nifU Gene Product from *Azotobacter vinelandii* Is a Homodimer That Contains Two Identical [2Fe-2S] Clusters†

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ABSTRACT: The *nifU* gene product is required for the full activation of the metalloenzyme nitrogenase, the catalytic component of biological nitrogen fixation. In the present work, a hybrid plasmid that contains the *Azotobacter vinelandii nifU* gene was constructed and used to hyperexpress the NIFU protein in *Escherichia coli*. Recombinant NIFU was purified to homogeneity and was found to be a homodimer of 33-kDa subunits with approximately two Fe atoms per subunit. The combination of UV/visible absorption, variable-temperature magnetic circular dichroism, EPR, and resonance Raman spectroscopies shows the presence of a $[2Fe\text{-}2S]^{2+,+}$ center ($E_m = -254$ mV) with complete cysteinyl coordination in each subunit. The electronic, magnetic, and vibrational properties of the $[2Fe\text{-}2S]^{2+,+}$ center do not conform to those established for any of the spectroscopically distinct types of 2Fe ferredoxins. These distinctive properties appear to be a consequence of a novel arrangement of coordinating cysteinyl residues in NIFU, and the residues likely to be involved in cluster coordination are discussed in light of primary sequence comparisons to other putative [2Fe-2S] proteins. The observed physicochemical properties of NIFU and its constituent [2Fe-2S] cluster also provide insight into the role of this protein in nitrogenase metallocluster biosynthesis.

The reduction of dinitrogen to yield ammonia is called nitrogen fixation, and the biological process is catalyzed by nitrogenase, a complex metalloenzyme comprising two separately purifiable component proteins commonly referred to as the Fe protein and the MoFe protein [reviewed in Dean and Jacobson (1992)]. Nitrogenase turnover involves sequential single-electron deliveries from the Fe protein to the MoFe protein by a process that involves hydrolysis of at least two MgATP for each electron transferred and is accompanied by an association and dissociation of the component proteins. Reduction of the Fe protein is accomplished by transfer of electrons from either a flavodoxin or a ferredoxin *in vivo* or by dithionite in *in vitro* assays. The primary sites for MgATP binding and hydrolysis are located on the Fe protein (a γ_2 homodimer that contains a single cubane-type Fe_4S_4 cluster), whereas the sites for substrate binding and reduction are located on the MoFe protein (an $\alpha_2\beta_2$ heterotetramer that contains two Fe_8S_{7-8} clusters and two $MoFe_7S_9$:homocitrate cofactors). The individual metalloclusters contained within the nitrogenase component proteins are very likely to participate in either the inter- or the intramolecular electron transfers required for substrate reduction and/or to provide the site of substrate reduction. See Kim and Rees (1994) or Dean et al. (1993) for brief reviews on the structures of the nitrogenase component proteins and their associated metal clusters.

The biochemical complexity of nitrogenase is reflected in the number and organization of genes required to form the active complex. For example, in addition to those genes that encode the nitrogenase structural components, there is an associated consortium of *nif*-specific genes whose products are required for formation and insertion of the appropriate metalloclusters into their complementary protein partners [see Dean and Jacobson (1992) and Dean et al. (1993) for reviews]. Included among these genes are *nifS* and *nifU*, which in *Azotobacter vinelandii* and *Klebsiella pneumoniae* are cotranscribed and adjacently located on their respective genomes (Beynon et al., 1987). The *A. vinelandii nifU* and *nifS* gene products (hereinafter referred to as NIFU and NIFS, respectively)[1] are distinguished from other *nif* gene products from this organism in that the activities of both are required for the full activation of both nitrogenase component proteins. Thus, because the acquisition of iron and inorganic sulfide for Fe-S core formation is the one feature required for activation of both of the nitrogenase component proteins, we have suggested that NIFS and NIFU might be involved in the mobilization of the iron and sulfide necessary for nitrogenase metallocluster core formation (Zheng et al., 1993; Dean et al., 1993). Indirect support for this idea comes from the pale tan color of crude extracts prepared from *A. vinelandii nifU* or *nifS* mutants (Jacobson et al., 1989b) that contrasts with the deep chocolate brown hue characteristic of crude extracts prepared from diazotrophically grown wild-

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[1] Abbreviations: NIFU, *A. vinelandii nifU* gene product; NIFS, *A. vinelandii nifS* gene product; NHE, normal hydrogen electrode; Fd(s), ferredoxin(s); (VT)MCD, (variable-temperature) magnetic circular dichroism; RR, resonance Raman; S^b, bridging or inorganic S; S^t, terminal or cysteinyl S.

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type cells. The dark color of diazotrophically grown wild-type *A. vinelandii* is attributed to the metalloclusters contained within the nitrogenase component proteins.

As an approach to determine the respective functions of NIFS and NIFU, we have constructed hybrid plasmids for the purpose of hyperexpressing the *A. vinelandii* proteins in *Escherichia coli* to facilitate their purification and biochemical analyses. Studies of this nature have led to the discovery that NIFS is a pyridoxal phosphate-containing homodimer that catalyzes the desulfurization of L-cysteine (Zheng et al., 1993). An enzyme-bound persulfide is an intermediate in that reaction (Zheng et al., 1994), and we have proposed that this species is the likely sulfur donor in nitrogenase metallocluster core formation. In the present work we describe complementary studies which involve the hyperproduction, isolation, and initial biophysical characterization of NIFU.

