density of the reconstructions from XMIPP (in blue and to the left) and ISAC/SPARX (in pink to the right). The correlation values show that the fits are reasonably well. B. Hexamer crystal structure of NtrC1\(^C\) (shown in cyan) has been fitted in the ATPase density of the reconstructions from XMIPP (in blue and to the left) and ISAC/SPARX (in pink to the right). The correlation values show that the fits are almost similar to that of the heptamer crystal structure.
Figure 2.7. The gap in the NtrC1 ATPase hexamer structure matches with the gap in the ATPase ring density in the EM reconstructions and the density for σ54 and promoter DNA makes contact with the ATPase density opposite to the gap. In spite of close correlation of fit of the heptamer and hexamer crystal structures of NtrC1 into the EM density of the complex, the major difference lies in the match between the cleft in the EM density to the gap in the hexamer crystal structure. The gap in the EM density is made prominent by reducing the iso-surface threshold and the red arrow shows the gap. This suggests that the ATPase exists as a gapped hexamer in the complex. The density for σ54 and promoter DNA are located diametrically opposite to the gap in the ATPase ring.
Figure 2.8. Asymmetric ATPase ring can be observed in PspF when in complex with σ54 or with σ54 and RNAP. A-C is the EM reconstruction of PspF ATPase in complex with σ54 (EMD-1109) (Rappas et al., 2005). A. Top and side view of the complex with σ54 density positioned on top of the ATPase ring. B. Upon reducing the iso-surface threshold a gap in the ATPase ring appears. Note the quasi-helical organization of the ATPase density. C. Upon increasing the iso-surface threshold level from (B), σ54 density makes contact with the ATPase at the diametrically opposite side from where the gap was observed in (B). D. EM reconstruction of PspF ATPase in complex with σ54 and RNAP (EMD-1566) (Bose et al., 2005). Note the asymmetric PspF ATPase ring, with a gap marked with red arrow. RNAP- σ54 density makes off centered contact with the ATPase.
2.4 Discussions

Polymorphism in the structure of the bEBP ATPases have been observed in the past. This can be in terms of the oligomeric state – heptamer or hexamer or in asymmetric arrangement of the subunits of the ATPase ring. Prior structural studies on NtrC1\textsuperscript{C} under saturating nucleotide concentrations showed its existence as a closed heptamer. EM studies by Chen and De Carlo (Chen et al., 2007) with NtrC1\textsuperscript{C}, showed the existence of NtrC1\textsuperscript{C} mostly as heptamers and some hexamers. Recent crystal structure and X-ray scattering experiments have shown that NtrC1\textsuperscript{C} can exist as hexamers (Sysoeva thesis and manuscript in preparation). Wemmer’s group, studying of the NtrC4 ATPase of A. aeolicus, made similar observations, where isolated ATPase domain was seen by crystallography and mass spectroscopy to be a heptamer, but mass spectroscopy showed that activated or full length NtrC4 exists as a hexamer. On the other hand, the majority of bEBP ATPases like ZraR and PspF have been observed as hexamers.

Furthermore, these homomeric ATPases do not always exist as symmetric rings. Crystal structure of PspF ATPase has shown that it can form spiral filaments in the crystal lattice. As mentioned above, NtrC1 ATPase under sub-saturating or stoichiometric amount of nucleotides exists as gapped, asymmetric hexamer. However the later seems to differ from the former in the ATPase ring being more planer than helical. Moreover the handedness of the PspF spiral is different from that observed in NtrC1\textsuperscript{C} and can possibly be a
crystallographic artifact. EM reconstructions show that NtrC1 ATPase is present as asymmetric ring in complex with σ54 and promoter DNA. The gap in the ATPase density matches with the gap in the hexamer crystal structure, suggesting that the oligomeric state of the ATPase in the complex is an asymmetric, gapped hexamer. EM structures of PspF ATPase in complex with σ54 and RNAP also appear to be a gapped hexamer ring (Figure: 2.8). Moreover in Chapter 4, I have shown the existence of gapped ring in NtrC1 ATPase in complex with the full transcription complex where RNAP is present. Novel contrast matching SANS experiments on complex between NtrC1C and σ54 shows that the ATPase ring acquires its asymmetry before interacting with σ54 (Sysoeva, manuscript in preparation). This has been presented in Chapter 3 of this thesis. Therefore from these observations we can conclude that the functional morphology of bEBP ATPase is its asymmetric hexamer form and the asymmetry guides the rings interaction with its asymmetric target, σ54.

One of the remarkable observations that can be made in the hexamer crystal structure of NtrC1C is that in spite of the overall ring being planar the L1 or GAFTGA loops are arranged in a helical staircase like fashion. Even though this unique staircase like arrangement of the L1 loops has not been observed in bEBP ATPases before, similar observations were made in the distantly related AAA+ ATPases Rho and E1, RNA and DNA translocases, respectively. Works of Berger and Joshua-Tor show that these ATPase form hexamer rings with functional loops protruding into the ring’s pore. While the loops in the translocases emerge from a different part of the ATPase fold than do the loops of
bEBPs, the translocase loops have similar helical distribution around the pore as observed for the L1 loops in the NtrC1\textsuperscript{C} hexamer. In these translocases the helical arrangement of the loops is crucial for translocation of single stranded helical nucleic acids. However it is still unclear how this helical arrangement of the L1 loops helps bEBPs to remodel σ54-closed complexes. Observations from recent work by Jolly (Joly et al., 2011), with covalent dimmers and trimers of PspF ATPase, suggest that a possible asymmetric geometry of the ATPase is necessary for activating σ54 dependent transcription. Further by selectively mutating the threonine residue in some of the GAFTGA loops and by using various combinations of mutated and non-mutated loop bearing residues, they were able to propose that two GAFTGA loops are necessary for interacting with single copy of σ54 and three loops are necessary for open complex formation. Careful observation of the extended density of σ54 and promoter DNA on top of the NtrC1 ATPase ring and close to the central pore, but distal to the gap, may reveal that the three subunits B, C and D make contact with this density (Figure: 2.9). This is consistent with to the interpretation made by Jolly et al. whereby three GAFTGA loops are necessary for open complex formation.

In hexamer crystal structure of the NtrC1 ATPase the gap between subunits A and F can be either apo, ADP or in ATP bound state. However the actual significance of the gap is unknown. Similar gaps can be observed E1 and Rho ATPases. In the later the gap is believed to be associated with release of ADP and uptake of ATP. Furthermore, in comparing the B-factors of all the subunits in the NtrC1\textsuperscript{C} hexamer crystal structure Sysoeva found that the subunits
flanking to the gap had more flexibility. This dynamics could be necessary for facilitating nucleotide exchange. Also the co-operative arrangement of the loops can be a direct consequence of the nucleotide state at the interface between two subunits.

The exact ATP hydrolysis mechanism in the NtrC1 ATPase ring is unknown. Given the asymmetric structure of the ATPase, its subunits are not in the same state, so a concerted ATP hydrolysis mechanism seems unlikely. Furthermore, it is unlikely to be stochastic since the tight linkage between the L1 and L2 loops seems to reflect high co-operativity between the subunits A through E when they are all bound to ATP. Given these considerations, it seems likely that the mechanism of ATP hydrolysis in bEBPs is sequential.

Presuming a sequential hydrolysis mechanism, the following speculations can be made for the NtrC1 hexamer ring. The gap between subunits A and F is necessary for the release of ADP and uptake of ATP by subunit F. Analysis of the distances between the subunits in this crystal structure by Sysoeva (Sysoeva thesis and manuscript in preparation) shows that the smallest distances occur between subunit pairs E-F and D-E. Prior large zone gel filtration studies by Chen (Chen et al., 2007) have shown the formation of more stable complex between NtrC1 ATPase with 54 in presence of ATP transition state analogue ADP-AIF₃/Mg²⁺ than ATP ground state analogue ADP-BeF₃/Mg²⁺. Therefore it is possible that the more tightly defined active sites may be closer to the transition states for hydrolysis of ATP. Hydrolysis of ATP in subunit E could cause the disengagement of the arginine-finger from subunit F, which could cause the F
subunit with newly bound ATP to move towards subunit A. This movement could then cause a new gap to be created between subunits E and F that could then allow nucleotide exchange at this interface. Proceeding further, this action would lead to a counter clockwise propagation of the gap with sequential ATP hydrolysis. If such a rotation happens, it will be very interesting to learn when work is done on the sigma factor – with each hydrolysis event, or only when a specific juxtaposition exists between bound sigma and firing interface? It also seems possible that subunit A could move to bind to the $\gamma$-phosphate of freshly bound ATP on subunit F, making the gap to rotate clockwise around the ring. This would seem to require breaking the interaction between arginine-finger and ATP between subunits B and A, so it may be less likely than movement of subunit F to join subunit A. An alternative possibility is that with each reloading of ATP at subunit F, contact with the closed complex is renegotiated, such that the gap, while moving with respect to the prior ring of ATPase subunits, remains stationary with respect to the target macromolecule. In this case, work may be delivered to remodel the RNAP from the structural context present in the crystal structure and EM density map of the complex trapped by ADPAIF$_x$/Mg$^{2+}$. Perhaps the ring closes after ATP is bound to subunit F, and that closure propagates a conformational change through the linked rigid bodies of L1 and L2 loops that mediates work on the polymerase.

Indeed, by any of the above scenarios the lateral movements of subunits near the gap and the rigid body roll that occurs between ATPase sub-domains,
upon changing from occupancy by ADP to occupancy by ATP, can generate the mechanical force necessary for remodeling σ54 closed transcription complex.

