CHARACTERIZATION OF HUMAN NFU AND ITS INTERACTION WITH THE MOLECULAR CHAPERONE SYSTEM

DISSertation

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By

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ABSTRACT

How iron-sulfur clusters are synthesized in vivo remains an interesting topic to explore. In this dissertation, studies were focused on human NFU, an important protein that participates in iron-sulfur cluster biosynthesis. Studies were first carried out on NFU’s functional characterization. Due to the presence of the conserved CXXC motif in NFU, this protein was proposed and demonstrated to cleave the persulfide bond on NifS. In the presence of L-cysteine, a catalytic amount of NifS, ferrous iron and NFU, apo proteins lacking iron-sulfur clusters can be successfully reconstituted. Both human ISU and Thermotoga maritima IscU were used as the scaffold. The successfully reconstituted IscU was confirmed by both UV and EPR spectroscopy. A kinetic study of this process indicated similar results for both human ISU and T. maritima IscU. The $k_{\text{obs}}$ was 0.0165 min$^{-1}$ and 0.015 min$^{-1}$, respectively when data were fitted to a first-order process. The C-terminal domain of human NFU was also cloned and purified. Similar studies as described above on the C-terminal domain yielded similar results as the study on full-length NFU. This supports the idea that the C-terminal domain in full-length NFU is the functional domain. The function of human NFU led to the hypothesis that this protein can interact with NifS. The binding of NFU to NifS was quantified by ITC (isothermal titration calorimetry). The binding affinity was fitted to 9.7 μM and 17 μM for NFU and
the C-terminal domain, respectively. The temperature-dependence of binding was also examined to determine the underlying thermodynamics. A positive change in enthalpy with increased temperature suggested dominance of polar charged residues in the binding.

The interaction of human NFU and the chaperone system was also studied \textit{in vitro}. Both NFU and its C-terminal domain can stimulate the ATPase activity of the chaperone HscA and exhibit a binding affinity of 50 μM for both. However, NFU stimulates the ATPase activity by a maximal 4.7-fold in contrast to 3.4-fold of the truncated C-terminal domain. With the addition of the co-chaperone HscB, the binding affinities obtained from the ATPase experiment increased to 18 μM and 15.7 μM, respectively. This led to the assessment of the role of the co-chaperone HscB: helping NFU form a tighter complex with HscA. The ITC study was also used to explore the binding of NFU to the chaperone system. Both NFU and truncated C-terminal domain can form a complex with HscB. The ADP form of HscA binds to both NFU and the C-terminal domain more tightly than the ATP form, providing additional evidence for NFU interacting with the chaperone system. The binding of NFU to the HscA/HscB complex is tighter than binding to HscA alone, which is consistent with previous ATPase results.

Besides functional characterization, structural characterization was also carried out. The secondary structures of both NFU and its C-terminal domain were deconvoluted from their CD (circular dichroism) spectra. The fitted data indicates 31% α-helix, 12% β-sheet, and 57% random coil for full-length NFU and 30% α-helix, 17% β-sheet, 53% random coil for the C-terminal domain. The near UV CD data were almost negligible, suggesting a possible molten globule property of human NFU. Results from ANS binding
and tryptic digestion experiments were consistent with molten globule type proteins. During the tryptic digestion experiment, unlike the truncated C-terminal domain which disappeared quickly, full-length NFU showed an intermediate band on the SDS PAGE gel. The LC-MS experiment identified the band as the N-terminal part of the full-length NFU. Thus the N-terminal domain was cloned and purified. The N-terminal domain was confirmed to possess a tertiary structure by the ANS experiment and the tryptic digestion experiment. Finally the well-structured N-terminal domain and the molten globule C-terminal domain were confirmed by high-resolution $^{1}H^{15}N$ HSQC experiments. The molten globule property of the C-terminal part of full-length NFU may provide the docking site to interact with different partners for function.
DEDICATED TO MY PARENTS
I am greatly thankful to my advisor, Dr. James A. Cowan. Without his valuable advice and encouragement, I could not have completed so many experiments successfully during my doctoral study. He taught me not only technical skills but also how to think creatively. All these are priceless gifts and will benefit me in my future career.

I would like to thank all the professors on my committee, Ross Dalbey, Russ Hille, and Mark Foster. Their advice on my projects always led to big breakthroughs. I also learned a lot from them in various classes that I took.

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Special thanks also go to all my friends who gave me help when needed. Without them, my life here would have been much more difficult. And I greatly appreciate the funding I received from the Department of Chemistry and The Ohio State Biochemistry Program.
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FIELD OF STUDY

Major Field: Ohio State Biochemistry Program
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CHAPTER 1

OVERVIEW

1.1 IRON HOMEOSTASIS

1.1.1 Iron and its Functions in Vivo

Iron is one of the most abundant elements that make up the earth. And among the elements, iron is the only transition metal (Table 1.1). Naturally occurring iron includes four isotopes: radioactive $^{54}$Fe 5.845%, stable $^{56}$Fe 91.754%, stable $^{57}$Fe 2.119%, stable $^{58}$Fe 0.282 % [1]. Although iron is abundant on earth, it is difficult to use directly due to its low solubility at pH 7. In cells, this essential element can be incorporated into a variety of proteins to perform different functions. Consequently, iron is involved in many biological functions such as oxygen transport and nitrogen-fixation [2].

<table>
<thead>
<tr>
<th>Element</th>
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<tr>
<td>Oxygen</td>
<td>46.1</td>
</tr>
<tr>
<td>Silicon</td>
<td>28.2</td>
</tr>
<tr>
<td>Aluminum</td>
<td>8.23</td>
</tr>
<tr>
<td>Iron</td>
<td>5.63</td>
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Table 1. 1 The most abundant elements in the earth’s crust and their approximate weights. (Taken from CRC Handbook of Physics and Chemistry, 77th Edition)
1.1.2 Introduction to Iron-containing Proteins

Many animals and humans use hemoglobin, an iron-containing protein, to carry oxygen in the blood [3]. The well-characterized hemoglobin is an $\alpha_2\beta_2$ tetramer protein [3]. Each subunit in hemoglobin contains a heme cofactor composed of a phorphyrin with a central bound iron atom (Figure 1.1) [3]. The transport of oxygen is achieved by reversibly binding to ferrous hemoglobin [4]. Hemoglobin carries oxygen through a concerted process, in which the binding of the first oxygen will induce the confirmation change that facilitates the binding of oxygen to other subunits [3]. Further studies indicate that lowering the oxygen binding affinity can be achieved by increasing the concentrations of $H^+$, $CO_2$, and 2, 3-D-diphosphoglycerate (DPG) [3]. This iron-containing protein is so crucial that many identified diseases are found to associate with it, such as anemia. Sickle cell anemia is associated with the mutation in hemoglobin characterized by the polymerization of the abnormal sickle hemoglobin [5].

![Figure 1.1 Structure of heme (Fe-protoporphyrin IX).](image-url)
Besides facilitating the transportation of oxygen, iron can also form iron-sulfur clusters, which act as the prosthetic group incorporated into a variety of proteins. Nitrogenase is an example of this class, which uses iron-sulfur clusters for its catalysis. Nitrogenase can reduce molecular $\text{N}_2$ to NH$_3$ as summarized in scheme 1 [3].

$$\text{N}_2 + 8\text{H}^+ + 8\text{e}^- + 16\text{MgATP} \rightarrow 2\text{NH}_3 + \text{H}_2 + 16\text{MgADP} + 16\text{Pi}$$

(1)

The study of nitrogenase reveals the presence of two component proteins: an ‘iron protein’ ($\gamma_2$ dimer encoded by $nifH$) containing a [4Fe-4S] cluster and can bind two MgATP [6]. The ‘iron protein’ can couple ATP hydrolysis to electron transfer [3]. The second component is a ‘molybdenum-iron protein’ ($\alpha_2\beta_2$ heterotetramer encoded by $nifD$ and $nifK$) [3,6]. The ‘molybdenum-iron protein’ harbors the site for substrate binding and reduction [3]. It binds two FeMo clusters (M-clusters) and four unusual [8Fe-8S] centers (P-clusters) (Figure 1.2) [7].
Figure 1. 2 The structure of P-cluster and M-cluster in nitrogenase. Label (a) refers to residues from iron-binding protein and (b) refers to residues from molybdenum-iron protein of nitrogenase.

The proposed mechanism for N₂ reduction is as follows: The binding of MgATP to the Fe-protein initially incurs the conformation change [3]. This change can facilitate the formation of the Fe-protein and MoFe-protein complex. The complex formation then
leads to the hydrolysis of ATP to ADP and to electron transfer [3]. Subsequently, the complex dissociates to inhibit the backflow of the electron transfer reaction [3]. Each time, one electron is transferred from iron protein to molybdenum-iron protein and the process is repeated eight times [3]. With the provided reducing equivalents, \( \text{N}_2 \) is reduced to \( \text{NH}_3 \). However, the detailed mechanism behind this is still in debate [3].

1.2 IRON-SULFUR CLUSTER PROTEINS

1.2.1 The Type and Function of Iron-sulfur Clusters

With the discovery of ferredoxin in the 1960s, people have paid more attention to iron-sulfur clusters [8]. With the development of technology and great interest, more and more iron-sulfur cluster containing proteins have been discovered and studied. Here we only introduce three types of iron-sulfur clusters frequently seen: rubredoxin-like cluster, [2Fe-2S] cluster, and [4Fe-4S] cluster (Figure 1.3). Commonly, cysteine is the residue that provides the sulfur coordination for iron to accommodate the iron-sulfur cluster. However, cysteine can be replaced by aspartate, histidine, or serine which can modify the redox potential to facilitate the function of iron-sulfur clusters [9]. Iron-sulfur clusters participate in a variety of different functions \textit{in vivo}. The function includes the following:

(1) electron transfer: In total, 13 iron-sulfur clusters are involved in the mitochondrial electron transfer chain (ETC) [10]. Most of the iron-sulfur clusters act as a one-electron carrier. In contrast, nitrogenase containing the [8Fe-7S] cluster can be a two-electron carrier [11].
Figure 1. Three types of common iron-sulfur clusters in nature.

(2) Sensing: Iron-sulfur cluster containing proteins can be involved in the transcriptional regulation when stimulated by the environment factor [12]. A well-characterized example is the SoxR /SoxS system. SoxR contains a [2Fe-2S] cluster [13,14]. Although the [2Fe-2S] cluster is +1 charge under normal conditions, it can be oxidized to +2 when superoxide or nitric oxide is present [15]. This will lead to the transcriptional activation of SoxS, ultimately resulting in the expression of enzymes to protect the organisms from oxidative stress [13]. Another iron-sulfur cluster containing
protein called FNR (Fumarate and Nitrate Reduction) is responsible for switching between aerobic and anaerobic metabolism in *Escherichia coli* [12]. In the absence of oxygen, FNR can adapt a $[4\text{Fe}-4\text{S}]^{2+}$ cluster with DNA-binding affinity [12]. The $[4\text{Fe}-4\text{S}]^{2+}$ cluster is oxygen sensitive and can be degraded to a $[2\text{Fe}-2\text{S}]^{2+}$ cluster which deprives its DNA-binding affinity to further regulate the gene expression [12,16,17].

(3) Substrate binding: In the case of aconitase, an enzyme responsible for the conversion of citrate to isocitrate in the TCA cycle (tricarboxylic acid cycle) [18,19], the noncysteinylligated Fe can bind to the substrate and facilitate the catalytic reaction [19]. Another example is the substrate binding of CO dehydrogenase, different from aconitase, where a nickel ion is incorporated to implement this function [20].

### 1.2.2 Operons Related to Iron-sulfur Cluster Assembly

Although iron-sulfur cluster proteins play essential roles, how iron-sulfur cluster is assembled *in vivo* is far from being understood. Due to the toxicity of both iron and sulfur, iron and sulfur cannot float around freely to form an iron-sulfur cluster. Because of the importance of iron-sulfur cluster proteins, researchers have already started the assembly machinery study. Current evidence indicates three operons may be responsible for this phenomenon: *nif* operon; *isc* operon and *suf* operon [21].

Iron-sulfur cluster assembly mechanism was initially studied in *nif* operon of *Azotobacter venelanderii* by Dean and his co-workers [22]. They found that gene deletion of *nifU* or *nifS* would cause the loss of enzymatic activity of nitrogenase [22]. This suggested that the translated products of both *nifU* or *nifS* were involved in the
iron-sulfur cluster biosynthesis [23,24]. Both proteins were purified and characterized. NifS was a PLP (pyridoxal L-phosphate) containing homodimer [23]. It can convert L-cysteine to L-alanine via a persulfide bond intermediate (Figure 1.4) [23]. Cys$^{325}$ was critical during the catalysis [25]. It was suggested that L-cysteine formed a ketimine adduct with PLP and was then attacked by thiolate anion of Cys$^{325}$ in the active site [8]. Since this protein can provide sulfur atom for the iron-sulfur cluster biosynthesis, it is often referred to as a sulfur donor [26,27].

![Figure 1.4](image.png)

Figure 1.4 The formation of persulfide bond during NifS catalysis [8].

NifU is another protein responsible for the formation of iron-sulfur clusters on nitrogenase and may work as an iron-sulfur cluster scaffold protein [8,28]. Purification of NifU shows that it contains three domains [29,30]. The N-terminal domain contains three conserved cysteines (Cys$^{35}$, Cys$^{62}$, Cys$^{106}$) which can bind a transient cluster [31,32]. The central domain contains four conserved cysteine residues (Cys$^{137}$, Cys$^{139}$, Cys$^{172}$, Cys$^{174}$).
Cys^{175}) hosting a permanent iron-sulfur cluster [33]. The function of the permanent cluster may lie in the formation or the release of the transient cluster within the N-terminus [33]. The C-terminal domain contains two conserved cysteine residues (Cys^{272}, Cys^{275}) [33]. The function of this domain is still unknown.

Although nifU and nifS are important for the iron-sulfur cluster biosynthesis, deletion of nifU or nifS strains are able to produce a very low amount of active nitrogenase, indicating that other pathways exist to produce iron-sulfur clusters [22]. Later, another operon was identified which included nine linked genes: cysE2, iscR, iscS, iscU, iscA, hscB, hscA, fdx, and orf3 [33]. Since this operon may be involved in the iron-sulfur cluster biosynthesis, it is designated as isc operon (iron-sulfur cluster formation) [8]. When the isc operon was discovered in A. vinelandii, its homologs were correspondingly found in many other bacteria, suggesting its more prevalent role regarding the iron-sulfur cluster biosynthesis [8]. In this operon, iscU encodes a protein homologous to the N-terminal domain of NifU [33]. The translated product of iscU serves as iron-sulfur cluster scaffold [34,35]. An interesting aspect regarding IscU is its D37A mutant, in which the iron-sulfur cluster bound is more stable than on native protein [34-36]. A similar phenomenon was also observed on the N-terminus of NifU, suggesting that this residue may be critical in controlling the stability or the transfer of the iron-sulfur cluster [31]. IscA encoded by iscA was once thought to be an iron donor for the iron-sulfur cluster assembly [37,38]. However, an iron-binding study on IscA indicates that Fe^{3+} does not bind to IscA and Fe^{2+} binds to IscA very weakly [39]. Thus, the iron donor role of IscA is less favored. Now more data suggest that IscA is an iron-sulfur cluster scaffold protein [39-41]. However, the type of iron-sulfur cluster
bound to IscA is still a puzzling question. It is reported that both $[2\text{Fe}-2\text{S}]^{2+}$ and $[4\text{Fe}-4\text{S}]^{2+}$ can be assembled on *A.vinelandii* IscA and *E.coli* IscA, but only $[2\text{Fe}-2\text{S}]^{2+}$ can form on *S.pombe* IscA and *synechocystis* IscA1 [39,41-44]. Ferredoxin encoded in *isc* operon still has an unclear function. Experiments show that the apo form of ferredoxin can be reconstituted by the IscU or IscA proving the idea of intact cluster transfer [36,45,46]. The intact cluster transfer mechanism may allow further assessment of the role of ferredoxin. IscS encoded by *iscS* is also a PLP-dependent enzyme, which can convert cysteine to alanine, serving as the sulfur donor for the iron-sulfur cluster formation [47]. *cysE2* encodes a protein homologous to O-acetylserine synthetetase, which catalyzes the rate-limiting step of cysteine biosynthesis [33]. Thus, it may provide the substrate for the sulfur donor IscS. IscR is possibly a regulatory protein. Kiley and his colleagues demonstrated that deletion of *iscR* would lead to the elevated expression of iron-sulfur cluster assembly proteins in vivo [48]. Strikingly, two proteins encoded by *hscA* and *hscB* were identified as chaperone and co-chaperone, based on their primary sequences [33]. But the detailed mechanism of how the chaperone and co-chaperone are involved in the iron-sulfur cluster biosynthesis needs to be explored further.

However, deletion of *iscS* or *iscU* does not cause a lethal effect in *E.coli* [8]. Due to the importance of iron-sulfur clusters, the organism must have an alternative way to build iron-sulfur clusters. This leads to the discovery of *suf* (sulfur utilization factor) operon. The function of *suf* operon has been studied. The deletion of *suf* operon in *E.coli* does not have any apparent phenotype under normal conditions, but overexpression of *suf* operon can restore the phenotype and activity of iron-sulfur cluster proteins of *E.coli* mutant absent of *isc* genes [49]. The *in vivo* study shows that *suf* operon has a special role
under iron-starved conditions [50]. Although both *isc* and *suf* operon were found to be induced when exposed to hydrogen peroxide (H$_2$O$_2$), unlike *isc* operon regulated by iscR, *suf* operon regulation is dependent on Fur, whose induction is a response to oxidative stress [51].

The three identified the iron-sulfur cluster biosynthesis operons may perform distinct functions *in vivo*. This may account for the discrepancy in the phenotypes when the same gene is deleted in different organisms. *Nif* system was once thought to be specialized in the maturation of nitrogenase of nitrogen-fixing organisms. Overexpression of a *Nif*-like system can restore the phenotype of *E.coli isc* and *suf* mutant, but only under anoxic conditions [52]. This provides evidence that a *Nif*-like system cannot replace the function of *isc* or *suf* operons *in vivo*. However, recent data support that *Nif*-like operon is also present in nonnitrogen-fixing organism for the generalized role of the iron-sulfur cluster biosynthesis such as *Helicobacter pylori* [53]. Most nonnitrogen-fixing organisms contain either *isc* system or *suf* system, or both to assemble the iron-sulfur cluster *in vivo*. Of the two systems, the *isc* system may be responsible for the iron-sulfur cluster biosynthesis under normal conditions and the *suf* system may possibly work under iron deficient conditions [8]. Figure 1.5 shows a comparison of *nif*, *isc*, and *suf* operon.
Figure 1. The comparison of nif, isc and suf operons.

1.2.3 *The Iron-sulfur Cluster Biosynthesis Mechanism*

Iron-sulfur cluster can be reconstituted by incubating the apoprotein with excess of Fe$^{2+}$ and S$^{2-}$ in the presence of reducing reagent *in vitro* [54]. Since the discovery of NifS as a desulfurase, iron-sulfur cluster can be reconstituted by using NifS and cysteine in place of sulfide, and usually a higher rate or better yield of iron-sulfur cluster can be observed [55,56]. This leads to the conclusion that enzymatic synthesis of iron-sulfur cluster is more efficient than the inorganic method. And due to the potential toxicity of free iron and free sulfur, the study of iron-sulfur cluster assembly mechanism has been carried out. The current model suggests that Fe binds to the scaffold protein prior to
sulfur transfer [57]. Subsequently, NifS/IscS acts as a sulfur donor, which converts cysteine to alanine, labeling itself with a persulfide bond [58]. And the sulfur atom is next delivered to form an iron-sulfur cluster. The whole scheme is shown in Chapter 2 Figure 2.13 since it is the picture of iron-sulfur cluster assembly is related to the functional study of human NFU.

1.2.4 Iron-sulfur Cluster Biosynthesis in Eukaryotes

Research on how iron-sulfur cluster is synthesized in vivo is carried out in prokaryotes as well as in eukaryotes. Homologs of isc operon are also identified in eukaryotes, mainly in the mitochondria [59-61]. From an evolutionary view, the hypothesis of mitochondria evolving from prokaryotes cells may account for the similarity between the iron-sulfur cluster biosynthesis machinery in prokaryotes and in eukaryotes [62-64]. Even though eukaryotes are similar to prokaryotes in the iron-sulfur cluster biosynthesis machinery, the machinery for eukaryotes is possibly much more complex than for prokaryotes. For example, yeast, a widely studied eukaryotic system, not only contains homologs to IscU, IscS, IscA HscB, HscA, and the C-terminal domain of NifU, but also contains proteins in the intermembrane space of mitochondria, such as Atm1p, which may possibly involve the transport of iron-sulfur cluster proteins to cytosol [59]. Moreover, Nar1p residing in cytoplasm is shown to involve the maturation of iron-sulfur cluster proteins in the nucleus [65]. As research makes progress in cluster assembly machinery of eukaryotic cells, more proteins are possibly found to be
implicated in this process and the general mechanism of cluster assembly in eukaryotes will be finally revealed.

**1.3 INTRODUCTION TO NFU, HSCA/HSCB AND NIFS-LIKE PROTEINS**

**1.3.1 Introduction to NFU**

Although all three operons (Nif, suf, isc) contribute greatly to the understanding of the iron-sulfur cluster biosynthesis, proteins outside the operon are reported to be involved in the iron-sulfur cluster biosynthesis process. For example, frataxin is shown to be involved in the maturation of cellular [Fe-S] proteins in yeast [66,67]. Frataxin can bind 6-8 Fe ions with binding affinity about 10 µM, as demonstrated by the ITC (isothermal titration calorimetry) experiment and the fluorescence quench assay [68]. Human frataxin can also form a tight complex with IscU and substitute inorganic ferrous iron in the iron-sulfur cluster assembly process in vitro [68]. Therefore, frataxin is suggested to be the iron donor for the iron-sulfur cluster biosynthesis in vivo.

Similar to frataxin, NFU is another protein involved in the iron-sulfur cluster biosynthesis outside the isc operon. When comparing the nif and isc operon, although both the N-terminal and central domain of NifU from the nif system have homologs in the isc system, the C-terminal domain of NifU does not have any homologs in isc operon. However, homologs of the C-terminal domain of NifU are encoded in the genome, but somewhere outside the operon. Evidence that the C-terminal domain is involved in the iron-sulfur cluster biosynthesis was obtained through a synthetic lethal screen study in yeast [69]. The study demonstrated that the homolog of NifU C-terminal domain can
interact with \textit{SSQ1} homolog of HscA in yeast genetically and this protein is designated as NFU [69]. Deletion of the \textit{nfu} gene in yeast will cause the cells with iron to overload in mitochondria, decrease iron-sulfur cluster enzymatic activity, and result in poor growth under oxidative stress [69].

Research about human NFU is extensively carried out since NFU may participate in different biological processes in humans. Evidence shows that human NFU can interact with HIRA (Histone Interaction Regulator A) via yeast two hybrid system and the interaction is confirmed \textit{in vitro} [70]. HIRA can interact with core histone and transcriptional regulators, implicating its role in the DiGeorge syndrome and the velocardifacial syndrome [71-73]. So a study of human NFU (also designated as HIRIP5 [HIRA-interacting protein 5]) may contribute to the understanding of the DiGeorge syndrome or the velocardifacial syndrome at the molecular level although the significance behind the interaction between human NFU and HIRA still needs to be revealed.

Besides the DiGeorge syndrome, human NFU is possibly related to other disorders, such as PME (progressive myoclonus epilepsy). Progressive myoclonus epilepsy of Lafora type is a fatal autosomal recessive disorder [74]. Patients with this disorder usually display tonic-clonic seizures, absences, drop attacks, or partial visual seizures [75]. In the later stage, patients have the syndrome of progressive dementia with apraxia, aphasia and visual loss [76]. And more importantly, the onset of this disorder usually leads to death or a vegetative state within ten years [76]. In 1911 Gonzalo Lafora first described the existence of polyglucosan intracellular inclusion bodies, which were later called lafora bodies both in the brain and in the spinal cord of his patients [76].
Later research indicated that lafora bodies were similar to glycogen with reduced branching in composition [77,78]. However, the mechanism for lafora disorder is still far from understood. In 1998, the gene responsible for 80% of lafora disorder is identified on 6q24 designated as EPM2A [79,80]. The EPM2A encoded product is a 331-amino acid-long protein named laforin, containing a carbohydrate-binding domain at the N-terminus and a dual-specificity phosphatase domain at its C-terminus [81]. The identified Laforin is not homologous to any known protein. In order to reveal the mechanisms of lafora disease, proteins possibly interact with laforin are identified via yeast two hybrid system. And human NFU is among the candidates [82]. Although the significance behind this interaction is largely unknown, this may provide a way to unveil lafora disease at the molecular level, ultimately leading to a cure method.