MATERIALS AND METHODS

Hyperproduction of NifU in E. coli. Hyperproduction of NIFU in *E. coli* was accomplished by constructing a *nifU* gene cartridge *in vitro* and cloning this cartridge into the pT_7-7 plasmid (Tabor & Richardson, 1985) such that *nifU* gene expression was controlled by the T_7 phage transcriptional and translational regulatory elements. The *nifU* gene cartridge was constructed by using an isolated 3.4-kb DNA fragment that was generated by *Bam*HI restriction enzyme digestion of the hybrid plasmid pDB133 (Jacobson et al., 1989a) as a template for polymerase chain reaction (PCR) amplification of the *nifU* coding sequence. PCR amplification of *nifU* was performed essentially as recommended by the suppliers of Amplitaq (Perkin-Elmer, Norwalk, CT). Cycling temperatures for PCR amplification of *nifU* were as follows: 95 °C for 1 min, 60 °C for 2 min, and 72 °C for 3 min. The two PCR primers used for *nifU* amplification had the following sequences: 5′ATGCATATGTGGGATTATTCGGAA3′ and 5′ATCGGATCCCGGGTGGTGGCGTTG3′. The amino terminal coding region for NIFU is underlined in the first primer sequence shown above. Following *nifU* amplification, the gene cartridge was digested with the restriction enzymes *Nde*I and *Bam*HI and ligated to *Nde*I- and *Bam*HI-digested pT_7-7 DNA. Proper orientation of *nifU* within the resulting hybrid plasmid (pDB525) such that the T_7 gene-10 promoter directs *nifU* transcription was confirmed by restriction enzyme digestion of isolated plasmid DNA. The functional integrity of the cloned *nifU* gene contained within pDB525 was demonstrated by transforming the *A. vinelandii nifU* deletion strain DJ105 (Jacobson et al., 1989b) to prototrophy using isolated pDB525 DNA. Transformation of *A. vinelandii* was performed as previously described (Page & von Tigerstrom, 1979). For hyperproduction of NIFU in *E. coli*, pDB525 was transformed into the host strain BL21 (DE3) and the transformed cells were maintained on LB media supplemented with 100 μg/mL ampicillin. Cells were grown in 500-mL batches in LB media at 30 °C and were induced for NIFU production at approximately 160 Klett units (red filter) by the addition of lactose to 1% final concentration. Following the addition of lactose, cells were cultured for an additional 2 h, and after this induction period cells were harvested by centrifugation and frozen at −20 °C.

Purification and Biochemical Analysis of NIFU. NIFU was purified anaerobically under a purified argon atmosphere with 2 mM dithiothreitol added to all buffers. Crude extracts were prepared by resuspending cells in 25 mM Tris buffer (pH 7.4, 2.5 mL of buffer per gram of wet weight cells) and disruption by sonication, followed by centrifugation at 20 000 rpm for 20 min in a Beckman Type 35 rotor. The crude extract was fractionated by addition of solid streptomycin sulfate to 1%, w/v, incubation at ambient temperature for 20 min, and centrifugation as above. NIFU was then precipitated from the streptomycin sulfate-treated crude extract by bringing it to 30% saturation with solid ammonium sulfate. NIFU precipitated in this way was collected by centrifugation as above and resuspended in buffer at a volume equal to the original supernatant volume. The sample was then loaded onto a 2.5 × 20 cm Q Sepharose column (Pharmacia, Piscataway, NJ) using a peristaltic pump and eluted using a 600-mL 0.1 to 0.6 M NaCl gradient controlled by FPLC pumps. NIFU eluted between 0.41 and 0.46 M NaCl and was judged to be greater than 95% pure at this stage on the basis of Coomassie-stained polyacrylamide gels. Further purification was achieved using a 5 × 200 cm Sephacryl-300 gel-filtration column equilibrated with the above buffer containing 0.2 M KCl. The flow rate of the column was 10 mL/min. Metal analyses were performed on highly purified NIFU samples that were passed over the Sephacryl-300 column, whereas other experiments were performed on samples purified by Q Sepharose chromatography. Protein concentrations were determined by the biuret reaction (Gornall et al., 1949). That authentic NIFU was obtained by this scheme was demonstrated by N-terminal sequence analysis performed using an Applied Biosystems Model 477A protein sequenator operated by the Virginia Tech sequencing facility. The M_r of denatured NIFU was determined by gel electrophoresis (Laemmli, 1970), whereas the subunit composition was determined by gel-filtration chromatography using either a 1 × 30 cm Superose-12 FPLC column or a 2.5 × 50 cm Bio Gel A-0.5 m, 100−200 mesh gel-filtration column. The buffer used for column chromatography was 25 mM Tris·HCl (pH 7.4), 0.2 M KCl, and 10 mM $MgCl_2$. The iron content of NIFU was determined using the *o*-phenanthroline method of Fortune and Mellon (1938) using ferrous ammonium sulfate solutions as standards. Attempts to chelate Fe from isolated NIFU were performed using α,α′-dipyridyl as described previously (Walker & Mortenson, 1974). For chelation experiments, α,α′-dipyridyl was added to a final concentration of 4 mM to a 1.5-mL sample of 0.15 μmol of purified NIFU, and the release of Fe was monitored at A_{520}.

Chemical and Electrochemical Redox Titration of NIFU Metal Cluster. Chemical redox titration of the Fe-S center in NIFU was performed using dithionite as the reductant as previously described (Mayhew, 1978). In these experiments dithionite solutions were freshly prepared by adding 0.087 g of dithionite to an anaerobic 5-mL solution of 25 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid to which 0.15 mL of 3 M NaOH had been added. Anaerobiosis of solutions described here and elsewhere was achieved by repeated degassing under purified argon using a Schlenk apparatus. The dithionite solution was diluted 1:10 in the same buffer and then standardized spectrophotometrically using a degassed solution of 0.02 mM methylene blue as described previously (Schultz et al., 1985). Titrations were performed on 1-mL samples containing 0.1 μmol of NIFU in an argon-flushed cuvette sealed with a rubber serum

stopper. Redox titration of NIFU was performed by sequential additions of 1 or 2 μL of the dithionite solution using a gas tight 10-μL Hamilton syringe followed by gentle mixing. The absorbance change at A_{464} following each incremental dithionite addition was recorded using a Beckman DU 7400 diode array spectrophotometer.

The use of redox mediators for determining the midpoint potential of metalloproteins has been described (Watt, 1979). Briefly, 0.12 μmol of NIFU was prepared in a stoppered degassed vessel that contained 25 mM Tris (pH 7.4), 0.2 M KCl, and 2 mM dithiothreitol containing 10 μM safranine O or benzyl viologen as the redox mediator. The working electrode consisted of vitreous reticulated carbon glued to a silver wire that was passed through the rubber stopper. The auxiliary electrode was a platinum wire immersed in 3 M KCl in a Luggin tube fitted with a Vycor tip. The reference electrode was a Ag/AgCl microelectrode (Microelectrodes, Inc., Londonderry, NH). The protein solution was continuously stirred throughout the experiment and maintained under a constant flow of purified argon supplied by a tube fitted through a port in the stopper. Potentials were applied using a Bioanalytical Systems CV-27 voltammograph. Once the potential stabilized, the absorbance was recorded using a Hewlett-Packard 8452A diode array spectrophotometer. Potentials are reported vs the normal hydrogen electrode (NHE) and are therefore corrected by +0.231 V to compensate for the potential of the Ag/AgCl reference electrode vs the NHE. The percentage change in A_{426} was used to monitor the reduction of NIFU. Safranin O does not undergo a redox-associated change in absorption at this wavelength and therefore does not interfere with determination of the NIFU Fe-S cluster midpoint potential. Similar results were obtained using either safranin O or benzyl viologen as redox mediators, although only experimental data obtained with safranin O is presented herein.

