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Iron−Sulfur Cluster Biosynthesis: Characterization of a Molten Globule Domain in Human NFU[†]

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ABSTRACT: Human NFU (also known as HIRIP5) has been implicated in cellular iron−sulfur cluster biosynthesis. Bacterial and yeast forms are smaller than the human protein and are homologous to the C-terminal domain of human NFU. This C-terminal domain contains a pair of redox active cysteines and demonstrates thioredoxin-like activity by both binding to and mediating persulfide bond cleavage of sulfur-loaded IscS, the sulfide donor for [2Fe-2S] cluster assembly on ISU-type scaffold proteins. Herein, human NFU is shown to possess a novel combination of a molten globule-type C-terminal domain and an N-terminal domain with a fully folded regular tertiary structure. The molten globule characteristics of the C-terminal domain have been evaluated by 1-anilino-8-naphthalenesulfonic acid binding, the kinetics of trypsin digestion, and heteronuclear single-quantum coherence nuclear magnetic resonance studies. Human NFU is a functionally competent reducing agent for cysteinyl persulfide bond cleavage, releasing inorganic sulfide for incorporation into the ISU-bound [2Fe-2S] cluster, a reactivity that might be facilitated by the flexibility of the C-terminal domain.

NFU has highly conserved homologues in both prokaryotes and eukaryotes (Figure 1) (*1*), suggesting a significant cellular role for this protein. Two distinct isoforms of human NFU have been identified, with one isoform localized in the mitochondrion and another localized in the cytosol and nucleus (*2*). As a multifunctional protein, human NFU has been found to interact with HIRA,[1] and therefore named HiRIP5 (HIRA interacting protein, where HIRA is the histone cell cycle regulation homologue A), and has been suggested to play a role in transcriptional regulation at the level of local chromatin structure (*1*, *3*). Recent studies also show that human NFU could interact with laforin, mutations of which lead to lafora disease (*4*), while genetic evidence had earlier suggested a role for NFU in iron homeostasis, perhaps in iron−sulfur cluster assembly (*5*).

Prior studies have begun to probe the functional chemistry of this protein in a variety of organisms (*1*, *2*, *6*), while human NFU has been implicated in several physiological pathways and may be multifunctional (*1*, *2*, *4*). While earlier reports have suggested that NFU serves as an alternative scaffold protein in iron−sulfur cluster assembly (*2*, *6*, *7*), we have recently implicated thioredoxin-like activity for the C-terminal domain of human NFU, which appears to act as a persulfide reductase associated with the sulfide donor protein IscS in iron−sulfur cluster biosynthesis (*8*, *9*). This domain was found both to bind to IscS with micromolar affinity and to mediate cleavage of the IscS persulfide bond. The competency of this domain to mediate sulfide delivery in a [2Fe-2S] cluster assembly assay using ISU-type Fe−S cluster scaffold proteins was also demonstrated. It should be noted that the term ISU is used for the human iron−sulfur cluster scaffold protein while the term IscU is used for bacterial homologues.

In this paper, we report experiments that investigate the conformational properties of human NFU. In particular, the C-terminal domain of human NFU is demonstrated to show molten globule-type structural behavior that may be significant for function, while the N-terminal domain is well-structured. The behavior of each domain is also reflected in the properties of full-length NFU.

MATERIALS AND METHODS

General Chemicals. All restriction enzymes were obtained from Invitrogen (Carlsbad, CA). The Qiaquick gel extraction kit and Ni-NTA columns were from Qiagen (Valencia, CA). BL21-(DE3) cells and pET28 vectors were from Novagen (Madison, WI). Primers were obtained from Integrated DNA Technologies Inc. (Coralville, IA), and CM 32 and DE 52 ion exchange resins were from Whatman (Aston, PA). Homogeneous-20 precast polyacrylamide gels and G-25 resin were obtained from Pharmacia (Peapack, NJ), and ANS was from Invitrogen Inc. (Eugene, OR). Carbonic anhydrase and trypsin were obtained from Sigma Aldrich Inc. (St. Louis, MO). Ammonium chloride (99% ^{15}N) was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA).

Proteins. Experimental details of the cloning, expression, and purification of NFU and the N- and C-terminal domains have

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[1]Abbreviations: ANS, 1-anilino-8-naphthalenesulfonic acid; CD, circular dichroism; DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; HIRA, histone cell cycle regulation homologue A; IPTG, isopropyl β-D-thiogalactopyranoside; *isc*, iron sulfur cluster; ITC, isothermal titration calorimetry; LB, Luria-Bertani; M_w, molecular weight; *nif*, nitrogen fixation; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; LC−MS, liquid chromatography and mass spectrometry; Ni-NTA, nickel nitriloacetic acid.