Figure 2.9. Density for σ54-promoter DNA makes contact with subunits B, C and D of the NtrC1 ATPase at the opposite interface to where the gap is located. On the left-hand side the hexamer crystal structure of NtrC1 has been fitted into the EM density of the ATPase. This helps in annotating the various blobs in the EM density corresponding to the subunits of the ATPase. On the right hand side I have highlighted the extension of the density of σ54 and DNA, with yellow ring, which makes contact with subunits B, C and D of the ATPase. Subunit C lies directly below this extended density of σ54 and DNA and makes maximum contact with it.
2.5 Conclusions

The EM reconstruction of the complex of NtrC1 ATPase with σ54 and promoter DNA shows the existence of ATPase as a split, asymmetric hexamer ring in the complex. This asymmetric morphology is believed to direct asymmetric force for remodeling σ54. Fitting of the asymmetric NtrC1\(^C\) hexamer crystal structure into the ATPase density of the EM reconstruction suggests that the ATPase acquires the asymmetric morphology prior to interacting with its asymmetric target. Further maximum contacts between the ATPase density with the densities of σ54 and promoter DNA occurs distal to the gap. A possible explanation for this can be based on one of the several possible variations of sequential nucleotide hydrolysis mechanism. These hypothesis needs to be tested in future to have precise understanding of the mechanism.
CHAPTER 3

NOVEL DEUTERATION PROTOCOL AND SOLUTION SCATTERING APPROACH FOR STUDYING INTERACTION OF NTRC1 AAA+ ATPASE WITH σ54

Summary

In Chapter 2, I showed that NtrC1C is present as an asymmetric hexamer, splitting in complex with σ54 and closed promoter DNA. As an alternative approach, I simultaneously explored using novel contrast matching small angle neutron scattering technique combined with small angle X-ray scattering to obtain structural information about binary and ternary complexes of ATPase plus σ54, or ATPase, σ54 and promoter DNA. Unlike EM, these solution scattering methods are useful for smaller objects such as the binary complex. My efforts to obtain ab initio structures were not successful in the end, but the methods I developed did allow my lab-mate Sysoeva to successfully explore conformational change in the ATPase as it moves from solution to binary complex with σ54 (Sysoeva and Nixon manuscript in preparation). In order to perform such experiments it is crucial to have purified isotopically labeled (here deuterated) proteins. In this chapter I present an improved method of expressing deuterated proteins, by which one can expect to have significant improvement in yields. At
the end of this chapter I provide new solution structure of the NtrC1\textsuperscript{C}-\sigma54 complex from prior solution X-ray scattering data collected by Chen and Nixon. For this solution I have used the new asymmetric, hexamer NtrC1\textsuperscript{C} crystal structure as a known object (Sysoeva thesis and manuscript in preparation) and then modeled the \sigma54 density based on the scattering profile of the complex.

**3.1 Introduction**

Obtaining low-resolution structures of macromolecular complexes by using solution scattering techniques is becoming more common. This has helped us to study molecules in solutions that are closer to physiological conditions than can be accessed by crystallography or NMR (Nuclear Magnetic Resonance). The most commonly used solution scattering technique is X-ray scattering (SAXS/WAXS). In a multi protein complex, obtaining structural information about a subset of the proteins is not possible by X-ray scattering. Changing the X-ray scattering length density of one subunit or subset of protein from the others is not feasible, as it depends on the electron density, which is more or less uniform for all proteins. In contrast this can be achieved by solution neutron scattering technique (SANS), where differential isotope labeling of subunits can vary scattering length density (SLD) of that subunit. This can change the contrast (which is the difference between the SLD of the molecule and that of the solvent) of the labeled subunit from the unlabeled ones. Therefore by matching the SLD of a subunit with the solvent one can obtain scattering data from the unmatched
subunits. This is known as the contrast matching technique. For neutron scattering experiments this is achieved by selective deuteration of proteins. In order to perform SANS experiments, sufficient quantity of deuterated protein is necessary, and often limiting due to high costs that are encountered for expressing these proteins.

In this chapter I have described a feed-batch fermentation protocol for expressing large quantities of deuterated proteins. By increasing the Mg$^{2+}$ concentration in the feed, I was able to improve the yield of deuterated NtrC1$^C$ by five fold. This can significantly reduce the cost of production of deuterated proteins, as D$_2$O is expensive. Further, Sysoeva used this deuterated NtrC1$^C$ to perform contrast matching SANS experiments on the complex of NtrC1$^C$ with σ54. She matched out the scattering from non-deuterated σ54 in the complex by changing the D$_2$O percentage in the buffer. The neutron scattering data thus had just the scattering contribution from NtrC1$^C$. This experiment showed that the NtrC1$^C$ structure did not change much from its free state, compared to when it was in complex with σ54. Since these experiments were performed at a nucleotide concentration where the NtrC1$^C$ is known to exist as an asymmetric hexamer ring, the above observation suggests that in complex with σ54, NtrC1$^C$ persists in the same structural state.

In order to see how σ54 would be oriented with respect to the NtrC1$^C$ asymmetric ring, I modeled unpublished solution X-ray scattering data of NtrC1$^C$ and σ54 complex, collected by Chen and Nixon. This was done by creating and fixing a bead model of the Cα atoms of the asymmetric NtrC1$^C$ hexamer crystal
structure and then modeling the density for σ54 by simulated annealing using the program Credo (Petukhov and Svergun; EMBL, Hamburg). The final model obtained is also presented in this chapter.

3.2 Materials and methods

Protein expression and purification

Expression and purification of non-deuterated NtrC1\textsuperscript{C} and σ54 have been described in Chapter 2, section 2.2. Composition of media and feed for expression of deuterated NtrC1\textsuperscript{C} is provided in Table: 3.1.

Expression and purification of deuterated NtrC1\textsuperscript{C}

The deuteration protocol described here was done at the Penn State Shared Fermentation Facility (Huck Institutes of the Life Sciences). This protocol was modified from my training at the Center for Structural and Molecular Biology, Oak Ridge National Laboratory (CSMB, ORNL).

Plasmid containing the NtrC1 ATPase (NtrC1\textsuperscript{C}) gene from *Aquifex aeolicus* was transformed in *E.coli* RosettaR(DE3) ultra-competent cells (Novagen, USA), as described (Inoue et al., 1990). The cells were then plated on LB agar plate containing 50 μg/ml of ampicillin and 50 μg/ml of chloramphenicol and then kept in a 37°C incubator for 16hrs. In order to adapt the transformed *E. coli* cells to grow on minimal media, a single colony from the LB agar plate was
picked and streaked on a water based minimal media (MM) agar plate and this was kept for incubation at 37°C for 24hrs. Next, for adapting the cells to grow in deuterated minimal media (DMM), single colony from the MM agar plate was picked and streaked on DMM agar plate and incubated for 48hrs at 37°C. Then, a single colony from the DMM agar plate was used to inoculate a 5ml liquid DMM culture and grown at 37°C and 300r.p.m to an OD<sub>600nm</sub> of 0.6. One milliliter of the culture was then centrifuged at 3K r.p.m and the pellet was re-suspended in fresh 10ml DMM and grown at 37°C and 300r.p.m to an OD<sub>600nm</sub> of 0.6. The entire 10ml culture was then centrifuged at 3K r.p.m and the pellet was now suspended in 100ml of DMM and grown at 37°C and 300r.p.m to an OD<sub>600nm</sub> of 0.6. This culture was centrifuged at 3K r.p.m in Sorvall SS-34 rotor and the cell pellet was re-suspended in 10ml of fresh DMM. This suspension was used as the inoculum for the larger scale fermentation.

The fermentation was carried out in a 2L BIOFLO 3000 fermentor (New Brunswick). Before fermentation the vessel, pH probe and two feed bottles were autoclaved. These were then dried in a 60°C oven prior to adding media or feed. 85% (v/v) DMM (1.4L) was added into the fermentor. To this 500μl of sterile PPG-200 antifoam was added. After the temperature in the fermentor stabilized at 37°C, 100ml of the suspended cells were added to the fermentor. Due to non-availability of a dissolved oxygen probe, a constant agitation at the maximum limit of 700 r.p.m was maintained throughout the fermentation. A dessicator column was attached before the air inlet filter to allow dry air to enter the fermentation vessel. The 10% (w/v) glycerol feed was started at a flow rate of
100μl/min when the OD\textsubscript{600nm} reached 0.25. At the beginning of the fermentation, the pH/pD of the media was 8.2. In order to maintain the pH/pD of the media between 7.0 and 8.2 during the fermentation a 4M NaOH feed was also started. When the cell OD\textsubscript{600nm} was at 6, the temperature was reduced from 37°C to 15°C. Induction of protein expression was done by adding 0.5mM IPTG. In order to avoid nutrient depletion, the 10% (w/v) glycerol feed rate was increased to 110μl/min. After 15hrs the cells were harvested and expression was verified by SDS-PAGE (Figure: 3.2). The total 10% (w/v) glycerol feed used for this process was 250ml.

The entire process described above was first tested with water based minimal media, before D\textsubscript{2}O based media was used. The percent D\textsubscript{2}O in the media was decided based on percent deuteration of the protein to be achieved. This decision was based on the work of Leiting et al., 1998 (Figure: 3.1). For the contrast matching SANS experiments we needed to have 80-83% deuteration of NtrC\textsubscript{1C}, which called for using 85% (v/v) D\textsubscript{2}O in the medium.