Spectroscopic Characterization of NIFU. The sample concentrations given in the figure captions and used in quantifying spectroscopic results are based on protein concentrations and are expressed per monomer (M_r = 33 000). UV/visible absorption spectra were recorded on either a Beckman DU 7400 or a Hewlett-Packard 8452A diode array spectrophotometer. Variable-temperature magnetic circular dichroism (VTMCD) spectra were recorded on samples containing 50% (v/v) glycerol using an Oxford Instruments SM3 split coil superconducting magnet (1.5−300 K and 0−5 T) mated to the Jasco J-500C spectropolarimeter. The experimental protocols used in VTMCD studies for accurate sample temperature and magnetic field measurement, anaerobic sample handling, and assessment of residual strain in frozen samples have been described in detail elsewhere (Johnson, 1988; Thomson et al., 1993). X-band (~9.5 GHz) EPR spectra were recorded on a Bruker ESP-300E EPR spectrometer equipped with an Oxford Instruments ESR-9 flow cryostat. Spin quantitations were carried out under nonsaturating conditions using 1 mM Cu(II)EDTA as the standard and the procedures outlined by Aasa and Vänngård (1975).

Resonance Raman (RR) spectra were recorded using an Instruments SA Ramanor U1000 spectrometer fitted with a cooled RCA 31034 photomultiplier tube with 90° scattering geometry. Spectra were recorded digitally using photon-counting electronics, and improvements in signal-to-noise were achieved by signal averaging multiple scans. Band positions were calibrated using the excitation frequency and CCl_4 and are accurate to ± 1 cm^{-1}. Lines from a Coherent Innova 100 10-W argon ion laser or a Coherent Innova 200-K2 krypton ion laser were used for excitation, and plasma lines were removed using a Pellin Broca Prism premonochromator. Using a custom-designed sample cell (Drozdzewski & Johnson, 1988), samples under an Ar atmosphere were placed on the end of a cold finger of an Air Products Displex Model CSA-202E closed-cycle refrigerator. Scattering was collected from the surface of a frozen 10-μL droplet. This enabled the samples to be cooled to 17 K, which facilitated improved spectral resolution and prevented laser-induced sample degradation.

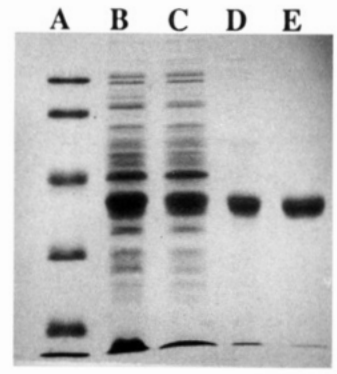

FIGURE 1: Polyacrylamide gel electrophoretic analysis of the stages of NIFU purification. Lane A, molecular weight standards phosporylase *b*, $M_r \approx 97\,400$; bovine serum albumin, $M_r \approx 66\,200$; ovalbumin, $M_r \approx 42\,700$; carbonic anhydrase, $M_r \approx 31\,000$; and soybean trypsin inhibitor, $M_r \approx 21\,500$. Lane B, crude extract. Lane C, streptomycin sulfate supernatant fraction. Lane D, ammonium sulfate precipitate fraction. Lane E, Q Sepharose fraction.

RESULTS

Hyperproduction of NIFU from *A. vinelandii* was accomplished in *E. coli* by its controlled heterologous expression using a hybrid plasmid that contains the *nifU* structural gene fused to the cloned transcriptional and translational regulatory elements of the T_7 phage system. Accumulation of NIFU in crude extracts of BL21(DE3) cells harboring the *nifU* expression plasmid was demonstrated by denaturing gel electrophoresis (Figure 1, lane B). This analysis showed the accumulation of a soluble polypeptide having an M_r of ≈33,000 that was not produced in control cells that harbored the parental pT$_7$-7 plasmid. These results are in good agreement with the predicted M_r of 33 274 that was deduced for NIFU from gene sequence data (Beynon et al., 1987). Cell pellets of the *E. coli* strain that hyperproduced NIFU had a characteristic red color. This was the first indication that NIFU is an Fe-S protein and provided a convenient means for visually following NIFU purification. Because of the abundance of NIFU produced in the heterologous expression system, it was readily purified in a simple four-step procedure (Figure 1). The identity of isolated product as NIFU was confirmed by comparison of the N-terminal sequence determined from the isolated protein (Met-Trp-Asp-Tyr) to the predicted N-terminal sequence deduced from the *nifU* gene sequence (Beynon et al., 1987). On the basis of gel-filtration column chromatography, using cytochrome *c* ($M_r \approx 12\,400$), carbonic anhydrase ($M_r \approx 29\,000$), bovine serum albumin ($M_r \approx 66\,000$), and alcohol dehydrogenase ($M_r \approx 150\,000$) as molecular weight markers, native NIFU was shown to be a homodimer having an apparent $M_r \approx 63\,000$.

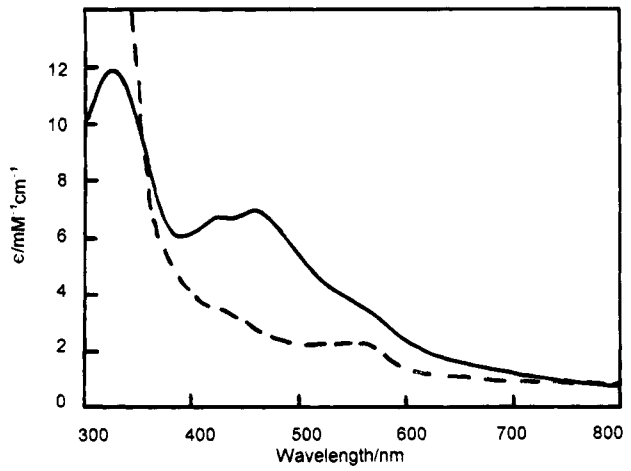

FIGURE 2: Room temperature UV-visible absorption spectra of NIFU. The solid line is the spectrum of NIFU as prepared, and the dashed line is the spectrum of NIFU after anaerobic reduction with a 15-fold stoichiometric excess of sodium dithionite. The protein concentration was 0.20 mM, and the buffering medium was 50 mM Tris·HCl, pH 8.0. The reduced spectrum is dominated by an intense absorption from dithionite centered at 314 nm.