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1 GSSHHHHHHS SGLVPRGSHM FIQTQDTPNP NSLKFIPGKP VLETRTMDFP TPAAAFRSPL

61 ARQLFRIEGV KSVFFGPDFI TVTKENEELD WNLLKPDIYA TIMDFFASGL PLVTEETPSG

121 EAGSEEDDEV VA**MIKELLDT RIRPTVQEDG GDVIYKGFED GIVQLKLQGS CTSCPSSIIT**

181 LKNGIQNMLQ FYIPEVEGVE QVMDDESDEK EANSP

FIGURE 1: Primary amino acid sequence of expressed recombinant full-length human NFU with an N-terminal His_6 tag. The underlined residues represent the region that is resistant to tryptic digestion, while the sequence in boldface corresponds to the C-terminal domain of human NFU. The residue numbering shown in Table 1 is based on this sequence.

Table 1: Summary of LC−MS Results following Tryptic Digestion[a]

eluted peak	mass (Da)	fragment by residue numbering (see Figure 1)
1	1768.93	1−16
2	1689.98	142−156
3	1256.82	35−45
4	2015.02	17−34
5	1635.88	167−182
6	1324.74	46−57
7	2776.60	142−166
8	13237.8	17−135
9	1457.84	72−84
10	5661.28	85−135

[a] The data are arranged according to the order of elution of the peptide fragments. The residue numbering was determined from the mass of the fragment.

been reported elsewhere (*9*), as has the purification of recombinant human ferredoxin (*10*, *11*).

ANS Binding. The binding of ANS to protein samples was monitored by use of a PerkinElmer Life Science LS50B luminescence spectrometer, with an excitation wavelength of 371 nm and a slit width of 3 nm, while the emission was monitored at 482 nm with a slit width of 10 nm at 25 °C. Samples were prepared in 50 mM Tris-HCl (pH 7.5), and a baseline was measured with 10 μM ANS alone. The fluorescence enhancement of 10 μM ANS when 100 μM protein samples were added was compared with the baseline. The ANS binding experiments were performed both on human full-length NFU and on the truncated N- and C-terminal domains.

Thermotoga maritima (*Tm*) IscU has previously been demonstrated to possess molten globule characteristics and served as a positive control (*12*). ANS binding experiments with the well-structured protein carbonic anhydrase served as a negative control, while ANS binding to human ISU was also examined. Reverse titration experiments were used to correlate the fluorescence response with the moles of ANS bound to the protein, as previously described (*13*), and the binding affinity was obtained through a Scatchard analysis of the variation of bound ANS with protein concentration (*14*).

Tryptic Digestion Monitored by SDS−PAGE and Mass Spectrometry. Reactions were conducted as previously described with a few minor modifications as detailed below (*12*). Digestion of the well-structured carbonic anhydrase served as a negative control, while digestion of apoferredoxin served as a positive control. All protein samples (NFU, truncated C-terminal NFU, carbonic anhydrase, and apoferredoxin) were adjusted to the same concentration (~800 μM) and dialyzed against 50 mM Tris-HCl (pH 7.5). The ratio of trypsin to protein was set at 1:200, and the mixture was incubated at 37 °C. Aliquots were removed at 1, 2, 5, 15, 30, and 60 min time intervals and then mixed with SDS−PAGE loading buffer containing phenylmethanesulfonyl fluoride (PMSF), before immediately being plunged into a hot water bath. After being boiled for 5 min, the sample was maintained on ice until all the samples were ready for loading. The first reference point was similarly treated but lacked the addition of trypsin. After being stained and destained, the SDS−PAGE gel was subjected to quantification by use of a Bio-Rad Gel-Doc imager. All tryptic digestion experiments were performed under identical conditions. The background was subtracted and each lane converted to the percentage of band remaining, relative to the intensity of the reference point, before the data were fitted to a first-order decay profile.

LC−MS Evaluation of Tryptic Digest Intermediates. The digestion of full-length NFU by trypsin resulted in formation of an intermediate species, so LC−MS was used to determine the part of the human NFU sequence to which this corresponded. The trypsin-digested sample was separated by reverse phase high-performance liquid chromatography (Waters) using water with 0.1% trifluoroacetic acid as mobile phase A and acetonitrile with 0.1% trifluoroacetic acid as mobile phase B. An aliquot (10 μL) of the sample was then injected and separated on a Vydac C18 MS column. The composition of mobile phase B was maintained at 2% for 2 min. Subsequently, the concentration was linearly increased from 2 to 95% over a period of 28 min. The column was washed with 98% B for 1 min and equilibrated with 2% B for 29 min, prior to injection of the next sample. The flow rate was adjusted to 50 μL/min, and the eluates were directly infused into a Micromass LCT (Micromass, Wythenshawe, U.K.) mass spectrometer with an orthogonal electrospray source (Z-spray) to determine the molecular weight. The optimized conditions for electrospray ionization mass spectrometry (ESI-MS) included a capillary voltage of 3000 V, a source temperature of 100 °C, and a cone voltage of 50 V. Data were acquired in continuum mode, and the rate was one scan per second. All spectra were acquired in the positive ion mode with NaI used for external mass calibration over an m/z range from 500 to 2500 Da.