The deuterated NtrC\textsubscript{1C} was purified in the same way as non-deuterated NtrC\textsubscript{1C}, in water-based buffers as described in Chapter 2, section 2.2. The percent deuteration of NtrC\textsubscript{1C} was determined by mass-spectrometry at the Huck Institutes of the Life Science’s Proteomics and Mass Spectroscopy Core Facility at University Park.
Contrast matching SANS studies of the complex of NtrC1\(^{C}\) with \(\sigma54\)

My former colleague Sysoeva carried out these experiments. In order to match scattering from protonated \(\sigma54\) protein with scattering from the buffer, solution neutron scattering was measured in buffers containing different percentages (v/v) of D\(_2\)O. Scattering from hydrogenated \(\sigma54\) in 100% (v/v) D\(_2\)O was seen to vanish at 42% (v/v) D\(_2\)O (Figure: 3.3). This was consistent with the predicted match point for non-deuterated protein, typically between 40%-45% D\(_2\)O (Wu et al., 2009). Having established the match point for protonated \(\sigma54\), the scattering profile from deuterated NtrC1\(^{C}\) was measured in the absence and presence of \(\sigma54\) in 42% (v/v) D\(_2\)O containing buffer that lacked ATP analog, and thus consisted of free \(\sigma54\) and free NtrC1\(^{C}\). The scattering profiles were identical (Figure: 3.2), revealing that the contribution to the scattering at 42% (v/v) D\(_2\)O was only from deuterated NtrC1\(^{C}\) and not from protonated \(\sigma54\). Next the complex of protonated \(\sigma54\) and deuterated NtrC1\(^{C}\) were prepared in 42%(v/v) and 100%(v/v) D\(_2\)O containing 1mM ADP-AlF\(_3\)/Mg\(^{2+}\) buffer (Composition of this buffer has been described in Chapter 2, Table: 2.2). Scattering profiles from both these complexes are shown in Figure: 3.2. Finally scattering from deuterated NtrC1\(^{C}\) and complex of protonated \(\sigma54\) with deuterated NtrC1\(^{C}\) were measured at 42% (v/v) D\(_2\)O containing 1mM ADP-AlF\(_3\)/Mg\(^{2+}\) buffer. This was done to compare the scattering from deuterated NtrC1\(^{C}\) by itself with the scattering from the complex of deuterated NtrC1\(^{C}\) with protonated \(\sigma54\), where the scattering from \(\sigma54\) has been matched out (Figure: 3.2).
**Modeling solution X-ray scattering data of the complex of NtrC1\(^C\) with \(\sigma54\)**

Chen and Nixon performed Solution X-ray scattering (SAXS/WAXS) experiments on the complex of NtrC1\(^C\) and \(\sigma54\) in 1mM ADP-AlF\(_x\)/Mg\(^{2+}\). These data were previously modeled using the program Credo from ATSAS suite (Svergun et al., EMBL), where a C\(\alpha\) atom model of the heptamer NtrC1\(^C\) (1NY6) was fixed and by simulated annealing the density for \(\sigma54\) was modeled. However from the contrast matching SANS experiments we found that the NtrC1\(^C\) ring structure essentially remains unchanged when it binds with \(\sigma54\) under conditions when it is hexameric rather than heptameric as assumed in the prior modeling (This has been discussed later in the next section). Therefore the SAXS/WAXS data were remodeled using Credo, where the C\(\alpha\) atom model of the new hexamer NtrC1\(^C\) crystal structure (Sysoeva thesis and manuscript in preparation) was fixed and density for \(\sigma54\) was modeled by simulated annealing of 496 scattering centers. One hundred independent runs of Credo were carried out. Output structures from the one hundred runs were then separated based on the position of the \(\sigma54\) density - either above or below the NtrC1\(^C\) ring. There were thirteen models where the \(\sigma54\) was placed on top of the ATPase ring and eighty-seven had the \(\sigma54\) placed below the ring. The models where \(\sigma54\) was placed on top of the ATPase ring had the gap positioned opposite to where the \(\sigma54\) was placed, and the models where the \(\sigma54\) was placed below the ring had the \(\sigma54\) placed below the gap region. In the majority of the output models the \(\sigma54\) density was placed below the gap to compensate for the lack of scattering...
centers at the gap region in the ATPase ring. Prior cross-linking and mutagenesis experiments (Bordes et al., 2003; Chen et al., 2008) have shown that σ54 interacts with the GAFTGA loops on top of the ATPase ring. Therefore output models containing σ54 on top of the ATPase ring were selected and further processed. They were then subjected to alignment in order to obtain the most likely consensus model. This was done using the DAMAVER suite from Svergun’s group in EMBL Hamburg (Kozin and Svergun, 2001). All possible pairs of models were first superimposed on each other and cross-correlations between them were computed. This cross-correlation is also known as normalized spatial discrepancy (NSD). The model with the lowest NSD value is considered the most probable model and models having two standard deviations above mean NSD values are discarded. In my case all thirteen models had close NSD values and the one with the lowest NSD was selected as the most probable consensus model.
Table 3.1. Media and buffers for protein expression and purification and complex formation.

<table>
<thead>
<tr>
<th><strong>Minimal Media (MM) Recipe (1L):</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Batch Media (for 1L Minimal Media):</strong></td>
<td>9.86g/l (NH₄)₂SO₄; 5.2g/l Na₂HPO₄; 1.57g/l KH₂PO₄; 0.491g/l Ammonium citrate; 1ml from 1M stock of MgSO₄; 5g/l Glycerol; 1ml of 1000x Trace Supplement.</td>
</tr>
<tr>
<td><strong>1000x Trace Supplement:</strong></td>
<td>0.378g/l CaCl₂; 0.098g/l CoCl₂; 0.102g/l CuSO₄; 16.7g/l FeCl₃.6H₂O; 0.114g/l MnSO₄.H₂O; 22.3g/l Na₂EDTA.2H₂O; 0.18g/l ZnSO₄.7H₂O.</td>
</tr>
<tr>
<td><strong>10% (w/v) Glycerol Feed (250ml):</strong></td>
<td>25g of Glycerol; 12.5μl from 1M stock of MgSO₄; MM is added to make up the volume to 250ml.</td>
</tr>
<tr>
<td><strong>4M NaOH (100ml):</strong></td>
<td>16 g of NaOH pellet dissolved in water or D₂O.</td>
</tr>
</tbody>
</table>

Minimal media was sterilized by filtration through 0.2μ filter. Autoclaving is avoided to prevent salts from precipitating.

For a certain volume percentage DMM (Deuterated Minimal Media) water was replaced with the appropriate volume of D₂O.
Figure 3.1. Relationship between percent deuteration of proteins expressed in *E. coli* and percent D$_2$O (v/v) in media and carbon source. This graph has been adapted from Leiting et al., 1998. It gives a relationship between percent (v/v) D$_2$O in the media to the % deuteration of the expressed protein. The bottom curve is when a non-deuterated carbon source like regular glucose is used. The dashed line above is a linear fit to the endpoints. The curve on the top of the graph with dark circles is for percent deuteration that can be achieved when a deuterated carbon source like deuterated glucose is used. This is essential for expressing per-deuterated proteins. The importance of this curve is that it provides an estimate of how much D$_2$O to use for expressing certain percent deuterated protein.
3.3 Results and Discussion

**Higher yield of deuterated proteins**

I first learned the deuteration protocol described above at CSMB, ORNL from Dr. Kevin Weiss. At ORNL I was able to obtain 15g of cell paste, from which I could purify 49mg of 80-85% deuterated NtrC1\textsuperscript{C}. Thus the yield at ORNL was 1.2g of cell paste or 3.9mg of purified deuterated NtrC1\textsuperscript{C} per gram of carbon in the medium. Back at Penn State by using the protocol described above I was able to get 40g of cell paste, from which I could purify 600mg of 82.5% deuterated NtrC1\textsuperscript{C}. Therefore the yield at Penn State was 3.15g of cell paste or 47.2mg of purified deuterated NtrC1\textsuperscript{C} per gram of carbon in the medium. The major difference between the two protocols was that at ORNL the 10\%(w/v) glycerol feed did not have any magnesium sulfate supplement, whereas at Penn State I added 50mM magnesium sulfate in the feed. In addition, lacking a probe for dissolved oxygen I provided maximal aeration throughout the fermentation. These changes lead to 2.6 fold increase in cell mass and a 12 fold increase in yield of expressed deuterated NtrC1\textsuperscript{C}. The total volume of feed and medium in both fermentations were the same. After my work was completed, Meilleur et al. reported for expression of per-deuterated proteins (Meilleur et al., 2009) that by using 16.6mM magnesium sulfate in the feed the yield of cell paste was 1g per gram of carbon in media; note that other culture conditions differ from those I used so direct comparison is not possible.
I have later used the modified deuteration protocol, developed at Penn State, for expressing deuterated human RCC1 (Regulator of Chromatin Complex 1) and LSD1/CoREST (Lysine De-methylase / Co-repressor of REST transcription factor), for a collaborative SANS project with Dr. Song Tan, to study their complexes with Nucleosome. In order to express LSD1/CoREST, I had to supplement the minimal media with Basal Eagle Vitamin concentrate (100X) (Life Technologies). This was because LSD1/CoREST had bound Nicotinamide co-factor and lack of Nicotinamide in the minimal media prevented expression of this protein.

**NtrC1** ring structure does not change upon binding with σ54

The contrast matching SANS experiments, performed by Sysoeva, clearly showed that protonated σ54 was matched out at 42% (v/v) D₂O containing buffer, whereas scattering from deuterated NtrC1** could be significantly observed in this buffer (Figure: 3.3). This information was used to study what happens to the NtrC1** when in complex with σ54. When scattering from NtrC1** ring was compared with the scattering from complex of NtrC1** with σ54, in 42% D₂O containing 1mM ADP·AlF₄⁻/Mg²⁺ buffer, no significant differences could be observed between the two scattering curves (Figure: 3.3). From this one can infer that the NtrC1** ring structure does not change significantly upon binding to σ54, or if it does change structure, there is very little evidence of this in the pair distribution function that dictates scattering. The minor variation that can be
observed upon super-positioning the scattering curve of the complex of deuterated NtrC1\textsuperscript{C} with σ54 in 42% (v/v) D\textsubscript{2}O buffer with the scattering from deuterated NtrC1\textsuperscript{C} in 42% (v/v) D\textsubscript{2}O buffer, suggests reordering of the GAFTGA loop upon binding to σ54. Therefore the observations from the contrast matching SANS experiments further supports the idea that NtrC1\textsuperscript{C} acquires its functional geometry before it interacts with its target σ54 to remodel it.