Research of NFU is carried out in different organisms. The conservation of this protein from prokaryotes to eukaryotes indicates that its function is important (Figure 1.6). Due to the presence of CXXC conserved motif in NFU, this protein is hypothesized to act as a thioredoxin-like protein. Despite all the progress made, how this protein is involved in the iron-sulfur cluster biosynthesis still remains an intriguing question.
**Figure 1.6** Sequence Comparison of NFU in different organisms. Sequence is aligned by the online software T-COFFEE. (http://www.ch.embnet.org/software/TCoffee.html) The organisms are indicated at the front part.
1.3.2 Introduction to HscA/HscB and Other Chaperone Systems

Heat shock chaperones and co-chaperones compromise a special system in cells. They were originally found to increase in expression under heat stress [83]. Later research showed that chaperone participated in a variety of biological processes, such as protein folding or refolding in a nucleotide-dependent way [84]. Currently, chaperones are named after their molecular weight to classify different families and their distinctive functions. Table 1.2 summarizes some of the current work on molecular chaperones.
<table>
<thead>
<tr>
<th>Chaperone Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp100</td>
<td>ATP-dependent disaggregation and unfolding to be degraded</td>
</tr>
<tr>
<td>Hsp90</td>
<td>Prevent aggregation and facilitate activation of regulated proteins</td>
</tr>
<tr>
<td>Hsp70(Dna K)</td>
<td>Assist the folding of newly translated proteins; control the biological activity of regulatory proteins; and guide the translocating proteins across membranes.</td>
</tr>
<tr>
<td>Hsp60 (GroEL)</td>
<td>Facilitate folding to its native state</td>
</tr>
<tr>
<td>small Hsp</td>
<td>Stabilization of proteins against heat stress</td>
</tr>
</tbody>
</table>

**Table 1.2** Summary of different chaperone classes and their functions [83].

Although its identification involves temperature stress, heat shock chaperones also function under normal conditions. Hsp70 is used here as an example to illustrate the unusual properties and structures of this type of protein. Hsp70 type chaperone facilitates the folding of proteins by hydrolysis of ATP. In detail, hsp70 chaperones can bind hydrophobic patches from a usually unfolded or misfolded polypeptide substrate, possess intrinsic low ATPase activity, which can be stimulated by binding the substrate [83]. The chaperone and substrate interaction can be regulated by the bound nucleotide [83]. Typically, when ADP molecule binds to the chaperone, the hsp70 and substrate complex is stable. In contrast, when ATP binds to the chaperone, the complex is less stable [83]. However, hsp70 chaperones do not act alone. Rather, the activity of hsp70 chaperone is regulated by its co-chaperone. These co-chaperones can occasionally interact with the...
substrate and help to deliver it to the chaperone [85]. The structure of hsp70 was also resolved. The identified hsp70 proteins all consist of an N-terminal ATPase domain and a C-terminal substrate binding domain [83]. In 1996, the C-terminal substrate-binding domain of DnaK was crystallized with a heptapeptide substrate NRLLLTG (Figure 1.7) [86]. In the structure, two sheets made of four strands each constitute a β sandwich. The β sandwich is followed by two α helices which fold back over the sandwich.

Figure 1. 7 The crystal structure of the substrate-binding domain (blue) from DnaK with the peptide NRLLLTG (green). The figure is generated by Jmol Viewer from the Protein Database Bank (PDB id: 1Q5L) [86].

The kinetics and the catalytic cycle of hsp70 proteins are also studied. The binding of ATP increases both the \( k_{\text{off}} \) and \( k_{\text{on}} \) rate; however, there is more magnitude in the \( k_{\text{on}} \) rate [87-89]. Thus, hsp70 proteins can be characterized by the alteration between the ATP-bound state with low substrate affinity and the ADP-bound state with high
substrate affinity [83]. The catalytic cycle of hsp70 chaperones is currently believed to initiate with the rapid binding of substrate to the ATP-bound form of chaperone with subsequent structural rearrangements [90,91]. With the hydrolysis of ATP, the chaperone substrate ATP complex is then converted to a more stable chaperone substrate ADP complex [87,88,92]. Subsequently, the ATP molecule will replace the ADP molecule, leading to the release of the substrate [85].

HscA and HscB belong to the family of hsp70. However, due to their presence in the isc operon, they are hypothesized to have special roles in the iron-sulfur cluster biosynthesis. The role of the HscA and HscB chaperone system is assessed in vivo. It was demonstrated that HscA was essential in A. vinelandii [93]. However, the HscA and HscB gene disruption experiment did not cause a lethal effect in E.coli [94]. Instead, the mutant strain had a retarded growth rate and a decreased activity of iron-sulfur cluster-containing enzymes [94]. This indicates that HscA and HscB may comprise a special chaperone system functioning in the iron-sulfur cluster biosynthesis in vivo. The chaperone proteins are also purified and studied in vitro. In E.coli, HscA is shown to interact with either apo or holo forms of IscU [95-97]. IscU can stimulate the intrinsic ATPase activity of HscA [97]. The co-chaperone HscB can interact with IscU and HscA [97]. The intrinsic ATPase activity of HscA can be stimulated to hundreds of folds by HscB and IscU synergically [97]. The conserved sequence LPPVK near the third conserved cysteine is responsible for the stimulation of ATPase activity of HscA [96]. Compared with the structure of IscU, the LPPVK motif is solvent-exposed and near the iron-sulfur cluster assembly site [96]. Mutagenesis in any residue of the motif
will cause a corresponding loss of ATPase activity stimulation and decreased binding between IscU and HscA, although in different folds [96].

Similar studies are also carried out in eukaryotes. In yeast, ISU1 can stimulate the ATPase activity of SSq1, yeast homolog of HscA [98]. Jac1, homolog of HscB can facilitate the interaction between ISU1 and Ssq1 in the presence of ATP [98]. Different from prokaryotes, Ssq1 needs the nucleotide exchange factor Mge1 to release the nucleotides [98]. Unlike hundreds of folds of ATPase activity stimulation in *E. coli*, the ATPase activity can only be stimulated up to several folds in yeast [98]. This implies that although a similar chaperone system is present in eukaryotes, it has significant differences from prokaryotes.

**Figure 1. 8** The crystal structure of the substrate-binding domain from HscA (blue) with the peptide ELPPIHCVK (green). The figure is generated by Jmol Viewer from the Protein Database Bank (PDB id: 1U00) [99]. Each label represents a different structure component.
The substrate-binding domain of \textit{E.coli} HscA was also crystallized and the structure was compared with DnaK (Figure 1.8). The substrate-binding domain of HscA contains two distinct subdomains: \(\alpha\) subdomain and \(\beta\) subdomain \[99\]. The \(\alpha\) subdomain (residue 506-515) is made up of five \(\alpha\) helices (A, B, C, D, E) \[99\]. Of the five helices, helix A is on the side of the \(\beta\) subdomain \[99\]. In contrast, helices B-E are above the peptide-binding pocket. The \(\beta\) subdomain (residue 389-498) is made up of two \(\beta\)-sheets (F and G) \[99\]. Each \(\beta\)-sheet is composed of four anti-parallel strands and the two \(\beta\) sheets form a sandwich structure together. The peptide-binding pocket is formed by two loops between \(\beta\) sheets A and B and side chains M401 and F426 \[99\]. The bound peptide is in the binding pocket in the \(\beta\) domain and extended across the whole domain \[99\]. In summary, the overall structure of HscA substrate-binding domain is similar to that of DnaK, except for the \(\alpha\)-helical part, which is 10Å more up relative to the \(\beta\)-sandwich part than DnaK \[99\]. Additionally, the orientation of the bound peptide is reversed compared to its counterpart in DnaK \[99\].

1.3.3 Introduction to \textit{NifS}-like Proteins

\textit{NifS} is the first identified cysteine desulfurase, utilizing PLP (Pyridoxal L-phosphate) \[23\]. Since PLP is a derivative of the vitamin B\(_6\) family, \textit{NifS}-like proteins are also classified as B\(_6\) enzymes. B\(_6\) enzymes are found in almost all organisms and their activity is related to the metabolism of amino acids, including transfer and decarboxylation of the amino group from the target amino acid and conversion of amino acids from the D configuration to the L configuration \[100,101\]. Thus, how \textit{NifS} is involved in the metabolism of amino acids was further explored and \textit{NifS} was
demonstrated to be the sulfur donor for the iron-sulfur cluster biosynthesis [23]. During the time of this discovery, NifS-like proteins were thought to be specific only in the iron-sulfur cluster biosynthesis. However, later in vivo evidence supported that NifS or NifS-like (IscS) proteins may act as a general sulfur donor [94,102,103]. Mutation of the iscS gene in either E.coli or Salmonella enterica could cause the deficiency of formation of thiamine or even thionucleotides [8,103]. A detailed study in thionucleotide formation pathway indicated that the sulfur atom incorporated into some thionucleotides came from IscS via intermediate carriers Thil or MnmA, while other thionucleotides required MiaB for their maturation [104-108]. The sulfur transfer of thiamine may involve either Thil or ThiH as the intermediate carrier [105,109,110]. No matter which protein served as the intermediate carrier, IscS was the ultimate sulfur source for thionucleotides and thiamine. Recently, IscS was demonstrated to be involved in the formation of biotin [111]. In detail, IscS was implicated in iron-sulfur cluster formation on BioB, followed by the sulfur atom from BioB inserted into dethiobiotin [111,112].

A structure study was carried out as well as the functional study on NifS-like protein. The structures of both Thermotoga maritima NifS and E.coli IscS were resolved. The foldings they exhibited were strikingly similar despite their low sequence alignment [113,114]. T. maritima NifS consists of two domains (Figure 1.9). The small domain contains 1-6 residues at the N-terminus and 257-378 residues at the C-terminus. The extended β-sheet motif in the small domain is composed of two parallel sheets and four anti-parallel sheets. The parallel sheets are designated as S1 and S12, and the anti-parallel sheets are +S10, -S13, +S11 and -S12 [115]. One side of it forms the active cleft, while the other side is harbored by three helices with topology H8, H11, and the
C-terminal part of H7, which makes it solvent inaccessible [115]. The active cysteine 324 in this domain is found in a disordered region. The flexibility in this region is proposed to be related to the multiple function of this protein [113].

The large domain contains residue 7-256. The conserved K203 is included in this region. The central sheet is composed of seven strands of parallel or anti-parallel sheets with topology (+S2, -S9, +S8, +S7, +S5, +S3, +S4). Two helices H5 (residues 128-135 connecting S4 and S5) and H6 (residues 158-168 connecting S5 and S6) are on either side of the central sheet, shielding it from the solvent [115]. Helices 3 (residues 70-85 between S2 and S3) and H4 (residues 100-112 connecting S3 and S4) contain most residues for the dimer interface [115]. K203, the co-factor-binding site is on S9, the only anti-parallel strand in the central β-sheet. The PLP is located between H99 and V79. And H7 composed of residue 241-278 is on the solvent-exposed side with a kink at Glu 257, which is the mark for the boundary between the two domains [113].
Figure 1. The crystal structure of the *T.maritima* NifS monomer (PDB id: 1ECX). Details as described in the text [113].
CHAPTER 2

HIRIP-5 MEDIATES THE SULFUR DELIVERY TO ISU IN THE FINAL STEP OF [2FE-2S] CLUSTER ASSEMBLY

2.1 INTRODUCTION

During the time when *isc* operon was first discovered, no homologs of the C-terminal domain of NifU were identified within the operon. However, with the tools of bioinformatics, the gene encoding the NifU C-terminal homolog was identified in the genome of different organisms outside the *isc* operon [116]. These homologs are named NFU. The assessment of the role of NFU *in vivo* was originally performed in *Saccharomyces cerevisiae* [69]. Although the deletion of *nfu* together with the *isu1* gene did not produce a lethal effect, the growth of the yeast was greatly compromised [69]. The phenotype of the mutated cell line included sensitivity to oxidative stress, abnormal accumulation of iron in mitochondria, and a decreased activity of several respiratory enzymes that contain iron-sulfur clusters [69]. The phenotype is similar to cells deficient in the iron-sulfur cluster biosynthesis, suggesting the role of NFU may be related to the cluster formation *in vivo*. And this conserved protein is ubiquitously present in different organisms [69]. The above evidence shows that this protein plays an important role in the life cycle of cells.
In addition, many organisms contain more than one form of NFU. Research on *Arabidopsis thaliana* attested the existence of five different isoforms [117]. They are designated as At NFU 1-5. AtNFU 1-3 can be classified as a new type of NFU, since each of them has two repeated NFU domains, one of which loses the conserved CXXC motif [117]. This class of NFU is believed to be unique in plants, localized in the plastids [117]. In contrast, AtNFU4-5 are the mitochondria type NFU and AtNFU4 is demonstrated to target mitochondria [117]. Human NFU is also identified and known as HIRIP-5 (Histone interaction regulator A interaction protein 5), given that it can interact with HIRA. However, the significance behind this has not been understood yet [70]. Two isoforms were found for human NFU, originating from alternative splicing of the same pre-mRNA [118]. The two isoforms target distinct localizations. Isoform 1, about 22 kD, is present in mitochondria where isoform 2, about 26 kD, is in the cytosol and nucleus [118]. Although the function was established to be related to the iron-sulfur cluster biosynthesis, the precise function of this protein still needs to be further addressed. Due to the conserved CXXC motif present and the lack of efficient reductant available for the NifS persulfide bond, this protein is proposed to act as a ‘thioredoxin-like’ protein [33]. Thioredoxin protein can catalyze the formation and cleavage of thio-disulfide bond via the reduction or oxidation of its cysteines in CXXC motif [119]. The demonstration of NFU as a ‘thioredoxin-like’ protein has a profound effect and can contribute to the completeness of the current picture of the iron-sulfur cluster biosynthesis.
2.2 EXPERIMENT

2.2.1 Materials

All restriction enzymes were from Invitrogen (Carlsbad, CA). Qiaquick gel extraction kit and Ni-NTA columns were from Qiagen (Valencia, CA). BL21 (DE3) cells and pET 28 vectors were from Novagen (Madison, WI). Primers were from Integrated DNA Technologies Inc (Coralville, IA). CM 32 and DE 52 ion exchange resins were from Whatman (Aston, PA). Homogenous-20 precast polyacrylamide gels and G-25 resin were obtained from Pharmacia (Peapack, NJ).

2.2.2 Cloning of Human NFU

Two designed primers were as follows: 5’-GGC CAT ATG TTT ATT CAA ACA CAA GAT ACC CC-3’ and 5’-GCG GGA TCC TTA AGG TGA GTT TGC TTC TT TT-3’, where the underlined regions represent the introduced Nde I and BamH I sites. Human Nfu gene was amplified with the PCR (Polymerase Chain Reaction) technique. The optimized condition for PCR reaction was high fidelity PCR buffer (1X), a 0.2 mM dNTP mixture with 2 mM MgSO₄, 100 ng human genome DNA, 0.2 µM amount of each primer, and 1.0 unit platinum Taq DNA polymerase with a total volume of 50µl. The thermocycler was set with the following parameters: denaturation at 94°C for 30 sec followed by annealing at 55°C for 30 sec and subsequently extended at 68°C for 1 min. The cycle was repeated 35 times and extended at 68°C for 10 min to complete the PCR reaction afterward. The PCR product and vector pET-28 were digested simultaneously with 10 units of BamH I and 20 units of Nde I in 1X React 3 buffer (Invitrogen). Then
Qiagen gel extraction kit was used to purify the digested samples from agarose gel. 10:1 ratio of digested insert to the digested vector with the addition of 1 µl of T4 ligase for every 10 µl of the mixture was used for ligation reaction. The whole ligation reaction was incubated at 16°C for 16 hours and the whole mixture was transformed into calcium chloride-treated DH5α competent cells by the heat shock method [120] and successfully transformed colonies were selected on the LB plate against 30 µg/ml kanamycin. Each picked colony was incubated overnight for plasmid extraction by Qiagen mini prep kit. 10 units of restriction enzyme Pst I was used to digest the extracted DNA at 37°C. The digested DNA sample mixed with 1X DNA loading buffer was running on one 0.8% agarose gel in 0.5X TAE buffer. The successful construct will show a 400 bp characteristic band. The sequence of positive construct was verified at The Ohio State University Plant-Microbe Genomics Facility. The translated product was fused with an N-terminal His-tag for purification.

2.2.3 Overexpression of Human NFU

The expression host for human NFU was BL21 pLysS (DE3) (Novagen). The BL21 pLysS (DE3) containing the *nfu* construct was grown in a 50ml Luria-Bertani culture (supplemented with 30 µg/ml kanamycin and 30 µg/ml chloramphenicol) as inoculum. The entire overnight culture was used for 4 L LB fermentation for the protein expression process. The cells were grown to OD_{600nm} ~ 0.6 and subsequently 1mM IPTG was supplemented for protein induction. Cells were harvested with 5,000 rpm centrifuge after 6 h induction and stored at -80°C for future use.
2.2.4 Protein Purification of human NFU

The frozen cell pellet was resuspended in 50 mM Tris-HCl pH 7.5 and sonicated. The cell lysate was centrifuged at 15,000 rpm for 30 min to remove cell debris and the cleared part was subsequently applied to a Ni-NTA column equilibrated with binding buffer (50 mM NaH$_2$PO$_4$, pH 7.9, 5 mM imidazole, 300 mM NaCl). With NFU which was bound to the Ni-NTA column, the impurities were washed off with 5 volumes of binding buffer + 15 mM imidazole. Finally, pure bound NFU was eluted with binding buffer + 295 mM imidazole. Amicon was used to remove all of the excess imidazole and NaCl. Due to the acidic nature of this protein, human NFU can be loaded onto an anion exchange column (DE-52) and washed with 5 column volumes of 50 mM sodium phosphate pH 7.5. The target protein can finally be eluted with 50 mM sodium phosphate containing 300 mM NaCl pH 7.5. All of the fractions containing NFU were collected and concentrated. The purity of the sample was confirmed by SDS-PAGE. The protein sample was stored at either -80 °C (long term) or 4 °C (short term).

2.2.5 Cloning of the C-terminal Domain of Human NFU

The PCR process for cloning the C-terminal domain of human NFU is almost identical to the above cloning procedure, except that the following primers were used: 5’-GAA GAA GAT GAT GAA GTT GTG CAT ATG ATT AGG G-3’ and 5’-GCG GGA TCC TTA AGG TGA GTT TGC TTC TTT TT-3’. The mixture for PCR reaction contained a high fidelity PCR buffer (1X), a 0.2 mM dNTP mixture, 2 mM MgSO$_4$, 100
ng human genome DNA, 0.2 µM amount of each primer and 1.0 unit platinum Taq DNA polymerase. The reaction was started with the denaturation at 94°C for 30 sec followed by annealing at 55°C for 30 sec and finally extended at 68°C for 1 min. The above cycle was repeated 35 times prior to incubation 10 min at 68°C for the completeness of the PCR reaction. Both truncated NFU PCR product and vector pET-28 were digested with 10 units of BamH I and 20 units of Nde I in 1X React 3 buffer (Invitrogen) at 37°C for 2 h. The DNA sample was then dissected and purified from agarose gel with the gel extraction kit (Qiagen). The extracted DNA samples were mixed at the recommended ratio of 10:1 (PCR product : pET 28) with 1 µl of T4 ligase for ligation. The ligation reaction was carried out at 16°C for 16 h with the whole mixture transformed into DH5α competent cells by the heat shock method [120]. When proper DNA construct was transformed, it could confer the kanamycin resistance property to DH5α cells. As a result, the transformed cells could grow as colonies on the LB plate supplemented with 30µg/ml kanamycin. The colonies were selected and cultured overnight. The DNA from each colony can be extracted by the DNA mini prep kit (Qiagen). The candidates were then screened by digestion with PstI and ApaI in 1X buffer 2 (Invitrogen) at 37°C. The digested DNA sample was mixed with 10X DNA loading buffer and loaded on 0.8% agarose gel for a correct digestion pattern. The candidate sequence was confirmed by The Ohio State University Plant-Microbe Genomics Facility with sequencing before transformed for overexpression.
2.2.6 Overexpression of the C-terminal Domain of Human NFU

BL21 CodonPlus (DE3) RIL was used for protein overexpression, since it can partially correct the codon bias problem. An overnight culture (supplemented with 30 µg/ml kanamycin and 30 µg/ml chloramphenicol) was grown as an inoculum. After inoculation, cells were fermented at 37°C until OD$_{600nm}$ ~ 0.6 with subsequent addition of 1 mM IPTG. The protein was induced for 6 h before harvesting. Cell harvesting was achieved by centrifugation at 5,000 rpm. The harvested cells were stored at -80 °C for future use.

2.2.7 Protein Purification of the C-terminal Domain of Human NFU

Cells containing the expressed truncated NFU were sonicated and centrifuged at 15,000 rpm, 4°C for cell debris removal. After centrifugation, the supernatant was then loaded onto a Ni-NTA column equilibrated with binding buffer (50 mM NaH$_2$PO$_4$, pH 7.9, 5 mM imidazole, 300 mM NaCl). The column was washed with 5 volumes of binding buffer + 15 mM imidazole to remove the non-specific bound impurities, and the target protein was eluted with binding buffer + 295 mM imidazole. The protein sample was concentrated and diluted repeatedly with 50 mM sodium phosphate pH 7.5 via ultrafiltration. After removing the excess NaCl and imidazole, the protein sample was applied to an anion exchange column (DE-52). The column was then washed with 5 column volumes of 50 mM sodium phosphate (pH 7.5) and followed by elution with 50 mM sodium phosphate + 300 mM NaCl (pH 7.5). All fractions containing truncated NFU were pooled and concentrated by amicon ultrafiltration. The purity of the concentrated
truncated NFU was confirmed by SDS-PAGE. The protein sample was stored either at
-80°C or 4°C for future use.

2.2.8 Mass Spectrometry

After purification to homogeneity, both full-length NFU and its truncated form
were dialyzed against the Barnstead purified water. The mass spectra were obtained at
CCIC (campus chemical instrument center) of The Ohio State University. ESI
(Electro-spray ionization) using a Micromass Q-TOF™ II (Micromass, Wythenshawe,
UK) mass spectrometer with an orthogonal electrospray source (Z-spray) operating in
positive ion mode was used to measure the molecular weight of both full-length NFU
and truncated NFU. Sodium iodide was used for mass calibration with m/z in a range of
100-2500. Salt was removed from the sample by manual syringe protein traps from
Michrom BioResources (Auburn CA). The concentration of the protein sample was 50
pmol/µl, which was prepared in 50% acetonitrile, 50% water, and 0.1% formic acid.
The sample was injected into the electrospray source with the injection rate at 5-10
µl/min. The ESI parameters were set as follows: source temperature 110°C, capillary
voltage 3,000 V, cone voltage 60 V and nitrogen as ESI gas. QI was adjusted to pass
ions whose mass charge ratio varied from 100-2000. All of the ions in the push part of
the TOF analyzer were scanned with mass to charge ratio from 100 to 3,000. The
integration time was 1 sec. All of the data were obtained in a continuous mode and
deconvoluted by the provided software MaxEnt1.
2.2.9 Circular Dichroism of NFU and its C-terminal Domain

Circular dichroism could be used to probe the secondary structure of human NFU. Vigorous dialysis was carried out to ensure that human NFU in 20 mM Na$_2$HPO$_4$ buffer pH 7.5. Spectra were collected on the Aviv model 202 circular dichroism spectrometer at 25°C every 1 nm. A 1cm path length cuvette containing 0.85 μM human NFU (or 1.53 μM truncated human NFU) was used for this study. The scanning wavelength varied from 200 nm to 280 nm. The measurement was performed five times and averaged. The CD spectrum for the protein sample was obtained by subtracting the background spectrum. Secondary structure components were deconvoluted with the online software K2D (http://www.embl-heidelberg.de/~andrade/k2d/). The percentage of each component was compared with the online prediction results from PSIPRED Protein Structure Prediction Server (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html).

2.2.10 Cloning and Mutagenesis of T. maritima D40A IscU

25 ng, 50 ng and 100 ng T.maritima genomic DNA (From American type culture collection) were seperately mixed with 0.3 μM primer, 2.5units Pfu, 1x cloned buffer and 0.2 mM of the nucleotide mix to amplify the iscU gene. The parameters of the thermocycler were set according to the Pfu manual (Stratagene). In order to increase its yield in E.coli, the first Val was converted to Met. The PCR product was purified and digested with Nde I and BamH I. The pET-21 and pET-28 vectors were digested similarly. Subsequently, the digested vector (pET-21 or pET-28) was incubated with the digested PCR product, the 1x T4 ligase buffer, and T4 ligase according to the Invitrogen
protocol. The reaction was carried out at 16°C for 16 h. The ligation mixture was electroporated into the DH5α strain and plated against antibiotics (ampicillin 100 µg/ml for pET-21 and kanamycin 50 µg/ml for pET-28) and the plate was incubated overnight. The colonies from the plate were screened. Plasmid was purified by Qiagen mini prep kit and digested with 10 units of EcoR I, 1x buffer 3. The reaction was incubated at 37°C for 1 h before loading the mixture onto agarose gel to check for the correct digestion pattern. The positive construct was confirmed by nucleotide sequencing at The Ohio State University Plant Microbe Genomics Facility. Cloning into pET 21 and pET-28 resulted in IscU protein without His tag and with His tag, respectively when translated. This cloning step was done by Dr. Sheref Mansy.

The D40A point mutation was performed following the Qiagen quickchange protocol. 50ng of DNA construct, 2.5 units of cloned Pfu polymerase, 1x cloned Pfu buffer, and 0.5 mM DTT were mixed properly. Both primers ordered from IDT were added to the mixture. The two primers included the following: 5’-GGGAAAGAACATCTCTTGTGGCGCAGAAATCACACTCTAC-3’ and also 5’-GTAGATGTGATTTTCGCCAACACAGAGATGGTTTTCCC-3’. Mutagenesis parameters for the thermocycler were adjusted according to the Qiagen quickchange manual. Then the thermocycle product was treated with Dpn I at 37°C for 2 h to remove the original DNA construct. The DNA was transformed into the CaCl2-treated DH5α competent cells via the heat shock method [120]. The selected construct was confirmed by nucleotide sequencing at The Ohio State University Plant Microbe-genomic facility. This step was also performed by Dr. Sheref Mansy.
2.2.11 Overexpression and Purification of T. maritima D40A IscU

The transformed BL21CodonPlus (DE3)-RIL expression host was used for the T.maritima D40A IscU protein expression. We inoculated and grew 10ml of LB supplemented with either 100 µg/ml ampicillin and 30 µg/ml chloramphenicol for non-His tagged IscU or 50 µg/ml kanamycin and 30 µg/ml chloramphenicol for the His tagged IscU as the starter culture. We transferred 10 ml overnight culture into a 2 L flask containing 1L of LB media supplemented with the same antibiotics of the starter culture. Cells were grown at 37°C to an OD$_{600nm}$ of 0.6, then 1mM IPTG was added to induce protein expression. The cells were harvested 5 hours post induction. The cells containing T.maritima D40A IscU were stored at -80°C for future use.

