NifS-directed assembly of a transient [2Fe-2S] cluster within the NifU protein

Pramvadee Yuvaniyama*, Jeffrey N. Agar†, Valerie L. Cash*, Michael K. Johnson†‡, and Dennis R. Dean*‡

*Department of Biochemistry, Virginia Tech, Blacksburg, VA 24061-0346; and †Department of Chemistry and Center for Metalloenzyme Studies, University of Georgia, Athens, GA 30602

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The NifS and NifU proteins from *Azotobacter vinelandii* are required for the full activation of nitrogenase. NifS is a homodimeric cysteine desulfurase that supplies the inorganic sulfide necessary for formation of the Fe-S clusters contained within the nitrogenase component proteins. NifU has been suggested to complement NifS either by mobilizing the Fe necessary for nitrogenase Fe-S cluster formation or by providing an intermediate Fe-S cluster assembly site. As isolated, the homodimeric NifU protein contains one $[2Fe\text{-}2S]^{2+,+}$ cluster per subunit, which is referred to as the permanent cluster. In this report, we show that NifU is able to interact with NifS and that a second, transient [2Fe-2S] cluster can be assembled within NifU *in vitro* when incubated in the presence of ferric ion, L-cysteine, and catalytic amounts of NifS. Approximately one transient [2Fe-2S] cluster is assembled per homodimer. The transient [2Fe-2S] cluster species is labile and rapidly released on reduction. We propose that transient [2Fe-2S] cluster units are formed on NifU and then released to supply the inorganic iron and sulfur necessary for maturation of the nitrogenase component proteins. The role of the permanent [2Fe-2S] clusters contained within NifU is not yet known, but they could have a redox function involving either the formation or release of transient [2Fe-2S] cluster units assembled on NifU. Because homologs to both NifU and NifS, respectively designated IscU and IscS, are found in non-nitrogen fixing organisms, it is possible that the function of NifU proposed here could represent a general mechanism for the maturation of Fe-S cluster-containing proteins.

Iron-sulfur clusters are found in numerous proteins that have important redox, catalytic, or regulatory properties (for a recent review, see ref. 1). Moreover, Fe-S clusters are intimately involved in the respective functions of these proteins. For example, Fe-S clusters are known to act as electron carriers or environmental sensors or to be involved in substrate binding and activation. Advances in our understanding of the structures, organization, and reactivity of certain biologically relevant Fe-S clusters have involved determination of the spectroscopic and electronic properties of protein-bound Fe-S clusters, characterization of clusters chemically extruded from their polypeptide matrices, and preparation of synthetic Fe-S clusters. Until recently, however, the biological mechanism by which Fe-S clusters are formed has received scant attention. It was shown many years ago that Fe-S clusters could be spontaneously incorporated into apo-forms of certain ferredoxins by simply incubating them *in vitro* in a solution that contains iron and sulfide (2). However, considering the toxicity of free iron and sulfide, it is unlikely that protein-bound Fe-S clusters are spontaneously formed *in vivo* from free iron and sulfide. It is more likely that the iron and sulfur necessary for Fe-S cluster formation are delivered to the cluster assembly site by intermediate carrier proteins.

Previous work has led to the proposal that the NifU and NifS nitrogen fixation-specific gene products are involved in the acquisition of iron and sulfur necessary for the maturation of the two nitrogenase component proteins, both of which contain Fe-S clusters (3). It was shown that NifS is a pyridoxal phosphate-dependent L-cysteine desulfurase and that an enzyme-bound persulfide is an intermediate in that reaction (4, 5). Thus, NifS has been targeted as the source of inorganic sulfide necessary for nitrogenase Fe-S cluster formation. We have recently found that NifU is a modular protein with two distinct types of iron-binding sites (see Fig. 1). One of these binding site types is located within the central third of the NifU primary sequence (6, 7) and binds a $[2Fe\text{-}2S]^{2+,+}$ cluster (one cluster binding site per subunit). The second type of site is a labile mononuclear iron-binding site (7) located within the N-terminal third of the NifU primary sequence (one mononuclear site per subunit). Because the $[2Fe\text{-}2S]^{2+,+}$ clusters present in isolated NifU are tightly bound to the protein (6), the labile mononuclear site is the likely source of iron for nitrogenase Fe-S cluster assembly. If NifS and NifU do have complementary functions in the mobilization of sulfur and iron for nitrogenase Fe-S cluster assembly, several different pathways for this process can be considered. For example, NifS and NifU could either operate independently during Fe-S cluster formation or they could function together. If NifU and NifS function together, then iron and sulfur could be separately released from each of them during cluster assembly or an Fe-S cluster precursor could be preformed and then released. In the current work we have addressed these issues by asking whether NifU and NifS are able to form a macromolecular complex and whether a labile Fe-S cluster species can be formed on NifU.