**Solution X-ray scattering models of complex of NtrC1\textsuperscript{C} with σ54**

A supplementary approach for defining the structure of the ATPase and sigma factor in a binary complex would be to use contrast matching to extract the form factors from each component as well as the entire complex. These could then be used to make *ab initio* models to determine at low resolution the juxtaposition of σ54 and NtrC1\textsuperscript{C}. While details would be lacking, data suggesting hexamer versus heptamer composition of the ring would be obtained. I spent a great deal of energy performing these experiments, and did collect contrast matched SANS data. However, the analysis failed to give believable models, and fitting scattering curves calculated from pseudo high resolution models that I created failed to provide good fits to the solution data (data and analyses not shown). At this point it is not clear if there are problems with the data, or the analysis.

As an alternative approach, I revisited solution X-ray scattering data previously collected for the complex of NtrC1\textsuperscript{C} and σ54 by Chen and Nixon.
Although in principle it lacks the required information to separately solve the structures of the two components, there is a way that involves including \textit{a priori} information about the ring structure that lets one accomplish this goal. This was done previously (by Chen and Nixon, unpublished) by imposing a heptamer model for the ATPase, because at the time there were only heptameric crystal structures available. In my analysis, I imposed six-fold symmetry, and used the Cα atoms of the asymmetric hexamer ring as a fixed portion of the model to which scattering centers were added to represent σ54 to best fit the data. The rationale for fixing the ring structure is as follows. The contrast matching neutron scattering data described above for the complex can be interpreted to mean that in responding to ATP binding the ATPase acquires its functional conformation and shape before interacting with its target macromolecule. Since σ54 is asymmetric, it’s highly likely that NtrC1 C ring may be asymmetric while interacting with σ54. Fixing the ring Cα atoms as scattering centers representing the ATPase in its asymmetric hexamer ring form incorporates this \textit{a priori} knowledge in the modeling effort. During this modeling process 87% of the output models had σ54 placed at the bottom of the ATPase ring as the top and bottom of the ring does not have major differences at low resolution. In these models the σ54 density was placed at the gap region, to compensate for the missing density at the gap. As explained earlier, since these models were not biologically relevant they were discarded and consensus model was determined by comparing the remaining 13% of the output models where the σ54 density is placed on top of the NtrC1 C ring. Comparison between these models showed that all 13 of them
had close NSD values and they were all within the acceptable range (Table: 3.2). The highly likely model is the one with the lowest NSD value (Average NSD value for this model is 0.829). In this model the σ54 density was placed off-centered with respect to the ring and opposite to the gap. A density map for this model (Figure: 3.4) was created using Sculptor (Birmanns et al., 2010) at 23Å resolution (same resolution as the EM reconstruction of the complex of NtrC1σ, σ54 and promoter DNA, presented in Chapter 2).
Figure 3.2. Glycerol-based fermentations to produce deuterated NtrC1\textsuperscript{C}. Optical density (light scatter at 600 nm) is plotted versus age of culture for water based minimal medium (blue) and medium containing 15% water and 85% deuterium oxide (black); IPTG (0.5 mM) was added to induce expression at the times of the oversized data points - the pH/pD (red) was monitored for the deuterium oxide based fermentation to verify that induction was done before pH/pD began to increase, as we have seen that optimal induction occurs ~45 min prior to such an upward shift in pH. The concentration of NtrC1\textsuperscript{C} (percentage of total cellular protein) was measured by scanning the inset SDS-PAGE gels - 11% at the end of the water-based culture (blue arrow) and 16% for the deuterium oxide based culture (green arrow). Lane labels indicate successive data points beginning at time of induction with IPTG, or for the water based culture: 4 – 100,000g supernatant after lysis, 5 – mwt markers; and the deuterium oxide based culture: 11 – supernatant after harvesting cell paste to monitor for lysis, 12 – mwt markers.
Figure 3.3. Neutron scattering by hydrogenated σ54 and deuterated NtrC1C. A. Scattering by hydrogenated σ54 in buffer containing 100% (v/v) D$_2$O (red) and 42% (v/v) D$_2$O (blue). Note the scattering from σ54 is matched out completely at 42% (v/v) D$_2$O buffer B. Scattering by deuterated NtrC1C (red) and that plus hydrogenated σ54 (green) in buffer containing 42% (v/v) D$_2$O. No nucleotide was present in these buffers; hence there was no complex of σ54 with NtrC1C. Note that σ54 was matched out and scattering in both the cases were from free NtrC1C. C. Scattering from complex of hydrogenated σ54 with deuterated NtrC1C in the presence of 1mM ADPBeF$_6$/Mg$^{2+}$ in 42% (v/v) D$_2$O buffer (red) and 100% (v/v) D$_2$O buffer (blue). At 100% (v/v) D$_2$O the scattering is from both σ54 and deuterated NtrC1C present in the complex. Whereas the scattering from the bound σ54 is matched out at 42% (v/v) D2O. This is the reason for difference between the two scattering curves. D. Comparison between neutron scattering curves from 1mM ADPBeF$_6$/Mg$^{2+}$-stabilized complex of hydrogenated σ54 and deuterated NtrC1C in 42% D$_2$O buffer (red; scattering from only NtrC1C, with σ54 matched out) with the scattering from mixture of σ54 and deuterated Ntrc1C in apo 42% D$_2$O buffer with no complex formation (blue; scattering from only NtrC1C, with σ54 matched out). Note the curves are almost identical, except for minor difference, which has been magnified in the inset. This distance region corresponds to the distance where the GAFTGA loops would be from the edge of the ATPase ring and the variation in intensity may be attributed to the ordering of the loop when bound to σ54.
Table 3.2. Cross correlation NSD table between the Credo Models having σ54 placed on top of the NtrC1\(^c\) ring. Note the NSD values for all 13 models are close to each other and all are within the acceptable range. “25top.pdb” (marked in brown) is the consensus model, having the lowest average NSD.

<table>
<thead>
<tr>
<th>File</th>
<th>Aver</th>
<th>14</th>
<th>16</th>
<th>24</th>
<th>25</th>
<th>30</th>
<th>3</th>
<th>57</th>
<th>63</th>
<th>69</th>
<th>74</th>
<th>75</th>
<th>85</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>14top.pdb</td>
<td>0.92</td>
<td>0.00</td>
<td>0.84</td>
<td>0.95</td>
<td>0.84</td>
<td>0.94</td>
<td>0.72</td>
<td>0.84</td>
<td>0.69</td>
<td>1.10</td>
<td>1.20</td>
<td>1.12</td>
<td>0.96</td>
<td>0.96</td>
</tr>
<tr>
<td>16top.pdb</td>
<td>0.89</td>
<td>0.84</td>
<td>0.00</td>
<td>0.93</td>
<td>0.69</td>
<td>0.87</td>
<td>0.85</td>
<td>0.68</td>
<td>0.84</td>
<td>0.85</td>
<td>1.14</td>
<td>1.15</td>
<td>1.15</td>
<td>0.99</td>
</tr>
<tr>
<td>24top.pdb</td>
<td>0.93</td>
<td>0.95</td>
<td>0.93</td>
<td>0.00</td>
<td>0.93</td>
<td>0.91</td>
<td>0.82</td>
<td>0.91</td>
<td>0.95</td>
<td>0.95</td>
<td>0.95</td>
<td>0.94</td>
<td>1.00</td>
<td>0.93</td>
</tr>
<tr>
<td>25top.pdb</td>
<td>0.83</td>
<td>0.84</td>
<td>0.69</td>
<td>0.93</td>
<td>0.00</td>
<td>0.72</td>
<td>0.87</td>
<td>0.75</td>
<td>0.86</td>
<td>0.71</td>
<td>0.69</td>
<td>0.86</td>
<td>1.13</td>
<td>0.89</td>
</tr>
<tr>
<td>30top.pdb</td>
<td>0.86</td>
<td>0.94</td>
<td>0.87</td>
<td>0.91</td>
<td>0.72</td>
<td>0.00</td>
<td>1.02</td>
<td>0.66</td>
<td>0.95</td>
<td>0.75</td>
<td>0.77</td>
<td>0.95</td>
<td>0.81</td>
<td>0.93</td>
</tr>
<tr>
<td>3top.pdb</td>
<td>0.91</td>
<td>0.72</td>
<td>0.85</td>
<td>0.82</td>
<td>0.87</td>
<td>1.02</td>
<td>0.00</td>
<td>0.95</td>
<td>0.68</td>
<td>0.95</td>
<td>0.90</td>
<td>0.99</td>
<td>0.96</td>
<td>1.25</td>
</tr>
<tr>
<td>57top.pdb</td>
<td>0.84</td>
<td>0.84</td>
<td>0.68</td>
<td>0.91</td>
<td>0.75</td>
<td>0.66</td>
<td>0.95</td>
<td>0.00</td>
<td>0.86</td>
<td>0.76</td>
<td>0.79</td>
<td>0.97</td>
<td>0.97</td>
<td>0.96</td>
</tr>
<tr>
<td>63top.pdb</td>
<td>0.92</td>
<td>0.69</td>
<td>0.84</td>
<td>0.95</td>
<td>0.86</td>
<td>0.95</td>
<td>0.68</td>
<td>0.86</td>
<td>0.00</td>
<td>1.14</td>
<td>1.17</td>
<td>0.96</td>
<td>0.95</td>
<td>1.02</td>
</tr>
<tr>
<td>69top.pdb</td>
<td>0.87</td>
<td>1.20</td>
<td>0.85</td>
<td>0.95</td>
<td>0.71</td>
<td>0.75</td>
<td>0.95</td>
<td>0.76</td>
<td>1.14</td>
<td>0.00</td>
<td>0.71</td>
<td>0.86</td>
<td>0.69</td>
<td>0.90</td>
</tr>
<tr>
<td>74top.pdb</td>
<td>0.90</td>
<td>1.12</td>
<td>1.14</td>
<td>0.95</td>
<td>0.69</td>
<td>0.77</td>
<td>0.90</td>
<td>0.79</td>
<td>1.17</td>
<td>0.71</td>
<td>0.00</td>
<td>0.87</td>
<td>0.73</td>
<td>0.89</td>
</tr>
<tr>
<td>75top.pdb</td>
<td>0.94</td>
<td>0.96</td>
<td>1.15</td>
<td>0.94</td>
<td>0.86</td>
<td>0.95</td>
<td>0.99</td>
<td>0.97</td>
<td>0.96</td>
<td>0.86</td>
<td>0.87</td>
<td>0.00</td>
<td>0.94</td>
<td>0.80</td>
</tr>
<tr>
<td>85top.pdb</td>
<td>0.91</td>
<td>0.96</td>
<td>0.87</td>
<td>1.00</td>
<td>1.13</td>
<td>0.81</td>
<td>0.96</td>
<td>0.97</td>
<td>0.95</td>
<td>0.69</td>
<td>0.73</td>
<td>0.94</td>
<td>0.00</td>
<td>0.87</td>
</tr>
<tr>
<td>8top.pdb</td>
<td>0.95</td>
<td>1.01</td>
<td>0.99</td>
<td>0.93</td>
<td>0.89</td>
<td>0.93</td>
<td>1.25</td>
<td>0.96</td>
<td>1.02</td>
<td>0.90</td>
<td>0.89</td>
<td>0.80</td>
<td>0.87</td>
<td>0.00</td>
</tr>
<tr>
<td>Aver</td>
<td>0.90</td>
<td>0.92</td>
<td>0.89</td>
<td>0.93</td>
<td>0.83</td>
<td>0.86</td>
<td>0.91</td>
<td>0.84</td>
<td>0.92</td>
<td>0.87</td>
<td>0.90</td>
<td>0.94</td>
<td>0.91</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Mean value of NSD = **0.898**
Variation of NSD = **0.038**