Purified samples of NIFU exhibited a single band on electrophoresis gels (Figure 1, lane E), and colorimetric iron analyses indicated approximately two irons per subunit (average of 1.8 Fe/subunit from four determinations using three independent NIFU preparations). In accord with the Fe analyses, the UV/visible absorption spectra of NIFU as initially prepared and of dithionite-reduced NIFU (Figure 2) are indicative of a single, dithionite-reducible $[2Fe\text{-}2S]^{2+,+}$ cluster in each subunit. Biological $[2Fe\text{-}2S]^{2+,+}$ centers have characteristic visible absorption spectra. Oxidized clusters have bands centered around 330 nm (ϵ in the range 11 000–16 000 M^{-1} cm^{-1}), 420 nm (ϵ in the range 6 000–11 000 M^{-1} cm^{-1}, not well resolved in some proteins, *e.g.*, Rieske-type proteins), and 460 nm (ϵ in the range 6000–10 000 M^{-1} cm^{-1}), with a broad shoulder centered around 550 nm (ϵ in the range 3000–6000 M^{-1} cm^{-1}) (Dailey et al., 1994). Reduced clusters have relatively featureless spectra that increase in intensity at shorter wavelengths except for a prominent feature centered near 550 nm (ϵ in the range 2000–4000 M^{-1} cm^{-1}) that has been tentatively assigned to an Fe(II) → Fe(III) intervalence band of the localized valence $[2Fe\text{-}2S]^{+}$ core (Fu et al., 1992). The spectra for NIFU conform to this general pattern, with bands at 330 nm (ϵ = 12 000 M^{-1} cm^{-1}) 420 nm (6600 M^{-1} cm^{-1}), and 460 nm (7000 M^{-1} cm^{-1}) and a shoulder at approximately 550 nm ($\epsilon \approx$ 4000 M^{-1} cm^{-1}) for the protein as initially prepared and a pronounced feature centered at 570 nm (ϵ = 2500 M^{-1} cm^{-1}) for the reduced protein. Moreover, the extinction coefficients expressed per 33-kDa subunit are consistent with one $[2Fe\text{-}2S]^{2+,+}$ center.

The change in absorption on reduction was used to assess the number of electrons required to effect reduction via quantitative chemical titrations with sodium dithionite and dye-mediated electrochemical redox titrations. Chemical titrations indicated approximately one electron per subunit (average of 0.84 reducing equivalent per subunit from three different experiments using two independent NIFU preparations). Electrochemical redox titrations mediated by safranin O were well fit by one-electron Nernst plots and indicate a midpoint around −254 ± 20 mV vs NHE (Figure 3). The ability of Fe chelators to effect cluster degradation was

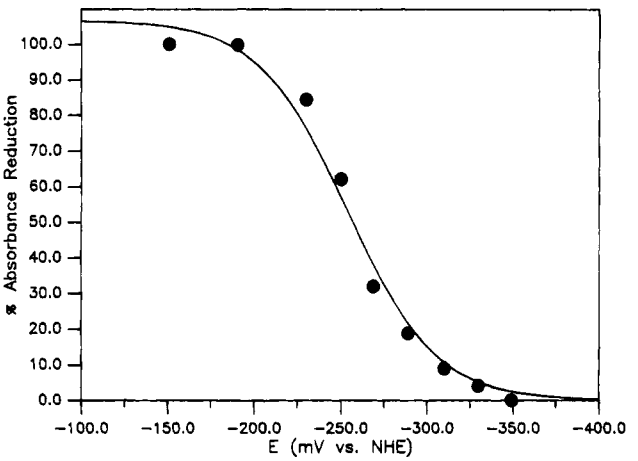

FIGURE 3: Optically monitored electrochemical reduction of NIFU. Conditions are described in Materials and Methods. The solid line is the best fit to a one-electron Nernst equation with E_m = −254 mV *vs* NHE.

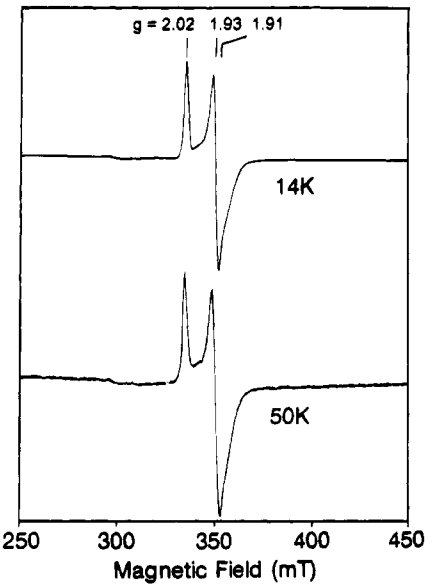

FIGURE 4: X-band EPR spectra of dithionite-reduced NIFU. Conditions of measurement: microwave frequency, 9.46 GHz; modulation amplitude, 1 mT; microwave power, 2 mW; temperatures as indicated.

assessed by experiments in which oxidized and reduced samples of NIFU were treated with a 40-fold excess of α,α′-dipyridyl. The absorption of the $[Fe(\alpha,\alpha'\text{-dipyridyl})_3]^{2+}$ complex at 520 nm was monitored at room temperature as a function of time.[2] Little if any chelation of Fe by α,α′-dipyridyl was observed over 2 h, indicating that the cluster is either inherently stable or inaccessible to solvent.

The magnetic, electronic, and vibrational properties of the Fe-S center in NIFU were investigated by EPR (Figure 4), VTMCD (Figure 5), and RR (Figures 6 and 7) spectroscopies. In addition to providing unambiguous confirmation of the presence of a single $[2Fe\text{-}2S]^{2+,+}$ center, these techniques facilitated assessment of the ligation at the reducible and nonreducible Fe sites and detailed comparison

[2] Although both Fe^{2+} and Fe^{3+} are chelated by α,α′-dipyridyl, the colorimetric assay is only for the ferrous state. However, quantitative removal of Fe as Fe^{2+} has been reported for oxidized Fe-S clusters under denaturing conditions with inorganic sulfide released on cluster degradation as the likely reductant (Anderson & Howard, 1984).

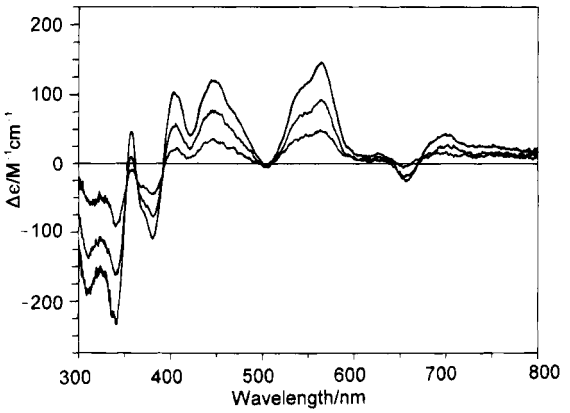

FIGURE 5: VTMCD spectra of dithionite-reduced NIFU. The protein concentration was 0.16 mM, and the buffering medium was 50 mM Tris·HCl, pH 8.0, with 50% (v/v) glycerol. MCD spectra were recorded in 1-mm cuvettes with a magnetic field of 4.5 T, at 1.66, 4.22, and 10.7 K. All MCD bands increase in intensity with decreasing temperature.