Experimental Procedures

Plasmids and Strains. Construction of plasmids used for the heterologous expression of altered forms of NifU in *Escherichia coli* has been described (7). Plasmid pDB822 was used to express a full-length version of NifU for which the Cys^{137} residue is substituted by Ala. This form of NifU is designated NifU(Cys^{137}Ala). Plasmid pDB1041 was used to express a full-length version of NifU for which the Asp^{37} residue was substituted by Ala [designated NifU(Asp^{37}Ala)]. Plasmid pDB937 was used to express a truncated form of NifU that includes the first 131 residues of NifU. This truncated form of NifU is designated NifU-1. Plasmid pDB1044 was obtained by the oligonucleotide-directed mutagenesis of pDB937. This plasmid expresses a form of NifU-1 that has the Asp^{37} residue substituted by Ala and is designated NifU-1(Asp^{37}Ala). Fig. 1 is a schematic representation of NifU and different forms of NifU used in the current work.

Biochemical Manipulations. The purification of NifS and altered forms of NifU was performed as previously described (4, 7). The optimized *in vitro* Fe-S cluster biosynthetic system includes the following: 0.05 mM NifU-1 dimer (or other form of NifU-1 or NifU)/0.1 mM ferric ammonium citrate/5 mM β-mercaptoethanol/1 mM scap]l-cysteine/1 μM NifS/0.1 M NaCl in a 25 mM Tris·HCl (pH 7.4) buffer. All volumes were 1.0 ml, and reactions

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‡To whom reprint requests should be addressed. E-mail: deandr@vt.edu or johnson@sunchem.chem.uga.edu.

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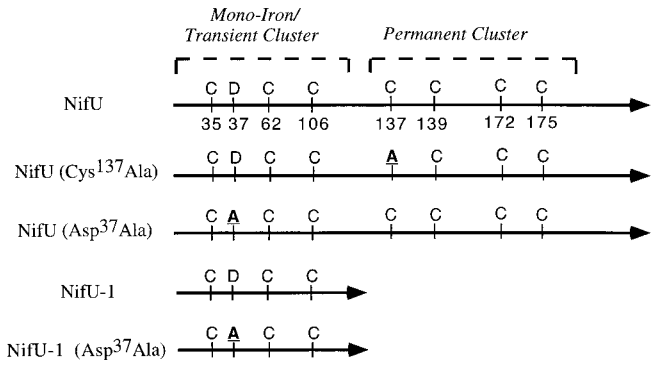

Fig. 1. Schematic representation of NifU and different forms of NifU. The uppermost line represents full-length NifU. Above the line are indicated the cysteine (C) and aspartate (D) residues relevant to the present work. The numerical residue positions are indicated below the line. Substituting residues present in altered forms of NifU or NifU-1 are indicated by a bold, underlined letter at the appropriate position. Dashed brackets at the top of the figure indicate the respective mononuclear iron/transient [2Fe-2S] cluster domain and permanent [2Fe-2S] cluster domain within the NifU primary sequence.

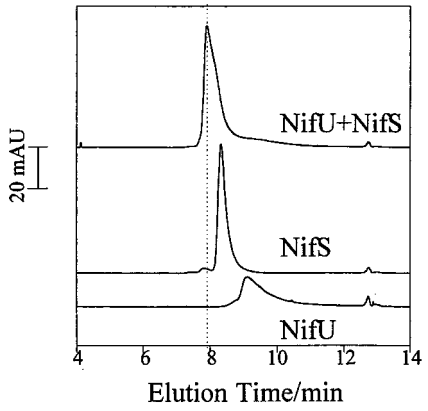

Fig. 2. Complex formation between NifU and NifS. The figure shows the elution profiles of NifS, NifU, or an equimolar mixture of NifU and NifS, by using size exclusion chromatography. Conditions used are described in *Materials and Methods*.

were carried out anoxically in a septum-sealed cuvette under an Ar atmosphere. Protein samples were purified under Ar, and buffers used were extensively degassed and sparged with Ar before use. Anoxic conditions were maintained with a Schlenk apparatus and/or an anaerobic glove box. Reactions were initiated by the addition of L-cysteine and were monitored by UV-visible spectroscopy as described below.