Recommend to discard files with NSD > Mean + 2*Variation

25top.pdb  --- Reference // Aver NSD = 0.829
57top.pdb  --- Include // Aver NSD = 0.843
30top.pdb  --- Include // Aver NSD = 0.859
69top.pdb  --- Include // Aver NSD = 0.873
16top.pdb  --- Include // Aver NSD = 0.892
74top.pdb  --- Include // Aver NSD = 0.895
85top.pdb  --- Include // Aver NSD = 0.907
3top.pdb   --- Include // Aver NSD = 0.913
14top.pdb  --- Include // Aver NSD = 0.922
63top.pdb  --- Include // Aver NSD = 0.922
24top.pdb  --- Include // Aver NSD = 0.931
75top.pdb  --- Include // Aver NSD = 0.939
8top.pdb   --- Include // Aver NSD = 0.954
Figure 3.4. SAXS models of complex of NtrC$^1_\mathrm{C}$ with $\sigma_{54}$. The density for $\sigma_{54}$ is in blue and for NtrC$^1_\mathrm{C}$ is in light brown. A. Consensus model of the complex of $\sigma_{54}$ with NtrC$^1_\mathrm{C}$, where the C$\alpha$ model of the heptamer NtrC$^1_\mathrm{C}$ ring was fixed and density for $\sigma_{54}$ was modeled from the solution X-ray scattering curve using Credo (Chen and Nixon unpublished work). B. Consensus model of the complex of $\sigma_{54}$ with NtrC$^1_\mathrm{C}$, where the C$\alpha$ model of the hexamer NtrC$^1_\mathrm{C}$ ring was fixed and density for $\sigma_{54}$ was modeled from the solution X-ray scattering curve using Credo. This model has been blurred to 23Å resolution to make it at the same resolution level as the EM reconstruction of the complex of NtrC$^1_\mathrm{C}$, $\sigma_{54}$ and promoter DNA presented in Chapter 2. C. Upon reducing the iso-surface threshold in the density of the model, the gap in the NtrC$^1_\mathrm{C}$ becomes prominent. The density for $\sigma_{54}$ is located opposite to this gap. This arrangement of $\sigma_{54}$ on the NtrC$^1_\mathrm{C}$ ring is similar to what was found in the EM reconstruction of the complex of NtrC$^1_\mathrm{C}$, $\sigma_{54}$ and promoter DNA, presented in Chapter 2.
3.4 Conclusions

In this chapter I have described a modified feed batch fermentation technique, where the addition of magnesium allows one to expect a several fold increased yield of deuterated proteins expressed in *E. coli*. It can be recapitulated from Studier's work (Studier, 2005) that heterologous protein expression in *E. coli*, growing in LB or rich media, can be significantly improved by supplementing the media with magnesium. Such higher yields of deuterated proteins are important, as they are necessary for doing SANS experiments and also for NMR. As D$_2$O is expensive [~$300 per liter (Cambridge Isotope Laboratories)], the cost of producing deuterated proteins can be significantly reduced by this technique, making experiments feasible that were never considered in the past. Moreover, if in place of D$_2$O one uses water based minimal media and does the same feed batch fermentation, the cost of heterologous expression for similar or better yield of proteins will be much less than if using rich LB media (~$40 per liter (Life Technologies)).

Using the culture system I developed, deuterated NtrC1$^{\text{C}}$ protein was produced and successfully used by my colleague Sysoeva to perform contrast matching SANS experiments under conditions expected to generate asymmetric, hexamer rings. Interpreting her observation of little if any change in scattering between free and $\sigma$54-bound ATPase to mean that the ring remains hexameric in complex with $\sigma$54, and bolstered by my prior EM data suggesting the same, I decided to revisit the approach of *ab initio* modeling of the NtrC1 ATPase/ $\sigma$ 54 complex using SAXS data and fixing the ring structure to reduce the complexity
of the modeling problem to one that current algorithms (implemented in the program CREDO) can handle. The results showed the density for σ54 to be positioned off-centered with respect to the NtrC1 \(^C\) ring and opposite to the gap in the ring. This observation is similar to what has been observed in the EM reconstructions of NtrC1 \(^C\), σ54 and promoter DNA complex. Therefore from these observations the conclusion that can be drawn is that NtrC1 \(^C\) acquires the asymmetric structure to work on asymmetric σ54.
CHAPTER 4

NTRC1 AAA+ ATPASE INTERACTS WITH CLOSED COMPLEX OF σ54-RNAP AND PROMOTER DNA AS AN ASYMMETRIC SPLIT RING

Summary

In Chapter 2 the structure of the complex of NtrC1C with σ54 and promoter DNA was reported. The ATPase in the complex was present as an asymmetric hexamer ring. Class averages and the 3D reconstructions showed a gap in the ATPase ring and diametrically opposite to the gap the density of σ54 and DNA made maximum contact with the ring density. Contrast matching solution scattering studies showed that the NtrC1C asymmetric ring structure does not change significantly when in complex with σ54 (Sysoeva manuscript in preparation; Chapter 3). In order to understand what happens to the NtrC1 AAA+ ATPase, in the complete σ54-transcription activation complex, Transmission Electron Microscopic studies of the complex of NtrC1C, σ54, promoter DNA and RNAP is presented in this chapter. The EM studies reveal the persistence of NtrC1C in similar asymmetric ring structure in the full complex, thus suggesting that this asymmetry is indeed necessary for bEBP ATPase for remodeling σ54.
4.1 Introduction

The uniqueness of σ54 dependent bacterial transcription initiation lies in its reliance on a bEFP AAA+ ATPase to couple ATP hydrolysis to remodel σ54. The important question that I address is how do these ATPases made up of chemically identical subunits work on asymmetric target macromolecule? As discussed earlier in Chapter 2, sub-saturating or stoichiometric amount of ATP leads to a transition of symmetric, heptamer NtrC1 C ring to an asymmetric, hexamer ring structure. My EM reconstructions of the complex of NtrC1 C with σ54 and closed promoter DNA showed that in this complex the ATPase exists as an asymmetric split ring. Further a reasonable match between the gaps of the asymmetric hexamer crystal structure of NtrC1 C with that in the doughnut shaped portion of the EM density in the complex suggests the oligomeric state of the ATPase in the complex to be a gapped hexamer. The gap in the ATPase ring in the complex appears at the opposite interface where the σ54 and promoter DNA density makes maximum contact with the ATPase density. This is consistent with observations that can be made in models generated from SAXS data presented in Chapter 3. Further recent contrast matching SANS experiments by Sysoeva shows that the hexamer ring structure of the NtrC1 C remains unchanged upon binding with σ54.

In this chapter I present electron microscopy data on the full σ54-transcription complex made up of NtrC1 C, σ54, closed promoter DNA and RNAP. The EM data shows the persistence of asymmetry in the ATPase ring in the
complex with a prominently displayed gap. This strongly suggests that bEBPs are obliged to acquire this asymmetry to remodel their asymmetric target, the $\sigma^{54}$-form of bacterial RNAP, in closed complex with promoter DNA.

### 4.2 Materials and methods

**Protein expression and purification**

Expression and purification of NtrC1$^C$ and $\sigma^{54}$ have been described in Chapter 2, section 2.2. Details about the $nifH$ promoter DNA that is present in the complex has also been provided in Chapter 2, section 2.2.

**Expression and purification of RNAP**

The RNAP core construct used has two $\alpha$, single copy each of $\beta$ and $\beta'$ subunits and lacks the $\omega$ subunit (kindly provided by K. Murakami). It was transformed into *E.coli* BL21(DE3) ultra-competent cells, as described (Inoue et al., 1990). These cells were grown in 16 L of rich LB media containing 50 $\mu$g/ml of ampicillin in a 20L New Brunswick Fermentor (at Penn State Shared Fermentation Facility) at 37°C with constant agitation of 700 r.p.m, up to a cell density ($OD_{600nm}$) of 6. The temperature was then reduced to 15°C and expression induced with 0.5mM IPTG. After 10-12 hours of expression, cells were harvested and stored at -80°C.