Circular Dichroism of Full-Length NFU and the N- and C-Terminal Domains. Circular dichroism data were collected as previously reported (*9*).

HSQC Study of Human NFU. BL21 lysozyme plus (DE3) served as the protein expression host. The tertiary structure of full-length NFU, the C-terminal domain, and the N-terminal domain of NFU were probed by comparison of the $^{1}H-^{15}N$ HSQC spectra. The expression conditions for the three proteins were as follows. Cell mass from a 10 mL overnight culture was collected by centrifugation at 4000 rpm and transferred to 1 L of M9 medium supplemented with ammonium chloride (99% ^{15}N). The cells were subsequently grown at 37 °C for 8 h before addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and then grown at 30 °C for an additional 18 h. Both cell

harvesting and protein purification procedures were carried out as described previously (*8*). NMR measurements were taken in 20 mM sodium phosphate and 50 mM NaCl (pH 7.5) in 10% D_2O. $^1H-^{15}N$ HSQC spectra were recorded at The Ohio State University Campus Chemical Instrument Center on a Bruker DMX-600 spectrometer equipped with a triple-resonance probe and three-axis gradient coils at 298 K (*15*). A data matrix of 2048 × 256 was recorded for each sample with an experimental time of ~1.5 h. Data were processed with XWINNMR3.5 (Bruker, Inc.).

RESULTS

Near-UV Circular Dichroism of NFU and Its N- and C-Terminal Domains. Results from a prior circular dichroism (CD) analysis of NFU secondary structure have previously been described and discussed (*9*). The near-UV region of the CD spectrum of a protein is sensitive to the tertiary structural environment of the aromatic residues, with only fully folded proteins showing dominant signals in this region, while fully unfolded protein and molten globule proteins do not show such prominent features (*16*). CD spectra for full-length NFU and each of the N- and C-terminal domains (Figure 2) show that while the C-terminal domain exhibited almost no signal in the near-UV region, data for the N-terminal domain and full-length NFU display pronounced signals that suggest the presence of at least, in part, a well-defined tertiary structure. Accordingly, additional experiments were performed to probe the structural properties of each domain.

ANS Binding. Binding of ANS has frequently been used to probe the structure of molten globule proteins, since the fluorescence response is sensitive to environment. ANS fluorescence is negligible in strongly polar aqueous environments; however, in the case of molten globule-type proteins, where ANS can penetrate and bind to the hydrophobic core of the protein with shielding from the polar exterior, the fluorescence is greatly increased and the emission maximum shifts to ~480 nm (*17*). Addition of either full-length human NFU or its C-terminal domain to a solution of ANS resulted in a significant enhancement of fluorescence, suggesting molten globule-type properties (Figure 3). The dissociation constants (K_D) for dissociation of ANS from both full-length human NFU and the C-terminal domains were obtained from a Scatchard plot, with values of 4 ± 1 and 9 ± 2 μM, respectively (Figure 3). The ratio of protein to ANS was determined to be 1:1.6 ± 0.1 for full-length NFU and 1:1.3 ± 0.1 for the C-terminal domain (*18*). The dissociation constants are apparent K_D values since varying numbers of ANS molecules can bind with discrete quantum yields and are reflected by the noninteger ANS binding stoichiometry. The clear increase in ANS emission is, however, diagnostic of molten globule behavior. Similar studies of the N-terminal domain provided no evidence of significant fluorescence enhancement, so it appears that human NFU possesses a tertiary structure with a flexible ANS binding pocket in its C-terminal domain.