NifU and NifS complex formation was monitored by gel exclusion HPLC (Beckman System Gold) column chromatography with a Zorbax GF-250 column. Volumes of 100 μl containing 6 nmol of NifU or NifS (or 6 nmol of NifU plus 6 nmol of NifS) in a 25 mM Tris·HCl (pH 7.4) buffer containing 20 mM NaCl/1.0 mM DTT were injected onto the column. Samples that contained a mixture of NifU and NifS were preincubated at room temperature for 8 min before loading the column. For sample mixtures in which a molar excess of NifU or NifS was used, the sample contained approximately 6 nmol of one protein and 12 nmol of the other. Elution of the protein samples was monitored by visible *A* at 405 nm. Results obtained by using the Beckman System Gold HPLC were also independently confirmed with an Amersham-Pharmacia FPLC chromatography system fitted with a Superose 12 column.

Spectroscopic Methods. All sample concentrations are based on protein determinations and are expressed per NifU or NifU-1 monomer. UV-visible absorption spectra were recorded under anoxic conditions in septum-sealed 1-mm and 1-cm cuvettes, by using a Shimadzu 3101PC scanning spectrophotometer or a Cary diode array spectrophotometer. X-band ($\approx$9.6 GHz) EPR spectra were recorded by using a Bruker ESP-300E EPR spectrometer equipped with a dual-mode ER-4116 cavity and an Oxford Instruments ESR-9 flow cryostat.

Resonance Raman spectra were recorded by using an Instruments SA (Edison, NJ) Ramanor U1000 spectrometer fitted with a cooled RCA 31034 photomultiplier tube with 90° scattering geometry and lines from a Coherent Radiation (Palo Alto, CA) Sabre10-W argon ion laser. Spectra were recorded digitally with photon-counting electronics, and the signal/noise ratio was improved by signal averaging multiple scans. Band positions were calibrated by using the excitation frequency and CCl_4, and these positions are accurate to ± 1 cm^{-1}. Samples consisted of 12-μl droplets of concentrated protein (2–4 mM) that were placed in a custom designed sample cell attached to the cold finger of an Air Products Displex model CSA-202E closed-cycle refrigerator. The sample temperature was maintained at 18 K during scanning to minimize laser-induced sample degradation. Bands caused by the frozen buffer solutions have been subtracted from all of the spectra shown in this work after normalization of lattice modes of ice centered at 229 cm^{-1}.

Results

NifU and NifS Complex Formation. That NifU and NifS do not form a tight complex was determined in two different ways. First, NifU was not found to copurify with NifS when NifS was isolated from crude extracts prepared from nitrogen-fixing *Azotobacter vinelandii* cells. Second, specific immunoprecipitation of either NifU or NifS from *A. vinelandii* crude extracts did not result in the coprecipitation of the complementary protein. However, NifU and NifS can form a transient complex because an equimolar mixture of NifU and NifS results in the appearance of a new peak during size-exclusion column chromatography when compared with individually chromatographed samples of either NifU or NifS (Fig. 2). Calibration of the column and denaturing gel electrophoresis of the peak fraction of the NifU-NifS complex indicate formation of a heterotetrameric complex. When a twofold molar excess of NifU was mixed with NifS, a peak corresponding to the NifU-NifS complex and a peak corresponding to uncomplexed NifU could be resolved by gel exclusion chromatography. The converse experiment involving the addition of a twofold molar excess of NifS also resulted in the appearance of two peaks during chromatography of the sample, one corresponding to NifS and the other corresponding to the NifU-NifS complex. The shoulder recognized in the NifU-NifS complex fraction shown in Fig. 2 is reproducible and probably indicates that dissociation of the complex occurs during chromatography.