T. maritima D40A IscU was purified in a similar way to what has been reported previously [36]. The cultured cells containing the T. maritima D40A IscU were resuspended in five volumes of 50 mM Tris-HCl pH 7.5 and sonicated. The cell lysate were centrifuged (15,000rpm) for cell debris removal. Due to the thermophilic nature of T. maritima D40A IscU, the supernatant was heated at 85°C for 0.5 h to precipitate other heat-sensitive proteins. The denatured protein was separated by centrifuging at 15,000 rpm for 10 min. The cleared portion containing mainly IscU was loaded onto the Ni-NTA column equilibrated with binding buffer (50 mM NaH$_2$PO$_4$, pH 7.9, 5 mM imidazole, 300 mM NaCl) and then washed with 5 volumes of binding buffer + 15 mM imidazole. The bound T. maritima D40A IscU can be eluted with binding buffer + 295 mM imidazole. The elution portion was red, indicating that some of the protein still existed in holo form. When the apo form of IscU was desired, DTT and EDTA were added and
incubated at 50°C to remove the bound cluster. All excess imidazole, NaCl, and EDTA were removed by the G-25 column.

The IscU without His-tag can be purified to homogeneity in a different way. The nonHis-tagged *T. maritima* D40A IscU cell was lysated as usual and then centrifuged at 15,000 rpm to remove all of the insoluble materials. The cleared supernatant was heated up to 85°C for 0.5 h, since the impurities vulnerable to heat would be denatured and aggregated. The denatured protein could be separated by centrifuging at 15,000 rpm, 4°C for 10 min. As previously discovered [36], nonHis-tagged *T. maritima* D40A IscU did not bind to either the cation exchange column CM32 or the anion exchange column DE 52. Therefore, the two columns can be used to remove the impurity proteins during the purification process. The supernatant was loaded to a cation exchange column (CM32), which was equilibrated with 50 mM sodium phosphate pH 7.5. The column was washed with 2 volumes of sodium phosphate buffer. The flowthrough and washed fractions were combined and then applied to a pre-packed anion exchange column (DE-52). The column was then washed with 3 volumes of 50 mM sodium phosphate buffer pH 7.5. The flowthrough and wash fractions were collected and concentrated. The concentrated nonHis-tagged D40A IscU was loaded to the G-75 gel filtration column. All of the tubes containing the non-His tagged D40A IscU were collected and concentrated by amicon ultrafiltration. The protein was stored at 4°C for future use. When apo *T.maritima* D40A IscU was preferred, the fractions containing holo *T. maritima* D40A IscU were treated with 25 mM DTT and 100 mM EDTA. The whole mixture was heated at 50°C for 30 min and subsequently loaded to the G-25 column to remove the excess ions.
2.2.12 Overexpression and Purification of *T. maritima* NifS

The overexpression and purification procedure for *T. maritima* NifS were similar to the previously described procedure [113]. BL21 strain harboring the *T. maritima* NifS construct was used to express the target protein. We used 10 ml overnight LB culture with 100 µg/ml ampicillin to inoculate 1L LB media. The culture was then grown at 37°C. When its O.D. reached 0.6, 1 mM ITPG was added to induce the expression of the target protein. The cells were centrifuged at 5,000 rpm and stored at -80°C.

The cultured cells were resuspended in 50 mM Tris buffer pH 7.4 containing 1 µg/ml PMSF and 100 mM EDTA then lysated by sonicator. The mixture was centrifuged at 15,000 rpm to remove cell debris. The cleared supernatant was heated to 85 °C for 20 min. The denatured proteins were removed by centrifugation at 15,000 rpm for about 10 min. 1% of streptomycin sulfate was added and the precipitate was removed by centrifugation. Ammonium sulfate was added stepwise to precipitate the *T. maritima* NifS. The precipitate from 0-0.5M ammonium sulfate was discarded. And the precipitate from 1-3M ammonium sulfate was collected and resuspended in 50 mM Tris pH 7.4. Also, PLP (pyridoxal-L-phosphate) was added to reconstitute the NifS enzyme for one hour. All of the excess PLP and ammonium sulfate were separated by loading the sample to the G-75 gel filtration column. All fractions containing the target protein were collected and concentrated by an amicon concentrator. The protein was stored at -80 °C or 4°C for future use.
2.2.13 Overexpression and Purification of Human D37A ISU

Cloning of human isu gene into pET-28 construct was performed by Dr. Matthew Foster and D37A mutagenesis was generated according to the QuikChange Mutagenesis procedure (Stratagene) by Dr. Sheref Mansy. The plasmid was transformed into BL21 pLysS (DE3) for protein expression. Also, 10 ml overnight culture was used to inoculate every liter of LB media with 30 µg/ml kanamycin. The cells were grown to an O.D. 1.0 before 1 mM IPTG was added for protein induction. The protein was expressed for 4-6 hours before harvested and stored at -80°C for future use.

The purification procedure of human ISU is similar to what was described previously [45]. The cells were resuspended in the binding buffer (5 mM imidazole, 300 mM NaCl, 50 mM NaH$_2$PO$_4$, pH 7.9) and sonicated. All of the insoluble cellular debris was removed by centrifugation at 15,000 rpm and 4°C for 30 min, and the supernatant was passed through a Ni-NTA column equilibrated with binding buffer and followed by washing with 10 volumes of wash buffer (40 mM imidazole, 300 mM NaCl, 50 mM NaH$_2$PO$_4$, pH 7.9). The human D37A ISU was eluted with elution buffer (400 mM imidazole, 300 mM NaCl, 50 mM NaH$_2$PO$_4$, pH 7.9). Ultrafiltration (amicon) was used to concentrate the target protein. Since the purified human ISU was partially in holo form, the bound cluster was removed by the following procedure. The D37A ISU was treated with EDTA (100 mM) overnight to degrade the iron-sulfur cluster. Then the concentrated ISU was loaded to the G-25 column equilibrated with 50 mM Tris-HCl pH 7.5 for desalting. And the sample was kept at 4°C for future use.
2.2.14 UV Spectrum of NFU Promoted T.maritma D40A IscU Reconstitution

Both NFU and IscU were dialyzed vigorously in the same buffer (50 mM Tris-HCl 50mM NaCl pH 7.5). NFU and IscU were reduced with excess DTT. Under restricted anaerobic conditions DTT was removed by ultrafiltration. Then 400 μM NFU and 200μM IscU were mixed. 800μM Fe^{2+}. 2mM L-Cysteine were then injected into the mixture. Finally, 7μM NifS was added to initiate the reaction. After incubating for 2 h, the mixture was loaded to a G-25 gel filtration column equilibrated with argon-purged 50 mM Tris-HCl pH 7.5 for desalting. The colored fractions containing holo IscU were collected and concentrated. If non-His tagged IscU was used, NFU could be separated by binding to the Ni-NTA column. So only the nonHis-tagged IscU was present in the solution. The protein fractions from the desalting column were loaded onto the Ni-NTA column, and washed with 3 volumes of argon purged 50 mM Tris-HCl pH 7.5 buffer. The washed and flowthrough fractions were combined and passed through an anion exchange column (DE-52). This was because only holo T. maritima IscU bound to the DE-52 column while apo T.m. IscU did not [121]. Also, 50 mM Tris-HCl + 300 mM NaCl pH 7.5 buffer was used to elute the reconstituted non-His tagged T.maritima IscU. The colored portions containing holo IscU were pooled and concentrated. The high concentration of salt was removed by repeated ultrafiltration.

UV-visible spectra were recorded on a Hewlett-Packard 8425A diode array spectrophotometer using the On-Line Instrument Systems (OLIS) 4300S operating system software. The blank reference was the 50 mM Tris-HCl pH 7.5 buffer. A 1.0 cm path length curvette was used to record the spectrum. The scanning wavelength varied from 300 nm to 800 nm.
2.2.15 *EPR Spectroscopy of the Reconstituted T. maritima D40A IscU*

As described above, the reconstitution mixture was loaded onto a G-25 column to remove excess salt. The colored fractions containing holo *T. maritima* IscU were pooled together and concentrated. Then the concentrated holo IscU was quickly loaded onto an EPR tube containing saturated sodium dithionite and frozen in liquid nitrogen. Thus the EPR silent [2Fe-2S] cluster was partially degraded and showed signal. The holo *T. maritima* IscU which was reconstituted directly with iron and sulfide was used as a positive control for the EPR experiment. Similar reconstitution with no addition of *T. maritima* IscU was used as a negative control. EPR spectra were acquired with an X-band Bruker ESP 300 spectrometer equipped with an Oxford liquid helium cryostat at 15 K by C. Hemann in Dr. R. Hille’s laboratory.

2.2.16 *Kinetic Analysis of T. maritima D40A IscU Reconstitution Promoted by both Human NFU and the C-terminal Domain of NFU*

Both NFU and IscU were in 50 mM Tris-HCl + 50 mM NaCl pH 7.5. NFU and IscU were treated with excess of DTT for disulfide bond reduction, respectively. Then repeated ultrafiltration was used to remove all excess DTT. All of these steps were carried out under strict anaerobic conditions with argon-purged buffers. Also, 50 μM IscU and 100 μM NFU (or truncated NFU) were incubated in the absence of air and 200 μM ferrous iron and a 500 μM L-cysteine were injected into the mixture. The reaction was initiated with the addition of 3.6 μM NifS. The time dependence of iron-sulfur cluster assembly on IscU was monitored by the Hewlett-Packard 8425A diode array.
spectrophotometer. Control experiments without the components NFU, IscU, and NifS separately were also performed. The absorbance at 412 nm was recorded at 1 min intervals for 120 min. Data were fitted to first-order process by the software Origin 6.0. The fitted equation is 

\[ y = y_0 + A_1 \times (1 - e^{-x/t_1}) \]

The \( k_{obs} \) was obtained from \( 1/t_1 \).

2.2.17 Kinetic Analysis of Human D37A ISU Reconstitution Promoted by both Human NFU and the C-terminal Domain of NFU

In the case of human D37A ISU, both NFU and ISU were dialyzed into 50 mM Tris-HCl + 50 mM NaCl pH 7.5. NFU and ISU were incubated with excess DTT and repeated ultrafiltration was used to remove DTT under anaerobic conditions. Then, 90 \( \mu \)M ISU and 180 \( \mu \)M NFU (or truncated NFU) were mixed with the addition of 200 \( \mu \)M ferrous iron and 500 \( \mu \)M L-cysteine. Finally the 3.6 \( \mu \)M NifS was supplemented into the mixture to start the reaction. The Hewlett-Packard 8425A diode array spectrophotometer was used to monitor the iron-sulfur cluster assembly on ISU over a period of 120 min at 412 nm. Control experiments without NFU, ISU, or NifS separately were also conducted. Kinetic data were fitted to first-order process.

2.2.18 Oxygen Sensitivity of Human NFU

Since human NFU has CXXC motif, the stability of reduced NFU needs to be measured. As previously described, the human NFU (or truncated NFU) was treated with DTT for disulfide bond reduction. Then DTT was removed by repeated ultrafiltration. Free thiols could be quantified by reacting reduced human NFU with DTNB. The moles
of free cysteines were calculated based on the absorbance at 412 nm as previously described [122]. The ratio of moles of free thiol: moles of protein was calculated for different time intervals under a variety of different storage conditions.

2.2.19 Methylene Blue Assay of Sulfide Reduction

Methylene blue assay was also used to detect the persulfide bond cleavage on NifS as described by Siegel [123]. All reactions were carried out in sealed glass tubes. Human NFU was treated with DTT anaerobically and excess DTT was removed by ultrafiltration. In the experiment, 600 nM *T. maritima* NifS, 1μM pyridoxal phosphate, 1mM L-cysteine, 600μM reduced human NFU were mixed and incubated for different time scales. The experiment in the absence of human NFU was used as a negative control and the experiment in which DTT substituted human NFU was used as a positive control. All buffers used in this reaction were argon-purged 50 mM Tris pH 7.5. The reaction was carried out for different time intervals and terminated by the addition of 100 μl of 20 mM N, N-dimethyl-p-phenylenediamine and 100 μl of 30 mM ferric chloride for methylene blue color development. The quantity of the methylene blue production in each experiment was determined by its corresponding UV absorbance at 670 nm compared with the standard curve generated from different known concentrations of sulfide.
2.2.20 Quantitation of Iron Binding to Human NFU by Fluorimetry

Previous research measured the iron binding affinity by intrinsic fluorescence quenching [57]. The basis for this method is the intrinsic fluorescence is quenched in iron bound protein molecules. Thus, the real-time change of fluorescence could serve as an index to differentiate the iron bound NFU and the free NFU. The quench was due to the formation of iron bound NFU. This was also supported by the following experiment, since the N-terminal domain of NFU alone did not bind iron and did not change significant change of fluorescence. The change in the fluorescence intensity was measured by a Perkin-Elmer LS50B luminescence spectrometer with the excitation wavelength at 291 nm and monitoring wavelength at 341 nm, respectively. 8 μM human NFU was reduced as described above and the fluorescence intensity was measured on the Perkin-Elmer Life Science LS50B luminescence spectrometer. Ferrous iron was added until final concentration of 200 μM and ferric iron was added until final concentration of 400 μM. Under strict anaerobic conditions, with the addition of ferric or ferrous ions, the iron-bound NFU showed a decrease in fluorescence intensity. Thus, the fluorescence served to differentiate between free NFU and iron-bound NFU. All buffers used were argon-purged 50 mM HEPES buffer pH 7.5. The plotting of decreased fluorescence intensity vs. the iron concentration generated a binding function as previously described [68,124]. Then the binding affinity was obtained with the data fitting to one-site binding model.
2.2.21 Quantitation of Iron Binding to the N-terminal Domain of NFU by Fluorimetry

The cloning and purification of the N-terminal domain of NFU was described in Chapter 4. To study whether iron binds to the N-terminal domain or the C-terminal domain, the N-terminal domain of NFU was dialyzed in 50 mM HEPES buffer pH 7.5 and the intrinsic fluorescence intensity was measured on the Perkin-Elmer Life Science LS50B luminescence spectrometer. The parameters were similar to the fluorescence quench assay for full-length NFU. With the addition of ferrous iron to final concentration of 200 μM or ferric iron to final concentration of 400 μM, the fluorescence intensity of the N-terminal domain of human NFU (6 μM) was measured. The data were compared with the full-length human NFU.

2.2.22 Human D37A ISU Reconstitution by the N-terminal Domain of Human NFU

Human D37A ISU and N-terminal domain of human NFU were purified and dialyzed to 50 mM Tris-HCl +50mM NaCl pH 7.5. Both proteins were treated with DTT and all excess DTT was removed by ultrafiltration under strict anaerobic conditions. In this experiment, 90 μM ISU and 180 μM N-terminal domain of NFU were mixed. Subsequently, 200 μM ferrous iron and 500 μM L-cysteine were injected. The cuvette containing all of the above was thoroughly degassed. The addition of 3.6 μM NifS finally initiated the reaction. The reaction was recorded for 120 min at 412 nm on the Hewlett-Packar 8425A diode array spectrophotometer. The control experiments in the absence of the N-terminal domain NFU, ISU, or NifS were performed separately. To test whether the N-terminal domain has any effect on the reconstitution process promoted by
the C-terminal domain NFU, 180 μM C-terminal domain of NFU was also added to the mixture with the N-terminal domain. The data recorded were fitted to the first order decay by software Origin 6.0.

2.2.23 Attempts of Iron-sulfur Cluster Reconstitution on Human NFU

As previously described [118], the reconstitution of iron-sulfur cluster was also attempted on human NFU or truncated NFU. The reconstitution was carried out with the cell lysate or purified proteins. Both chemical and enzymatic methods were used. In brief, for the chemical reconstitution method, the concentrated human NFU (or truncated NFU) was incubated with DTT anaerobically, followed by an injection of different folds of ferric and ferrous ions. Then the freshly prepared sulfide was added to the mixture. The reaction was carried out for 30 min. Then the mixture was loaded to a G-25 column equilibrated with argon-purged 50 mM Tris.HCl pH 7.5. The fraction containing human NFU was collected and concentrated. In the case of enzymatic methods, a catalytic amount of NifS was added to the DTT-treated concentrated NFU (or truncated NFU). Ferrous or ferric ions were injected and also L-cysteine was added as the ultimate sulfur source for iron-sulfur cluster reconstitution. The reaction was carried out for 2 hours and desalted with the G-25 column as described above.
2.2.24 Quantitation of Human NFU Binding to NifS by Isothermal Titration

Calorimetry

ITC (Isothermal titration calorimetry) was a routine way for protein interaction study. ITC experiments were performed on a MicroCal VP-ITC at 25°C. The titrant was 750 µM human NFU in syringe and the sample solution was 50 µM NifS in the cell. Both proteins were dialyzed in the same buffer solution (20 mM HEPES pH 7.5). The buffer used was argon-purged. Before loading onto the ITC system, both NFU and NifS were thoroughly degassed with the addition of 1 mM TCEP. Each time, 10 µl titrant was injected into the sample cell in 20 sec. In order to ensure the rapidity and completeness of the reaction, the sample cell was stirred at 300 rpm, and a 300-sec interval was pre-set between each injection. In order to ensure the saturation and no further NFU-NifS complex formation, 3.5 equivalents of human NFU were added. The background heat in which the titrant was injected into the cell containing only the buffer solution was performed and subtracted. The data were then fitted to a one-site binding model with the software Origin 7.0.

A similar experiment was performed when NFU truncation form was used. Truncated NFU and NifS solution were in the same buffer solution (20 mM HEPES pH 7.5). All solutions were thoroughly degassed and all buffers were purged by argon. Both the titrant (human truncated NFU) and the sample solution (NifS) were supplemented with 1 mM TCEP. In this experiment, 10 µl titrant was injected into the cell in 20 sec for each titration. A 300-sec interval ensured the equilibration and completeness of the reaction before the next injection. The titration continued until no heat was generated from the formation of NifS-truncated NFU complex. The control reaction in which the
titrant was injected into the blank buffer solution was performed and the heat generated by sole dilution was subtracted. The data was fitted to the one-site binding model.

2.2.25 Temperature-Dependence of NifS and truncated NFU Interaction

Similar conditions as described above were used in the following experiments. Instead of 25°C, the experiments were carried out at 10°C, 35°C, and 45°C. For a typical ITC experiment, 25 μM NifS (in the sample cell) and 1.1 mM truncated NFU (in the syringe) with the addition of 1mM TCEP (tris(carboxymethyl)phosphine) were used. Both truncated NFU and NifS were degassed for 10 min. 10 μL titrant was injected each time. In order to ensure the equilibration and the complete formation of NifS-truncated NFU complex formation, more than 5 equivalents of titrant were added into the cell. For each reaction, the dilution heat generated in the control reaction was subtracted. The data were fitted to a one-site model with the included software Origin 7. The change in the molar heat capacity (ΔCp) was calculated from each of the titrations above.

2.2.26 ITC Study of the Interactions between the N-terminal Domain NFU and the C-terminal Domain NFU (or N-terminal Domain NFU and NifS)

Possible interactions between the N-terminal domain and the C-terminal domain were also attempted on the ITC system. Both the N-terminal domain (1.1 mM) and the C-terminal domain (0.025mM) were dialyzed against 20 mM HEPES pH 7.5 buffer. Then, 1 mM TCEP was added to both titrant and sample. In this experiment, 10 μl of titrant (N-terminal domain of NFU) was added into the cell each time. In order to
guarantee the complete saturation, more than 10 equivalents of the N-terminal domain were added into the system. The data were integrated and the background dilution heat was subtracted.

When the possibility of the N-terminal domain of NFU interacting with NifS is considered, the measurement of this possible interaction was carried out on ITC. TCEP was added to each solution used and all solutions were argon-purged. In this experiment, 10 µl titrant (N-terminal domain of NFU) was added within 20 sec for each injection. In total, about 7 equivalents of the N-terminal domain of NFU (0.72 mM) were supplemented into the cell containing NifS (0.03 mM). A control experiment in which the N-terminal domain of NFU was injected into a blank buffer was performed. The dilution heat was subtracted from the titration experiment.

To test whether the N-terminal domain NFU has any effect on the interaction between C-terminal domain NFU and NifS, 5 equivalents of the N-terminal domain NFU (0.125 mM) were pre-mixed with NifS (0.025 mM) and added to the cell. The syringe was filled with the C-terminal domain of NFU (0.8 mM) and injected into the cell. The parameters were set according to the ITC study of NifS and truncated NFU. More than 5 equivalents of the C-terminal domain NFU were added to ensure the saturation of reaction. The background heat was integrated and subtracted. The heat from the reaction was fitted to the one-site binding model with the software Orgin 7.0.
2.3 RESULTS

2.3.1 Cloning of human NFU

PCR technique was used to amplify the human *nfu* gene successfully from the human heart cDNA library (Figure 2.1). The size of the human *nfu* gene (606 bp) was further confirmed by comparing with the 1kb DNA ladder on agrose gel. pET-28 plasmid (Qiagen) was commercially available to express His-tagged NFU when the *nfu* gene was inserted. Moreover, pET-28 plasmid endows the transformed competent cells with kanamycin resistance to allow selection on the plate. When pET-28 was purified, it existed as a supercoiled form and migrated about 3,000 bp on agrose gel, whereas when digested, it was converted to linear form and migrated between 5,000 bp and 6,000 bp (Figure 2.2). During the screening step, a 400 bp band was produced after digestion with Pst I (Figure 2.3). The sequence of the construct was confirmed by nucleotide sequencing.
Figure 2. 1  PCR products under different conditions. Lane 1 is a 1kb DNA ladder. Lanes 2, 3, 4, and 5 are aliquots of different PCR conditions with 0.05 µM primers and 50ng cDNA library, 0.2 µM primers and 100ng cDNA library, 0.05 µM primers and 50ng cDNA library, 0.2 µM primers and 100ng cDNA library, respectively.

Figure 2. 2  Purification of pET-28 and digestion confirmation. Lane 1 is a 1 kb DNA marker, Lanes 2 and 3 are pET-28 digested by Nde I and BamH I, respectively and Lane 4 is the supercoiled form of pET-28 plasmid.
Figure 2.3 Screening of the correct construct by Pst I. Lanes 7, 11, and 14 are loaded with the 1kb DNA marker; others are digestion patterns from different colonies. Lane 3 shows the desired 400bp band and was confirmed by nucleotide sequencing.

2.3.2 Overexpression of Human NFU

The construct was transformed into BL21 CondonPlus (DE3)-RIL expression host. The reason behind this is that BL21 CondonPlus (DE3) –RIL has tRNAs for rare codons in E.coli such as codons for Arg, Ile, and Leu. This may partially resolve the codon bias problem and occasionally result in better yield for human proteins. A different time scale (5h, 8h and overnight) was used to optimize the expression conditions. However, no significant difference was found. Therefore, the cells were harvested 5 h post induction.
2.3.3 **Protein Purification of Human NFU**

Since the study of the iron-sulfur cluster biosynthesis process is mainly carried out in mitochondria [60], the cloned human NFU is actually the mitochondria type that lacks the N-terminal signal. The calculated mass of cloned NFU was 21801.6 Da with a predicted pI 4.2. With the fusion of the His-tag, the mass and the pI of NFU increased to 23833.7 Da and 4.6, respectively. With the introduced N-terminal His-tag, the protein was purified by the Ni-NTA column. It could also be further purified with the DE 52 column when necessary. Following the purification, the purity was confirmed by SDS-PAGE. Only one band was observed, and the size was between the 20.1 kD and 30.0 kD protein markers (**Figure 2.4**).

Blast search (http://www.ncbi.nlm.nih.gov/BLAST/) indicated that the highly conserved domain was the C-terminal domain (**Figure 2.5**). The human NFU had a pre-sequence before the conserved C-terminal domain. The human NFU sequence was shown in Figure 2.6 with the underlined methionine indicating the start of the conserved C-terminal domain.
Figure 2.4 Purification of human NFU by the Ni-NTA column and the DE 52 column. Lane 1 is the protein molecular marker. Lanes 2, 3, 4, 5, 6, 7 are different fractions of the Ni-NTA column elution and Lane 8 is the concentrated NFU after elution from the DE 52 column.

Figure 2.5 Conserved domain search from the NCBI website. (http://www.ncbi.nlm.nih.gov) The red indicates the conserved NFU domain present in full-length human NFU (mature mitochondria form). The numbers indicate the number of residues.
The nucleotide sequence and primary amino acid sequence of human NFU. The underlined methionine is the start of the C-terminal domain. The figure is generated by Protparam. (http://expasy.org/tools/protparam.html)

2.3.4 Cloning of the C-terminal Domain of Human NFU

Since the conserved NFU domain is located in the C-terminal part of full-length human NFU, it is interesting to study this conserved domain alone. Cloning the C-terminal domain of human NFU (designated as truncated NFU) was similar to the cloning process of the full-length NFU. The desired part of the **nfu** gene was amplified by PCR with the designed primer (Figure 2.7). The pET-28 plasmid was used to express truncated NFU. After inserting the PCR product into the pET-28 plasmid by double digestion and ligation, the DNA sample was transformed into DH5α competent cells. The transformed cells were then selected by kanamycin resistance on the LB plate. The screening process was performed to select the correct construct. Here, restriction enzymes PestI and Apa I were used for plasmid digestion. The correct pattern contained one band about the size of 1,200 bp (Figure 2.8). The screened plasmids were sent for nucleotide sequencing and were confirmed.
2.3.5 Overexpression of the C-terminal Domain of Human NFU

The correct construct was transformed into different *E. coli* expression hosts for best production. BL21 CodonPlus (DE3)–RIL was demonstrated to have the highest yield due to the same codon bias reason stated previously. Different time scales (5 h, 8 h, overnight) were used to optimize the expression conditions. No significant difference was observed for truncated NFU.