Additional positive and negative controls were performed. ANS displays a significant enhancement of fluorescence at 482 nm (Figure 3B) following binding of *T. maritima* IscU, as previously observed (*12*) and consistent with conformational flexibility. Carbonic anhydrase, a well-structured protein under normal conditions, was used as a negative control (Figure 3B), and the enhancement of ANS fluorescence at 482 nm was found to be a relatively small fraction of that observed for NFU, which is again

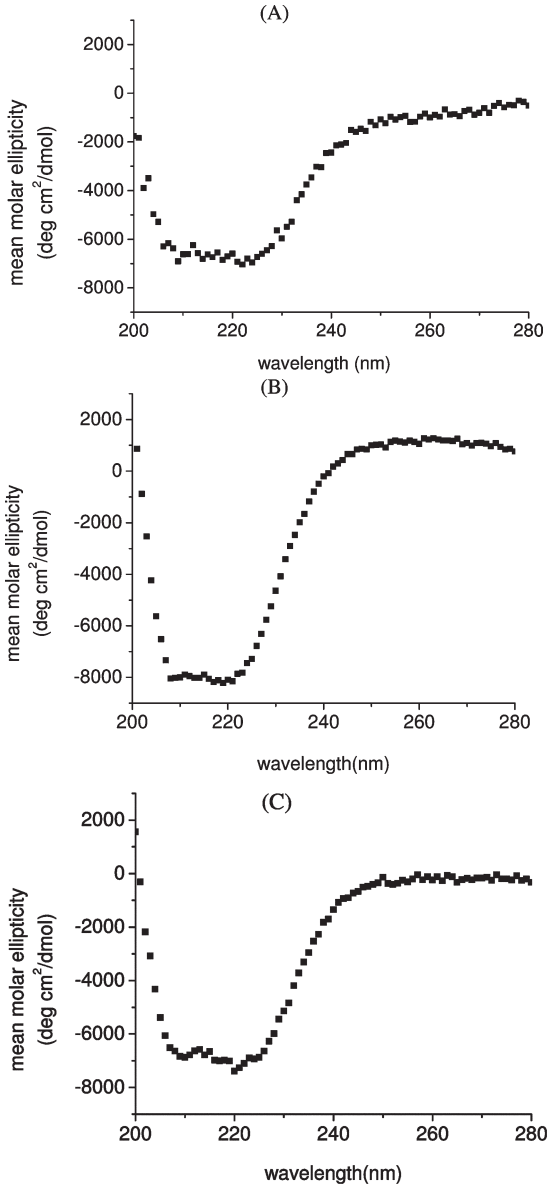

FIGURE 2: CD spectra of human NFU and its domains. The CD signal was converted to the mean residue ellipticity based on the protein concentration and the number of residues. Spectra were collected in 10 mM sodium phosphate buffer (pH 7.5) as described in ref *8*: (A) N-terminal domain, (B) C-terminal domain, and (C) full length NFU.

consistent with expectations based on previous research (*19*). ANS binding experiments were also conducted on human ISU. Similar to *T. maritima* IscU, the ANS−human ISU complex was also found to exhibit a significant enhancement of fluorescence yield (Figure 3A). While it has previously been suggested that the conformational flexibility associated with *Tm* IscU could be unique (*20*), the data described here for human ISU suggest that this behavior is likely to be common to this family and functionally important for the role of a scaffold protein in iron−sulfur cluster biosynthesis and interactions with various partner proteins. Such flexibility is also consistent with difficulties in obtaining structural information (by NMR or X-ray) in the absence of bound metal cofactors. While several reports have described the structural characterization of IscUs from a variety of organisms (*21*, *22*), all of these were of the zinc-bound [or recently a cluster-bound (*23*)] form, consistent with the conformational flexibility that we have previously described