Experimental Rationale. In previous work we found that seven cysteines contained within NifU are required for its full *in vivo* function (7). Four of these cysteines (Cys^{137}, Cys^{139}, Cys^{172}, and Cys^{175}) are contained within a central segment of NifU and provide the ligands for the [2Fe-2S] cluster present in each monomer of the dimeric NifU protein as isolated. Because the [2Fe-2S] clusters contained within isolated NifU are tightly bound and cannot be released by chelating reagents, we refer to them as the permanent clusters and consider it unlikely that they represent precursors destined for assembly of the nitrogenase metalloclusters. We therefore became interested in whether a second, more labile cluster might be assembled elsewhere on NifU. The three other cysteines (Cys^{35}, Cys^{62}, and Cys^{106})

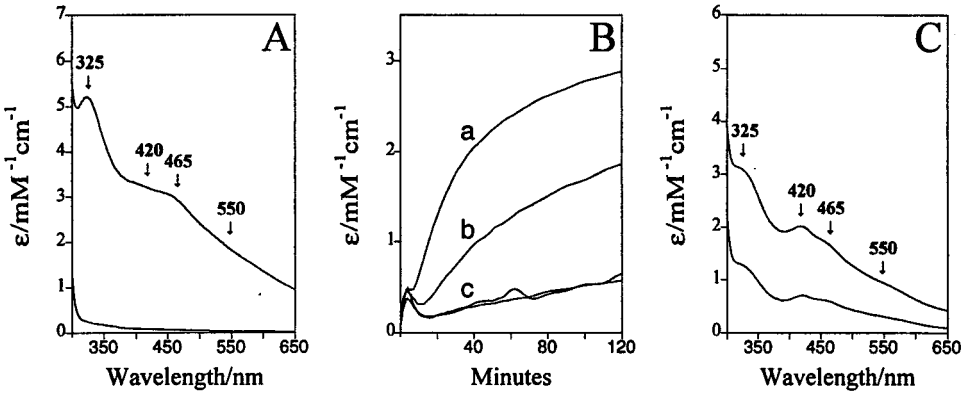

Fig. 3. NifS-dependent *in vitro* Fe-S cluster assembly. (*A*) UV-visible spectrum of NifU-1 before *in vitro* Fe-S cluster assembly [before L-cysteine was added to initiate assembly (lower spectrum) and after 140 min of *in vitro* cluster assembly (upper spectrum)]. The postassembly spectrum shown in *A* is the maximum that could be obtained. (*C*) UV-visible spectrum of NifU-1(Asp^{37}Ala) before *in vitro* cluster assembly (lower spectrum) and after 80 min of *in vitro* Fe-S cluster assembly (upper spectrum). The postassembly spectrum shown in *C* represents approximately 60% of the maximum that could be obtained. (*B*) Time dependence of Fe-S cluster assembly as monitored by the change in extinction coefficient at 465 nm vs. time after initiation of the Fe-S cluster assembly reaction. The time dependence for cluster assembly shown in line a of panel *B* corresponds to the same sample shown in panel *A*. Line b of panel *B* shows cluster assembly under the same conditions as for line a, except that half of the amount of NifS was added to the assembly cocktail. Data shown in the lines labeled c are controls. One data set corresponds to conditions that are the same as for line *a* except that an altered form of NifS having the active-site Cys^{325} residue substituted by alanine was used. The other data set in *C* corresponds to conditions that are the same as used for line a, except that an altered form of NifU-1 having the Cys^{62} residue substituted for by alanine was used. Conditions for Fe-S cluster assembly are described in *Materials and Methods*.

required for full *in vivo* NifU function were targeted as the most likely residues to participate in providing such an assembly site for formation of a transient cluster. The possibility for assembly of a second Fe-S cluster on NifU was also considered because we recently obtained evidence for the presence of one mononuclear Fe-binding site within each subunit of the NifU protein as isolated (7). Thus, we considered the possibility that binding of Fe at the mononuclear site could represent an intermediate stage in the assembly of a transient Fe-S cluster on NifU. Binding of Fe at the mononuclear site(s) requires residues Cys^{35}, Cys^{62}, and Cys^{106}.

Our ability to test whether a transient Fe-S cluster could be assembled on NifU was complicated by the presence of one permanent [2Fe-2S] cluster in each monomer of intact isolated NifU. This problem was circumvented in two different ways. First, a portion of NifU was recombinantly expressed and then isolated that corresponds only to the N-terminal third of the NifU coding sequence. This truncated form of NifU is referred to as NifU-1, and it does not include those cysteines that provide the coordinating ligands to the permanent [2Fe-2S] cluster contained within full-length NifU. Thus, the isolated form of NifU-1, which is a homodimer, does not contain the permanent [2Fe-2S] clusters contained within full-length NifU (see Fig. 1). Second, a full-length form of altered NifU that carries an alanine substitution for one of the 2Fe-2S cluster-coordinating residues (residue Cys^{137}) was also recombinantly produced and isolated. This altered NifU protein is referred to as NifU(Cys^{137}Ala) and also does not contain the permanent [2Fe-2S] cluster in its isolated form (Fig. 1). Purified samples of NifU-1 and NifU(Cys^{137}Ala) were then used in experiments described below, which demonstrate that they can serve as scaffolds for NifS-catalyzed formation of [2Fe-2S]$^{2+}$ clusters.