![Image](image.png)

**Figure 2.7** The PCR product of truncated NFU gene from the human cDNA library under different conditions. Lane 1 is a 1 kb DNA marker. Lanes 2, 3, 4, and 5 are aliquots of different PCR conditions with 0.05 µM primers and 50 ng cDNA library, 0.2 µM primers and 100 ng cDNA library, 0.05 µM primers and 50 ng cDNA library, 0.2 µM primers and 100 ng cDNA library for Lanes 2, 3, 4, and 5, respectively.
2.3.6 **Protein Purification of the C-terminal Domain of Human NFU**

Similar to full-length human NFU, fused His-tag allowed the purification of truncated C-terminal NFU by SDS-PAGE. The purity and size of the protein were checked by SDS-PAGE (Figure 2.9). The parameters calculated from ProtParam (http://us.expasy.org/tools/protparam.html) for the native truncation form of NFU are 83 amino acid, pI 4.18, and Mw 9235.4 Da. With the His-tag introduced into its amino-terminus, both the molecular weight and pI slightly increased to 11,398 Da and 5.17. Similar to full-length human NFU, the truncated C-terminal domain NFU can be further purified by binding to and eluting from the DE-52 column (Figure 2.10).
Figure 2. 9 Purification of the C-terminal domain of human NFU by the Ni-NTA column. Lane 1 is the protein molecular marker. Lanes 2, 3, 4, and 5 are different fractions of the elute from the Ni-NTA column. Lane 6 is the wash fraction. Lane 7 is the supernatant of cell lysate after binding to the Ni-NTA column. Lane 8 is the cell lysate.

Figure 2. 10 Different fractions of the C-terminal domain of human NFU eluted from the DE-52 column. Lane 1 is the protein marker. Lanes 2-5 are the fractions containing truncated human NFU.
2.3.7 **Mass Spectrometry**

Mass spectroscopic analysis of full-length human NFU showed one major peak at 23831 Da, matching the molecular weight of human NFU which lacks the amino-terminal Met (23833.7 Da). In the case of the truncated human NFU, ESI-MS showed a major peak of 11265 Da also in the absence of the N-terminal Met. The exact reason for the N-terminal Met cleavage was still unknown. Under aerobic conditions, the intra-disulfide bond could form between the two cysteins in the CXXC motif of both full-length and truncated human NFU. This could account for the 2 Da difference between the measured and calculated mass.

2.3.8 **Circular Dichroism of NFU and its C-terminal Domain**

The CD spectra are shown in Figures 2.11 and 2.12. The online software K2D could calculate the percentage of different secondary structure components. Resolving both CD spectra provided a secondary structure composition of 31% α-helix, 12% β-sheet, and 57% random coil for full-length NFU. For the C-terminal domain, 30% α-helix, 17% β-sheet, and 53% random coil were resolved. The secondary structure prediction was performed based on its sequence. It showed a composition of 32.5% α-helix, 17.6% β-sheet, and 49.7% random coil for human NFU, while the truncated C-terminal domain had a composition of 32.3% α-helix, 17.6% β-sheet, and 50% random coil. This was consistent with the secondary structure component calculated from the CD spectrum (Table 2.1). Also the near UV CD spectra are known to be sensitive to the protein tertiary structure environment of the aromatic residues and only fully folded proteins show dominant signals in this region; in contrast, both fully unfolded protein and
molten globule do not show dominance [125]. Both human NFU and its corresponding C-terminal domain had almost no signal in the near UV region. This leads to the hypothesis of abnormal structural properties for human NFU. Thus, more experiments were performed to probe the structure of both proteins in Chapter 4.

Figure 2.11 CD Spectrum of full-length human NFU. Experiment was performed as in ‘material and methods’ and the protein was dissolved in 10 mM sodium phosphate buffer with pH 7.5.
Figure 2. 12 CD spectrum of the C-terminal domain of human NFU. Experiment was performed as in ‘material and methods’ and the protein was dissolved in 10 mM sodium phosphate buffer with pH 7.5.

<table>
<thead>
<tr>
<th></th>
<th>Full-length NFU (From CD)</th>
<th>Full-length NFU (Predicted)</th>
<th>Truncated NFU (From CD)</th>
<th>Truncated NFU (Predicted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helix</td>
<td>31%</td>
<td>33%</td>
<td>30%</td>
<td>32%</td>
</tr>
<tr>
<td>β-sheet</td>
<td>12%</td>
<td>17%</td>
<td>17%</td>
<td>18%</td>
</tr>
<tr>
<td>random coil</td>
<td>57%</td>
<td>50%</td>
<td>53%</td>
<td>50%</td>
</tr>
</tbody>
</table>

Table 2. 1 The summary of predicted and deconvoluted secondary structure. The CD spectrum was deconvoluted with K2D (http://www.embl-heidelberg.de/~andrade/k2d) and the predicted with PSIPRED (http://bioinf.cs.usl.ac.uk/psipred/psiform.html).

2.3.9 Cloning and Mutagenesis of T. maritima D40A IscU and its Purification

IscU is a well-known iron-sulfur cluster scaffold protein [36]. However, the iron-sulfur cluster on IscU is more labile than on D40A mutant [34-36]. It is easier to build an iron-sulfur cluster on this mutant and monitor the cluster assembly process.
Moreover, the thermophilic property of *T.maritima* IscU makes the purification procedure easier. Most *E.coli* proteins could be removed by simple boiling with the *T.maritima* IscU intact. Due to this, *T.maritima* IscU was used as the indictor to test the function of NFU.

### 2.3.10 Overexpression and Purification of *T. maritima* NifS

Human Nfs1 was also cloned and expressed. However, the expressed protein existed as inclusion bodies. Different procedures were used to solubilize and purify this protein. But human Nfs1 was not readily dissolved in proper buffer and thus was not used for further experiments. In this case, *T.maritima* NifS was used as a substitute for human Nfs1. This substitution is based on the fact that NifS-like proteins belong to the vitamine B6 family and this family of proteins usually exhibits similar overall folding [115]. Recently, the structures of *T.m.* NifS and *E.coli* IscS were resolved [113,114]. Despite their low sequence identity, they showed an almost superimposable tertiary structure [113,114]. For this reason, it is reasonable to use *T.maritima* Nifs in place of human Nfs1. *T.maritima* NifS was a generous gift from Kaiser [113]. The *T.maritima* NifS did not possess an N-terminal His tag. The construct was transformed into an appropriate expression host by the heat shock method and the protein was purified as described in Kaiser’s ‘experimental’ section [120]. After purification, the yield of pure *T.maritima* NifS was calculated to be about 30 mg from one liter fermentation.
2.3.11 Cloning and Mutagenesis of Human D37A ISU and its Purification

The human ISU mutant could also be purified to homogeneity by the Ni-NTA column due to the infused His tag. However, the eluted portion from the Ni-NTA column was red, indicating that some ISU molecules still bound to the iron-sulfur clusters. For human ISU, the iron-sulfur clusters could be removed by EDTA treatment. After overnight treatment, the iron-sulfur clusters were degraded and all excess salts were removed by the G-25 column. Human ISU remained the most important candidate for this study, since human NFU was possibly involving the iron-sulfur cluster assembly on human ISU in vivo.

2.3.12 UV Spectrum of NFU Promoted T.maritima D40A IscU Reconstitution

Fitting our hypothesis into the general picture of the iron-sulfur cluster biosynthesis mechanism, iron-bound frataxin can act as an iron donor and deliver the iron to the cluster scaffold protein ISU [68]. Subsequently, the desulfurase NifS can form a persulfide bond with the conversion of L-cysteine to L-alanine [58]. This persulfide bond formed on NifS is hypothesized to be cleaved by NFU, the thioredoxin-like protein. Thus, the available sulfur can be provided to build iron-sulfur clusters (Figure 2.13).

In the description above, all other steps were demonstrated, except the cleavage of persulfide bond on NifS. The demonstration of persulfide bond cleavage by NFU was attempted as follows. When the proper amount of NFU, IscU, NifS, and L-cysteines was mixed, an iron-sulfur cluster was formed on the scaffold protein IscU according to the proposed mechanism. The G-25 column was used to remove extra salt and the Ni-NTA
column was used to remove the His-tagged NFU. Only the non-His tagged IscU and trace amount of NifS flew through the Ni-NTA column and were collected. UV spectrum was measured as shown in Figure 2.14. The purified holo D40A *T.maritima* IscU had a characteristic peak at about 412 nm and shoulder about 450 nm and 600 nm in agreement with the published data for IscU [121].

**Proposed Model:**

![Proposed Model Diagram](image)

**Figure 2.13** The general proposed mechanism for the iron-sulfur cluster biosynthesis with the proposed function for NFU.
2.3.13 EPR Spectroscopy of the Reconstituted *T.maritima* D40A IscU

EPR spectroscopy of *T.maritima* D40A IscU was attempted and failed before [121]. This was possibly due to the difficulty in the management of rapid degradation of the labile cluster. In this experiment, dithionite was used to reduce the iron-sulfur cluster, since the [2Fe-2S] cluster on *T.maritima* IscU was EPR silent. The rapid freezing made this study possible, since the cluster was partially degraded and EPR active. EPR signals arise from the fact that in the presence of the magnetic field, the two possible orientations of the spin vector of the unpaired electron (\(m_s = \pm 1/2\)) are no longer equivalent. The energy difference depends on the magnitude of the applied field \(H\). When the energy absorbed matches the transition of the electrons, the signal is observed. *T.maritima*
IscU, which was reconstituted by incubating Fe^{2+} and S^{2-} in the presence of DTT, served as a positive control. A similar experiment in which human NFU incubated with NifS, L-cysteine, and Fe^{2+} acted as a negative control. The EPR spectrum of the IscU reconstituted by NFU (Figure 2.16 a) was similar to the positive control (Figure 2.16 b). Both of the two EPR spectrum were consistent with the previously observed [2Fe-2S] EPR spectrum demonstrating the correct form of iron-sulfur cluster on IscU [126]. The reaction without IscU did not show a signal of reconstituted [2Fe-2S], and only Fe^{2+} signal was observed (Figure 2.16 c). The EPR results supported human NFU as the persulfide bond cleavage reagent on NifS, since iron-sulfur clusters can be reconstituted on the scaffold protein IscU.
Figure 2.15 EPR spectrum of reconstituted *T. maritima* IscU by NFU. (a) represents the spectrum of [2Fe-2S] IscU reconstituted by NFU; (b) represents the spectrum of [2Fe-2S] IscU reconstituted with Fe$^{2+}$ and S$^{2-}$ and the data were consistent with previously published [2Fe-2S] EPR spectrum [126]; (c) represents the spectrum of the reaction without the scaffold protein IscU. The sharp spike during (b) and (c) was due to the reusage of the EPR tubes. The reconstituted holo IscU were treated with saturated dithionite solution and frozen quickly in liquid nitrogen. The EPR spectra were acquired with an X-band Bruker ESP 300 spectrometer at 15 K.
2.3.14 Kinetic Analysis of *T. maritima* D40A IscU Reconstitution Promoted by both Human NFU and the C-terminal Domain of NFU

The time dependence kinetics study by UV spectrometer indicated that iron-sulfur cluster assembly was unsuccessful in the absence of NifS, IscU, or NFU. A slight increase in UV absorbance with time for the experiment without IscU was observed as a result of the formation of ferrous sulfide produced by the system. This experiment demonstrated the essential role of NFU in the iron-sulfur cluster biosynthesis. Kinetic data obtained were fitted to first order process and summarized in Table 2.2. Both human NFU and the C-terminal domain of NFU yielded similar results, suggesting that this domain was enough to fulfill the cleavage function (Figure 2.16). When the efficiency was concerned, 58% and 69% of *T. maritima* IscU were reconstituted by
truncated NFU and full-length NFU, respectively based on the published extinction coefficient [34,36].

<table>
<thead>
<tr>
<th></th>
<th>$k_{obs}$ (min$^{-1}$)</th>
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</tr>
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<tbody>
<tr>
<td>Full-length NFU/T.m IscU</td>
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<td>0.006</td>
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<tr>
<td>Truncated NFU/T.m IscU</td>
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<td>0.003</td>
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</table>

**Table 2.2** Summary of NFU reconstitution on *T. maritima* IscU.

(a)

**Figure 2.16** Time-dependent experiment for iron-sulfur cluster reconstitution on *T.maritima*. D40A IscU. (a) is the reconstitution process and control reaction by the truncated C-terminal domain of human NFU. ▼ represents the process when all of the components in ‘material and methods’ are present. ■ represents the control reaction in the absence of truncated NFU. ● represents the control reaction in the absence of the scaffold protein IscU. ▲ represents the control reaction in the absence of the sulfur donor NifS. (b) indicates the similar reconstitution process by full-length human NFU. The ■ indicates the reconstitution process by full-length NFU. ● indicates the control reaction in the absence of full-length NFU. ▲ indicates the control reaction in the absence of IscU. ▼ indicates the control reaction in the absence of NifS.
2.3.15 Kinetic Analysis of Human D37A ISU Reconstitution Promoted by both Human NFU and the C-terminal Domain of NFU

In order to test whether a similar process happened in humans, we substituted *T. maritima* IscU with human D37A ISU. Both NFU and its truncated form were used to reconstitute human ISU. This process was similar to the reconstitution of *T. maritima* IscU and the results could be fitted to first order decay. Both the C-terminal domain of human NFU and the full-length NFU can contribute to complete the iron-sulfur cluster assembly process on human ISU. In the absence of any components in the system, the iron-sulfur cluster assembly process was greatly diminished (Figure 2.17). In total, 53% and 79% of human ISU were reconstituted to holo form with truncated NFU and
full-length NFU, respectively. The kinetic results were summarized as shown in Table 2.3.

<table>
<thead>
<tr>
<th></th>
<th>$k_{\text{obs}} \text{ (min}^{-1}\text{)}$</th>
<th>Standard deviation</th>
</tr>
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<tr>
<td>NFU/human ISU</td>
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<td>Truncated NFU/human ISU</td>
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**Table 2.3** Summary of NFU reconstitution on human ISU.
Figure 2. 17 A time-dependent experiment for iron-sulfur cluster reconstitution on human D37A ISU by NFU. (a) represents the reconstitution process by full-length human NFU. ▲ represents the cluster reconstitution process when all the components in ‘material and methods’ are present. ■ represents the control reaction in which the NifS is absent. ● represents the control reaction in the absence of the scaffold protein ISU. ▼ represents the control reaction in the absence of NFU. (b) shows a similar process of reconstitution by the truncated human NFU. ▼ shows the control reaction in the absence of NifS. ■ shows the reconstitution process by truncated NFU. ● shows the control reaction in the absence of truncated NFU. ▲ shows the control reaction in the absence of human ISU.
2.3.16 Oxygen Sensitivity of Human NFU

The oxygen sensitivity assay was used to determine the best storage condition for reduced NFU. After reducing human NFU with DTT and DTT removal by untrafiltration, human NFU reacted with DTNB to determine the moles of free thiol. The ratio of the moles of free thiol to moles of protein was initially determined around 2. When left in the air at room temperature, the ratio decreased to 0.1, indicating the oxidation of human NFU. The reduced human NFU may be stored about 8 h without being oxidized if it is stored anaerobically and at 4°C. This condition was used for storage of reduced human NFU in all kinetic studies.

2.3.17 Methylene Blue Assay of Sulfide Reduction

Methylene blue assay was traditionally used to quantitate the sulfide production in a biological system. However, due to unknown reasons, the reaction did not have a very good reproducibility. Although the result was not subject to quantitative analysis, the experiment showed that the reaction produced more sulfide compared with the negative control.

2.3.18 Quantitation of Iron Binding to Human NFU by Fluorimetry

The binding affinity of iron to human NFU was measured by quenching its intrinsic fluorescence in the concentration-dependent manner of iron. Fitting of the data to one site binding model \( y = \frac{x}{k_d + x} \), \( y \) is the binding function \( r = \frac{F - F_{\text{min}}}{F_{\text{max}} - F_{\text{min}}} \), \( x \) is the Fe\(^{2+}\) or Fe\(^{3+}\) concentration) showed a binding affinity of \( k_d \approx 75.9 \mu\text{M} \) for ferric iron.
and $k_d$=36.6 μM for ferrous ion (Figure 2.18). The weak binding of iron to human NFU suggested that human NFU may not be related to the iron pathway.

Figure 2.18 Quantitation of iron binding to human NFU by fluorimetry. (a) indicates the fitted data of $\text{Fe}^{2+}$ binding to human NFU. The inlet reflects the decrease in fluorescence intensity with the addition of $\text{Fe}^{2+}$. (b) shows the fitted data of $\text{Fe}^{3+}$ binding to human NFU. The inlet reflects the decrease in intrinsic fluorescence intensity with the addition of $\text{Fe}^{3+}$. 

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2.3.19 Quantitation of Iron Binding to the N-terminal Domain of NFU by Fluorimetry

Iron binding on full-length NFU was measured by a fluorescence quench assay. The intrinsic fluorophore tryptophan was within the N-terminal domain. A similar fluorescence quench assay was also attempted, and unlike the full-length NFU fluorescence assay, the fluorescence of the N-terminal domain was not quenched with the addition of iron. This could be rationalized by the explanation that iron binding occurred on the C-terminal domain of human NFU and that the N-terminal domain did not bind to iron (Figure 2.19).

Figure 2.19 Quantitation of iron binding to the N-terminal domain human NFU by fluorimetry. (a) reflects almost no change in the fluorescence intensity with the addition of different concentrations of Fe$^{2+}$. (b) reflects almost no change in the fluorescence intensity with the addition of different concentrations of Fe$^{3+}$.
2.3.20 Human D37A ISU Reconstitution by the N-terminal domain of Human NFU

The N-terminal domain of NFU was also used to reconstitute human D37A ISU. However, unlike the C-terminal domain or full-length NFU, the UV signal did not show a significant increase with time. This implied that N-terminal domain was not the functional domain to promote the iron-sulfur cluster assembly on ISU. This was consistent with the previous result that the C-terminal domain and full-length NFU both promoted the iron-sulfur cluster assembly on ISU in a similar way. Whether the N-terminal domain had any effect on the reconstitution process promoted by C-terminal NFU remained an interesting topic to explore. With the addition of both the N-terminal and C-terminal domain of NFU, the reconstitution process was similar to the reconstitution promoted by the C-terminal domain alone (Figure 2.20). Control experiments were also performed, yielding similar results to what was observed for the reconstitution by the C-terminal domain NFU. Therefore, the control experiments were
not shown here to simplify the graph. This led us to conclude that the N-terminal domain did not have any effect in the reconstitution process.

**Figure 2.** The reconstitution of human D37A ISU with the N-terminal domain of NFU. □ shows the reconstitution without the addition of the N-terminal domain of NFU or the C-terminal domain of NFU. ▼ shows the reconstitution process by the N-terminal domain of NFU. ★ shows the reconstitution process by the C-terminal domain of NFU alone. ▲ shows the reconstitution process by both the C-terminal domain and the N-terminal domain NFU.

### 2.3.21 Attempts of Iron-sulfur Cluster Reconstitution on Human NFU

As previously reported [118], human NFU can serve as an iron-sulfur cluster scaffold. Human NFU was reconstituted with the incubation of cell lysate, L-cysteine, DTT, and ferrous ion.[118] Thus, cysteine was converted to sulfide and alanine with the catalytic activity of the basal amount of *E.coli* IscS.[118] With the presence of sulfide and ferrous ions, the iron-sulfur clusters were reconstituted on NFU. Due to unknown reasons, iron-sulfur clusters can not be successfully reconstituted on the target protein. Since the reconstitution was performed in cell lysate with excess iron and L-cysteine, it
was very difficult to carry out a single reaction in such a complex system. There was no additional evidence to indicate that the target protein formed a homodimer when harboring an iron-sulfur cluster. Possibly other proteins from *E. coli* contributed to the formation of an iron-sulfur cluster with human NFU. Both UV spectrum and EPR experiments were used. They did not show the characteristic band of an iron-sulfur cluster. In the EPR experiment, only a signal from iron was observed. The unsuccessful reconstitution suggests that human NFU may play a role rather than as iron-sulfur cluster scaffold.

### 2.3.22 Quantitation of Human NFU Binding to NifS by Isothermal Titration Calorimetry

Since NFU could cleave the persulfide bond formed on NifS, NFU is proposed to interact with NifS to facilitate this cleavage. The interaction between NFU and NifS was quantitified by isothermal titration calorimetry. The fitted data indicated the binding affinity of about 9.7 µM for this interaction and one molecule of human NFU approximately bound to one molecule NifS (Figure 2.21). Entropy and enthalpy were also calculated and summarized in Table 2.4. The interaction between NifS and truncated NFU was also confirmed. This suggested that the binding site was within the C-terminal domain of NFU. Truncated NFU could bind to NifS with an approximate ratio of 1:1. The measured $K_D$ was 17 µM for truncated NFU-NifS complex (Figure 2.21). This interaction also supported the hypothesis that NFU could interact with the sulfur donor NifS and cleave the persulfide bond on it.
Table 2.4 Summary of the thermodynamic parameters for the binding between NFU and NifS.

<table>
<thead>
<tr>
<th></th>
<th>Binding sites</th>
<th>$K_d$ (µM)</th>
<th>$ΔH$ (kcal/mol)</th>
<th>$ΔS$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-length NFU/T.m NifS</td>
<td>0.81±0.02</td>
<td>9.7±0.7</td>
<td>-1.69±0.45</td>
<td>15.3</td>
</tr>
<tr>
<td>Truncated NFU/T.m NifS</td>
<td>1.04±0.054</td>
<td>17±1.4</td>
<td>-4.36±0.27</td>
<td>7.22</td>
</tr>
</tbody>
</table>

Figure 2.21 Interaction between NifS and NFU (or truncated NFU). (a) represents the calorimetric analysis of the binding between truncated NFU and NifS. The heat caused by the binding was integrated and fitted to one binding site model. The plot represents the best fitting curve with 1.04±0.054 binding sites, $K_d \approx 17±1.4$ µM, $ΔH= -4.36±0.27$kcal/mol, $ΔS=7.22$ entropy units. (b) represents the calorimetric analysis of the binding between NFU and NifS. The plot represents the best fitting curve of one binding site model with 0.81±0.02 binding sites, $K_d \approx 9.7±0.7$µM, $ΔH= -1.69±0.45$kcal/mol,$ΔS=15.3$ entropy units.
2.3.23 Temperature Dependence of NifS and truncated NFU Interaction

Since the interaction between NifS and NFU was established, the underlying force for the binding of NifS and NFU was further investigated. However, due to the lacking of the structure information for human NFU, details about residues involving in the protonation and forming the NFU-NifS complex remained unresolved. Thus, a temperature-dependent experiment was performed to determine whether more polar or more apolar residues were involved in this process. The change in molar heat capacity could account for the reorganization of solvent molecules due to the ligand binding. The negative change in molar heat capacity changes associated with protein-protein interaction was usually attributed to the hydrophobic interaction [115,127,128]. And the
positive change in molar heat capacity changes associated with protein-protein interactions was rare [129-131]. Truncated NFU was used as a model to investigate this topic, since it was easier to obtain higher yield than when using full-length NFU. ITC experiments were performed with the same pre-set parameters; the only difference was the temperature. In this case, only the binding enthalpy and entropy were affected by temperature, while the binding affinity remained almost the same (Figure 2.22 a,b,c). Three plots were constructed to determine the possible underlying forces. The first graph was constructed with temperature (in Kelvin) as the X-axis and binding enthalpy as the Y-axis. The graph indicated that the change in binding enthalpy was dependent on temperature. The data were fitted by linear regression analysis with the equation \[ \Delta H_m = \Delta C_p T + \Delta H_0 \] [131]. The positive \( \Delta C_p = 138 \text{ cal/mol.K} \) was determined from the plot (Figure 2.22 d). Similarly, since \( \Delta C_p \) can also be generated from the equation \[ \Delta S_m = \Delta C_p \ln T + \Delta S_0 \] [131], the change of entropy was also plotted against the log of temperature (in Kelvin) (Figure 2.22 e). \( \Delta C_p = 178 \text{ cal/mol.K} \) was also determined with the linear regression analysis of the plot. Positive molar heat capacity changes usually attribute to the reduction in the reduction of surface exposed polar groups [131]. Negative enthalpy and positive entropy in the temperature dependent experiments suggested negative Gibbs free energy for spontaneous reactions. Favorable change in entropy was possibly due to electrostatic interaction by increasing the effective encounters [131,132]. Another factor that also contributed to the positive entropy is the loss of the solvent molecules on the binding surface when the complex was formed. In the case of enthalpy, it was determined by the relative stability of the reactant and product. When negative enthalpy was concerned, the heat was released when converting from th
reactant to the product indicating the product was in a lower energy state than the reactant. \[ \Delta H_m = T^* \Delta S_m + \Delta G \] was also used when the plot of \( \Delta H_m \) vs. \( T^* \Delta S_m \) resulted in a straight line (Figure 2.22 f). The fitted data indicated that \( \Delta G \) was -5.77 kcal/mol and was not sensitive to the variation of the temperature.