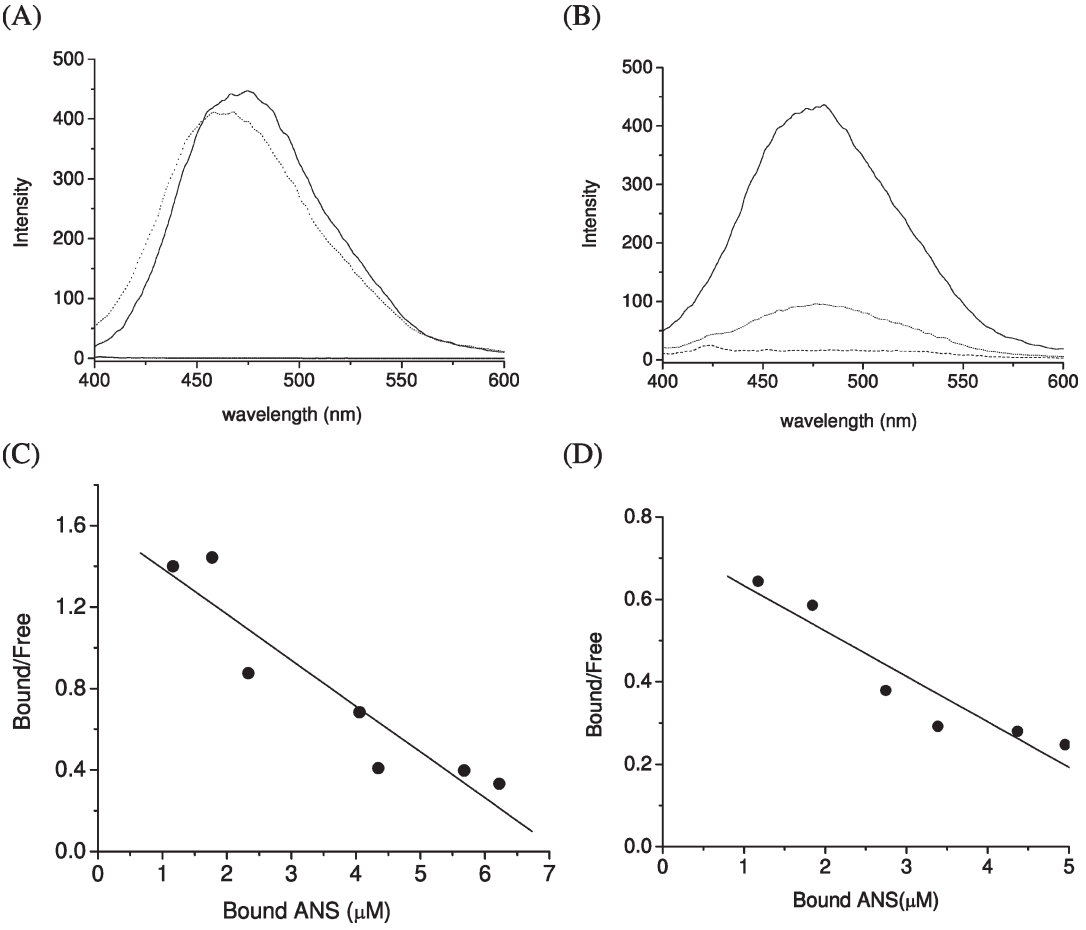

FIGURE 3: NFU ANS binding experiments. For each experiment, the sample was prepared by dialysis in 50 mM Tris-HCl (pH 7.5). (A) Addition of human NFU to ANS, where the dashed line represents the baseline of ANS alone and the solid and dotted lines show the ANS fluorescence response following addition of full-length NFU and its C-terminal domain, respectively. (B) Control reactions, where the dashed line represents the baseline of ANS alone, the dotted line represents the addition of carbonic anhydrase to ANS (negative control), and the solid line represents the addition of *T. maritima* IscU to ANS (positive control). (C) Scatchard plot for ANS binding to NFU. (D) Scatchard plot for ANS binding to the C-terminal domain of NFU.

as a functionally important attribute for this class of protein (*12*, *24*).

Tryptic Digestion Monitored by SDS−PAGE and Mass Spectrometry. Tryptic digestion is a widely used technique for probing the native structure of proteins. Rigid structured proteins are not readily digested by trypsin (*18*), while in the case of molten globule and unstructured proteins the structural flexibility allows facile access and more rapid cleavage. Rapid tryptic digestion of the C-terminal domain of NFU was observed (Figure 4) and supports the intrinsic flexibility of this domain. Fitting of the first-order decay yielded a rate constant k of ~0.54 min^{-1} with almost complete loss of the protein within 5 min (Figure 4). In contrast, the well-structured protein carbonic anhydrase was digested only to the extent of ~20% within 1 h with a measured rate constant k of ~0.004 min^{-1} (Figure 4). Holoferredoxin, a well-structured protein, and apoferredoxin, an unstructured protein, were also used as negative and positive controls, respectively. Fitting the digestion profile for holoferredoxin yielded a rate constant k of ~0.045 min^{-1}, while the digestion profile for apoferredoxin yielded a k of ~0.53 min^{-1} (Figure 4). These results are consistent with the mechanism of tryptic digestion, with the noticeably faster disappearance of the band for holoferredoxin, relative to carbonic anhydrase, most likely reflecting loss of the iron−sulfur cluster following backbone cleavage and subsequent loss of tertiary structure.

An interesting phenomenon was noted in the case of full-length NFU. During digestion of full-length NFU, the starting band disappeared within 2 min ($k \sim 0.96$ min^{-1}) and an intermediate product appeared. Resistance to trypsin digestion implied the possession of a rigid tertiary structure. LC−MS studies demonstrated that this intermediate product, with a mass of 13237.8 Da (Figure 4), corresponded to the N-terminal domain (from residue 17 to 135 of the His-tagged NFU). A tryptic digestion experiment was also performed, and only 10% of the N-terminal domain was digested within 2 h (Figure 4), consistent with a well-defined tertiary fold for the N-terminal domain of human NFU.