Twenty-four grams of the frozen cell paste were suspended in 50ml of Lysis buffer containing one tablet of EDTA free protease inhibitor (Roche) in 50ml
Falcon tube and the cells were disrupted by ultra-sonication over ice. The cell lysate was then subjected to centrifugation at 45K r.p.m, in Ti-70 rotor in Beckman Ultra-centrifuge, for 1hr to remove cell debris. To the supernatant 10% (v/v) Polymin P (Polyethylene amine) (Sigma Aldrich), pH 7.9 was slowly added to a final concentration of 0.4 to 0.6 % (v/v). This mixture was gently stirred for 15min. It was then subjected to centrifugation at 100,000 x g for 15mins and the supernatant was discarded. The pellet was then re-suspended in Buffer H containing 0.5M NaCl and then centrifuged at 30K r.p.m, in Ti-70 rotor, for 15 mins. This step was repeated twice. The pellet was then re-suspended in 50ml of Buffer H containing 1M NaCl. After 15mins, it was then centrifuged at 30K r.p.m, in Ti-70 rotor for 15 mins. The supernatant was retained and this process was repeated again. After pooling the supernatant, RNAP was precipitated by bringing the solution to 60% saturation with ammonium sulfate at 4°C. This was followed by centrifugation at 100,000 x g for 15mins at 4°C and the pellet was then re-dissolved in 30ml Buffer H. The re-dissolved protein was then subjected to dialysis for 16hrs at 4°C in 4L of Buffer H containing 0.1M NaCl. The dialyzed protein was purified by anion exchange chromatography in a 50 ml BioRex70 column (BioRad). RNAP was eluted from the column with a NaCl gradient from 0.2M to 0.8M NaCl containing buffer H. Ammonium sulfate precipitation at 60% saturation at 4°C was then carried out on the pooled fractions. It was then subjected to centrifugation at 100,000 x g for 15 mins in Ti-70 rotor in Beckman Ultracentrifuge and the pellet was re-suspended in 10ml of Buffer H containing 0.5M NaCl. This was then subjected to size exclusion chromatography over
Sephacryl S-300 (GE Healthcare) equilibrated with Buffer H containing 0.5M NaCl. Fractions between 115ml to 140ml were pooled after verifying with SDS-PAGE that they contained the RNAP. To the pooled fraction 5mM TCEP was added and was dialyzed in 4L of Buffer I for 12hrs at 4°C. To the dialyzed sample glycerol was added to a final concentration of 20% (v/v) and 5mM TCEP was also added. The final concentration of pure RNAP obtained was 4.3 mg/ml and it was stored in 1.5 ml aliquots at -80 °C until further use. Prior to use the RNAP would be thawed on ice.

**Preparation of complex**

5.5nmols NtrC1C, 7.8nmols σ54, 3.9nmols RNAP and 11.7nmols promoter DNA were added to 10ml of Buffer GF2 containing 1mM ADP-AlF₄/Mg²⁺ and concentrated to 150μl using 10KDa Amicon Concentrator (Millipore). The concentrated protein was then subjected to size exclusion chromatography in a 24 ml Superdex-200 column (GE Healthcare), which was pre-equilibrated with buffer GF2. The complex of NtrC1C, σ54, RNAP and DNA eluted at 9.8ml (Figure: 4.1) and the concentration of the complex was about 0.69mg/ml by Bradford estimation (Bradford, 1976).
Table 4.1. Media and buffers for protein expression and purification and complex formation.

| Fermentation Media (10 L): | 200 g peptone; 400 g yeast extract; 300 g glycerol; 80 g NaH₂PO₄; 70 g K₂HPO₄; 0.6ml Poly Propylene Glycol-200 (antifoaming agent; more may be added later); Antibiotics (0.5 g kanamycin, 0.5 g ampicillin or 0.34 g chloramphenicol) are added after autoclaving. |
| Buffers for RNAP purification: |  |
| Lysis buffer: | 50 mM TrisHCl pH 8.0; 1 mM EDTA; 5mM DTT. |
| Buffer H: | 10 mM TrisHCl, pH 8.0; 0.1mM EDTA; 1mM DTT; 5%(w/v) glycerol. |
| Buffer I: | 20 mM TrisHCl, pH 8.0; 200 mM KCl; 5%(w/v) glycerol. |
| Buffers for Gel-filtration: |  |
| The nucleotide buffer was prepared by mixing components in the following proportions: 1 [ADP]:1 [AlCl₃]:8 [NaF]:1 [MgCl₂]. The buffer after preparation was filtered with 0.45μ filter and re-filtered again right before Gel-filtration. |
| Buffer GF2: | 20 mM TrisHCl, pH 7.8; 200 mM KCl; 1mM TCEP; 1mM ADP; 1mM AlCl₃; 8mM NaF; 1mM MgCl₂. |
Figure 4.1. Purification of NtrC1\(^C\), σ54, RNAP and DNA complex by Gel-filtration. Gel-filtration over Superdex-200 for purification of NtrC1\(^C\), σ54, RNAP and promoter DNA closed complex in 1mM ADP-AlF\(_x\)/Mg\(^{2+}\) buffer. The elution peak of the complex is at 9.8ml and has been shown by an arrow. Elution profiles of the individual components and smaller sub-complexes have been shown in order to show separation of the complex peak. The SDS-PAGE of the complex is shown inset, with the dashed box highlighting the full complex lane. Lane labels are as follows: 1 – molecular weight markers; 2 – σ54; 3 – NtrC1\(^C\); 4 – RNAP; 5 – promoter DNA (runs at dye front; stained with ethidium bromide); 6 – full complex. This lane is marked with dotted box.
Single particle reconstruction of the complex

Preparation of grids for electron microscopy

1. Negative stained samples

The purified complex was further diluted one hundred fold with Buffer GF1. Thin carbon coated 300 mesh grids were used. These grids were prepared by the method described in Chapter 1, Section 1.7. They were first plasma cleaned for 30s with hydrogen and oxygen in a Solarus plasma cleaner. Five micro liters of protein sample were absorbed on the grid and stained with 1% (w/v) Uranyl Acetate and air-dried. The detailed procedure for negative staining is described in Chapter1, Section 1.7.

2. Cryo samples

In order to overcome stain artifacts and for collecting data in the frozen, hydrated, close to native condition, cryo data of the complex were collected. The purified complex was further diluted sixty, seventy, eighty and one hundred fold with Buffer GF2. Three micro liters of sample were added on plasma cleaned thin carbon coated lacey carbon grids (Ted Pella). After blotting off excess sample for 3s using ash-less Whatman-1 filter paper, the grid was plunge frozen in liquid ethane at -179°C. Prior to using these grids, I placed samples on regular holey carbon grids (C-Flats), but they tended to stick to the carbon edges and failed to distribute into the holes. The thin carbon coated grids provided a more uniform distribution of particles in the holes, thus enabling imagining of the samples.
Data collection and processing

1. Negative stained sample

Twenty-five micrographs (Figure: 4.2 (a)) were collected at 0° in a Tecnai F20 TEM, operated at 200kV at a nominal magnification of 50,000X, at the New York Structural Biology Center (NYSBC), New York. These micrographs were collected over a defocus range from 1.5μ to 2.5μ on a 4K X 4K CCD Gatan camera with 15μ pixel size. The actual calibrated magnification on the CCD was 88,000X (calibrated with TMV (Tobacco Mosaic Virus)). The pixel size of the micrographs at this magnification was 2.5 Å/pixel. Using a box size of 96x96 pixels in the EMAN boxer program, 7,600 particles were manually picked.. The boxed out particles were then converted into a stack that is compatible with the program Spider (Frank et al., 1996). Particles in the stack were then centered using Spider. For reference-based classification, the EM reconstruction of PspF, σ54 and RNAP complex (EMD-1566; Bose et al., 2008) was low pass filtered to 50Å resolution and then projections were generated from it. These projections were then used for aligning the particles, followed by correspondence analysis and hierarchical ascendant classification with Ward’s criterion (Carazo et al., 1990). The class averages were promising (Figure: 4.2 (b)), so the first 3D reconstructions were prepared. This was done by angular and projection-matching refinement of the low pass filtered PspF, σ54 and RNAP complex mentioned above. The final reconstruction was then low pass filtered with a cut-off of 20Å (Figure: 4.6 (A)).
In order to obtain reference free reconstructions and overcome possible bias from using a blurred version of an existing model, 200 tilted / un-tilted micrograph pairs were then collected at a nominal magnification of 50,000X in JEOL-2100 TEM operated at 200kV. Tilted data were collected by tilting the grid at 55° to 65° angles. These data were then preprocessed and CTF parameters were estimated for each micrograph. A total of 13,213 tilt pair particles were then manually picked using XMIPP and boxed out in 46X46 pixel boxes. The tilted and the un-tilted particles were saved as separate image stacks. The un-tilted particles were then aligned and classified using reference free, maximum likelihood algorithm of the ML2D program of XMIPP. This classification was repeated over several cycles with different number of classes. 3D reconstructions of the class averages were carried out using the tilted data set. This was done using XMIPP’s RCT module. The basic principle of RCT reconstruction was explained in Chapter 1, Section 1.7. The volume of the complete complex had a split ring architecture for the ATPase with RNAP, σ54 and promoter DNA density on top. (Figure: 4.4).

The un-tilted data set was re-analyzed using a recently described EQ-K mean based Iterative Stable Alignment and Classification (ISAC) program of SPARX/EMAN2 (Yang et al., 2012). The XMIPP picked particle stacks were first converted to a format compatible with SPARX. All the un-tilted particles were centered and aligned by reference free alignment in SPARX. After aligning and centering the particles, ISAC runs were performed to achieve stable classification. The class averages generated by ISAC showed several different
orientations of the particles and also other smaller disintegrated parts of the complex.