Figure 2.22  A temperature-dependent experiment of the interaction between truncated NFU and NifS quantified by ITC. (a), (b), and (c) represent the ITC experiments performed at 10°C, 35°C, and 45°C, respectively as in ‘material and method’. The ITC data were integrated and fitted to one binding site model. (d) represents the resulting \( \Delta H \) which was plotted against the temperature. (e) represents the resulting \( \Delta S \) plotting against the temperature. (f) represents the resulting \( \Delta H \) plotting against \( T^* \Delta S \)
(Continue)
(d) 

\[ \Delta H (\text{Kcal/mol}) \]

\[ \text{Temperature (K)} \]

(e) 

\[ \Delta S (\text{cal mol}^{-1} \text{K}^{-1}) \]

\[ \ln(T) \text{ (Temperature in Kelvin)} \]
2.3.24 ITC Study of the Interaction between the N-terminal Domain NFU and the C-terminal Domain NFU (or the N-terminal Domain NFU and NifS)

Both the N-terminal domain and the C-terminal domain of human NFU were cloned and purified. An ITC study was carried out to see whether these two domains could interact. However, no heat was released or absorbed (data not shown). Thus, the C-terminal domain and the N-terminal domain may not interact with each other, although the possibility that the reaction did not produce heat cannot be ruled out. In Chapter 4, the HSQC experiments were performed and the spectra of the N-terminal and the C-terminal domains overlapped with the spectrum of full-length NFU. This told us that the N-terminal domain and the C-terminal domain of NFU were independent of each other. Otherwise, dramatic shifts would be observed. This was consistent with the ITC study.
and demonstrated that the C-terminal domain of NFU did not interact with the N-terminal domain.

An experiment to determine whether the N-terminal domain and NifS can interact with each other was also carried out on ITC. No heat from the reaction was observed (data not shown). This suggested that the N-terminal domain possibly did not interact with NifS.

Therefore, the next question was whether the N-terminal domain could interfere with the NifS and the C-terminal domain NFU interaction. An ITC study of the C-terminal domain interacting with NifS was carried out in the presence of the N-terminal domain. The heat was integrated and fitted. No significant differences were observed from the reaction without the addition of the N-terminal domain (Figure 2.23).

![Figure 2.23](image)

**Figure 2.23** The interaction between truncated NFU and NifS in the presence of the N-terminal domain of NFU. The best fitting curve was with the following parameters: 0.939±0.017 binding sites, $K_d \approx 6.25 \pm 0.56 \mu M$, $\Delta H=-1.908 \pm 0.045$ kcal/mol, $\Delta S=17.4$ entropy units.
2.4 DISCUSSION

2.4.1 Significance of This Work

Currently, two different theories exist regarding the iron-sulfur cluster biosynthesis process. The major difference between these theories is on whether iron or sulfur binding occurs first. The NMR study of *E.coli* IscU failed to show evidence that Fe$^{3+}$ or Fe$^{2+}$ can bind to IscU [133]. Moreover, no evidence supported that Fe$^{3+}$ or Fe$^{2+}$ binds the cysteine residues on *A. vinelandii* IscU [134]. Mass spectrometry indicated that direct transfer of sulfane sulfur S$^0$ on persulfide bond of IscS to the cysteine residues of IscU [134]. However, the iron-sulfur clusters cannot be reconstituted on IscU with the incubation of IscU’s presulfurated form with Fe$^{2+}$, indicating that the sulfuration process on IscU may be artifactual [57].

Thus, a second theory is proposed that the iron binding step precedes sulfur binding. This idea is supported by the high binding affinity of Fe$^{3+}$ or Fe$^{2+}$ for *T.maritima* IscU quantified by ITC and fluorescence quench [57]. In the case of iron binding first, the persulfide bond needs to be cleaved for sulfur delivery. Due to lack of components to fulfill this function and the presence of the CXXC motif in this ‘thioredoxin-like’ protein, human NFU is hypothesized to cleave the persulfide bond on human NFU. Previously, NFU was proposed to act as an alternative scaffold [118,135]. However, no solid evidence was present to support this function. Human NFU was reconstituted within cell lysate [118]. In this case, the possibility of NFU binding a cluster with other impurity proteins could not be ruled out.
Now the theory of iron binding first is favored. Nontheless, this theory can not explain all the phenomena. For example, when the scaffold protein was incubated with persufide bond labeled NifS, sulfur was automatically transferred to the scaffold protein[134]. For the theory of iron binding first, how sulfur was transferred from NifS to the scaffold protein with bound iron remained an enigma until the discovery of NFU. NFU possesses the ‘thioredoxin-like’ motif and consequently the hypothesis that this protein acts to cleave the persufide bond is reasonable. It can fulfill the gap for the iron-occurrence-first theory. If NFU was used as a scaffold to build iron-sulfur clusters, the question, why two kinds of proteins (NFU and IscU) were needed to fulfill the same function, was raised. This would result in a waste of material and energy. Therefore, the ‘thioredoxin-like’ theory seems to be in conflict with the ‘iron-sulfur cluster scaffold’ theory. Interestingly, in 2005, one important discovery about human glutaredoxin 2 may help to resolve the conflict. Human glutaredoxin could harbor a nonoxidizable \( [2Fe-2S]^{2+} \) as a homodimer via the cysteines in the CXXC motif [136]. When binding the clusters, the human glutaredoxin 2 lost its oxidoreductase activity [136]. This was later explained as the binding of iron-sulfur clusters served to regulate the activity of human glutaredoxin 2 [136]. Similar explanation may apply to the situation of human NFU. If the binding of iron-sulfur clusters is not artificial, the formation of iron-sulfur cluster on NFU may act as a regulatory pathway of its thioredoxin activity and thus provides a way to reconcile all the conflicts.

The \textit{in vivo} data were not in conflict with the proposed ‘thioredoxin-like’ function. The deletion of \textit{nfu} gene in yeast showed the phenotype of lacking iron-sulfur cluster biosynthesis[69]. Nonetheless, this does not mean NFU serves as the iron-sulfur
cluster scaffold. It only means NFU is somehow implicated in the iron-sulfur cluster biosynthesis pathway. If NFU involves the persulfide bond cleavage on NifS, the knockout phenotype will also show similar phenomena.

The NFU activity was measured by reconstitution of the scaffold protein IscU. Without NFU, the iron-sulfur clusters can not be reconstituted successfully on IscU in the presence of NifS, Fe\(^{2+}\), and L-cysteines. When Fe\(^{2+}\) and S\(^{-2}\) were used to reconstitute the same amount of IscU, the reaction was fitted with t1~ 5 min and the reaction finished about 10 min. In the presence of L-cysteines and catalytic amount of NifS, the reaction lasted much longer; with t1~ 35 min and the reaction finished around 2 hr. Comparing the above two sets of data, we can draw the conclusion that in the presence of NifS, NFU, Fe\(^{2+}\), and L-cysteines, the reconstitution is carried out in two steps: the release of sulfur from NifS by NFU and the reconstitution of iron-sulfur clusters on IscU in the presence of Fe\(^{2+}\) and S\(^{-2}\). Since the reconstitution of IscU in the presence of Fe\(^{2+}\) and S\(^{-2}\) is carried out much faster than the reaction in the presence of NifS, NFU, Fe\(^{2+}\), and L-cysteines. Therefore, the reaction of sulfur release from NifS by NFU is the rate-limiting step. Both the C-terminal domain NFU and full length NFU were used and yielded similar results indicating the C-terminal domain of NFU was the functional domain in the persulfide bond cleavage. Both human ISU and *T.maritima* IscU were used as the indicator. The fitted results were similar. Considering the rate-limiting step was the sulfur release NFU cleaved the persulfide bond on NifS were nearly identical in all the tested reactions.

The homolog of NFU is absent in the *isc* operon in prokaryotes. However, the interaction between NFU and NifS demonstrated close association between NFU and the
iron-sulfur cluster biosynthesis. This is the first time when NFU was found to interact with proteins translated from the *isc* operon. Thus the involvement of NFU in iron-sulfur cluster biosynthesis is reasonable. Considering the conserved sequence of NFU in different organisms, the ‘thioredoxin-like’ role of NFU may be universal suggesting the conserved iron-sulfur cluster biosynthesis mechanism.

The identified ‘thioredoxin’ role of NFU may be implicated in multiple pathways. Previous work showed that NFU interacted with laforin and the mechanism behind this was still unknown [82]. The demonstration of the ‘thioredoxin’ role of NFU may help to reveal how the function of NFU is correlated with lafora disease at molecular level.

2.4.2 Co-localization of Components for Iron-sulfur Cluster Biosynthesis

Interestingly, the ISC biosynthesis machinery has only been identified in mitochondria [59]. However, the proteins containing iron-sulfur clusters are present not only in mitochondria, but also in the cytosol and nucleus [137]. This indicates that the iron-sulfur cluster biosynthesis takes place inside mitochondria and the cluster is transported into different compartments. The subcellular localization of human NFU is studied, indicating that its pre-mRNA can be processed in two different ways: one form can encode the cytosolic form of human NFU, the other can encode the NFU targeting the mitochondria [118]. Both mammalian Nfs1 and yeast Nfs1 were identified and demonstrated to be localized in mitochondria [138-140]. ISU was also localized in the mitochondria matrix in *Saccharomyces cerevisiae* [69]. The co-localization of these proteins allows the possible interaction for each component during the iron-sulfur cluster biosynthesis. The iron-sulfur cluster is an ancient structure present in both prokaryotes
and eukaryotes [141]. The presence of ISC machinery in mitochondria can be rationalized with the endosymbiotic hypothesis that the residing bacteria inside the mammalian cells can confer the iron-sulfur cluster biosynthesis ability to the cell.

For plants and algae, plastid is another place when iron-sulfur cluster biosynthesis takes place. This was demonstrated by iron-sulfur cluster assembly on ferredoxin in isolated chloroplasts with the presence of L-cysteines as sulfur donor, ATP, and NADH [142]. This is explicable, since chloroplasts are thought to be derived from cyanobacteria. Thus, iron-sulfur cluster assembly in the non-nitrogen fixing cyanobacteria may mimic similar process in chloroplasts [135]. However, the mitochondria and chloroplast may have big differences in the iron-sulfur cluster biosynthesis, since they are derived from different ancestors. In chloroplasts, most proteins were encoded and synthesized in the nuclear with a cleavable N-terminal signal sequence [143]. The iron-sulfur cluster cofactor is reconstituted on proteins after their import into chloroplasts [144,145]. Currently, only homologs of IscS, NFU, and the homologs of *E.coli Suf* operon were identified in chloroplasts regarding the iron-sulfur cluster biosynthesis process. Most research about iron-sulfur cluster biosynthesis is carried out in *Arabidopsis*. The encoded NifS-like proteins are in two forms: one is present in mitochondria, the other is present in plastids [146-148]. Interestingly, CpNifS can utilize both Cys and SeCys as substrates unlike its bacterial counterparts [148]. Its cysteine desulfurase activity is 300 fold lower when comparing with the selenocysteine lyase activity [148]. Although it is demonstrated that Se is essential in animals, its involvement in plants are still to be determined [47]. Thus, the sulfur donor for iron-sulfur cluster biosynthesis in plastids was identified. Nonetheless, the homolog of
IscU or the N-terminal domain of NifU is missing. This leads to question which protein acts as the iron-sulfur cluster scaffold. The homolog of SufA called CpIscA is the most promising candidate [149]. NFU is another candidate for the iron-sulfur cluster scaffold [117]. It is redundant to have two different kinds of proteins as the scaffold. NFU is possibly involved in the cleavage of the persulfide bond on NifS which makes the role of NFU inseperatable.
CHAPTER 3

EXPLORATION OF THE LINKAGE BETWEEN NFU AND THE MOLECULAR CHAPERONE SYSTEM HSCA/HSCB IN HUMANS

3.1 INTRODUCTION

During the study of *isc* operon, the DnaK type chaperone and DnaJ type co-chaperone were identified [93,116]. The presence of the two chaperone genes within the *isc* operon suggests that the corresponding encoded products are potentially involved in the iron-sulfur cluster biosynthesis [150]. The DnaK type chaperone is designated Hsc66 in *E.coli*, Ssq1 in yeast and HscA in human, respectively. This type of protein belongs to the Hsc70 family involving the protein folding/refolding or non-specific aggregation by the nucleotide-dependent protein-protein interaction [84]. This type of chaperone contains two domains: The N-terminal nucleotide-binding domain showing ATPase activity and the C-terminal substrate-binding domain [83]. The encoded J-type co-chaperone may facilitate the function of the corresponding chaperone by stimulating the ATPase activity of the chaperone and enhance its substrate binding affinity [151]. The co-chaperones HscB are classified as a new family of the J-type chaperones designated as ‘small Jac’s’ (small J-type accessory chaperones) [152]. Unlike other J-type co-chaperones, HscB does not have the cystein-rich zinc finger, and the mass (~20 kD) is different from the classic DnaJ chaperone (~45 kD) [151,153]. Moreover, the
HscA/HscB system does not have an overlapping function with the DnaK/DnaJ system. Disruption of \textit{hscA} gene in \textit{E.coli} did not produce gross phenotype changes in contrast to the growth defect when DnaK was mutated [154-156].

In the last decade, researchers have started to understand how the chaperone system was involved in the iron-sulfur cluster biosynthesis. The ATPase activity of HscA could be stimulated by IscU, and HscB can facilitate the interaction between IscU and HscA [96,157]. However, this phenomenon still remains to be further explored. Later genetic evidence suggested that the chaperone system possibly interacted with NFU, which is the C-terminal domain homolog of NifU in yeast [69]. However, the supporting data \textit{in vitro} are still lacking. The purpose of this chapter is to further explore the linkage between NFU and the chaperone system, which may contribute to the understanding of the iron-sulfur cluster biosynthesis process.

We initiated this study in humans. Thus, the understanding of human HscA is essential for this study. Human HscA (also known as mortalin/Grp75/mthsp70) has different subcellular localizations in different cell lines [158,159]. Despite multiple subcellular sites of Grp75, the mitochondrion was the major localization for the target protein [160-162]. Grp75 was also studied in mice. Different from humans in which only one Grp 75 was present, two isoforms were identified in mouse cells: One was expressed in normal mouse cells; the other was expressed in immortal mouse cells [163]. When the sequence of the two isolated proteins was compared, only 2 amino acids were different in the C-terminus of Grp75 [164].

Since the wide expression of Grp75 in different tissues, Grp75 is correlated with essential functions [158]. Muscle activity and mitochondrial activity have impact on the
expression of Grp75 [165-167]. Many conditions such as low level of ionizing radiation, glucose deprivation, and hyperthyroidism can all induce the expression of Grp75 [168-170]. The functional study of this protein yields many interesting results. For example, it was shown that Grp75 could transcriptionally inactivate p53 [171]. The detection of Grp75-p53 complexes in the mitochondria suggested that its role was implicated in the p53-induced apoptosis [172]. Other roles including antigen processing, radioresistance, and cell fate determination were also suggested based on a variety of results [168,169,173-176]. The yeast two hybrid system was used to study the interaction partner for Grp75. The partners included mitochondrial reduced form of NADH dehydrogenase (an inner mitochondrial membrane protein), mevalonate pyruvate decarboxylase (a peroxisomal protein) [163]. However, the importance behind these interactions is still unknown.

In summary, due to the importance of both NFU and the chaperone system, it is essential to study the interaction of the chaperone system with NFU. The current study provides insight into the iron-sulfur cluster biosynthesis.

3.2 EXPERIMENT

3.2.1 Materials

BL21 (DE3) cells and pET 28 vectors were obtained from Novagen (Madison, WI). DE 52 ion exchange resins were obtained from Whatman (Aston, PA). Homogenous-20 precast polyacrylamide gels and G-25 resin were obtained from Pharmacia (Peapack, NJ). The Ni-NTA columns were obtained from Qiagen (Valencia, CA). EnzCheck phosphate kit was obtained from Invitrogen (Carlsbad, CA). TCEP was
obtained from Pierce (Rockford, IL). ATP and ADP were obtained from Sigma (St. Louis, MO).

3.2.2 Molecular Cloning of Human HscB

The human HscB was cloned by Dr. Taejin Yoon in the Department of Chemistry at the Ohio State University. Two primers were constructed to amplify the hscB gene without the mitochondrial targeting sequences (residues 1-71).

5’ Primer 5’-GGA AGG CCA TAT GGA CTA CTT CAG CCTTAT GGA CTG CAA CC-3’

Nde I

3’Primer 5’-ACC GCT CGA GCC ACA ATT AAA GGG GAA TCT TCT TTA ACT T-3’

Xho I

NdeI and XhoI were introduced for ligation with pET-28 plasmid. After amplifying the gene hscB from the human cDNA library by PCR, both PCR product and pET-28 plasmid were digested with a proper amount of NdeI and XhoI (Invitrogen) at 37°C. The digested products were cleaned on 0.8% agarose gel by Qiagen gel extraction kit. Subsequently, both digested products were mixed and ligated by T4 ligase. The entire ligation product was used to transform the competent DH5α cells and screened with NdeI and XhoI. The positive candidate was confirmed by nucleotide sequencing at the Plant Microbe Genomics Facility.
3.2.3 **Protein Overexpression of Human HscB**

The correct construct was transformed into the BL21 (DE3) expression vector. Luria-Bertani overnight culture (supplemented with 30 µg/ml kanamycin) was used as the starter culture. Each 1 L culture was inoculated with 10 ml overnight culture. Cells were fermented at 37°C until the O.D. of the culture reached 0.6. Subsequently, 1 mM IPTG was added for human HscB induction. Then the cells were continued to be cultured for another 5 hours. Immediately the cells were harvested by centrifuging at 5,000 rpm and the cells were freeze at -80°C for future use.

3.2.4 **Protein Purification of Human HscB**

The cells were lysated by sonication. The lysate were centrifuged at 15,000 rpm for 30 min to remove all cell debris. The cleared supernatant was loaded onto the Ni-NTA column with binding buffer equilibration (50 mM Tris, pH 7.9, 5 mM imidazole, 300 mM NaCl). Then the Ni-NTA column was washed with 10 volumes of binding buffer + 25 mM imidazole. The bound human HscB was eluted with binding buffer + 295 mM imidazole. The human HscB was concentrated by ultrafiltration (Amicon). The concentrated sample was loaded onto the G-25 column equilibrated with 20mM HEPES buffer pH 7.5 for excess salt removal. The fractions containing human HscB were collected. The purity of HscB was confirmed by SDS-PAGE. The purified HscB was stored at -80°C for future use.
3.2.5 Molecular Cloning of Human HscA

The human HscA was also cloned by Dr. Taejin Yoon. Primers were constructed to amplify the hscA gene in the absence of the mitochondrial targeting sequence (Residue1-50). However, traditional PCR did not have a significant yield. Touchdown PCR was used to amplify the sequence. The touchdown PCR program parameters were set as follows:

- 94°C 2 min 30 sec
- 94°C 0 min 45 sec
- 63°C 0 min 30 sec
- 68°C 2 min 30 sec ---- 5 cycles
- 94°C 0 min 45 sec
- 61°C 0 min 30 sec
- 68°C 2 min 30 sec ---- 5 cycles
- 94°C 0 min 45 sec
- 59°C 0 min 30 sec
- 68°C 2 min 30 sec ---- 5 cycles
- 94°C 0 min 45 sec
- 57°C 0 min 30 sec
- 68°C 2 min 30 sec ----30 cycles
- 72°C 10 min 00 sec

5’ Primer 5’-GGA AGG CCA TAT GAT CAA GGG AGC AGT TGT TGG TAT TGA TTT-3’

Nde I
3’Primer 5’-ACC GCT CGA GTG TCC TTC TGG CTT CAA AAT TTC TGC TA -3’

Xho I

Touchdown PCR resulted in two similar bands of about 2,000 bp on agrose gel. Both bands were dissected and purified from the gel following the protocol of Qiagen Gel extraction kit and digested separately with NdeI and XhoI overnight. The digested PCR product was mixed with the similarly digested pET-28 plasmid for the ligation reaction. The ligated product was transformed into the competent DH5α cells. The possible colonies were screened with NdeI and XhoI. And the positive construct was confirmed by nucleotide sequencing.

3.2.6 Protein Overexpression of Human HscA

E.coli BL21 (DE3) was used to express human HscA. To inoculate 1 L cell culture, a 10 ml Luria-Bertani overnight starter culture (supplemented with 30 µg/ml kanamycin) was used. 1 mM IPTG was supplemented when OD$_{600nm}$ reached about 0.6. The cells were centrifuged down and collected 5 h post induction. The harvested cells were stored at -80°C.

3.2.7 Protein Purification of Human HscA

To avoid cleavage, cells were resuspended in 20 mM HEPES buffer pH 7.5, containing cocktail protease inhibitor. After sonication, the cell lystate was centrifuged at 15,000 rpm for 30 min. The cell debris was discarded followed by the supernatant loaded
onto the Ni-NTA column equilibrated with the binding buffer (50 mM Tris, pH 7.9, 5 mM imidazole, 300 mM NaCl). The column with bound HscA was then washed with 5 volumes of binding buffer + 25 mM imidazole. Finally, pure human HscA was eluted with binding buffer + 295 mM imidazole. HscA was concentrated via ultrafiltration (amicon) and loaded onto the G-25 column for desalting. Thus, the purified human HscA was in 20 mM HEPES pH 7.5. The homogenous-20 precast polyacrylamide gel was used to check the purity of human HscA.

3.2.8 ATP Concentration-dependent ATPase Activity of Human HscA

The intrinsic ATPase activity of human HscA with different concentrations of ATP was measured by Molecular Probe EnzChek phosphate kit (Invitrogen) as was previously reported [177]. The mixture for the measurement contained 1 μM HscA, MESG (2-amino-6-mercaptop-7-methyl purine riboside) substrate, 1 mM DTT, and PNP (purine nucleoside phosphorylase) in HKM buffer (50 mM HEPES buffer, 150 mM KCl, 10 mM MgCl$_2$ pH 7.5). Then, different concentrations of ATP (0 μM~200 μM) were injected to start the reaction. Phosphate was produced by the hydrolysis of ATP. The standard curve was constructed beforehand with different concentrations of potassium phosphate (0~100 μM). The standard curve correlated the UV signal with the concentration of phosphate in the cell. With the conversion of the UV signal to the ATP hydrolysis rate, the ATP concentration-dependent experiment was fitted to the Michaelis-Menten equation $V= V_{\text{max}} *[S]/(k_m+[S])$ with the software Origin 6.0. In the
above equation, V stands for ATP hydrolysis rate, \( V_{\text{max}} \) stands for the maximum ATP hydrolysis rate for the reaction. \([S]\) stands for the concentration of ATP.

### 3.2.9 NFU (or Truncated NFU) Concentration-dependent ATPase Activity Assays of HscA

Similar to what was previously reported [97,177], the ATP hydrolysis rates were measured at 25°C in HKM buffer, using coupled enzyme assays by the Molecular Probe EnzChek phosphate assay kit (Invitrogen). The reaction was pre-mixed with 1 \( \mu \text{M} \) HscA, MESG (2-amino-6-mercapto-7 -methylpurine riboside), substrate PNP (purine nucleoside phosphorylase), 1mM DTT, and different concentrations (0–150\( \mu \text{M}\)) of NFU (or truncated NFU). To initiate the reaction, 1 mM ATP injected. The ATPase activity was recorded by the UV spectrophotometer. The increased ATP hydrolysis rate by different concentrations of human NFU (or truncated NFU) was converted to the fold of stimulation with reference to the basal intrinsic ATPase activity of human HscA. Binding affinity was obtained when data were plotted and fitted with Origin software.

### 3.2.10 NFU (or Truncated NFU) Concentration-dependent ATPase Activity Assays of HscA in the Presence of HscB

In order to study HscB involvement in the stimulation effect of HscA by NFU (or truncated NFU), a similar experiment is used to measure the ATP hydrolysis rate. In detail, 1 \( \mu \text{M} \) HscA, MESG (2-amino-6-mercapto-7 -methylpurine riboside), substrate PNP (purine nucleoside phosphorylase), 1mM DTT, and 50 \( \mu \text{M} \) human HscB were
pre-mixed in the HKM buffer (50 mM HEPES buffer, 150 mM KCl, 10 mM MgCl$_2$ pH 7.5). Then different concentrations (0~150 μM) of human NFU (or truncated NFU) were added. The whole system was degassed and initiated with the addition of 1mM ATP. The ATPase activity was recorded by the UV signal at 360 nm and 25°C. The stimulated ATPase activity converted to the fold of stimulation based on the ATPase activity of HscA alone was plotted against different concentrations of human NFU (or truncated NFU). The data were plotted to obtain the binding affinity.

### 3.2.11 Quantitation of Human NFU Binding to Human HscA (ADP form) by Isothermal Titration Calorimetry

The possible interaction between HscA (ADP form) and human NFU was studied by ITC. The measurements were carried out on VP-ITC (MicroCal) system at 25°C. All reagents and solutions were thoroughly argon purged. Both protein samples were treated with 1 mM TCEP (tris(carboxyethyl)phosphine) and incubated for disulfide bond reduction. During the experiment, 10 μl 650 μM NFU (or 680 μM truncated NFU) used as titrant was injected into the sample cell, containing 11 μM human HscA. Each time, 300 sec interval was used to allow complete reaction between each injection. The titration continued until no further formation of the protein complex. The heat of dilution was subtracted as background and the data were fitted to one site binding model by the software Origin 7.
3.2.12 Quantitation of Human NFU Binding to Human HscA (AMPPNP form) by Isothermal Titration Calorimetry

The hydrolysis of ATP could influence the ITC results. The non-hydrolyzable substitute AMPPNP was used instead of ATP. Prior to the experiment, 1 mM AMPPNP was supplemented to HKM buffer (50 mM HEPES buffer, 150 mM KCl, 10 mM MgCl₂ pH 7.5). Both protein samples were incubated with 1 mM TCEP and degassed to avoid air bubbles before loading onto the ITC system. The binding between HscA and human NFU (or its C-terminal domain) was also characterized by the ITC study as described above with the exception that 1.5 mM truncated human NFU (or 1 mM human NFU) was titrated into 20 μM human HscA. All of the other parameters were set the same. The background heat was subtracted and the heat generated by the interaction was fitted to one site binding model.