$^{1}H-^{15}N$ HSQC Study of the Tertiary Structure of Human NFU. High-resolution $^{1}H-^{15}N$ heteronuclear single-quantum coherence (HSQC) spectra were obtained for the three individual proteins (full-length NFU, the C-terminal domain, and the N-terminal domain). Approximately 120 peaks were observed in the $^{1}H-^{15}N$ HSQC spectrum of the C-terminal domain, which is consistent with the predicted number. A lack of significant dispersion was observed in certain parts of the spectrum of the C-terminal domain (or full-length NFU), consistent with a partially folded protein (Figure 5). The demonstration of secondary structure through circular dichroism experiments, but apparent partial loss of tertiary structure, is consistent with a molten globule-like state. In contrast, the spectrum of the N-terminal domain showed significant signal dispersion, consistent

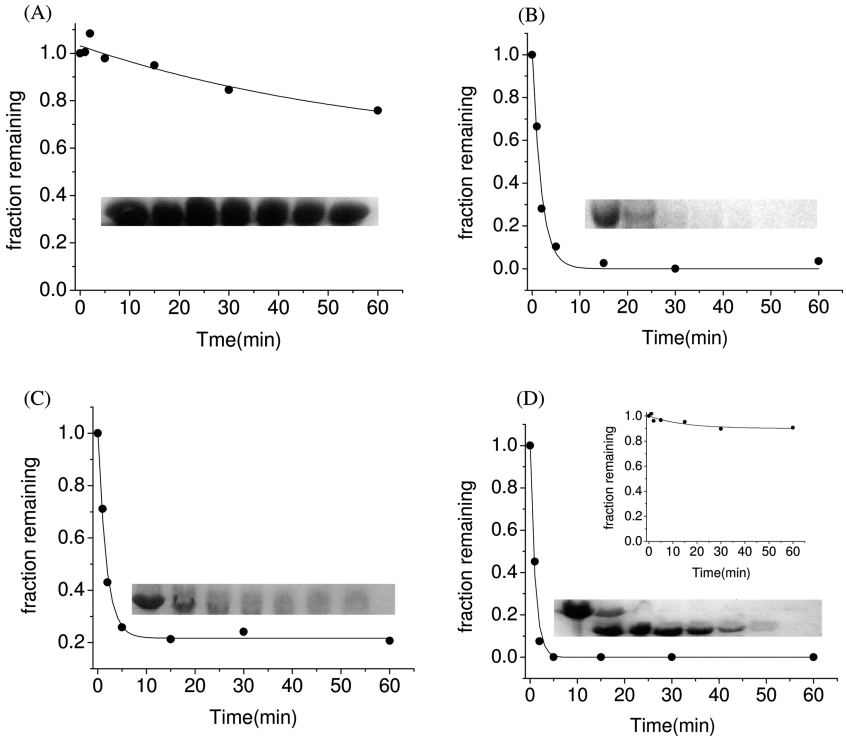

FIGURE 4: Limited trypsin digestion of various proteins. All protein concentrations were similar, and the ratio of protein to trypsin was 200:1. Protein samples were maintained in 50 mM Tris-HCl (pH 7.5). (A) Results of trypsin digestion of carbonic anhydrase. (B) Results of the limited trypsin digestion of apoferredoxin. (C) Results of trypsin digestion of the truncated C-terminal domain of human NFU. (D) Results of trypsin digestion of full-length human NFU. The protein concentration at each time point was converted to the fraction remaining, based on the protein intensity prior to addition of trypsin, and the resulting decay profile was fit to a first-order decay. The inset shows the formation of a relatively stable intermediate arising from a rapid initial digestion of the full-length protein. Mass spectrometric analysis reveals this band to correspond to the N-terminal domain. The recombinantly expressed N-terminal domain is even more stable with respect to tryptic digestion (see the inset), as noted in Discussion.

with a well-defined tertiary structure (Figure 5). Interestingly, prior secondary structure analysis by circular dichroism has indicated that both the N-terminal and C-terminal domains of human NFU exhibit equivalent degrees of secondary structure (*9*). However, they clearly show significant differences in the definition of their tertiary structure, as suggested by comparison of their $^{1}H-^{15}N$ HSQC spectra.