NifU-1 and NifU(Ala^{137}) Can Serve as Scaffolds for Assembly of a [2Fe-2S]$^{2+}$ Cluster. It was possible to develop an optimized *in vitro* Fe-S cluster biosynthetic system that includes a 0.05 mM NifU-1 dimer or NifU(Cys^{137}Ala)/0.1 mM ferric ammonium citrate/5 mM β-mercaptoethanol/1 mM L-cysteine/1 μM NifS. In this biosynthetic system, NifS is present at only very low levels when compared with NifU-1 or NifU(Cys^{137}Ala). One reason for performing the biosynthetic assay in this way was to ensure that the pyridoxal-phosphate chromophore present in NifS would not interfere with the ability to detect Fe-S cluster formation by UV-visible absorption and resonance Raman spectroscopies. Also, by using only catalytic amounts of NifS, it was possible to monitor the time-dependent formation of Fe-S clusters. A typical experiment in which NifU-1 was used as a scaffold for Fe-S cluster assembly is shown in Fig. 3*A* and *B*. These data show that there is a time-dependent assembly of a chromophoric species in NifU-1 that exhibits absorbance inflections at 325, 420, 465, and 550 nm (Fig. 3*A*). This UV-visible absorption spectrum is characteristic of [2Fe-2S]$^{2+}$ cluster-containing proteins (8), although the absorption peaks are less well defined in our sample when compared with similar spectra from typical [2Fe-2S]$^{2+}$ cluster-containing proteins. The most likely explanation for the relatively featureless nature of the absorbtion spectrum is that, once formed, the [2Fe-2S]$^{2+}$ cluster is relatively unstable at ambient temperature. This instability is manifested by a concomitant accumulation of a colloidal precipitate of iron sulfide that is responsible for the spectral inflections becoming increasingly less well defined upon incubation of the sample beyond about 200 min. A colloidal iron-sulfide precipitate also accumulates when NifU-1 is omitted from the reaction mixture, but this occurs at a much slower rate than the rate observed for Fe-S cluster biosynthesis in the complete system.

Fig. 3*B* (curves a and b) shows the time dependence of [2Fe-2S] cluster assembly on the NifU-1 scaffold. These data also show that cluster formation is dependent on the concentration of NifS, with an approximate doubling in the rate of cluster formation when the amount of NifS in the reaction mixture is doubled. The results of control experiments presented in Fig. 3*B* also show that an altered form of NifS, for which the active site Cys^{325} residue was substituted by alanine (5), is not active in cluster formation. Altered forms of NifU-1, in which any one of the three cysteine residues (Cys^{35}, Cys^{62}, and Cys^{106}) has been substituted by alanine, were also ineffective in cluster assembly. Finally, no [2Fe-2S] cluster formation occurred if any of the above-mentioned components of the biosynthetic mixture was omitted. When NifU(Cys^{137}Ala) was substituted for NifU-1 in the assembly mixture, a spectrum having a very similar line shape and intensity and the same absorbance maxima was observed (data not shown).