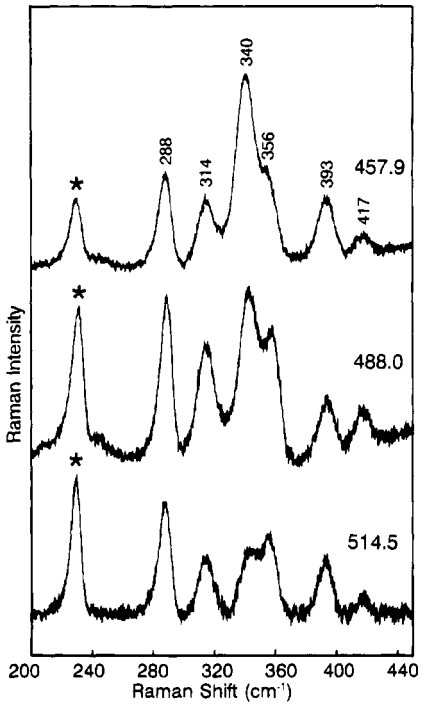

FIGURE 6: Low-temperature resonance Raman spectra of NIFU as prepared. The protein concentration was 1.8 mM, and the buffering medium was 50 mM Tris·HCl, pH 8.0. The spectra were obtained at 17 K using 457.9-, 488.0-, and 514.5-nm laser excitation and are the sum of 36, 28, or 33 scans, respectively. Each scan involved advancing the spectrometer in 0.2-cm^{-1} increments and photon counting for 1 s/point with 6-cm^{-1} spectral resolution. The asterisks indicate lattice modes of ice.

with other well-characterized [2Fe-2S]-containing proteins (Fu et al., 1992; Crouse et al., 1994). In common with other [2Fe-2S] proteins, oxidized NIFU did not exhibit any clearly discernible EPR signals, indicating that the oxidized cluster is diamagnetic at low temperatures as a result of strong antiferromagnetic exchange interaction between the high-spin Fe^{3+} ions. Reduced NIFU exhibited an almost axial EPR signal, $g = 2.02, 1.93, 1.91$, that is observable without significant broadening at 50 K (Figure 4). Spin quantitation of this $S = 1/2$ resonance for two different samples gave values between 0.8 and 0.9 spin/subunit. Taken together, the g-values and the relaxation properties are uniquely indicative of $[2Fe\text{-}2S]^{+}$ centers (Orme-Johnson & Orme-

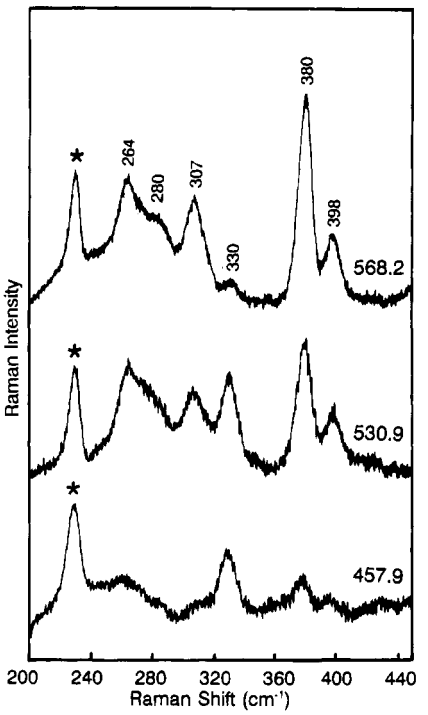

FIGURE 7: Low-temperature resonance Raman spectra of dithionite-reduced NIFU. The sample, with a protein concentration of 2.4 mM in 50 mM Tris·HCl buffer, pH 8.0, was reduced anaerobically with a 20-fold excess of sodium dithionite. The spectra were obtained at 17 K using 568.2-, 530.9-, and 457.9-nm laser excitation and are the sum of 25, 66, or 68 scans, respectively. The data collection parameters are the same as given in Figure 7, and the asterisks indicate lattice modes of ice.

Johnson, 1982) that exhibit $S = 1/2$ ground states as a result of antiferromagnetic exchange interaction between localized valence high-spin Fe^{2+} and Fe^{3+} ions (Bearden & Dunham, 1973).

Biological [2Fe-2S] centers can be divided into two classes termed ferredoxin-type (Fd-type) and Rieske-type that differ in terms of midpoint potentials and the EPR properties of the reduced clusters: Fd-type, $E_m = +70$ to -470 mV, $g_{av} \approx 1.96$; Rieske-type, $E_m = +350$ to -160 mV, $g_{av} \approx 1.90$ (Cammack, 1992, Johnson, 1994). On the basis of the X-ray crystal structures of several highly homologous plant-type Fds (Tsukihara et al., 1990; Jacobson et al., 1993) and/or mutagenesis or chemical modification studies of conserved cysteines (Cupp & Vickery, 1988; Werth et al., 1990; Gerber et al., 1990; Uhlmann et al., 1992; Fujinaga et al., 1993; Cheng et al., 1994), Fd-type clusters are generally considered to have complete cysteinyl coordination, whereas spectroscopic studies of Rieske-type centers have provided strong evidence in favor of coordination by two histidyl residues at the reducible Fe site and two cysteinyl residues at the nonreducible Fe site (Fee et al., 1984; Gurbiel et al., 1989; Kuila et al., 1992). Clearly the EPR and redox properties of the Fe-S center in NIFU, $g_{av} = 1.95$ and $E_m = -260$ mV, are indicative of a Fd-type [2Fe-2S] cluster. Moreover, since the g_{av} value and the g-value anisotropy of the $S = 1/2$ resonance of $[2Fe\text{-}2S]^{+}$ clusters are largely dependent on the ligand field at the localized Fe^{2+} site (Bertrand et al., 1985; Werth et al., 1990), the EPR properties of NIFU are consistent with coordination by two cysteinyl residues at the reducible Fe site. Such arguments must be used with caution, however, since the EPR g-values will depend on both the nature of the ligands and the distortion in the ligand field at

the Fe^{2+} site and require confirmation via techniques such as VTMCD and RR that are sensitive to the nature of the coordinating residues at both Fe sites (Fu et al., 1992).