2. Cryo sample:

After screening through various diluted samples the eighty fold diluted sample had well separated and uniformly distributed particles. A total of 2,049 micrographs were collected at a nominal magnification of 50,000X in a JEOL-2100 TEM operated at 200kV. These micrographs were collected over a range of 2μ to 4.5μ defocus on 2K X 2K CCD Teitz camera. As a preliminary assessment of image quality, 30,000 particles were manually boxed, with a box size of 150x150 pixels using the EMAN2 boxer program. The boxed particles were then converted into a stack that is compatible with the program Spider (Frank et al., 1996). Particles in the stack were then centered using Spider. Reference-based alignment and classification was carried out, with class averages obtained earlier during negative stained reconstruction and projections from the negative stained reconstruction as references. Classification was carried out by correspondence analysis and hierarchical ascendant classification with Ward’s criterion (Carazo et al., 1990). The class averages from the cryo data showed split ring architecture for the ATPase ring (Figure: 4.7)
2.3 Results

The class averages from reference based classification had a “Christmas tree” shaped structure for the complex. Majority of the particles lay along their longitudinal axis on the carbon film. This resulted in majority of the class averages showing a preferred orientation of the complex with their longitudinal axis parallel to the carbon layer. The 3D reconstructed volume from this negative stained data showed split ring architecture of the ATPase and the RNA, σ54 and promoter DNA density made major contacts with the ATPase ring diametrically opposite to the face having the gap.

Reference free class averages of the un-tilted particle set, from the RCT data, by XMIPP showed similar class averages as observed before. This eliminated the possibility of reference bias when the first classification and reconstruction was carried out with a reference. This was further supported when reference-free classification of the un-tilted data set was carried out using ISAC/SPARX. The class averages obtained by ISAC could separate out more conformational variants and partial complexes into different classes, than the classes obtained by XMIPP. The XMIPP RCT reconstructions of the class averages clearly showed a gapped ring in the complex. In future these can be used as initial models and further refined using larger cryo data sets.

Despite the cryo data (Figure 4.7) having lower particle contrast as compared to the stained dataset (Figure 4.3), class averages of these complexes did reveal “Christmas tree” shaped complexes as observed with the negative
stained data. Further, in the cryo class averages a gap in the NtrC1 ATPase ring was prominent. A larger similar cryo data set can now be used for refining the initial negative stained reconstructions from Spider or the RCT reconstructions from XMIPP. The full set of 2049 micrographs will generate roughly 200,000 particle images, and require increased computational power being sought for use at Penn State High Performance Computing Center, University Park.

4.4 Discussions

The NtrC1 AAA+ ATPase in sub-saturating ATP concentrations exists as an asymmetric, hexamer ring structure. EM reconstructions of the complex of NtrC1\( ^\text{C} \) with \( \sigma54 \) and promoter DNA show persistence of similar asymmetry in the ATPase ring architecture. Although, the authors did not describe it, a similar gapped helical arrangement of subunits is present in the PspF ATPase when in complex with \( \sigma54 \) or \( \sigma54\)-RNAP (Bose et al., 2008) (Figure: 4.6(B)). Further, contrast-matching-SANS experiments have shown that the ATPase ring structure does not change significantly from its free state when compared with it in complex with \( \sigma54 \) (Chapter 3). Recent biochemical studies by Buck’s group (Joly and Buck, 2010) with covalently linked dimers and trimers of PspF ATPase suggest that the asymmetry and flexibility between ATPase subunits is necessary for its interaction with RNAP-closed complex. These observations strongly support the hypothesis that this asymmetric ATPase structure is
necessary for targeting the force necessary for remodeling asymmetric closed σ54 transcription complex.

Figure 4.2. Negative stained micrograph of the NtrC1<sup>C</sup>, σ54, RNAP and DNA complex. a) Micrograph with stained particles. Majority of the particles have preferred orientation with their longitudinal axis parallel to the plane of the carbon. b) Class averages showing the complex in different orientation. c) One of the class averages magnified to show the individual components in the complex. Note the gap in the NtrC1<sup>C</sup> ring.
Figure 4.3. Tilt pair micrographs of the NtrC1<sup>C</sup>, σ54, RNAP and DNA complex. On the left hand side is a tilted micrograph (tilt angle 65°) and on the right is the corresponding un-tilted micrograph (tilt angle 0°) of the same area. This is representative of the complete set of tilted and untilted micrographs for RCT data collection.
Figure 4.4. RCT reconstructions of class averages of the complex using ML2D/XMIPP.

Class averages obtained by maximum likelihood based classification of the un tilted particles from the RCT data are shown on top of each row and the corresponding 3D reconstruction of the class averages are shown at the bottom. Note that by RCT the different partial complexes and different conformational variants of the complete complexes can be separated into different homogeneous classes. Some of the full complexes like numbers 3, 5, 10, 11, 12 and 14 can be used in the future as initial models for refinement against larger cryo EM datasets of the complex. Even in these low-resolution, noisy reconstructions of the complete complexes the ATPase ring appears to be highly asymmetric.
Figure 4.5. Classification of the untilted particles from the RCT data using ISAC/SPARX. Class averages obtained by using ISAC/SPARX on the same data set that has been dealt with in Figure: 4.4. Different class averages show more orientations of the particles. This method of classification has been able to separate out bad particles, partial complexes, free RNAP or dimers of RNAP into separate classes. In some of the class averages the gap in the ATPase ring is more prominent (Numbers. 17, 26, 41and 45).
Figure 4.6. 3D reconstruction of the complex of NtrC1<sup>C</sup>, σ54, RNAP and DNA (negative stained untilted data set) and its comparison with the 3D reconstruction of PspF, σ54 and RNAP complex. A. 3D reconstruction of the NtrC1<sup>C</sup>, σ54, RNAP and DNA complex shows a prominent gap in the ATPase ring. Densities of promoter DNA and σ54 can not be annotated with certainty as they are indistinguishable from the RNAP density. This will be possible in future with higher resolution reconstructions by using large cryo data set. B. Although not noted by the authors, a similar gap in the ATPase ring can be observed in the 20Å cryo EM reconstruction of PspF, σ54 and RNAP complex (EMD-1566; Bose et al., 2008). This reconstruction lacks the promoter DNA and has less detail as compared to the NtrC1 complex reconstruction. The presence of gapped asymmetric bEBP ATPase rings in both complexes suggests that it is essential for the function of the bEBP ATPase for remodeling σ54.
Figure 4.7. Initial Cryo EM data of NtrC1C, σ54, RNAP and closed DNA complex. A. Typical cryo micrograph at 2.5μ defocus with the particles barely visible as darker specs. Some of the particles have been boxed in yellow and they are shown on the right of the micrograph. Absence of stain and thin carbon film over the holes contribute to poor contrast and background noise in the data. B. Some class averages from the cryo data showing the full complex. C. One of the class averages magnified to shown the individual components in the complex. Notice the presence of gap in the ATPase ring. The RCT reconstructions of the full complex shown in Figure: 4.4 can be refined against similar larger cryo dataset to obtain better resolution in future 3D reconstructions of the complex.
4.5 Conclusions

The NtrC1 AAA+ ATPase transitions to an asymmetric structure that is crucial for its interaction with asymmetric target, σ54. I interpret the persistence of this asymmetric gapped ring architecture in the EM reconstructions of complexes in this chapter and in Chapter 2, along with similar structure of PspF AAA+ ATPase when in complex with σ54 and RNAP, to mean that this structural organization is common to all bEBP ATPases. The asymmetry may in fact reflect coordinated changes occurring across various subunits during different stages of nucleotide hydrolysis that leads to rigid body sub domain roll and GAFTGA loop movements to conduct mechanical work on σ54. This mechanical work can be envisioned to lead to removal of the inhibition caused by σ54 and facilitate the loading of the transcribing DNA strand into the active site of RNAP. Therefore in this chapter I have been able to provide structural data to further frame the question: How does a bEBP ATPase interacts with an asymmetric σ54-closed complex? The answer is: By the ATPase approaching the asymmetric target with an asymmetry of its own. The structural details, though low in resolution, provide restraints that must be satisfied with future explanations for activation of σ54-dependent transcription by bEBPs.
CHAPTER 5

SIGNIFICANCE AND FUTURE DIRECTIONS

All prior studies on the bEBP NtrC1 were done on the isolated AAA+ ATPase domain. Missing are studies in the presence of the other two domains that comprise the full-length protein, and studies in the presence of the closed complex of σ54-RNAP and promoter DNA. The work in this thesis contributes towards understanding the latter set of interactions, addressing what happens to the ATPase in context of the σ54 dependent closed transcription complex. By using transmission electron microscopy and single particle reconstructions I have presented the first structure of the complex of NtrC1C with σ54 and promoter DNA; plus the first initial EM reconstruction of the complex of NtrC1C with σ54, RNAP and closed promoter DNA. These studies suggest that the NtrC1 ATPase persists as an asymmetric ring while interacting with its asymmetric target, σ54. In addition to these advances, I have also described a novel way of expressing deuterated proteins, whereby one can expect to have three to five times higher yield. Deuterated NtrC1C expressed by this method was used by my colleague Tatyana Sysoeva for novel contrast matching SANS experiments on complexes of NtrC1C with σ54. She was able to observe that the NtrC1C ring structure remains unchanged upon interacting with σ54, which is entirely consistent with my observations.
In this thesis for the first time we present the asymmetric functional state of bEBP while interacting with σ54 closed transcription machinery. However there are still many improvements that can be done in the future with better technology and image processing algorithms. Many more intriguing questions still remain unanswered or are in speculative stages. In this chapter in addition to summarizing the significance of the work done by me, I have proposed future directions for seeking answers to these questions.