Isolation of an NifU-1 Variant That Contains a Stabilized $[2Fe\text{-}2S]^{2+}$ Cluster. The $[2Fe\text{-}2S]^{2+}$ cluster assembled onto NifU-1 or NifU($Cys^{137}Ala$) is labile *in vitro*. For example, as judged by A_{420}/A_{280} ratios, more than 50% of the cluster was lost when gel filtration was used to remove excess reagents. Such lability (also see below) was not unexpected considering that the proposed physiological function of the cluster is to supply the iron and sulfur necessary for nitrogenase metallocluster assembly. In other words, the transient 2Fe-2S cluster must have a mechanism to escape from the NifU scaffold during the maturation of the nitrogenase component proteins and therefore should not be tightly bound to NifU. During the course of our studies with altered forms of NifU-1 as an approach to examining the nature of the mononuclear iron-binding site, we fortuitously identified an altered form of NifU-1 that contains some $[2Fe\text{-}2S]^{2+}$ cluster in its isolated state (Fig. 3*C*). This altered form of NifU-1 has the Asp^{37} residue substituted by alanine and is referred to as NifU-1($Asp^{37}Ala$). The isolated form of NifU-1 ($Asp^{37}Ala$) contained ≈0.15 Fe per monomer. Moreover, the $[2Fe\text{-}2S]^{2+}$ cluster contained within isolated NifU-1($Asp^{37}Ala$) is stable with no loss or change in the UV-visible spectrum even after incubation at ambient temperature for 12 h. Stabilization of an Fe-S cluster resulting from an amino acid substitution has precedence for the FNR protein from *E. coli*. In this case a variant form was identified that contains a more stable 4Fe-4S cluster and is affected in signal transduction events dependent on cluster assembly and disassembly (9).

When NifU-1($Asp^{37}Ala$) was used as a scaffold for cluster assembly (Fig. 3*F*), a threefold increase in visible absorption intensity over 200 min was observed. The resulting spectrum is characteristic of a biological $[2Fe\text{-}2S]^{2+}$ center and, in agreement with the Fe analyses of the isolated sample, the extinction coefficients (e.g., A_{420} = 3.0 mM^{-1} cm^{-1}) are indicative of approximately 0.5 clusters per monomer or 1.0 cluster per homodimer. This conclusion is based on the range of extinction coefficients for typical $[2Fe\text{-}2S]^{2+}$ proteins [A_{420} = 6–11 mM^{-1} cm^{-1} (8)]. Analogous biosynthetic cluster reconstitution experiments were also carried out with a full-length form of NifU for which the Asp^{37} residue is substituted by alanine [designated NifU($Asp^{37}Ala$)]. The resulting UV-visible absorption spectrum was indistinguishable from that of the starting spectrum originating from the permanent $[2Fe\text{-}2S]^{2+,+}$ clusters, except for a uniform 30% increase in absorption intensity in the 300- to 800-nm region. This result is consistent with the formation of about one additional $[2Fe\text{-}2S]^{2+}$ cluster per homodimer (data not shown). It should be noted that, in the biosynthetic system described here, only one ferric ion is added per each protein monomer. Consequently, no more than one $[2Fe\text{-}2S]^{2+}$ cluster could be formed per homodimer. Nevertheless, during the development of the biosynthetic system, we found that the addition of higher levels of ferric iron did not increase the amount of transient cluster accumulated when any of the various forms of NifU was used in the assembly cocktail. The main consequence of a twofold or fivefold increase in the ferric ammonium citrate concentration was to decrease the quality of the absorption data owing to an increase in the accumulation of colloidal iron sulfide.

Resonance Raman Evidence for Assembly of the Transient $[2Fe\text{-}2S]^{2+}$ Cluster. Resonance Raman spectroscopy was used to identify and further characterize the transient $[2Fe\text{-}2S]^{2+}$ clusters present in the various forms of NifU proteins investigated in this work. A comparison of the low-temperature resonance Raman spectra of the permanent $[2Fe\text{-}2S]^{2+,+}$ clusters in NifU and of the transient $[2Fe\text{-}2S]^{2+}$ assembled in NifU-1 by using 488-nm excitation is shown in Fig. 4*A* and *B*. In both spectra, the pattern and frequency of bands in the Fe-S stretching region are uniquely indicative of $[2Fe\text{-}2S]^{2+}$ clusters (6, 10–12). The relative intensities of equivalent bands are remarkably similar for both clusters, but the frequencies are all up-shifted by 6–8 cm^{-1} for the transient $[2Fe\text{-}2S]^{2+}$ cluster in NifU-1 when compared with the permanent clusters in NifU. Hence, the vibrational assignments made for the permanent clusters in NifU (6) can be transferred directly to the transient cluster. Also, the relative intensities and frequencies of the bands in NifU-1 are almost identical to those of the $[2Fe\text{-}2S]^{2+}$cluster in human ferrochelatase (ref. 12; Fig. 4), which has recently been shown to have complete cysteinyl ligation by amino acid substitution (13) and crystallographic studies (H. A. Dailey, personal communication). This result strongly suggests that the cluster assembled on NifU-1 has complete cysteinyl ligation. Because there are only three cysteines available in each monomer, this situation indicates that the transient cluster is most likely bridged between the subunits. The higher Fe-S stretching frequencies for the transient cluster in NifU-1 compared with the permanent clusters in NifU indicate stronger Fe-S bonds for the transient structure. Thus, the lability of the transient cluster is more likely to be a consequence of enhanced solvent accessibility resulting from its location at the subunit interface, rather than from an intrinsic thermodynamic instability.