Three distinct subclasses of Fd-type $[2Fe\text{-}2S]^{2+,+}$ clusters have been identified in simple Fds on the basis of distinctive EPR, VTMCD, and RR spectra (Fu et al., 1992; Han et al., 1989b) and the arrangement of coordinating cysteine residues (Meyer et al., 1986; Crouse et al., 1994). These are plant- or chloroplast-type ($g = 2.05, 1.96, 1.89$), hydroxylase-type such as putidaredoxin and adrenodoxin that donate electrons to cytochrome P450s ($g = 2.02, 1.94, 1.94$), and a small group of Fds of unknown function from nitrogen-fixing bacteria such as *Clostridium pasteurianum* ($g = 2.00, 1.95, 1.92$). The EPR properties of the cluster in NIFU do not correspond to a particular subclass and are intermediate between those of hydroxylase-type Fds and *C. pasteurianum* 2Fe Fd.

Fe-S centers with paramagnetic ($S > 0$) ground states invariably give rise to temperature-dependent MCD spectra, and the pattern of positive and negative bands provides a sensitive probe of the electronic structure and a discriminating method for determining cluster type (Johnson et al., 1982; Johnson, 1988). Comparison of the VTMCD spectra reported for reduced spinach Fd (plant type), putidaredoxin (hydroxylase type), and *C. pasteurianum* 2Fe Fd (Fu et al., 1992) with those of reduced NIFU (Figure 5) unambiguously identifies the Fe-S cluster as a Fd-type $[2Fe\text{-}2S]^{+}$ center, and the intensity of equivalent bands under comparable conditions is consistent with approximately one cluster per subunit. Furthermore, magnetization data (Thomson & Johnson, 1980) collected for each of the prominent bands (data not shown) were well fit by theoretical curves constructed for $g_{\parallel} = 2.02$ and $g_{\perp} = 1.92$. This confirms that the transitions all originate from the $S = 1/2$ ground state responsible for the EPR signal. Each of the three known subclasses of Fd-type centers can be distinguished on the basis of its low-temperature MCD spectra (Fu et al., 1992). Within specific spectral regions, NIFU shows close correspondence to one or the other of these archetypical proteins, but overall the MCD spectrum cannot be identified with any particular subclass.

Despite small variations in the energies of individual bands among Fd-type $[2Fe\text{-}2S]^{+}$ centers, overall they show a one-to-one correspondence in their positive and negative MCD bands in the UV/visible region. The spectra have been tentatively assigned on the basis of MCD studies of oxidized and reduced rubredoxins and resonance Raman excitation profiles (Fu et al., 1992). The MCD bands above 600 nm are attributed to ligand field transitions in view of their low absorption intensities. The positive MCD band attributed to the $Fe^{2+} \rightarrow Fe^{3+}$ intervalence band has shifted to 570 nm in the NIFU spectrum compared to 530 nm in spinach Fd, 540 nm in putidaredoxin, and 550 nm in *C. pasteurianum* 2Fe Fd, and this facilitates clearer observation of the $+ - + + - +$ pattern of MCD bands in the 350−550-nm region arising from $S \rightarrow Fe^{3+}$ charge transfer at the localized valence Fe^{3+} site. By analogy with the crystallographically defined plant-type Fds, this indicates coordination of the Fe^{3+} site of the $[2Fe\text{-}2S]^{+}$ cluster in NIFU by two cysteine residues. In common with *C. pasteurianum* 2Fe Fd, the negative bands between 300 and 350 nm in the NIFU spectrum that are attributed to $Fe^{2+} \rightarrow S$ charge transfers at the localized valence Fe^{2+} site are more resolved than in plant- or hydroxylase-type Fds. However, the number and sign of the MCD bands in this region is highly conserved, indicating that the Fe^{2+} site is also coordinated by two cysteine residues. The validity of this interpretation of the MCD spectra and the spectroscopic consequences of non-cysteinyl coordination at either of the localized valence Fe^{3+} and Fe^{2+} sites of the $[2Fe\text{-}2S]^{+}$ center has recently been demonstrated in this laboratory via VTMCD studies of Rieske proteins[3] and of mutants of the plant-type Fd from *Anabaena* in which coordinated cysteines have been individually changed to serines.[4]

Table 1: Fe−S Stretching Frequencies and Vibrational Assignments for the $[2Fe\text{-}2S]^{2+,+}$ Clusters in NIFU

mode[a]			
D_{2h}	C_{2v}	oxidized (cm^{-1})	reduced (cm^{-1})
$B_{2u}{}^{b}$	B_1	417	398
$A_g{}^{b}$	A_1	393	380
$B_{3u}{}^{b}$	A_1	356	307
$B_{1u}{}^{t}$	B_2	n.o.[b]	330
$B_{2g}{}^{t}$	B_2	n.o.[b]	n.o.[b]
$A_g{}^{t}$	A_1	340	307
$B_{1g}{}^{b}$	B_1	314	280
$B_{3u}{}^{t}$	A_1	288	264

[a] Symmetry labels under idealized D_{2h} symmetry for an oxidized $Fe_2S_2{}^{b}S_4{}^{t}$ unit and under idealized C_{2v} symmetry for a localized-valence reduced $Fe_2S_2{}^{b}S_4{}^{t}$ unit. [b] Not observed with the excitation wavelengths used in this work.

Resonance Raman spectroscopy has emerged as a sensitive structural probe of Fe-S centers and a discriminating method for determining Fe-S cluster type (Spiro et al., 1988). It is particularly useful for investigating and identifying Fe-S centers with diamagnetic ground states, such as oxidized $[2Fe\text{-}2S]^{2+}$ clusters, which are not amenable to EPR or VTMCD investigation. All of the eight predicted Fe−S stretching modes of the $Fe_2S_2{}^{b}S_4{}^{t}$ unit of $[2Fe\text{-}2S]^{2+}$ centers have been assigned to predominantly $Fe-S^{b}$ or $Fe-S^{t}$ modes under idealized D_{2h} symmetry on the basis of extensive isotope shift data for proteins and synthetic analogs and normal mode calculations (Han et al., 1989a,b; Fu et al., 1992). The RR spectra obtained for oxidized NIFU (Figure 6) conform to the general pattern of frequencies established for $[2Fe\text{-}2S]^{2+}$ clusters, and vibrational assignments can be inferred by analogy with the published assignments (Table 1). It was not possible to obtain high-quality spectra with 406.7-nm excitation due to high fluorescence backgrounds. This excitation wavelength is usually required to observe the nearly degenerate $B_{1u}{}^{t}$ and $B_{2g}{}^{t}$ modes that differ only in the phases of the $Fe-S^{t}$ stretches on opposite sides of the $Fe_2S_2{}^{b}$ core.