Overlaying the spectra of the N-terminal and C-terminal domains with the full-length version of NFU (Supporting Information) demonstrated that cross-peaks from the N-terminal domain matched well those from full-length NFU. The overall spectrum of full-length NFU and the C-terminal domain were also found to show significant overlap; however, some minor shifts were observed. Future assignment of the HSQC spectra should allow a clearer understanding of the location and significance of these small shift differences, which most likely reflect the absence of the rigid N-terminal flexible domain and the impact on the flexibility and microenvironment of the C-terminal domain. Considering the sensitivity of the HSQC technique to the microenvironment, the data suggest that the structural forms that emerge for the isolated N-terminal and C-terminal domains are likely to be very similar to those structures adopted in full-length NFU. Previous isothermal titration calorimetry (ITC) experiments did not detect any interaction between the N-terminal and C-terminal domains (*9*). The apparent structural similarity of the discrete N- and C-terminal domains, relative to full-length NFU, also supports the independence of the two domains within full-length NFU. Given the presence of two redox active Cys residues, $^{1}H-^{15}N$ HSQC spectra were also recorded following the addition of a reducing reagent, such as dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine hydrochloride (TCEP), to solutions of the C-terminal domain of NFU; however, the reduced NFU did not show any significant difference relative to the oxidized protein. The extent of Cys oxidation was verified by DTNB titration as previously described (*8*, *9*).

DISCUSSION

Since it is neither completely folded nor unfolded, a molten globule protein possesses unique biochemical characteristics that serve as markers of this state. These include ANS binding (*17*), far-UV CD signals that reflect a high degree of secondary structure [but lacking a significant response in the near-UV region (*25*)], and the efficiency of tryptic digestion (*26*, *27*). Comparison of these data between various molten globule proteins is, however, very difficult, since molten globule behavior falls into a continuum from almost fully folded to almost fully unfolded states. In the case of induced molten globule states, variations in solution conditions make it even more difficult to compare the intrinsic molten globule properties of proteins.

Nevertheless, the C-terminal domain of human NFU shows experimental results that are consistent with those expected from a molten globule protein. The CD spectrum shows a far-UV CD signal that reflects a high degree of secondary structure and contrasts with the absence of signal in the near-UV region, a hallmark of molten globule behavior (*25*). ANS binding to the C-terminal domain of NFU also demonstrated a significant enhancement of fluorescence, again consistent the molten globule state. To obtain further support for a molten globule state, tryptic