3.2.13 Quantitation of Human NFU Binding to Human HscB by Isothermal Titration Calorimetry

The experiments were carried out on VP-ITC (MicroCal) with all buffers thoroughly argon purged. Both proteins were dialyzed in the same buffer (50 mM HEPES buffer, 150 mM KCl, 10 mM MgCl₂ pH 7.5). For disulfide bond reduction, 1 mM TCEP was supplemented to 0.62 mM human NFU and 0.022 mM HscB human, respectively. In order to avoid air bubbles, the proteins were degassed for 5 min before loading them onto the ITC cell and syringe separately. In the experiment, 10 μl NFU from the syringe was injected into NFU each time with the duration of 20 sec. The cell
was stirred at 300 rpm to accelerate the reaction. More than 4 equivalents of NFU were titrated into the cell to guarantee the complete formation of NFU-HscB complex. The background in which NFU was injected into the blank buffer was subtracted. The integrated data was then fitted to a one site binding model with the software Origin 7.

Truncated NFU containing the C-terminal domain only (1.1 mM) was also used as titrant for human HscB (0.03 mM) while all other conditions were kept identical. And the results were also fitted to a one site binding model.

3.2.14 Quantitation of Human NFU Binding to Human HscA/HscB Complex by Isothermal Titration Calorimetry

The study of human NFU binding to human HscA (ADP form)/HscB complex was also carried out on ITC. To ensure both proteins in the same buffer conditions, 354 μM NFU and 9 μM HscA and HscB complex were dialyzed in HKM buffer with the addition of 1 mM ADP. The night before, 9 μM HscA and HscB were pre-mixed in 20 mM HEPES buffer pH 7.5. The next morning, prior to loading onto the ITC system, both protein samples were supplemented with TCEP and degassed to remove any air bubbles. In this study, 10 μl NFU was injected into the cell containing HscA and HscB complex for each injection. The pre-set 300-sec interval was used to allow the completeness of reaction for each injection. NFU was titrated into the cell until only heat of dilution was observed. The dilution heat was subtracted as the background. The heat was integrated and fitted to the one site binding model with the software Origin 7.0.
Similarly the binding of human NFU to HscA (AMPPNP form)/HscB complex was also studied. Both protein samples were dialyzed in the same 20mM HEPES buffer pH 7.5 to minimize the inconsistency of buffer. Both protein samples were degassed for 5 min and supplemented with 1 mM TCEP and 1mM AMPPNP. NFU was used as a titrant and loaded into the syringe and the HscA/HscB complex in AMPPNP form was loaded into the ITC titration cell. The reaction was initiated with the addition of 10 μl NFU into the ITC cell. 300 sec interval allowed the completeness of the reaction. The titration continued until no heat from the binding was observed. The background titration was performed when NFU was titrated into the cell containing buffer solution without HscA or HscB. The heat was integrated and the background dilution heat was subtracted and fitted to the one site binding model with the software Origin 7.0.

3.2.15 The Study of the Relationship between the N-terminal Domain NFU and the HscA/HscB Chaperone System

The effect of the N-terminal domain of human NFU on the ATPase activity of HscA was also studied. The reaction was measured with the coupled enzymatic system Enzchek phosphate kit (Invitrogen). The conditions of the reactions were as described above with the exception of the N-terminal domain of human NFU substituting full length NFU. The reaction was carried out in HKM buffer with the addition of the N-terminal domain of human NFU ranging from 0~150 μM. All the ATPase hydrolysis rates were converted based on the HscA basal ATPase activity without the addition of the
N-terminal domain of NFU. The fold of ATPase activity of HscA was plotted against the concentration of the N-terminal domain of NFU.

In order to study the effect of the N-terminal domain of human NFU in the presence of HscB, similar experiment was performed when HscA was pre-mixed with 150 μM HscB. Different amounts of the N-terminal domain of NFU were added and the ATPase activity was measured and recorded on the UV spectrophotometer. The folds of stimulation of HscA ATPase activity were converted based on the ATPase activity of HscA alone. The reference point is the measurement of HscA ATPase activity with the addition of 150 μM HscB and without the addition of the NFU N-terminal domain. The folds of stimulation were plotted against the concentration of the N-terminal domain of human NFU.

The N-terminal domain of human NFU did not reveal any stimulation on the ATPase activity of HscA. Whether the N-terminal domain can facilitate the stimulation of HscA ATPase activity by the C-terminal domain remained an interesting topic to explore. Hence, the stimulation of HscA ATPase activity by the C-terminal domain was performed as described above. The only modification is the 1 μM HscA was pre-mixed with 150 μM of the N-terminal domain of human NFU. The ATPase hydrolysis rate was converted with reference to the basal activity of HscA. The folds of stimulation were plotted against the concentration of the C-terminal domain of NFU.

The possible involvement of the N-terminal domain of human NFU on the ATPase activity stimulated by the C-terminal domain in the presence of HscB was also further explored. All the conditions were identical as the experiment of the stimulation of HscA ATPase activity by the C-terminal domain of NFU in the presence of HscB.
except both HscB and 150 μM of the N-terminal domain of human NFU were present beforehand. The ATPase activity of HscA was measured by the coupled enzymatic assay of the Enzchek phosphate kit (Invitrogen). The recorded ATPase activity was converted into folds of stimulation based on the control experiments, in which only HscA was present for the ATPase activity measurements. The point of zero concentration of the C-terminal domain of human NFU was actually the ATPase activity when the HscA ATPase activity was measured in the presence of 150 μM of HscB and the N-terminal domain of human NFU, respectively.

The possible involvement of the binding between the N-terminal domain of human NFU and HscA (ADP or AMPPNP form) or HscB was measured by ITC. Similar conditions as described above were used. The heat from each injection was integrated and the background heat was subtracted.

The possible involvement of the N-terminal domain in the binding between the C-terminal domain of human NFU and the HscA ADP form, the C-terminal domain of human NFU and the HscA AMPPNP form, or the C-terminal domain of NFU and HscB was also studied. The experiments were performed under the same conditions as described above. The only difference is the addition of 150 μM of the N-terminal domain inside the ITC cell. The heat of each injection was integrated and the background heat was subtracted. The data were plotted and fitted to the one site binding model with the software origin 7.0. The results were compared with the ones without the addition of the N-terminal domain of human NFU.
3.3 RESULTS

3.3.1 Overexpression and Purification of both HscA and HscB

Both HscA and HscB were overexpressed in *E. coli*, although the yield of human HscA was not high. The N-terminal infused His-tag was for purification purposes only. The purification of HscB was checked on SDS-PAGE and its migration position was consistent with the predicted mass of 21.7 kD on gel (Figure 3.1). The cloned human HscB contains 185 amino acids without the N-terminal signal sequence. ProtParam predicted its pI 6.12 and molecular weight 21729.6 Da based on its sequence as shown in Figure 3.2. (The software can be found at http://www.expasy.org/cgi-bin/protparam.) For human HscA, its mitochondrial targeting sequence was also absent in the cloning step. The optimization of HscA overexpression did not produce a higher yield. For purification of HscA, cocktail protease inhibitor could successfully inhibit protease activity during cell lysis. On SDS-PAGE, purified HscA showed only one band with an apparent molecular weight ~70 kD (Figure 3.3). This was also consistent with HscA’s predicted molecular weight. The human HscA contains 650 amino acids with the predicted pI 6.01 and molecular weight 70794.3 Da (Figure 3.4). Dr. Taejin Yoon confirmed the identity of both HscA and HscB by ESI-MS.
Figure 3.1  Purification of HscB by the Ni-NTA column. Lanes 1 and 2 represent the elution of HscB from the Ni-NTA column after purification. Lane 5 represents the low molecular weight marker.

Figure 3.2  The primary sequence of human HscB. The underlined part represents the introduced His-tag. The mitochondrial targeting sequence (residues 1-71) is not included.

Figure 3.3  Purification of HscA by the Ni-NTA column. Lane 1 represents the elution of HscA from the Ni-NTA column after purification. Lane 2 represents the low molecular marker.
The primary sequence of human HscA. The underlined part represents the introduced His-tag. The mitochondrial targeting sequence (residues 1-50) is not included.

### 3.3.2 ATP Concentration-dependent ATPase Activity of Human HscA

The mechanism of the Molecular Probe Invitrogen Kit works as follows: the substrate MESG (2-amino-6-mercaptopo-7-methylpurine riboside) has a maximum absorbance at 330 nm [177]. However, with the presence of inorganic phosphate, the substrate could be converted to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine by the enzyme PNP (purine nucleoside phosphorylase) [177]. The converted 1-phosphate and 2-amino-6-mercaptopo-7-methyl purine has a maximum absorbance at 360 nm instead of 330 nm [177]. Thus, the phosphate concentration can be continuously measured by this kit.

Prior to the measurement of the ATPase activity for human HscA, the UV absorbance at 360 nm needed to be correlated with the amount of phosphate produced in this reaction. To generate the standard curve, different concentrations of potassium...
phosphate solutions were used with the coupled system of Molecular Probe Enzchek kit as described in the protocol (Invitrogen) (Figure 3.5). This resulted in a linear relationship between OD$_{360nm}$ and the phosphate concentration over the range from 2 µM to 100 µM. In the HscA APTase activity experiment, 1µM human HscA in HKM buffer was degassed and the reaction was started with the addition of different concentrations of ATP (0~200 µM) coupling with the Enzchek phosphate kit reaction (Figure 3.6). The absorbance at 360 nm was recorded and converted to the rate of ATP hydrolysis based on the standard curve. Since ATP worked as the substrate and was converted to ADP and phosphate by ATPase activity of HscA, the ATPase activity was plotted against the ATP concentration and fitted to the Michaelis-Menton equation to obtain $K_m$ value and $V_{max}$. The value of $K_m$~12µM is similar to the previously reported $K_m$ for HscA from *Escherichia coli* [178]. And $V_{max}$ was about 0.544 µM/min. This experiment provided the basis for the ATP concentration used in later experiments: 1 mM ATP was more than enough to saturate the HscA.
Figure 3.5 The relationship of phosphate concentration and the corresponding OD$_{360\text{nm}}$. The standard curve was generated by the Molecular Probe EnzChek phosphate kit.

Figure 3.6 The ATP concentration-dependent ATPase activity of human HscA. Fitting the data to the Michaelis-Menten equation generated $K_m \sim 12$ μM. The maximum ATP hydrolysis rate was 0.544 μM/min.
3.3.3 NFU (or truncated NFU) Concentration-dependent ATPase Activity Assays of HscA

IscU is the only protein identified to stimulate the ATPase activity of HscA in the *isc* operon [97]. However, genetic evidence suggests that NFU can interact with SSQ1 *in vivo* [69]. In order to enrich our understanding of the iron-sulfur cluster biosynthesis and seek evidence of NFU interacting with the chaperone system *in vitro*, the stimulation effect of human NFU (or its C-terminal domain) on ATPase activity of human HscA was tested and confirmed.

The stimulation effect was converted into folds of stimulation based on the basal ATPase activity of HscA. The ATPase activity was plotted against different concentrations of NFU (or truncated NFU). As the concentration of NFU increased, the stimulation effect on the ATPase activity of HscA increased, as well. Consequently, the ATPase activity of HscA could be seen as the function for their interaction. And the fitting function for this interaction was modified from the original one site binding function, \( Y = \frac{B_{\text{max}} \times m}{k_1 + m} + k_2 \). Here, \( Y \) stands for the folds of HscA ATPase activity and \( m \) represents the concentration of NFU (or truncated NFU). \( B_{\text{max}} \) represents the maximum number of folds of stimulation. The apparent binding affinity for the interaction is \( k_1 \). To correct for the basal ATPase activity without the addition of NFU (or truncated NFU), \( k_2 \) is used here. Instead of an arbitrary 1-fold, \( k_2 \) was used because it took into account the possible error in the measurement.

The fitting indicated that the addition of NFU (or truncated NFU) stimulated the ATPase activity of HscA with a maximum of 4.7 folds and 3.44 folds respectively. Due to the higher number of folds of stimulation for full-length NFU, additional residue from
the N-terminal domain may be involved in the stimulation. The apparent binding affinity for NFU (and its truncated form) was also obtained (Figure 3.7 and 3.8). Binding affinity of 50 µM for both of them was low, suggesting other factors may participate in this process. This also supported the idea that the chaperone was involved in the iron-sulfur cluster biosynthesis. All of the fitted parameters were summarized as in Table 3.1.

![Figure 3.7](image)

**Figure 3. 7** The concentration-dependent effect of full-length NFU on the ATPase activity. Data were reported based on the increased fold stimulation of basal ATPase activity. The fitting shows a maximal stimulation of 4.7 folds with the binding affinity of 50 µM.
Figure 3.8  The concentration-dependent effect of truncated NFU on the ATPase activity. Data were reported based on the increased fold stimulation of basal ATPase activity. The fitting shows a maximal stimulation of 3.44 folds with the binding affinity of 50 µM.

<table>
<thead>
<tr>
<th></th>
<th>$B_{\text{max}}$ (fold)</th>
<th>$k_1$ (µM)</th>
<th>$k_2$ (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-length NFU/HscA</td>
<td>3.78±0.31</td>
<td>50.1±12.2</td>
<td>0.91±0.14</td>
</tr>
<tr>
<td>Truncated NFU/HscA</td>
<td>2.44±0.21</td>
<td>50.7±12.7</td>
<td>1.00±0.08</td>
</tr>
</tbody>
</table>

Table 3.1  Summary of the HscA ATPase activity stimulated by NFU. $B_{\text{max}}$ represents the maximum number of folds of stimulation based on the basal ATPase activity of HscA. The apparent binding affinity is $k_1$. $k_2$ here stands for the fold of basal ATPase activity in the fitting. All measurements were based on the basal ATPase activity of HscA (0.506 µM/min). The maximum ATPase activity stimulation=0.506*$B_{\text{max}}$ µM/min and basal ATPase activity from the fitting= 0.506*$k_2$ µM/min.
3.3.4  **NFU (or truncated NFU) Concentration-dependent ATPase Activity Assays of HscA in the Presence of HscB**

Although the stimulation effect of NFU on the ATPase activity of HscA was observed, the effect of HscB still needed to be studied. The HscB possibly involved substrate binding and delivery, or even stimulating HscA ATPase activity [97]. A similar HscA ATPase stimulation experiment as described above was performed in the presence of 50 µM HscB. To ensure full saturation of HscA, 50 µM HscB was used according to the experiment of the stimulation of HscA ATPase activity by HscB as previously reported [179]. 25 µM 50 µM and 100 µM of HscB were added to 1µM HscA in HKM buffer separately. The measurement of the ATPase activity was performed with the Molecular Probe Enzchek phosphate kit. These resulted in the same number of folds of stimulation of HscA ATPase activity. This was consistent with the previous result: 25µM HscB was enough to saturate 1µM HscA in the ATPase activity experiment [179].

The stimulated ATPase activity of HscA by NFU (or truncated NFU) was converted based on its basal activity. The data were plotted and fitted with the modified formula described above. This time, \(k_2\) had a different meaning: it was used to correct for the basal ATPase activity of HscA in the presence of HscB. The fitting indicated the binding affinity of 17.9 µM and 15.7 µM for NFU and truncated NFU, respectively when 50 µM HscB was supplemented (Figure 3.9 and 3.10). The detailed parameters were summarized in Table 3.2. This led to the conclusion that the co-chaperone HscB greatly increased the binding affinity of HscA to NFU (or its C-terminal domain). The maximal stimulation of HscA ATPase activity was 6.97 and 4.85 folds for full-length NFU and truncated NFU. However, during the study of the chaperone system interacting with IscU
in *E.coli*, the IscU-HscB complex co-stimulated the ATPase activity synergically [97]. In our study, human NFU (or truncated NFU) did not stimulate the HscA ATPase activity synergically with the co-chaperone.

![Graph](image)

**Figure 3.9** The concentration-dependent effect of truncated NFU on HscA ATPase activity in the presence of HscB. Data were reported based on the increased fold stimulation of basal ATPase activity. The fitting shows a maximal stimulation of 4.85 folds with the binding affinity of 15.7µM.
**Figure 3.** The concentration dependent effect of full-length NFU on the ATPase activity in the presence of HscB. Data were reported based on the increased fold stimulation of basal ATPase activity. The fitting shows a maximal stimulation of 6.97 folds with the binding affinity of 17.9 µM.

<table>
<thead>
<tr>
<th></th>
<th>B&lt;sub&gt;max&lt;/sub&gt;(fold)</th>
<th>k&lt;sub&gt;1&lt;/sub&gt;(µM)</th>
<th>k&lt;sub&gt;2&lt;/sub&gt;(fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-length NFU/HscA</td>
<td>3.67±0.16</td>
<td>17.9±2.62</td>
<td>3.30±0.11</td>
</tr>
<tr>
<td>Truncated NFU/HscA</td>
<td>1.64±0.09</td>
<td>15.7±2.79</td>
<td>3.20±0.06</td>
</tr>
</tbody>
</table>

**Table 3.** Summary of the HscA ATPase activity stimulated by NFU in the presence of HscB. B<sub>max</sub> represents the maximum number of folds of stimulation based on the basal ATPase activity of HscA. The apparent binding affinity is k<sub>1</sub>. k2 here stands for the fold of basal ATPase activity in the presence of HscB in the fitting. All measurements were based on the basal ATPase activity of HscA (0.440 µM/min). The maximum ATPase activity stimulation=0.440* B<sub>max</sub> µM/min and basal ATPase activity in the presence of HscB from the fitting= 0.440*k<sub>2</sub> µM/min.
3.3.5 Quantitation of Human NFU Binding to Human HscA (ADP Form) by Isothermal Titration Calorimetry

The interaction between HscA and NFU (or the truncated C-terminal domain) was proposed due to the reason that NFU (or its truncated form) stimulates the ATPase activity of HscA. ITC study results suggested that both NFU and truncated NFU could form a protein complex with HscA at 1:1 ratio in the presence of ADP in HKM buffer. All fitted parameters were summarized as shown in Table 3.3. The binding affinity of NFU to the ADP form of HscA was 8.3 µM, while the binding affinity of truncated NFU to the ADP form of HscA was 18 µM (Figure 3.11 and 3.12).

<table>
<thead>
<tr>
<th></th>
<th>binding sites</th>
<th>$K_d$ (µM)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$\Delta S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-length NFU/HscA ADP</td>
<td>0.90±0.08</td>
<td>8.3±1.4</td>
<td>-6.29±0.67</td>
<td>2.30</td>
</tr>
<tr>
<td>Truncated NFU/HscA ADP</td>
<td>1.00±0.16</td>
<td>18±1.3</td>
<td>-7.30±1.27</td>
<td>2.78</td>
</tr>
</tbody>
</table>

Table 3.3 Summary of binding between NFU and HscA (the ADP form).
Figure 3.11 Calorimetric analysis of the binding between full-length NFU and HscA (the ADP form). Conditions were as described in ‘Material and Methods’. The heat caused by the binding was integrated and fitted to one binding site model. The plot represents the best fitting curve with 0.90 binding sites, $K_d=8.3\mu M$, $\Delta H=-6.29\text{kcal/mol}$, $\Delta S=2.30$ entropy units.
Figure 3. 12 Calorimetric analysis of the binding between truncated NFU and HscA (the ADP form). Conditions were as described in ‘Material and Methods’. The heat caused by the binding was integrated and fitted to one binding site model. The plot represents the best fitting curve with 1.00 binding sites, $K_d=18\mu M$, $\Delta H=-7.30$ kcal/mol, $\Delta S=-2.78$ entropy units.

3.3.6 Quantitation of Human NFU Binding to Human HscA (AMPPNP Form) by Isothermal Titration Calorimetry

The property of chaperone includes the alteration between the high substrate binding affinity ADP form and the low substrate binding affinity ATP form [83]. However, due to the ATPase activity of HscA, the binding between HscA ATP form and the substrate NFU cannot be measured directly. AMPPNP here was used as the non-hydrolyzable substitute of ATP. The structure of AMPPNP was found to be almost identical to the structure of ATP, except the oxygen atom in ATP connecting $\beta$ and $\gamma$
phosphate is replaced by nitrogen (Figure 3.13). Thus, AMPPNP becomes a very useful tool to probe the structures of proteins that utilize ATP. In the presence of AMPPNP, ITC fitting showed that the binding affinity increased from 8.3 µM and 18 µM to 36.5 µM and 51 µM, respectively for human NFU and the truncated C-terminal domain (Figure 3.14 and 3.15). These results suggested that the ADP form of HscA bound to NFU (or truncated NFU) around 3 folds tighter than HscA ATP form binding to NFU (or truncated NFU). These parameters were summarized in Table 3.4.

In summary, the above results can be fitted into the general picture of the HscA catalytic cycle: during the catalytic cycle, when ATP is hydrolyzed to ADP, the substrate binds to the chaperone more tightly with the subsequent structural arrangement. To complete the catalytic cycle, ATP replaces ADP in HscA kinetically. The decreased binding affinity promotes the release of the substrate for the next cycle.

<table>
<thead>
<tr>
<th>Binding Sites</th>
<th>$K_d$ (µM)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$\Delta S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-length NFU / HscA AMPPNP form</td>
<td>0.86±0.22</td>
<td>36.5±3.5</td>
<td>-4.93±0.13</td>
</tr>
<tr>
<td>Truncated NFU / HscA AMPPNP form</td>
<td>1.05±0.36</td>
<td>51.0±6.2</td>
<td>-3.21±0.21</td>
</tr>
</tbody>
</table>

**Table 3.4** Summary of binding between NFU and HscA (the AMPPNP form).
Figure 3.13 Comparison of the structures of ATP and AMPPNP. Figure (a) depicts the structure of ATP and Figure (b) depicts its non-hydrolyzable homolog AMPPNP. AMPPNP can prevent the transfer of $\gamma$ phosphate.
**Figure 3.14** The calorimetric analysis of the binding between full-length NFU and HscA (the AMPPNP form). Conditions were as described in ‘Experiment’ part. The heat caused by the binding was integrated and fitted to one binding site model. The plot represents the best fitting curve with 0.86 binding sites, $K_d=36.5 \mu M$, $\Delta H=-4.93$ kcal/mol, $\Delta S=3.74$ entropy units.

**Figure 3.15** The calorimetric analysis of the binding between truncated NFU and HscA (the AMPPNP form). Conditions were as described in ‘Experiment’ part. The heat caused by the binding was integrated and fitted to one binding site model. The plot represents the best fitting curve with 1.05 binding sites, $K_d=51 \mu M$, $\Delta H=-3.21$ kcal/mol, $\Delta S=8.86$ entropy units.
3.3.7 Quantitation of Human NFU Binding to Human HscB by Isothermal Titration Calorimetry

HscB is also known as co-chaperone. This protein was proposed to assist the function of chaperones. The presence of HscB during the ATPase activity study indicated that HscB enhanced binding between HscA and NFU. So it was proposed that there was interaction between HscB and NFU (or its C-terminal domain). This was demonstrated by the ITC study. Figures 3.16 and 3.17 showed the ITC results of the interaction between NFU (or truncated NFU) and HscB. The parameters are summarized in Table 3.5. Both NFU and the truncated C-terminal domain can form a complex with HscB at 1:1 ratio with binding affinity of 10 µM and 11 µM, respectively. This also provided evidence to support the role of HscB in the substrate recognition and contribution to the tighter binding of the NFU-HscA complex.

<table>
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<tr>
<th>Binding sites</th>
<th>$K_d$ (µM)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$\Delta S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-length NFU/HscB</td>
<td>0.93±0.06</td>
<td>10±1.0</td>
<td>-0.90±0.05</td>
</tr>
<tr>
<td>Truncated NFU/HscB</td>
<td>0.94±0.06</td>
<td>11±1.3</td>
<td>-1.67±0.13</td>
</tr>
</tbody>
</table>

Table 3.5 Summary of the binding between NFU and HscB.
Figure 3. 16  Calorimetric analysis of the binding between full-length NFU and HscB. Conditions were as described in ‘Experiment’ part. The heat caused by the binding was integrated and fitted to the one binding site model. The plot represents the best fitting curve with 0.93 binding sites, $K_d=10\mu$M, $\Delta H=-0.9$ kcal/mol, $\Delta S=20$ entropy units.

Figure 3. 17  The calorimetric analysis of the binding between truncated NFU and HscB. Conditions were as described in ‘Experiment’ part. The heat caused by the binding was integrated and fitted to the one binding site model. The plot represents the best fitting curve with 0.93 binding sites, $K_d=11\mu$M, $\Delta H=-1.67$ kcal/mol, $\Delta S=17.1$ entropy units.
3.3.8 Quantitation of Human NFU Binding to the Human HscA/HscB Complex by Isothermal Titration Calorimetry

The ATPase activity experiment suggested that the binding affinity between NFU and HscA was increased with the involvement of HscB. With the demonstration of the interaction between NFU and HscA, NFU was titrated into the HscA/HscB complex. The first experiment was performed with the presence of ADP. The obtained parameters were summarized as shown in Table 3.6. The fitted binding curve showed a binding affinity of 0.3 μM. Compared with the binding affinity of 8.3 μM for the NFU-HscA complex, the binding between NFU and HscA/HscB complex was much tighter (Figure 3.18 a). Similarly in the second experiment, the binding between NFU and HscA (AMPPNP form)/HscB complex was determined. The fitted result showed a binding affinity of 9.43 μM. Comparing the result with the HscA (AMPPNP form) and NFU binding, the binding affinity decreased around 2.5 fold. This was consistent with the ATPase activity experiment results. It demonstrated thermodynamically that NFU can bind more tightly to HscA/HscB complex than HscA alone.
Figure 3.18 The calorimetric analysis of the binding between NFU and the HscA/HscB complex. Conditions were as described in ‘Experiment’ part. The heat caused by the binding was integrated and fitted to the one binding site model. The (a) plot represents the binding between NFU and the HscA (ADP form)/HscB complex. The best fitting curve shows 0.95 binding sites, $K_d=0.33 \, \mu M$, $\Delta H=-3.38 \, \text{kcal/mol}$, $\Delta S=18.3$ entropy units. The (b) plot represents the binding between NFU and the HscA (AMPPNP form)/HscB complex. The best fitting curve shows 0.985 binding sites, $K_d=9.43 \, \mu M$, $\Delta H=-1.66 \, \text{kcal/mol}$, $\Delta S=17.4$ entropy units.
<table>
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<th>$K_d$ ($\mu$M)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$\Delta S$</th>
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<tr>
<td>Full-length NFU/HscA/B (ADP form)</td>
<td>0.95±0.02</td>
<td>0.33±0.08</td>
<td>-3.38±0.14</td>
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<tr>
<td>Full-length NFU/HscA/B (AMPPNP form)</td>
<td>0.985±0.04</td>
<td>9.43±0.07</td>
<td>-1.66±0.09</td>
</tr>
</tbody>
</table>

Table 3.6  Summary of the binding between NFU and HscB/HscA.