5.1 EM studies on complexes of NtrC1\textsuperscript{C} with σ54, promoter DNA and also with RNAP

In Chapter 2, I presented the EM studies on negatively stained complexes of NtrC1\textsuperscript{C} with σ54 and promoter DNA. The class averages and the single particle reconstructions show the persistence of asymmetric NtrC1\textsuperscript{C} ring structure with a gap. Upon superimposing the asymmetric hexamer crystal structure of NtrC1\textsuperscript{C} into the doughnut shaped density of the ring, the gap between first and sixth subunit in the crystal structure was seen to match with the gap in the EM density. This discovery prompted re-evaluating EM reconstructions of PspF in complex with σ54 and RNAP, revealing a similar gap in that bEBP ring. This feature of that structure has not been noted previously. If, as proposed by Sysoeva, this gap is responsible for ADP release and ATP uptake between
subunits much as has been suggested for the E1 and Rho nucleic acid translocases (Enemark et al., 2006; Erzberger et al., 2006), it will be required for future research to explain how that exchange is integrated with actions of the inner subunits opposite the gap that engage and remodel σ54. In the negative stained EM reconstruction of the NtrC1\textsuperscript{C}, σ54 and promoter DNA complex it is difficult to accurately distinguish between the density of σ54 and DNA. For now, one can interpret the low-resolution structure to suggest that σ54 interacts with subunits B, C and D of the ring, with most contact being with subunit C. To visualize precise interactions, high-resolution structures of these complexes by X-ray crystallography will be necessary.

It is always preferred to have cryo reconstructions than negative stained reconstructions to avoid potential stain artifacts and collect data close to physiological conditions in frozen hydrated state. One of the limitations is the size of the NtrC1\textsuperscript{C}, σ54 and promoter DNA complex. It is difficult to do cryo EM on complexes or molecules smaller than 400 kDa due to poor contrast. With the introduction of new phase-plate technology in TEM along with energy filters, it is becoming possible to collect cryo data on smaller complexes. Phase plates help in increasing the phase contrast and energy filters reduce the background noise by selectively removing in-elastically scattered electrons. Phase plates can enable data collection at or close to focus, which can lead to improved resolution of reconstructions. In the future, with access to such advanced microscopes, it will be possible to collect cryo data for NtrC1\textsuperscript{C}, σ54 and promoter DNA.
complexes. The negative stained derived models can be used as initial ones to be further refined using the cryo data set.

A similar strategy can be used to collect better quality cryo data for the full complex also containing the RNAP. To avoid preferred orientations of the particles landing on the thin carbon film, cryo data for the complex should be collected on regular holey carbon grids not having the thin carbon film on it. An even distribution of the particles in the holes of the grid may be achieved by using small quantities of surfactants like detergents or bacitracin. This can be expected to give rise to more random orientations of the particles and thus provide more information for more precise reconstructions. The thin carbon film can also contribute to background noise. Therefore eliminating its necessity can improve the quality of micrographs and also yield better visibility of particles. Once a large cryo data set has been collected the negative stained reconstruction of the complex of NtrC1\textsuperscript{C}, σ54, RNAP and promoter DNA can be used as initial model for refinement against this data set.

In order to visualize the complete picture of the bEBP based activation of σ54 dependent transcription by DNA looping mechanism, bEBP containing DNA-binding domain along with the ATPase domain should be used. A longer piece of DNA with the σ54 promoter and the UAS sequence is required for this experiment. The complete complex will be much larger in size and would be easier for visualization by cryo EM. This can help us visualize the actual DNA looping mechanism that poises UAS bound EBPs for interaction with σ54-RNAP at promoter DNA, and reveal how bEBPs are bound to the UAS region. By using
combination of all these EM techniques we can obtain a more complete picture of the σ54 transcription complex and how bEBPs interact with the closed complex to remodel it.

5.2 Deuteration of proteins for SANS experiments

In Chapter 3 I have described a novel feed batch technique that can be used to obtain higher yield of deuterated proteins. This was achieved by altering the magnesium content in the feed. In order to perform SANS experiments it is necessary to have sufficient quantity of labeled proteins. I have established a protocol, which can now be used for expressing deuterated proteins to facilitate further SANS experiments. As the SANS user community is relatively new, it is currently limited to binary systems, but with time it will be possible to use this technique for ternary and perhaps even more complex systems. This deuteration protocol will be useful for doing different SANS experiments on protein complexes, as illustrated by Sysoeva’s observation that the NtrC1C ring structure essentially remained unchanged upon binding to σ54. This fairly simple observation strongly suggests that the ring acquires its asymmetric conformation before interacting with its asymmetric target σ54. As the method becomes more accessible, it will be used more and more for such observations. Deuterated proteins are also necessary for NMR and this protocol will be helpful for
expressing sufficient quantity of deuterated proteins with the utilization of less D₂O.

5.2 Crystallography of NtrC¹

Former colleague Tatyana Sysoeva also obtained asymmetric hexamer crystal structure of NtrC¹, under sub saturating or stoichiometric amounts of ATP analogue ADP-BeFₓ/Mg²⁺. The GAFTGA loop in the crystal structure had a staircase like arrangement. The crystal structure she obtained had a resolution of 3.6Å (collected at Brookhaven National Laboratory). In order to improve the resolution I re-crystallized NtrC¹ with sub-saturating or stoichiometric amounts of ADP-BeFₓ/Mg²⁺. I had even set up crystals with ADP-AlFₓ/Mg²⁺ and a mixture of ADP-BeFₓ and AlFₓ /Mg²⁺. I could obtain some crystals and observed that soaking the crystals in Fomblin oil (Propene 1,1,2,3,3,3-Hexafluoride; Hampton Research) before freezing them in liquid nitrogen improved the resolution to 3Å (at a home source, thus resolution below 3Å can be expected for synchrotron data) (Figure: 5.1). This strategy can now be used to further enhance the resolution of crystals obtained at different nucleotide concentrations and perhaps in mixed nucleotide states for NtrC¹. Even larger complexes may be crystallized in the future. I was able to obtain diffraction to about 8Å for complex of NtrC¹, σ54 and promoter DNA; despite many screening attempts, no diffracting crystals
were obtained for the larger complex containing RNAP core enzyme. That target may require more suitable materials, perhaps from a thermophilic bacterium.

In order to observe if the gap in the ring propagates around the ring, atomic force microscopy can be carried out on the complex to locate the gap with respect to the polymerase density. Remarkably, this can be done on aqueous samples with a scanning frequency of 30-60 msec (Ando et al., 2001; Yamamoto et al., 2010; Sugimoto et al., 2010), well within the time domains of conformational changes that have been seen in time resolved SAXS measurements by Sysoeva. This can provide direct structural evidence to support or refute the sequential hydrolysis mechanism proposed by Sysoeva.
Figure 5.1. Crystallography of NtrC1©. A. Crystals of NtrC1© in absence of stoichiometric amount of ADP-BeF₆/Mg²⁺ (Left) and ADP-AlF₆/Mg²⁺ (Right). B. a) X-ray diffraction from 2min exposure of crystal of NtrC1© in stoichiometric amount of ADP-BeF₆/Mg²⁺ (Sysoeva thesis and unpublished work). b) X-ray diffraction from 1min exposure of crystal of NtrC1© in stoichiometric amount of ADP-BeF₆/Mg²⁺. This crystal was soaked in Fomblin oil before freezing and diffraction. c) X-ray diffraction from 1min exposure of crystal of NtrC1© in stoichiometric amount of ADP-AlF₆/Mg²⁺. This crystal was also soaked in Fomblin oil prior to freezing and diffraction. Note in b) and c) the Fomblin oil soaked crystals diffract to higher resolution at home X-ray source than diffractions obtained at synchrotron for crystals not soaked in Fomblin oil (a).
To summarize, work in this thesis has demonstrated how combination of different structural techniques can be used to obtain an ensemble picture of how bEBP ATPase domain interact with closed σ54-RNAP transcription complex. For the first time I have presented the low resolution EM reconstruction of the complex of NtrC1C ATPase in complex with σ54 and promoter DNA. In this structure we can see how the asymmetric ATPase targets σ54 and DNA. Further initial negative stained reconstruction of the full complex with RNAP has also been presented. The next step would be to collect high quality cryo EM data of the complex and obtain higher resolution reconstructions. The necessary procedure for preparing cryo samples has also been described. In the future contrast variation and contrast matching SANS experiments can be performed with deuterated proteins, deuterated to different extents to add more information to the data so that the individual scattering profiles and interactions can be extracted by current methodologies. The next step for this project should be to obtain improved resolution reconstructions of the σ54 transcription complex and also obtain higher order reconstructions. Further by soaking crystals in Fomblin oil the resolution of hexamer NtrC1C ring should be improved. This will help in understanding how the different residues in the ATPase interact with each other.
REFERENCES


VITA

Saikat Chowdhury

Education
Postdoctoral Associate: The Scripps Research Institute, La Jolla, CA, USA. (September, 2012)
  Advisor: Dr. Francisco J. Asturias

Ph.D. Biochemistry & Molecular Biology, Penn State University, University Park, PA, USA. (2012)
  Advisor: Dr. B. Tracy Nixon


Publications


In Preparation:
Sysoeva, T.A, Chowdhury, S*, Nixon, B.T. Nucleotide-induced asymmetry within bEBP ATPase ring drives Sigma54 interaction and ATP hydrolysis. (* Co-first authors)

Chowdhury, S., Sysoeva, T.A., Chen, B., Nixon, B.T. Feed batch deuteration technique for enhanced yield of deuterated proteins and solution scattering experiments to study complex of bEBP with Sigma54.

Awards and honors
Robert T. Simpson Innovative Science Research Award by Penn State Department of Biochemistry and Molecular Biology. (2012)

Graduate and Postdoctoral travel award to attend ASBMB Annual Meeting at San Diego. (2012)

Finn World Travel Award to attend Protein Society Symposium, Boston. (Declined, as I was unable to attend the symposium). (2011)

Homer F. Braddock and Neil H. and Oscar L. Roberts Fellowship, for outstanding graduate application, by Penn State Department of Biochemistry and Molecular Biology. (2006-2008)

3rd. Prize for research presentation competition at the Indian Society for Technical Education meeting, India. (2004)

Outstanding student scholarship for Bioinformatics undergraduate degree program at Vellore Institute of Technology, Vellore, India. (2004-2006)