Despite the overall similarity in the RR spectra of Fd-type oxidized [2Fe-2S] centers, each subclass is readily distinguishable in terms of the differences in the relative intensities of bands as a function of excitation wavelength and the frequencies of corresponding Fe−S stretching modes (Han et al., 1989b; Crouse et al., 1994). The latter have been interpreted in terms of minor differences in Fe−S bond strengths and variability in the cysteine $Fe-S^{t}-C_{\beta}-C_{\alpha}$ dihedral angles that govern the extent of mixing of the $Fe-S^{t}$ vibrations with the internal bending modes of coordinated

[3] T. A. Link, B. R. Crouse, M. G. Finnegan, D. Kuila, J. A. Fee, and M. K. Johnson, manuscript in preparation.

[4] B. R. Crouse, H. Cheng, B. Xia, J. L. Markley, and M. K. Johnson, manuscript in preparation.

cysteines (Han et al., 1989a,b; Fu et al., 1992), *i.e.*, minimal coupling with a dihedral angle of 90° or 270° and maximal coupling with a dihedral angle of 180°. Normal mode calculations for crystallographically defined analog complexes (Han et al., 1989a) indicate that the extent of coupling can be assessed by the frequency separation between the B_{3u}^t mode and the A_g^t, B_{1u}^t, B_{2g}^t, and B_{3u}^b modes. For NIFU these frequency separations are intermediate between those of hydroxylase-type and plant-type Fds but similar to those of *C. pasteurianum* 2Fe Fd , suggesting similar cysteine dihedral angles to the [2Fe-2S] in these proteins. Overall, however, the RR spectra for oxidized NIFU, in accord with the EPR and VTMCD spectra of reduced NIFU, are not directly identifiable with any particular subclass of Fd-type centers. Taken together, the spectroscopic results suggest that NIFU constitutes a new subclass of Fd-type [2Fe-2S] centers with a unique arrangement of coordinating cysteines.

Reduced $[2Fe\text{-}2S]^+$ clusters show much greater uniformity in their RR spectra (Fu et al., 1992). Reduced NIFU is no exception, and the spectra are uniquely indicative of a protein-bound $[2Fe\text{-}2S]^+$ center (Figure 7). Assignments (Table 1) can be made by direct analogy with those established for reduced putidaredoxin via $^{34}S^b$-isotope shifts (Fu et al., 1992). Excitation into the putative $Fe^{2+} \rightarrow Fe^{3+}$ intervalence band results in strong enhancement of modes involving substantial contributions from $Fe-S^b$ stretching, 264, 280, 307, 380, and 398 cm^{-1} in reduced NIFU. These modes have similar frequencies and enhancement profiles in all reduced $[2Fe\text{-}2S]^+$ centers investigated thus far, which attests to analogous structures for the mixed valence $Fe_2(\mu_2\text{-}S)_2$ cores. Higher energy excitation (optimally 457.9 nm) is required for enhancement of $Fe-S^t$ stretching at the localized valence Fe^{3+} site, 330 cm^{-1} in reduced NIFU. A band with similar enhancement characteristics is observed at 317 cm^{-1} in adrenodoxin,[5] 319 cm^{-1} in putidaredoxin, 325 cm^{-1} in spinach Fd, and 328 cm^{-1} in *C. pasteurianum* 2Fe Fd (Fu et al., 1992). The appearance of this band is considered to be indicative of coordination by two cysteines at the nonreducible Fe site, and this has recently been confirmed by RR studies of reduced Cys → Ser site-specific mutants of the *Anabaena* plant-type Fd.[4] The frequency of this mode is likely to be critically dependent on $Fe-S^t-C_\beta-C_\alpha$ dihedral angles at the nonreducible Fe site. The higher frequencies, such as those observed for NIFU and *C. pasteurianum* 2Fe Fd, are indicative of stronger coupling with cysteinyl modes and hence of one or both of these dihedral angles approaching 180°.

DISCUSSION

The major conclusions from the present work are that NIFU is a homodimer with identical [2Fe-2S] clusters in each subunit. These [2Fe-2S] clusters have a potential of about −260 mV, and they are coordinated to the polypeptide entirely by cysteinyl thiolate ligands. The coordination of the NIFU [2Fe-2S] clusters by cysteinyl residues is consistent with comparative gene sequence data, which show that among NIFU polypeptide primary sequences deduced from gene sequence data from *A. vinelandii* (Beynon et al., 1987), *K. pneumoniae* (Beynon et al., 1987), and *Anabaena* (Mulligan & Haselkorn, 1989) there are nine conserved cysteinyl residues, and seven of these are contained within domains exhibiting extraordinary sequence conservation (Dean & Jacobson, 1992). NIFU from *A. vinelandii* contains 312 residues, of which residues 35, 62, 106, 137, 139, 172, 175, 272, and 275 are the interspecifically conserved cysteines. Other than the Cys-X_2-Cys motif characteristic of many Fe-S cluster coordinating domains, the spatial organization of the NIFU cysteines does not exhibit a pattern that is typically found for other [2Fe-2S] proteins. Indeed, a novel organization of the cluster coordinating ligands within NIFU is likely to contribute to the unique spectroscopic features when compared to other [2Fe-2S]-containing polypeptides.