### 3.3.9 The Study of the Relationship between the N-terminal Domain NFU and the HscA/HscB Chaperone System

The cloning and purification procedure of human NFU N-terminal domain is in Chapter 4. Thus, the relationship between the N-terminal domain of NFU and the chaperone system can be studied directly. However, different from the C-terminal domain of human NFU, the N-terminal domain does not show any stimulation of HscA ATPase activity (Figure 3.19 a). And the same experiment in the presence of HscB was attempted. The N-terminal domain does not have any stimulation effect of ATPase activity in the presence of HscB. Only the stimulation effect of HscB was observed (Figure 3.19 b). The N-terminal domain can not stimulate the ATPase activity of HscA may be explained on the structural basis. In Chapter 4, the C-terminal domain and full length NFU possess the molten globule property. In contrast, the N-terminal domain is a well-structured protein. The molten globule property means the flexible folding of the C-terminal domain and chaperone system tends to interact and facilitate the folding of unstructured or molten globule proteins. This is possibly the molecular basis why the C-terminal domain can stimulate the HscA ATPase activity while the N-terminal domain
can not. However, full length NFU can stimulate more ATPase activity than the C-terminal domain as observed above. This suggested that the N-terminal domain had some stimulation effect on HscA ATPase activity. In order to resolve the apparent conflict, approximate effect was used here. The idea that the N-terminal domain alone has different structure from the one within full length NFU is ruled out based on the HSQC experiment in Chapter 4. Possibly, the difference here can be explained as: the interaction of the C-terminal domain with HscA can bring the N-terminal domain near HscA. Hence, such close distance may make the N-terminal domain co-stimulate the ATPase activity of HscA.

The possible interaction between N-terminal domain of NFU and HscA (both ADP form and AMPPNP form) was further explored. ITC study did not reveal significant heat absorption or release (Data not shown). It is quite possible that the N-terminal domain does not bind the chaperone HscA (both ADP and AMPPNP form). Possible interaction of the N-terminal domain of NFU and HscB was also explored. No heat was detected from the titration also. This suggested possibly the N-terminal domain did not bind to the co-chaperone protein HscB. This is consistent with the ATPase activity assay of the N-terminal domain. It suggested the N-terminal domain of NFU alone may not have a role for the catalytic turnover of the chaperone system.

Although the N-terminal domain of NFU alone may not have any role in the chaperone system, it may have special roles together with the C-terminal domain in the catalytic turnover of the chaperone system. Thus, the ATPase activity assay stimulated by the C-terminal domain of human NFU and the binding of the C-terminal domain of human NFU with HscA (ADP form or AMPPNP form) or HscB were attempted in the
presence of the N-terminal domain of human NFU (Figure 3.19 c d e f g). All the results were summarized in Table 3.7 and 3.8. Briefly, both the kinetic ATPase activity assay and the thermodynamic experiments had almost identical results with or without the addition of the N-terminal domain of human NFU. Hence, the N-terminal domain if not within full length NFU may not involve in the catalytic turnover of the chaperone system and have no influence on the involvement of the C-terminal domain in the catalytic cycle of the chaperone system.
Figure 3. 19 The study of the involvement of the N-terminal domain of NFU in the catalytic turnover of the chaperone system. Figure (a) shows HscA ATPase activity stimulation by the N-terminal domain. The fold of stimulation was based on the intrinsic ATPase activity of HscA. Figure (b) shows HscA ATPase activity stimulation by the N-terminal domain in the presence of HscB. The ATPase activity was converted into folds of stimulation based on the measured intrinsic ATPase activity of HscA. The first reference point was the folds of stimulation by HscA in the presence of HscB without the addition of the N-terminal domain of human NFU. Figure (c) shows the stimulation of HscA ATPase activity by the C-terminal domain of NFU in the presence of the N-terminal domain. The fitting shows a maximum of 3.26 folds with the binding affinity of 35.3 μM. Figure (d) shows the stimulation of HscA ATPase activity by the C-terminal domain of human NFU in the presence of both the N-terminal domain NFU and the co-chaperone HscB. The fitting shows a maximum of 4.87 folds with the binding affinity of 13.9 μM. Figure (e) shows the calorimetric analysis of the binding between the C-terminal domain of human NFU and the HscA (ADP form) in the presence of the N-terminal domain of human NFU. The best fitting curve shows 0.88 binding sites, $K_d=14.9$ μM, $ΔH=-3.82$ kcal/mol, $ΔS=9.28$ entropy units. Figure (f) shows the calorimetric analysis of the binding between the C-terminal domain of human NFU and the HscA (AMPPNP form) in the presence of the N-terminal domain of human NFU. The best fitting curve shows 0.907 binding sites, $K_d=30.3$ μM, $ΔH=-2.00$ kcal/mol, $ΔS=14.1$ entropy units. Figure (g) shows the calorimetric analysis of the binding between the C-terminal domain of human NFU and the co-chaperone HscB in the presence of the N-terminal domain of human NFU. The calorimetric titration of the N-terminal domain of NFU into the HscA (both ADP and AMPPNP form) or HscB was not shown here due to the lack of significant heat release or absorption.
(Continued)

(c)

(d)

(Continued)
Table 3. 7 Summary of the HscA ATPase activity stimulated by the C-terminal domain of NFU in the presence of the N-terminal domain of NFU. B\textsubscript{max} represents the maximum number of folds of stimulation based on the basal ATPase activity of HscA. The apparent binding affinity is \( k_1 \). \( k_2 \) here stands for the fold of basal ATPase activity alone or in the presence of HscB in the fitting. All measurements were based on the basal ATPase activity of HscA (0.344 \( \mu \text{M/min} \)). The maximum ATPase activity stimulation=0.344* \( B_{\text{max}} \) \( \mu \text{M/min} \) and basal ATPase activity alone or in the presence of HscB from the fitting= 0.344*\( k_2 \) \( \mu \text{M/min} \).
<table>
<thead>
<tr>
<th>binding sites</th>
<th>Kₐ (µM)</th>
<th>ΔH (kcal/mol)</th>
<th>ΔS</th>
</tr>
</thead>
<tbody>
<tr>
<td>The C-terminal NFU/HscA (ADP form) in the presence of the N-terminal NFU domain</td>
<td>0.88±0.14</td>
<td>14.9±1.67</td>
<td>-3.82±0.67</td>
</tr>
<tr>
<td>The C-terminal NFU/HscA (AMPPNP form) in the presence of the N-terminal NFU domain</td>
<td>0.907±0.05</td>
<td>30.3±2.82</td>
<td>-2.00±0.13</td>
</tr>
<tr>
<td>The C-terminal NFU/HscB in the presence of the N-terminal NFU domain</td>
<td>0.79±0.03</td>
<td>10.0±1.22</td>
<td>-0.57±0.03</td>
</tr>
</tbody>
</table>

**Table 3. 8** Summary of the binding between the C-terminal domain of NFU and HscA (ADP form or AMPPNP form) or HscB in the presence of the N-terminal NFU domain.

3.4 DISCUSSION

3.4.1 Exploration of the Linkage between NFU and HscA

During the discovery of *isc* (iron-sulfur cluster) operon, chaperone HscA and co-chaperone HscB were identified. This close association suggests that both chaperones may have special roles in the iron-sulfur cluster biogenesis. However, the exact role of HscA and HscB is still unknown. How the chaperone system is involved in the iron-sulfur cluster biosynthesis remained intriguing until the interaction between IscU and both chaperones in a variety of different organisms was identified [98,157]. IscU was the only protein that could stimulate the ATPase activity of HscA and there was no further progress. In *E. coli*, IscU can interact with Hsc66 in ADP form and it can stimulate the ATPase activity of Hsc66 [97]. The Hsc20 co-chaperone plays auxillary roles and can form a protein complex with IscU [97]. Hsc20 and IscU can stimulate the ATPase
activity of Hsc66 synergistically and the binding affinity is correspondingly decreased comparing with the stimulation of HscA ATPase activity by IscU alone [97]. Thus, Hsc20 can co-stimulate the ATPase activity with IscU and contribute to form a tighter complex of Hsc66-IscU [97]. Phage display affinity panning experiments reveal a consensus sequence Hφ-Pro-Pro- Hφ-polar preference for HscA [95]. And the sequence LPPVK in IscU interacts with HscA [95]. Different from DnaK, which binds to hydrophobic residues, HscA type proteins tend to bind residues with centrally located Proline [95]. During the phage display affinity panning experiments, DnaK tends to bind hydrophobic residues which are buried inside IscU [95]. This is possibly the reason why IscU stimulates the ATPase activity of DnaK weakly and DnaK may bind the denatured IscU [95]. The LPPVK sequence is possibly in the unstructured region and surface exposed [95]. This sequence is conserved in all IscU family members [95]. However, the peptide LPPVK can not stimulate HscA ATPase activity with Hsc20 synergistically unlike IscU [95]. This may help to avoid Hsc66 forming unproductive complexes with peptides [95].

How chaperone system is involved in the iron-sulfur cluster biosynthesis is still unknown. The interaction of IscU with HscA chaperone system may provide insight about the function of HscA in the iron-sulfur cluster biosynthesis process. The LPPVK motif is near the iron-sulfur cluster binding site and suggests the chaperone system is possibly related to iron-sulfur cluster assembly, iron-sulfur cluster transfer, or the type of iron-sulfur cluster formed on IscU [95]. Recent data suggested that similar system in *T.maritima* had some inhibitory effects on iron-sulfur cluster transfer from IscU [180]. The effect mainly focused on reducing the rate of the formation of the IscU-ferredoxin
complex and had negligible effects on the intrinsic rate of transfer to ferredoxin [180]. In general, the effect of the chaperone system on iron-sulfur cluster biosynthesis needs to be further explored.

Now the acidic protein NFU was the only one related to the operon identified to bind and stimulate the ATPase activity of the chaperone other than IscU. NFU and truncated NFU can stimulate the ATPase activity of HscA although to different folds. The interaction of ADP form and AMPPNP form of HscA interacting with NFU, respectively was studied. The ADP form of HscA forms a tighter complex with NFU comparing with the AMPPNP form of HscA. The co-chaperone HscB can interact with NFU and the complex formation increases the binding of HscA with NFU. The C-terminal domain is the functional domain since the C-terminal domain alone can stimulate the HscA ATPase activity. The N-terminal domain effect was also studied. However, the N-terminal domain alone does not have any effect on the ATPase of HscA. It does not have any effect on the ATPase activity stimulation of HscA, different from full length NFU. This is explicable as the approximate effect. The C-terminal domain can interact with the HscA chaperone and the N-terminal domain is brought to the vicinity of HscA. This leads to the interaction between the N-terminal domain and the HscA chaperone. When the C-terminal domain is not present, the N-terminal domain alone is hard to target towards HscA. This can not stimulate the ATPase activity of HscA. Finally the interaction between NFU and HscA/HscB complex is confirmed by the ITC experiment. Although the ATPase activity and interaction was observed, some key differences still exist between the reaction of NFU-HscA and IscU-HscA. The presence of HscB enhances the binding between NFU and HscB. However, NFU does not
co-stimulate the ATPase activity of HscA with HscB synergistically. The binding site was also considered. However, there are in total 15 Proline residues in human NFU. There is not -Pro-Pro- sequence in human NFU. Consequently, it is hard to predict where the site that the chaperone system interacts is. There is a possibility that although prolines are not close to each other in primary sequence, they can be close to each other in tertiary structure. Thus, it is hard to predict which residues are really involved the stimulatory effect of HscA ATPase activity. The interaction needs to correlate with the function of NFU. The chaperone system may influence the production of sulfide for human NFU. However, testing such a system is too complicated. NFU is not the only protein that can interact with the chaperone system. IscU and NifS may also interact with the chaperone system [85]. As the knowledge about NFU and the chaperone system accumulates, the role of the chaperone system in iron-sulfur cluster biosynthesis will be finally revealed.

In summary, the identified interaction between NFU and the chaperone system may help to reveal the role of the chaperone system in the iron-sulfur cluster biosynthesis. This will allow further investigation of this interaction to address its psychological significance in vivo.

3.4.2 The Catalytical Cycle of the Chaperone System Interacting with NFU (or Truncated NFU)

The proposed model of the chaperone system interacting with NFU (or truncated NFU) may work as follows: The NFU interacting with the substrate binding domain stimulates the ATPase activity of HscA. The bound ATP is then hydrolyzed to ADP. The
ADP bound form of HscA has a higher substrate binding affinity than its ATP bound form. Subsequently, ADP is replaced by ATP kinetically, which leads to the structure rearrangement and decreases the substrate binding affinity. The decreased binding affinity promotes the release of the substrate and the chaperone is ready for the next cycle (Figure 3.20).

**Figure 3. 20** The scheme of the catalytic cycle by HscA chaperone interacting with NFU (or truncated NFU).

The binding affinity of NFU with HscA is further decreased when the co-chaperone HscB is also involved. HscB interacts with the NFU (or truncated NFU) and helps to decrease the binding affinity of HscA and NFU. This may mimic the process *in vivo* (Figure 3.21).
3.4.3 The Distinctive Feature of the Human Chaperone System Interacting with NFU

The interaction between NFU and the chaperone system always resulted in positive change in entropy and negative change in enthalpy. Negative change in enthalpy favours the binding. The bond formation during protein protein interaction such as hydrogen bonding formation, salt bridges formation results in negative change in enthalpy [131]. In contrast, some bonds are broken during binding such as the displacement of water and sometimes the conformation change [131]. ITC actually measures the overall effect of the enthalpy. Changes in entropy are more complex to explain. The formation of protein complex always leads to the decrease in entropy because of stabilization of the protein binding surface and the decrease of the rotation and translation of the proteins [131]. However, the displacement of water molecules from the

Figure 3.21 The scheme of the catalytic cycle by HscA chaperone interacting with NFU (or truncated NFU) in the presence of HscB.
binding surface increases the disorder of the system and leads to the increase in entropy. In the case of our study, both entropy and enthalpy have favorable changes that lead to the formation of the protein-protein interaction.

Interestingly, the human chaperone system shows several distinctive features: first of all, unlike the prokayotes [97], human NFU can stimulate the ATPase activity of the chaperone up to several folds. This is similar to what was observed for the eukaryotic chaperone system interacting with ISU in yeast [98]. Second, the co-chaperone HscB does not co-stimulate the ATPase activity of HscA with NFU, unlike other HscA/HscB chaperones interacting IscU [97,98], although HscB contributes to increasing the binding affinity of HscA. Last but not least, the nucleotide exchange factor GrpE is identified in yeast, marking the difference between prokaryote and eukaryote chaperone systems [98]. However, the nucleotide exchange factor for the human HscA/HscB chaperone system is not identified yet. Thus the possible function of human GrpE involving the chaperone system will remain an interesting topic to explore.
CHAPTER 4

BIOCHEMICAL CHARACTERIZATION OF HUMAN NFU INDICATES ITS MOLTEN GLOBULE PROPERTY

4.1 INTRODUCTION

Although the characterization of human NFU has been carried out widely, little information is known about the structure of human NFU. As described in Chapter 2, the secondary structure of human NFU has been characterized by circular dichorism. Interestingly, the near-UV region in the CD spectrum did not show any significant signals in contrast to the far-UV region signal. These data suggest that human NFU may act as a molten globule [125].

Molten globule is a term to describe a protein with a high degree of secondary structure, but without a rigid tertiary structure [181,182]. Molten globule was once thought to be the intermediate between the unfolded state and the folded state during protein folding [183]. It possesses the following characteristics: (a) less compact than the native state, but more compact than the unfolded state; (b) a high degree of secondary structure; (c) loose tertiary structure with no tight side-chain packing [184]. And most molten globule-type proteins were induced under mild denaturation conditions [184]. The conditions vary for different proteins [184]. The conformations of
molten globules were diverse and even different molten globule states can be induced from the same protein [185,186].

Although the molten globule state is mostly induced, some proteins do exist as molten globule in nature [187]. An example is clusterin, a well-known protein detergent that acts as a native molten globule to fulfill its function [187]. Interestingly, some proteins can even change from an ordered structure to a molten globule state, such as nucleosomes that become hyperacetylated during DNA replication [187]. *T. maritima* IscU was recently identified to possess the molten globule property [188]. This can be rationalized as the MDC (Multiple Discrete Conformers) model: *T. maritima* IscU has several undistinguishable well-structured conformers and different conformers may interact with different protein partners, endowing the molten globule property to IscU [188]. This type of molten globule does not qualify for the traditional definition. *T. maritima* IscU in solution is actually a dynamic mixture of well-structured conformers instead of proteins without a rigid tertiary structure [188].

The traditional method to study the molten globule was used here. These methods include: ANS binding, limited trypsin digestion, near UV-CD, and HSQC NMR experiments. Due to the reason that human NFU is a multifunctional protein interacting with different partners, we propose that human NFU may behave more like molten globule type proteins to facilitate protein-protein interaction [70,82]. The question of whether NFU acts more like a traditional molten globule or a non-traditional one like *T. maritima* IscU is also answered in this chapter. The study of the molten globule property may help to further illustrate the structural property of human NFU and contribute to our understanding of the iron-sulfur cluster biosynthesis.
4.2 EXPERIMENT

4.2.1 Material

ANS was obtained from Invitrogen Inc. (Eugene, OR). Carbonic anhydrase and trypsin were obtained from Sigma Aldrich Inc. (St. Louis, MO). Amonium chloride (15N 99%) was obtained from Cambridge Isotope Laborotories Inc. (Andover, MA). All protein samples were purified as previously described (Liu & Cowan, unpublished).

4.2.2 ANS Binding

The binding of ANS to protein samples was monitored by the PerkinElmer Life Science LS50B luminescence spectrometer. The excitation wavelength was 371 nm with the slit width of 3 nm and the emission was monitored at 482 nm with the slit width of 10 nm at 25°C. The buffer used was 50 mM Tris-HCl pH 7.5. The baseline was measured when 10 μM ANS was in solution alone. When 100 μM protein samples (NFU, truncated NFU, T.maritima IscU, and apo and holo ferredoxin) were added, the fluorescence enhancement of ANS was recorded and compared. In order to generate the binding affinity of ANS to human NFU and truncated NFU, reverse titration was used to correlate the arbitrary fluorescence unit with the moles of ANS bound on protein as previously described [189]. The binding affinity between ANS and NFU (or truncated NFU) was obtained by plotting the Scatchard plot of the bound ANS/free ANS ratio with the concentration of bound ANS [190]. The addition of ANS in human ISU solution was performed under the same conditions. The ANS binding experiment was also carried out on Zn-bound ISU (both human ISU and T.maritima IscU). To
determine the binding affinity, both Zn-bound and unbound human ISU and *T. maritima* IscU were titrated with ANS and the Scatchard plot was generated.

### 4.2.3 Tryptic Digestion Monitored by SDS-PAGE and Mass Spectrometry

Reaction was carried out similar to the previously described experiment with slight modification [188]. All protein samples (NFU, truncated NFU, apo and holo ferredoxin, and *T. maritima* IscU) were dialyzed with 50 mM Tris-HCl pH 7.5. All protein samples were adjusted to the same concentration. The ratio of trypsin to protein was kept at 1:200. Subsequently, the mixture was incubated at 37°C. Aliquots were removed at 1 min, 2 min, 5 min, 15 min, 30 min, and 60 min and immediately mixed with the SDS-PAGE loading buffer containing PMSF. They were then put into a hot water bath. After 5 min of boiling, the sample was kept on ice until all of the samples were ready. The first point was used as a reference. The sample of the first point was similarly treated without adding any trpsin. After staining and destaining, the SDS-PAGE gel was subject to be quantified by the BioRad Gel-Doc imager. The background was subtracted and the percentages of each lane were calculated according to the intensity of the reference point. Then the data were fitted to first order decay.

### 4.2.4 Full-length NFU Digestion Product Determined by LC-MS

Since the digestion of full-length NFU by trypsin resulted in an intermediate, it was hypothesized that this part of the protein was not flexible. LC-MS was used to determine which part of human NFU this one was. The submitted sample was digested
as described above. In reverse phase high-performance liquid chromatography (Waters, USA) water with 0.1% trifluoroacetic acid was used as mobile phase A, acetonitrile and 0.1% trifluoroacetic acid was used as mobile phase B. Subsequently 10 μl of the digested sample was injected into a Vydac C18 MS column. The composition of mobile phase B was maintained at 2% for the first 2 min and its concentration increased from 2% to 95% linearly in the next 28 min. The column was washed with 98% B for 1 min and equilibrated with 2% of B for 29 min during the injection interval. The sample flow rate was maintained at 50μl min⁻¹. The eluted portion was directly infused into a Micromass LCT (Micromass, Wythenshawe, UK) mass spectrometer with an orthogonal electrospray source (Z-spray) to determine the molecular weight. The optimal ESI conditions were 3,000V capillary voltage, 100°C source temperature and 50 V cone voltage. Data were acquired in continuum mode with the rate of 1 scan sec⁻¹. All spectra were acquired in the positive ion mode with the NaI as the external mass calibration over the m/z range 500-2,500.

4.2.5 Cloning and Expression of the Trypsin Digested Intermediate of Human NFU

LC-MS indicated the intermediate was residue 17-135 which was the N-terminal domain. The first 20 residues were the infused His-tag, and residues after 135 were exactly the C-terminal truncated domain. It was hypothesized that the N-terminal domain of human NFU was not flexible. In order to test this hypothesis, molecular cloning of the N-terminal domain was necessary. PCR amplification of the gene was achieved by use of a high fidelity PCR buffer (1X), a 0.2 mM dNTP mixture
containing 2 mM MgSO₄, 100 ng human genome DNA, 0.2 µM amount of each primer, and 1.0 unit of platinum Taq DNA polymerase as recommended in the Invitrogen manual. Primers included the following: 5’- CCA GTG AGA CAT ATG TTT ATT CAA ACA CAA GAT ACC -3’ and 5’- CGT ATT CTA GTA TCT AAG GAT CCC TAA ATC ATT GCC -3’, where the underlined regions were introduced the Nde I and BamH I sites. The PCR product was digested with 10 units of BamH I and 20 units of Nde I in 1X React 3 buffer (Invitrogen) for 2 h. Vector pET-28 was digested similarly at the same time. After digestion and running on agarose gel, the DNA samples were dissected and purified with the Qiagen gel extraction kit. The extracted PCR product and plasmid were ligated by T4 ligase according to the Invitrogen manual. Subsequently, the whole ligation mixture was transformed into DH5α competent cells and screened with restriction enzymes Pst I and Apa I. The positive candidate was confirmed by nucleotide sequencing at The Ohio State University Plant-Microbe Genomics Facility. An N-terminal His-tag was infused for purification purposes.

BL21 Lysozyme plus (DE3) containing the transformed construct was used for protein expression. A 50 ml Luria-Bertani culture (supplemented with 30 µg/ml kanamycin and 30 µg/ml chloramphenicol) was grown overnight as the starter culture. 10 ml of the starter culture was used as an inoculum for 1 L culture. The cells were fermented to OD₆₀₀nm ~ 0.6 and 1 mM IPTG was added to induce protein expression. The cells were harvested after 6 h induction and stored at -80°C for future use.
4.2.6 Purification of the N-terminal Domain of Human NFU

The frozen cells containing the N-terminal domain of NFU were resuspended in 50 mM Tris-HCl pH 7.5 and lysated by a sonicator. The cell debris was removed by centrifuging at 15,000 rpm and 4°C. The cleared portion was loaded onto the Ni-NTA column equilibrated with 50 mM Tris-HCl pH 7.5, followed by washing with 2 volumes of 50 mM Tris-HCl supplemented with 200 mM NaCl and 20mM imidazole pH 7.5. The bound protein was washed with 3 volumes of 50mM Tris-HCl supplemented with 200 mM NaCl and 300 mM imidazole pH 7.5. The eluted portion was concentrated by ultrafiltration. The target protein was then dialyzed into 20 mM Na$_2$HPO$_4$ pH 7.5. Some of the target protein was also dialyzed with Barnstead nanopure water and submitted to CCIC (Campus Chemical Instrument Center) for identity confirmation by ESI-MS.

4.2.7 Circular Dichroism of the N-terminal Domain of NFU

The Aviv model 202 circular dichroism spectrometer was used to obtain the CD spectrum characterizing the secondary structure of the N-terminal domain of human NFU. For this experiment, 1 cm quartz curvette containing 1.2 μM of the N-terminal domain of NFU in 10 mM NaH$_2$PO$_4$ pH 7.5 was used. The spectrum was obtained by 5 repeated scans. The wavelength ranged from 350 nm to 200 nm. The background spectrum was obtained similarly with only a buffer solution in the curvette. The 5 spectra were averaged and the background was subtracted. The circular dichroism spectrum was fitted with the online software K2D by inputting the corresponding mean
residue ellipticity (http://www.embl-heidelberg.de/~andrade/k2d/). The secondary structure of the N-terminal domain of human NFU was predicted with the input of its sequence on PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html). Each component of secondary structure of predicted and deconvoluted results was compared.