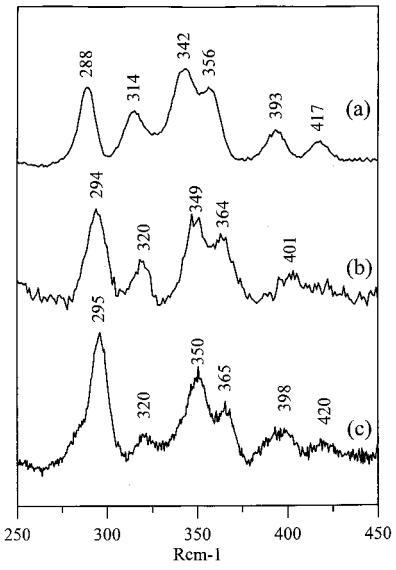

Fig. 4. Comparison of the low-temperature resonance Raman spectra of the $[2Fe\text{-}2S]^{2+}$ clusters in as-isolated NifU (*a*), reconstituted NifU-1 (*b*), and human ferrochelatase (*c*). All samples (2–4 mM in 100 mM Tris·HCl, pH 7.8, buffer) were in the form of concentrated frozen droplets maintained at 18 K. The spectra were recorded by using 488-nm excitation with 70-mW of laser power at the sample and are the sum of 19, 90, and 80 scans for *a*, *b*, and *c*, respectively. Each scan involved advancing the spectrometer in 1 cm^{-1} increments (0.5 cm^{-1} for *c*) and photon counting for 1 s/point with 6-cm^{-1} resolution.

Although the lability of the transient $[2Fe\text{-}2S]^{2+}$ cluster has thus far impeded our attempts to obtain resonance Raman spectra from reconstituted full-length NifU, the decreased lability in NifU($Asp^{37}Ala$) has provided an opportunity to assess the resonance Raman spectrum of the cluster in a full-length version of NifU (Fig. 5). The spectrum of the reconstituted form of NifU($Asp^{37}Ala$) (Fig. 5*a*) is clearly dominated by he permanent clusters of as-isolated NifU (Fig. 5*b*). Nevertheless, the difference between the reconstituted and as-isolated data sets (Fig. 5*c*) reveals a spectrum very similar to that of the reconstituted $[2Fe\text{-}2S]^{2+}$ cluster in NifU-1($Asp^{37}Ala$) (Fig. 5*d*) and having approximately half the intensity of the permanent clusters. Because Fe-S stretching frequencies are very sensitive to

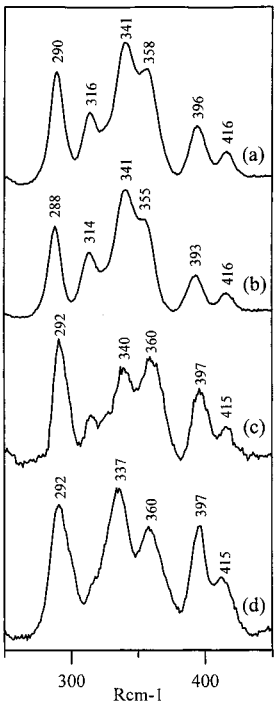

Fig. 5. Low-temperature resonance Raman spectra of transient $[2Fe\text{-}2S]^{2+}$ clusters. (*a*) NifU(Asp^{37}Ala) after treatment by the cluster biosynthetic system; (*b*) as-isolated NifU(Asp^{37}Ala); (*c*) difference spectrum (spectrum *a* minus spectrum *b*); (*d*) NifU-1(Asp^{37}Ala) after treatment with the cluster biosynthetic system. The conditions used are the same as those described in Fig. 4, and each spectrum is the sum of 33 scans.