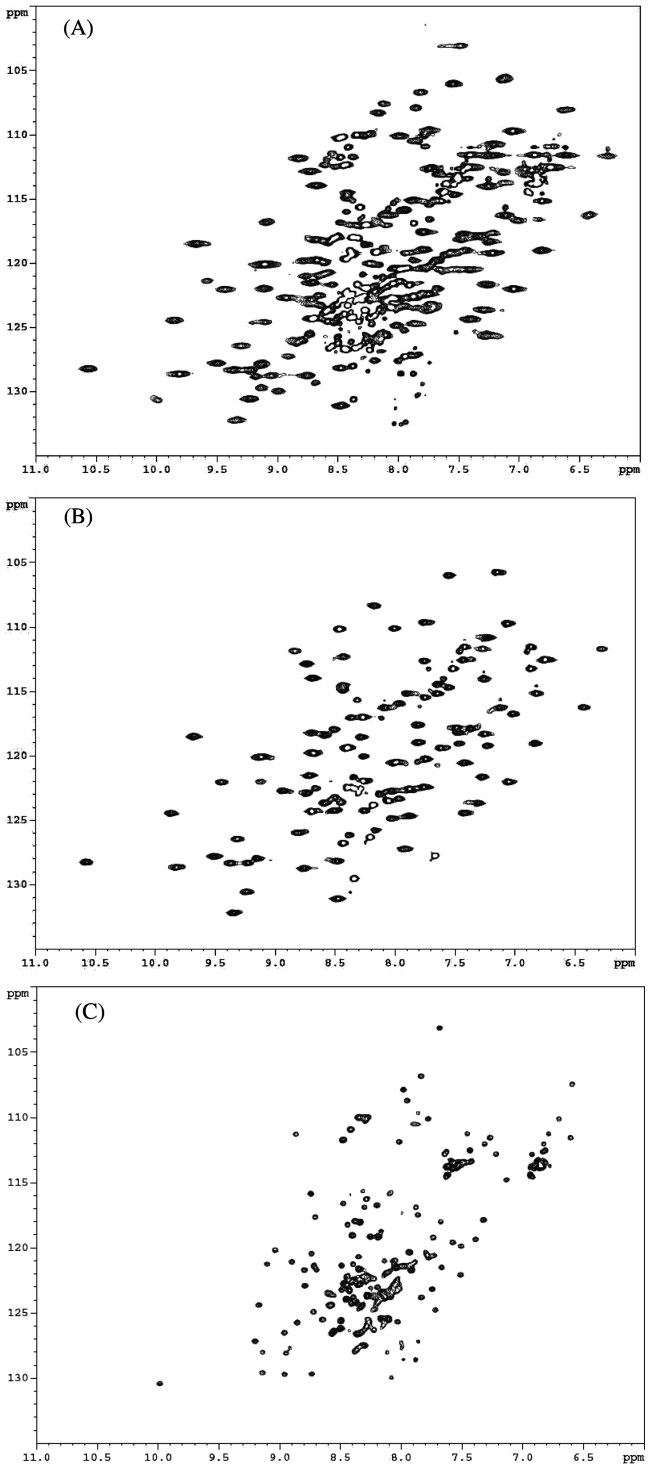

FIGURE 5: $^{1}H-^{15}N$ HSQC spectra obtained for (A) full-length human NFU, (B) the N-terminal domain of human NFU, and (C) the C-terminal domain of human NFU.

digestion experiments were performed where it was observed that the C-terminal domain of NFU was prone to protease digestion, consistent with its proposed molten globule property. When full-length NFU was used for the two experiments described above, the ANS binding experiment yielded results similar to those obtained for the C-terminal domain of NFU, while the tryptic digestion experiments showed formation of an intermediate species that was not observed in experiments with the C-terminal domain alone. The intermediate was confirmed by mass spectrometry to be the N-terminal part of human NFU.

Despite the overall consistency of the tryptic digestion/MS experiments, a possible discrepancy was noted. In contrast to the digestion experiment with full-length NFU, where the N-terminal domain finally disappeared within 1 h, the N-terminal domain alone seemed to be more resistant to tryptic digestion than the same domain within full-length NFU. This most likely reflects structural perturbation of the C-terminal end of the N-terminal domain by the presence of residual fragments following tryptic cleavage of the C-terminal domain between residues 135 and 136 (confirmed by mass spectrometry), making the N-terminal domain more accessible to trypsin digestion.

The N-terminal domain possesses a well-defined structure, as confirmed by ANS binding, tryptic digestion, and $^{1}H-^{15}N$ HSQC experiments, so human NFU is demonstrated to possess a structured N-terminal domain and molten globule C-terminal domain, which places human NFU in a unique position. Most molten globule-type proteins are induced under mild denaturation conditions and are currently thought to represent intermediate states during protein folding (*19*, *28*, *29*). Only a few proteins exist as native molten globules in their functional states (*12*, *18*). Even in the family of native molten globules, proteins seldom show such distinctive features between two separate domains, with the calmodulin-like skin protein (CLSP) as one of the few previously characterized examples of such (*30*).

While it is possible that the molten globule state could reflect the absence of stabilizing post-translational modifications in the recombinant protein, both the demonstrated ability of the C-terminal domain to bind to physiologically relevant partners and the persulfide reductase activity of this domain suggest that the protein is in a fully functional state (*9*).

Interestingly, both NFU and IscU display molten globule-type characteristics (*12*); however, significant differences are also noted. First, $^{1}H-^{15}N$ HSQC data for *T. maritima* IscU displayed a dispersion of cross-peaks that is typical of a well-structured protein, in contrast to NFU which lacked dispersion in certain regions (*12*). Second, human NFU was prone to trypsin digestion, while *T. maritima* IscU was resistant to tryptic digestion (*12*). These differences reflect the distinct structural characteristics of each protein. *T. maritima* IscU appears to undergo conformational isomerism between discrete well-structured states (*12*). The alternation between distinct conformer states is on the microsecond time scale, while common molten globule-like proteins exhibit a nanosecond to picosecond scale (*24*). In tryptic digestion experiments, the well-structured conformers of *T. maritima* IscU can withstand tryptic digestion, while in the case of human NFU, the partial unfolding that underlies the molten globule state facilitates rapid digestion. Such differences most likely reflect the greater functional complexity of human NFU, which has been implicated in pathways other than iron−sulfur cluster biosynthesis (*4*) and is required to interact with a larger pool of partner proteins.

In conclusion, we have characterized unusual and distinct structural behavior for the two-domain human NFU. The conformational mobility suggested for the C-terminal "persulfide reductase" domain of NFU appears to be consistent with the flexibility characterized in the ISU-type proteins that serve a distinct scaffolding role. It appears that conformational flexibility is a functional requirement for certain proteins involved in the biosynthesis of complex iron−sulfur clusters, and a feature that might be identified in proteins involved in the assembly of other families of metallocofactors.

SUPPORTING INFORMATION AVAILABLE

Experimental details and plots for selected $^{1}H-^{15}N$ HSQC experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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