Definitive evidence for the specific cysteines involved in the ligation of the [2Fe-2S] cluster in NIFU will require crystallographic and/or mutagenesis studies. However, it is interesting to note, as previously recognized by Ouzounis et al. (1994), that the domain containing the two pairs of closely spaced cysteines in NIFU proteins (Cys137, -139, -172, and -175 in *A. vinelandii* NIFU) bears a striking conservation in primary sequence and one-dimensional spatial organization when compared to tandemly repeated primary sequence domains located in nitrite reductases from *K. pneumoniae* (Lin et al., 1993), *E. coli* (Peakman et al., 1990), *Emericella nidulans* (Johnstone et al., 1990), and *Neurospora crassa* (Exley et al., 1993) and a single C-terminal domain in nitrate reductase from *K. pneumoniae* (Lin et al., 1993). Although it is not firmly established whether or not all these proteins contain [2Fe-2S] clusters, the available biophysical evidence, together with the gene sequence data, clearly indicates that nitrite reductases contain two [2Fe-2S] clusters (Cammack et al., 1982). Hence it is tempting to speculate that NIFU and these other proteins constitute a fourth subclass of Fd-type [2Fe-2S] centers that are coordinated by a C-X-C-X_n-C-X_2-C arrangement of cysteines. This contrasts to C-X_4-C-X_2-C-X_n-C in plant-type Fds (Tsukihara et al., 1990; Jacobson et al. 1993), C-X_5-C-X_2-C-X_n-C in hydroxylase-type Fds (Cupp & Vickery, 1988; Gerber et al., 1990; Uhlmann et al., 1992), and the C-X_2-C-X_n-C-X_3-C arrangement that has been proposed for *C. pasteurianum* Fd (Fujinaga et al., 1993; Crouse et al., 1994). The proposed spacing of coordinating residues in NIFU proteins is also similar to that established by mutagenesis studies for Rieske-type centers, *i.e.*, C-X-H-X_n-C-X_2-H (Johnson, 1994; Davidson et al., 1992), except that histidines replace the second cysteine in each pair. In this connection it is worth noting that the second repeat in each of the homologous nitrite reductase sequences has one histidine in place of a specific cysteine, *i.e.*, C-X-H-X_n-C-X_2-C (Ouzounis et al., 1994).

What is the physiological function of NIFU and what is the specific role of its [2Fe-2S] clusters? Several possible answers to these questions based on the biochemical phenotype of *nifU* mutants and on results of the present work can be considered. Upon first discovering that NIFU contains a redox-active metallocluster, we considered that NIFU might act as a surrogate electron donor to nitrogenase. This possibility seemed particularly attractive because it is known that the normal physiological electron donor to nitrogenase in *A. vinelandii* is a flavodoxin, but the gene encoding this flavodoxin, *nifF*, is not absolutely required for diazotrophic growth (Bennett et al., 1987). However, our results show that the redox potential of NIFU is not sufficiently low to accommodate reduction of the Fe protein. An alternative possibility is that NIFU, together with NIFS,

[5] C. Staples, B. R. Crouse, R. Bernhardt, and M. K. Johnson, unpublished observations.

functions in sequestering the Fe and sulfide required for formation of the Fe-S cores contained within the nitrogenase component proteins. This suggestion is supported by the observations that (i) the biochemical phenotypes of *A. vinelandii nifU* and *nifS* mutants are remarkably similar (Jacobson et al., 1989), (ii) nitrogenase component proteins containing a full complement of metalloclusters are apparently not accumulated in *A. vinelandii nifS* or *nifU* mutants, (iii) the *nifU* and *nifS* genes are cotranscribed (Beynon et al., 1987), and (iv) NIFS appears to have a role in sequestering the inorganic sulfide for nitrogenase Fe-S core formation (Zheng et al., 1993, 1994).

We have shown previously that NIFS is able to catalyze the desulfurization of L-cysteine and that an enzyme-bound persulfide is an intermediate in that reaction. Thus, NIFU might have a redox function which involves catalyzing the release of the sulfide from NIFS. In such a model NIFU might also be considered a candidate to serve as an intermediate sulfide carrier. Another possibility is that NIFU might complement the function of NIFS by sequestering the Fe required for nitrogenase Fe-S core formation. In this case the role of the [2Fe-2S] cluster could be to keep Fe bound at some other site located within NIFU in an oxidation state amenable to nitrogenase Fe-S core formation. As only four cysteines are required for coordination of each [2Fe-2S], it is noteworthy that there are five other cysteinyl residues per each NIFU subunit which have the potential to coordinate additional Fe atoms. If any of these cysteines do indeed coordinate Fe atoms destined for nitrogenase Fe-S core assembly, such Fe is not associated with the isolated protein. Namely, all of the Fe found in isolated NIFU can be accounted for by the two [2Fe-2S] clusters. Nevertheless, this feature is expected if it is considered that any NIFU-bound Fe atoms targeted for nitrogenase Fe-S core formation must be capable of being released during formation of the nitrogenase Fe-S cores and would, therefore, probably only be loosely associated with the polypeptide and readily lost during the purification scheme. Along these same lines we have also considered the possibility that the two [2Fe-2S] clusters contained within NIFU could be intermediates in nitrogenase Fe-S core formation. This consideration was prompted by previous reports that the irons contained within the [4Fe-4S] cluster of the Fe protein can be removed by using chelators (Walker & Mortenson, 1974) and that a [2Fe-2S] species of the Fe protein is an intermediate in that process (Anderson & Howard, 1984). However, because the NIFU [2Fe-2S] clusters are released only very slowly by treatment with the chelator α,α′-dipyridyl, they are unlikely to be precursors of the nitrogenase metalloclusters. In other words, if the [2Fe-2S] clusters from NIFU were capable of being transferred to another protein, facile chelation of their irons by α,α′-dipyridyl would be expected. Nevertheless, it remains an intriguing possibility that nitrogenase Fe-S core precursors could be assembled at one or more sites on NIFU, potentially using the available cysteinyl residues as nucleation sites, and that the [2Fe-2S] clusters could have a redox role in formation, stabilization, or release of such precursors during maturation of the nitrogenase component proteins. The possibility that NIFU might function to sequester the Fe necessary for nitrogenase metallocluster formation has an interesting parallel in the case of human ferrochelatase, which catalyzes the insertion of ferrous iron as the terminal step in heme biosynthesis and which has also recently been shown to be a [2Fe-2S]-containing homodimer (Dailey et al., 1994). However, the role of the [2Fe-2S] center in mammalian ferrochelatase has yet to be determined, and comparison of the primary sequences of NIFU and human ferrochelatase reveals no obvious sequence identity, particularly within regions surrounding cysteinyl residues.

In summary, although the specific function of NIFU is not yet known, its activity is required for the full activation of the nitrogenase component proteins, and the present evidence points to a role in formation of the Fe-S cores of the nitrogenase metalloclusters. Amino acid substitution studies, such as those already used to investigate the coordination of the nitrogenase metalloclusters (Brigle et al., 1987; Dean et al., 1990) as well as a variety of other metalloproteins, including [2Fe-2S] proteins (Werth et al., 1990; Gerber et al., 1990; Uhlmann et al., 1992, Fujinaga et al. 1993; Cheng et al., 1994), should provide insight concerning the organization and physiological function of the [2Fe-2S] clusters contained within NIFU. Also, because large amounts of purified NIFS and NIFU are now available, it is possible to initiate *in vitro* biochemical reconstitution experiments aimed at determining whether or not these proteins directly participate, either separately or in combination, in the mobilization of the inorganic Fe and sulfide required for maturation of the nitrogenase component proteins.

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