4.2.8 Investigation of the Structural Property of the N-terminal Domain of Human NFU

Exactly the same condition was used as previously described for ANS binding. The binding of 10 μM ANS to 100 μM the N-terminal domain of NFU was monitored by the PerkinElmer Life Science LS50B luminescence spectrometer. The excitation wavelength was 371 nm with the slit width of 3 nm. The emission was monitored at 482 nm with the slit width 10 nm at 25°C. The buffer used was 50 mM Tris-HCl pH 7.5. The tryptic digestion was performed under the same condition. The molecule ratio of trypsin to protein was 1:200. The mixture was incubated at 37°C. Aliquots were removed at different time intervals of 1 min, 2 min, 5 min, 15 min, 30 min, and 60 min and immediately mixed with the SDS-PAGE loading buffer containing PMSF. The mixture was then incubated in boiling water. After boiling for 5 min, the sample was kept on ice before loading. The first lane was loaded with the original amount of the intact N-terminal NFU. The intensity of the other lanes was converted to the remaining percentage based on the first lane as a reference.
4.2.9 **HSQC Study of Human NFU**

All three proteins (full-length NFU, the N-terminal domain NFU, and the C-terminal domain NFU) were labeled under the same conditions. The newly transformed BL21 Lys plus colony was picked and inoculated in 10 ml fresh LB media overnight. The inoculum was centrifuged at 4,000 rpm for 10 min, followed by resuspension in M9 media. The M9 media was supplemented with ammonium chloride (15N 99%). At this point, 30 µg/ml kanamycin and 30 µg/ml chloramphenicol were also added into the media. The cells were fermented at 37ºC until O.D. at 600 nm reached 0.8 and 1 mM IPTG was used to induce the protein expression. The cells were then grown at 30ºC for another 18 h. Finally, the cells were harvested and purified exactly as the unlabelled protein described previously. For the HSQC NMR experiments, all protein samples were dialyzed into 20 mM sodium phosphate 50 mM NaCl pH 7.5 10% D2O and 2D 1H-15N fast HSQC experiments were carried out on a Bruker DMX-600 spectrometer equipped with a triple-resonance probe and three-axis gradient coils at 298 K at CCIC (Campus Chemical Instrument Center) [191]. A data matrix of $2048 \times 256$ was recorded for each sample with the duration of 1.5 h. Data were processed with XWINNMR3.5 (Bruker Inc.).

4.3 **RESULTS**

4.3.1 **ANS Binding**

ANS was frequently used to probe the structure of molten globule proteins. Its fluorescence depends on its environment. When in water, its fluorescence is negligible in such a polar environment. For molten globule type proteins, where ANS can
penetrate and bind to its hydrophobic core shielding ANS from the polar environment, the fluorescence is greatly increased and the maximum absorbance shifts to around 480 nm [192]. For human NFU and its C-terminal domain, adding ANS to the protein solution caused greatly enhancement of ANS fluorescence indicating human NFU possibly acted as a molten globule type protein (Figure 4.1 a). The binding affinity of ANS and human NFU (or the C-terminal domain) was obtained from the Scatchard Plot. The fitted equation is 
\[
\frac{[B]}{[F]} = k_a[B_{max}] - k_a[B]
\]
[B] stands for the concentration of the protein bound ANS. [F] stands for the concentration of the free ANS. K_a represents the affinity constant of ANS. The [B]/[F] was plotted as y axis, while [B] alone was plotted as the x axis. The k_a was the negative slope and 1/k_a was k_d, the apparent binding affinity. [B_{max}] was the maximum concentration of protein bound ANS and could be obtained as the intercept of x axis. The k_d for ANS-NFU complex and ANS-truncated NFU were 4µM and 9µM, respectively (Figure 4.1 b and c). The ratio of ANS to protein was determined to be 1.6:1 for full-length NFU and 1.35:1 for truncated NFU, respectively. The ANS binding affinity of protein is about 20 µM or less, indicating the rigid structure with a flexible ANS-binding pocket [193]. Therefore, it was proposed that human NFU possesses a tertiary structure with a flexible ANS binding pocket in its C-terminus. Both positive and negative controls were performed. As previously observed, T.maritima IscU, which also showed the molten globule property, bound to ANS and greatly enhanced the fluorescence of ANS at 482 nm (Figure 4.1 d) [188]. Carbonic anhydrase, a well-structured protein under normal conditions, was used as a negative control (Figure 4.1 d). The enhancement of
ANS fluorescence at 482 nm was less than 5 folds, which was consistent with the results of previous research [194].

The ANS binding experiments were also carried out on human ISU. Similar to *T.maritima* IscU, the ANS-human ISU complex also showed great enhancement of fluorescence similar to what was observed for *T.maritima* IscU (Figure 4.1 e). This experiment indicates that the view that the molten globule property of *T. maritima* IscU is unique could be ruled out [8]. Possibly, the molten globule property is the common property of the IscU family, since this family serves as scaffolding for the iron-sulfur cluster biosynthesis and needs to interact with different partners for cluster transfer. Interestingly, previous experiments found Zn could bind to IscU and stabilize its structure [195]. Whether Zn-bound IscU still possessed molten globule property remains unclear. The study was carried out on both human ISU and *T.maritima* IscU. We observed that the addition of ANS had a similar effect on both Zn-bound and Zn-unbound IscU (Figure 4.1 f). The Scatchard plot for both *T.maritima* IscU and human ISU showed approximately 2 ANS binding sites and 20 μM for the binding affinity. With Zn bound to IscU (or ISU), the ANS binding affinity and the number of ANS binding sites were not influenced at all.
Figure 4. 1  ANS-binding experiment. In each experiment, the sample was dialyzed in the same solution of 50mM Tris-HCl pH 7.5. (a) depicts human NFU added to ANS. The black line represents the baseline of ANS alone; while the red and green line represent the addition of ANS to full length NFU and its C-terminal domain, respectively. (b) represents the Scatchard plot of ANS binding to NFU. (c) represents the Scatchard plot of ANS binding to the C-terminal domain of NFU. (d) represents the control reactions. The black line represents the baseline of ANS alone. The red line represents the addition of carbonic anhydrase to ANS (negative control). The green line represents the addition of *T. maritima* IscU to ANS (positive control). (e) depicts the addition of human ISU to ANS. The red line represents the addition of ANS to human ISU, while the green line represents the addition of ANS to Zn-bound ISU. (f) depicts the addition of *T. maritima* IscU to ANS. The red line represents the addition of ANS to *T. maritima* IscU, while the green line represents the addition of ANS to Zn-bound IscU.
4.3.2 Tryptic Digestion Monitored by SDS-PAGE and Mass Spectrometry

The tryptic digestion method has been a very traditional method to probe the native structure of proteins. If the structure of the target protein was rigid, it could not be easily induced to fit in the trypsin digestion site [193]. However, in the case of molten globule and unstructured proteins, due to their flexible structure, they could be easily fitted in the digestion site of trypsin and the digestion was fast [193].

All data were fitted to first order decay with the function \( y = y_0 + A_1 e^{-x/t_1} \). \( y \) represents the percentage left with reference to the control point. \( X \) is the time scale. The digestion of the C-terminal domain of NFU indicated its intrinsic flexible nature. The fitting of the first order decay showed a \( t_1 \sim 1.74 \) min and the protein almost dissapeared within 2 min (Figure 4.2 a and b). In contrast, the well-structured protein carbonic anhydrase was digested only about 20% within 2 h and \( t_1 \sim 61 \) min (Figure 4.2)
c). Holo ferredoxin, a well-structured protein, and apo ferredoxin, an unstructured protein, were also used as a positive control and negative control, respectively. The fitting of the digestion of holo ferredoxin showed $t_1 \sim 21.3$ min and the fitting of the digestion of apo ferredoxin showed $t_1 \sim 1.9$ min (Figure d and e). These results were consistent with the mechanism of the tryptic digestion. However, holo ferredoxin was digested noticeably faster than carbonic anhydrase. This was because as soon as holo ferredoxin was cut, it lost the bound iron-sulfur cluster. The degradation of the iron-sulfur cluster converted the structured holo ferredoxin to unstructured apo ferredoxin, which subsequently became prone to tryptic digestion.

In the case of full-length NFU, an interesting phenomenon was discovered. During the digestion, the full-length NFU disappeared within 2 min ($t_1 \sim 1.05$ min); an intermediate product appeared during the digestion. The resistance to trypsin digestion implied the possession of a rigid tertiary structure. Next, LC-MS was used to investigate where the fragment was located on full-length NFU. The MS result indicated the intermediate product with a mass of 13237.8 Da (Figure 4.3). This was inferred to be the residue 17-135 of the His-tagged NFU. Considering the first 20 residues were the infused His-tag, the tryptic digestion resistant part was exactly the N-terminal domain of human NFU. The mass of the trypsin-digested intermediate product was also calculated at roughly about 15 kD based on the position of the molecular weight marker. The rough estimation was consistent with the MS results. The other peptide fragments were also observed, and the results were summarized as shown in Table 4.1.
In order to test whether the N-terminal domain had a rigid tertiary structure or not, the N-terminal domain of human NFU was cloned into pET-28 and the translated products were purified to homogeneity by the Ni-NTA column (Figure 4.4). The calculated mass of the N-terminal human NFU lacking the initial methionine was 14,860 Da, consistent with 14,864 Da of the ESI-MS results.

<table>
<thead>
<tr>
<th>Number of eluted peaks</th>
<th>Mass</th>
<th>Residue number in the His-tagged NFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1768.93Da</td>
<td>1-16</td>
</tr>
<tr>
<td>2</td>
<td>1689.98Da</td>
<td>142-156</td>
</tr>
<tr>
<td>3</td>
<td>1256.82Da</td>
<td>35-45</td>
</tr>
<tr>
<td>4</td>
<td>2015.02Da</td>
<td>17-34</td>
</tr>
<tr>
<td>5</td>
<td>1635.88Da</td>
<td>167-182</td>
</tr>
<tr>
<td>6</td>
<td>1324.74Da</td>
<td>46-57</td>
</tr>
<tr>
<td>7</td>
<td>2776.60Da</td>
<td>142-166</td>
</tr>
<tr>
<td>8</td>
<td>13237.8 Da</td>
<td>17-135</td>
</tr>
<tr>
<td>9</td>
<td>1457.84Da</td>
<td>72-84</td>
</tr>
<tr>
<td>10</td>
<td>5661.28Da</td>
<td>85-135</td>
</tr>
</tbody>
</table>

Table 4.1 Summary of the LC-MS results. The above chart is in the order of the eluted peptide fragments and the residue number is figured out by the mass of the fragment.
Figure 4. 2  Limited trypsin digestion experiment. The protein/trypsin samples were under the same ratio of 200:1. The same buffer of 50 mM Tris·HCl pH 7.5 was used for all protein samples. Trypsin and protein samples were incubated for the same span of time (a) represents the full-length human NFU tryptic digestion. (b) represents tryptic digestion of the truncated C-terminal domain of human NFU. (c) represents the tryptic digestion of the carbonic anhydrase. (d) represents the tryptic digestion of apo ferredoxin. (e) represents the tryptic digestion of holo ferredoxin. The protein at each timepoint was converted to percentage based on the intensity of the control lane without the addition of trypsin. Then the data were fitted to first order decay.
Limited Trypsin digestion Holo ferredoxin omitting the first point

- Data: Data1_B
- Model: ExpDec1
- $\chi^2 = 0.00063$
- $R^2 = 0.9934$
- $y_0 = 0 \pm 0$
- $A_1 = 0.7201 \pm 0.01886$
- $t_1 = 21.31539 \pm 1.63373$

Percentage Left

Time (min)

---

Limited Trypsin digestion Holo ferredoxin omitting the first point
Figure 4.3 The LC-MS result of the tryptic digested intermediate of full-length human NFU. The inlet represents the disappearance of human NFU and appearance of the intermediate. The detailed digestion condition and LC-MS were as described in ‘Material and Methods’.

Figure 4.4 SDS-PAGE of the N-terminal NFU purification. Lane 1 represents the elution of the N-terminal NFU from the Ni-NTA column. Lane 2 represents the low molecular weight protein marker.
4.3.3 Circular Dichroism of the N-terminal Domain of NFU

The secondary structure of N-terminal domain of human NFU was investigated in this experiment. The far UV CD showed that the N-terminal domain had good secondary structure (Figure 4.5). The online software K2D was used to calculate different components of the secondary structure based on the CD spectrum. The deconvoluted result was about 30% α helix, 14% β sheets, and 56% random coil, consistent with our predictions using the PSIPRED software (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html): 31% α helix, 12% β sheets, and 57% random coil.

![CD wavelength experiment for N-terminal domain human NFU](image)

**Figure 4.5** CD spectrum of the N-terminal domain of human NFU. The CD signal is converted to mean residue elipticity based on the protein concentration and number of residues. The N-terminal domain of human NFU is in 10 mM sodium phosphate pH 7.5 buffer. All parameters are adjusted as in ‘Material and Methods’.
Investigation of the Structural Property of the N-terminal Domain of Human NFU

The ANS-binding experiment was performed in this experiment. Under exactly the same condition as in the previous ANS-binding experiment, no significant fluorescence change was observed (Figure 4.6 a). This probing suggested that the N-terminal domain may not possess the molten globule property, unlike full-length NFU or truncated NFU. This was also consistent with our previous findings: the C-terminal domain of human NFU behaved similarly to full-length human NFU in the ANS-binding experiment.

A tryptic digestion experiment was also performed and 10% of the N-terminal domain was digested within 2 h (Figure 4.6 b). This also supported the idea that the N-terminal domain of human NFU was well folded. Despite the similarity, discrepancy was also observed here. What was seen here was in contrast to the full-length NFU digestion experiment, where the N-terminal domain finally disappeared within 2 h. The N-terminal domain alone seemed to be more resistant than within the full-length NFU. This phenomenon was explained as approximate effect. When full-length NFU was digested, the C-terminal domain interacted with trypsin and was digested very quickly. The fast interaction also brought the N-terminal domain near the tryptic digestion site or even inside the digestion site. When the digestion of the N-terminal domain alone was concerned, the N-terminal domain needed to be delivered to the tryptic digestion site first, and then digested. Thus, the first digestion in the previous experiment was more efficient than the second one in the current experiment.
Figure 4.6 The N-terminal NFU structural property study. (a) represents the ANS-binding experiment of the N-terminal NFU. (b) represents the limited tryptic digestion experiment of the N-terminal NFU. The first point was the control point without the addition of any trypsin. All the rest points were digested by trpsin for different time periods and quantified based on the control point. The intensity was converted to percentage based on the intensity of the first point and fitted to first order decay. All conditions were described in ‘Experiment’ part. The Y axes in both graphs were left intentionally since the scale of Y axis was comparable with Figure 4.1 and 4.2.
4.3.5 HSQC Study of the Tertiary Structure of Human NFU

High-resolution HSQC spectra were obtained for the three individual proteins (full-length NFU, the C-terminal domain, and the N-terminal domain). A lack of significant dispersion was observed in certain parts of the spectrum of the C-terminal domain (or full-length NFU). This indicated that the C-terminal domain was only partially folded (Figure 4.7 a and c). Given that the C-terminal domain (or full-length NFU) had only partially tertiary structure and possessed secondary structure, it could be defined as molten globule-like state. There are about 120 peaks on the HSQC spectrum of the C-terminal domain and it was consistent with the predicted number. In contrast, the spectra of the N-terminal domain showed significant signal dispersion, demonstrating that this part had very good tertiary structure (Figure 4.7 b). Interestingly, previous secondary structure analysis indicated that the N-terminal domain of human NFU and C-terminal domain possessed nearly identical amount of each secondary structure components. However, they showed significant differences in the dynamics of the tertiary structure in HSQC spectrum.

The spectra of the N-terminal and the C-terminal domains were overlayed with full-length NFU (Figure 4.7 c d e). The peaks from the N-terminal domain could match the ones from the full length NFU identically. However, some minor shifts were observed when the C-terminal domain overlayed with the full length protein. The overall spectra of the full length NFU and the C-terminal domain could overlap. Possibly, the linker part connected the N-terminal domain and the C-terminal domain in the full length protein. The linker was flexible and included in the C-terminal domain. When the N-terminal domain of NFU was not present, the flexible linker may influence
the environments of the residues from the C-terminal domain. Considering the HSQC technique was very sensitive to the environment, the N-terminal domain and the C-terminal domain structures were very similar to their structures in the full-length NFU. Previous ITC experiments did not detect the N-terminal and C-terminal domain interaction. The intact N-terminal and C-terminal domain structures within full-length NFU also supported that the two domains within full-length NFU were rather independent of each other. Experiments with the addition of a reducing reagent were also performed on the C-terminal domain NFU. However, the reduced NFU did not show any significant difference as the oxidized NFU.
Figure 4.7 $^1$H-$^{15}$N HSQC spectra experiment of human NFU. (a) represents the $^1$H-$^{15}$N HSQC spectrum of the C-terminal domain human NFU. (b) represents the $^1$H-$^{15}$N HSQC spectrum of the N-terminal domain human NFU. (c) represents the $^1$H-$^{15}$N HSQC spectrum of full-length human NFU. (d) represents the overlap of the $^1$H-$^{15}$N HSQC spectrum of full-length human NFU with the N-terminal domain of human NFU. The black color represents the peaks from the full-length NFU while the red color represents the peaks from the N-terminal domain of human NFU. (e) represents the overlap of the $^1$H-$^{15}$N HSQC spectrum of full-length human NFU with the C-terminal domain of human NFU. The black color represents the peaks from the full-length NFU while the green color represents the peaks from the C-terminal domain of human NFU. All conditions were as described in ‘Experiment’ part.
4.4 DISSCUSSION

4.4.1 The Significance of the Molten Globule Property of Human NFU

The molten globule state possesses a unique position in protein families. It is not a completely unfolded or folded protein. This family of proteins has a variety of biochemical characteristics which can be used to detect new members that belong to this family. Currently the methods used to identify the molten globule state property of proteins include: ANS binding [192], far UV CD signals showing high degree of secondary structure without significant signals in near UV region [196], and protease tryptic digestion [197,198]. However, comparison of these data between different
molten globule proteins is very difficult due to the fact that molten globule type proteins vary a lot. It could be any stage between fully folded and fully unfolded states. Some of them are more similar to a rigid protein fold while others are more close to the unstructured state. In addition, different induced conditions make the above experiment results more wide ranging.

Despite of this situation, the C-terminal domain of human NFU showed experiment results consistent with the molten globule property. The CD spectrum showed far UV CD signal reflecting high degree of secondary structure in contrast to no signal in the near UV region. This spectrum was characteristic of molten globule type protein CD spectrum [196]. ANS binding on the C-terminal domain of NFU showed great enhancement of fluorescence indicating it served as the molten globule type protein. In order to rule out the possibility that the fluorescence enhancement was caused by ANS binding to the hydrophobic patches of NFU, tryptic digestion was carried out. Here, the C-terminal domain of NFU was prone to the protease digestion, consistent with its proposed molten globule property. When full length NFU was used for the above two experiments, ANS binding experiment yielded similar results as the C-terminal domain of NFU, while the tryptic digestion showed an intermediate, different from the C-terminal domain results. The intermediate was later confirmed to be the N-terminal part of human NFU. The N-terminal domain possessed good structure as confirmed by ANS experiment and the tryptic digestion experiment. Thus, human NFU was demonstrated to possess a partially unfolded and partially folded structure. This qualifies NFU as a molten globule-type protein. The good structure of the N-terminal domain and the molten globule property of the C-terminal domain put
human NFU to a unique position. Most molten globule type proteins are induced under mild denaturation conditions and are currently thought to be an intermediate during the protein folding process [183,187,194]. Only a few exist as native molten globule to fulfill their functions [188,193]. Even in the family of native molten globules, proteins seldom show such distinctive features between two domains.

Here we try to correlate this structural property with the function of NFU. Previously, human NFU was demonstrated as a multifunctional protein implicated in different pathways [70,82,135]. Thus interaction with different partners is necessary. The C-terminal domain was demonstrated to be the functional domain with the molten globule property. The unfolded region in the C-terminal domain may provide human NFU with the flexibility to interact with its partners and serve as the docking site. Due to the unavailability of the tertiary structure information, it is hard to know precisely where the unfolded region lies now. However, due to the reason that the active site of human is the CXXC motif in the C-terminal domain, it is possible that the flexible unfolded region is near the CXXC motif to fulfill its function. The molten globule property may also be used to explain a few phenomena. In Chapter 3, the C-terminal domain of NFU was shown to interact with the HscA/HscB chaperone system. In general, chaperones are believed to assist protein folding [83]. Both proteins IscU or NFU were demonstrated to possess molten globule property, different from fully folded state [188]. Consequently, we propose that the molten globule property is the ultimate reason for the stimulation of HscA ATPase activity and interaction with the chaperone system.
The N-terminal domain is a totally different story. It has good structure. This domain is found only in eukaryotes while the bacterial NFU contains only the homolog of the C-terminal domain of human NFU. Why the homolog of the N-terminal domain is not present in the prokaryotes is still unknown. Possibly, the N-terminal domain of NFU may assist other physiological functions. During the study of lafora disease, human NFU was the candidate that interacted with laforin [82]. Further study showed that laforin could act as dual dephosphatase and human NFU could be dephosphorylated [82]. However, human NFU in this study was translated \textit{in vitro} with phosphorylated serine and tyrosine in place of non-phosphorylated ones. Therefore, all the serine and tyrosine were phosphorylated. This may not mimic the true situation \textit{in vivo} since it is impossible for NFU to have all serine and tyrosine residues phosphorylated. Despite that, this study still offers great insight about human NFU at molecular level. Possibly the phosphorylation can take place within the N-terminus and leads to subsequent structural rearrangement. The structural rearrangement may help to stabilize the unfolded region in the C-terminal domain or the rearrangement may endow the N-terminal domain completely different properties to interact with proteins which it can not interact, during the non-phosphorylated state. It is also possible that the phosphorylation takes place in the C-terminal domain and the phosphorylation may help to stabilize the structure of the C-terminal domain. If this is the case, phosphorylation may be used to switch the on and off stage of human NFU.
4.4.2 Comparision of the Molten Globule Property between Human NFU and T. maritima IscU

Interestingly, both NFU and IscU were demonstrated to act as molten globule-type proteins. They showed similar results in ANS-binding experiments [188]. However, sharp differences were also observed between the two proteins. First of all, during the HSQC experiments, *T. maritima* IscU showed a good dispersion of significant cross peaks in contrast to NFU, which lacked dispersion in certain regions [188]. Second, human NFU was prone to trypsin digestion, while *T. maritima* IscU was resistant to tryptic digestion [188].

These differences arose from their intrinsic properties. *T. maritima* IscU is actually a slow motion conformer [188]. This means that several conformations exist simultaneously in the solution and each of them is well-structured. The alternation between distinct conformer states is on the millisecond scale while common molten globule-like proteins exhibit nanosecond-picosecond scale [188]. For ANS binding experiment, it is possible that ANS can penetrate the hydrophobic core during the transition between different conformer states [188,199]. Dynamic experiments, which are not influenced by the conformation flexibility such as near UV CD or dynamic light scattering, are in agreement with the well-folded protein [188]. For trypsic digestion experiments, *T. maritima* IscU can withstand for a long time indicating the good structure of *T. maritima* IscU or possibly most tryptic digestion sites are buried inside the protein [188]. This property possibly correlates with its function. *T. maritima* IscU needs to form protein complex with its apo target and transfer the intact [2Fe-2S] [188]. This requires IscU to have flexible parts and form intermediates before the iron-sulfur
cluster can be finally transferred [188]. Thus, it is quite possible that the binding apo
target proteins help to stabilize one conformation state [188]. Since the iron-sulfur
cluster transfer is a universal role for IscU family, it is quite possible that IscU
homologs from other organisms also possess the molten globule property [188]. A
contradictory view is T.maritima IscU is more like sufU and this makes its property
unusual [8]. In our work, we tested human ISU in ANS binding experiments. It showed
similar signals as the T.maritima IscU. This indicated that the molten globule like
property may be a general property for this family. Zn metal was found to stabilize the
tertiary structure of IscU [195]. Whether Zn metal can stabilize the confirmation of
IscU remained an interesting topic to explore. We tested the Zn bound IscU in ANS
binding experiment. Both human and T.maritima IscU showed no difference as the
unbound IscU. The possibility for Zn to stabilize IscU in one confirmation state is ruled
out.

In the case of human NFU, its molten globule property lies in the fact that it is
partially folded. This makes human NFU more like a traditional molten globule in
contrast to the MDC (multiple discrete conformers) model proteins, such as T. maritima
IscU. The ANS binding experiments and tryptic digestion experiments are in agreement
with the molten globule property of the C-terminal domain. Since NFU can bind iron,
whether iron can stop the molten globule property of NFU is explored here. The HSQC
spectra did not show any shift when supplemented with iron. Oxidized NFU and reduce
NFU HSQC spectra were also compared in the HSQC experiments. They did not show
any significant difference. Thus adding iron or reducing NFU did not contribute to
stopping its molten globule property. Possibly, interaction with other proteins may help
to stop the molten globule property of human NFU or phosphorylation may help as well. The molten globule property can be correlated with the function of NFU. Different from IscU, human NFU is possibly implicated in pathways other than iron-sulfur cluster biosynthesis [82]. Different partners may be needed to interact with the multifunctional protein NFU for functional purpose.
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