minor perturbations in the cluster environment, this result indicates a negligible change in the transient cluster environment on removal of the C-terminal domain. Moreover, there is a close similarity in the resonance Raman spectra of the transient clusters reconstituted in NifU-1 (Fig. 4*b*), NifU-1(Asp^{37}Ala) (Fig. 5*d*), and NifU(Asp^{37}Ala) (Fig. 5*c*). The main differences among these species lie in the frequencies of the total symmetric predominantly Fe-S(Cys) modes assigned at 349, 337, and 340 cm^{-1} in NifU-1, NifU-1(Asp^{37}Ala), and NifU(Asp^{37}Ala), respectively. This variability is likely to originate from differences between the wild-type and Asp^{37}Ala variant in terms of cysteinyl Fe-S-C-C dihedral angles and/or hydrogen bonding interactions involving cysteinyl-S atoms. An attractive possibility is that substitution of the Asp^{37} residue by alanine reduces cluster lability by decreasing solvent accessibility and that this feature is reflected in the resonance Raman as a result of perturbed hydrogen-bonding interactions involving the ligating cysteine S atoms. In summary, all of the resonance Raman spectra clearly demonstrate NifS-mediated assembly of a similar transient [2Fe-2S] cluster in a variety of different forms of NifU.

Release of the [2Fe-2S] Cluster on Its Reduction. UV-visible spectra and/or resonance Raman spectra of the Fe-S clusters formed in the biosynthetic system described here provides evidence for the catalytic formation of a labile $[2Fe\text{-}2S]^{2+}$ cluster within the NifU protein. Complementary spectroscopic evidence for this conclusion could not be obtained by using either electron paramagnetic resonance or variable-temperature magnetic circular dichroism spectroscopy, because of the extreme reductive lability of the cluster. For example, dithionite-mediated reduction of the transient $[2Fe\text{-}2S]^{2+}$ cluster resulted in an immediate, complete, and irreversible bleaching of the visible spectrum. Also, no paramagnetic form of the transient cluster could be trapped even by freezing the sample within 10 s after addition of a twofold excess of dithionite. The dithionite-reduced samples exhibited no $S = 1/2$ EPR signals over the temperature range 10–100 K and no temperature-dependent magnetic circular dichroism bands, suggesting that the $[2Fe\text{-}2S]^{+}$ form is unstable and degraded immediately on reduction. The lability of the transient [2Fe-2S] cluster on reduction was also shown by quantitative capture of the Fe^{2+} ion released during dithionite reduction by using the Fe^{2+}-chelating reagent α,α'-dipyridyl (data not shown).

Discussion

NifU contains two distinct types of iron-binding sites. In as-isolated NifU, one of these types of sites is occupied by a $[2Fe\text{-}2S]^{2+,+}$ cluster that we refer to as the permanent cluster (6). The other type of iron-binding site is a mononuclear site that is predominantly unoccupied in the as-isolated protein but can be filled *in vitro* by the addition of ferric ion (7). In the current work, we show that L-cysteine and catalytic amounts of NifS can be used to assemble an additional labile $[2Fe\text{-}2S]^{2+}$ cluster within a variety of different forms of the NifU protein. The lability of this cluster and the presence of the permanent clusters have so far prevented definitive identification of this transient cluster in full-length wild-type NifU. However, a combination of UV-visible absorption and resonance Raman studies has provided abundant evidence for the *in vitro* assembly of this cluster in samples of NifU-1 and the NifU(Cys^{137}Ala) variant, neither of which contains the permanent clusters. Other evidence has been obtained with the NifU-1(Asp^{37}Ala) and NifU(Asp^{37}Ala) variants, in which the transient cluster is less labile. The same three cysteine residues necessary for iron binding at the mononuclear site are also required for NifS-directed assembly of the transient [2Fe-2S] cluster (Fig. 1). Although we cannot yet rule out the possibility that a set of conditions exists whereby one transient $[2Fe\text{-}2S]^{2+}$ cluster can be assembled within each subunit, the available data are consistent with a model in which ferric ions bound at the individual mononuclear sites are rearranged on donation of sulfur by NifS, to form a $[2Fe\text{-}2S]^{2+}$ cluster that is bridged between the two NifU subunits. Evidence supporting this mode of binding includes (*i*) the lability of the transient [2Fe-2S] cluster; (*ii*) the iron-binding stoichiometry at the mononuclear sites; (*iii*) a maximal UV-visible absorption intensity consistent with no more than one transient [2Fe-2S] cluster per NifU homodimer, and (*iv*) a resonance Raman spectrum that is interpreted in terms of four cysteine ligands, owing to its close correspondence to the spectrum of human ferrochelatase.

The lability of the transient cluster and its release from the polypeptide matrix on reduction is consistent with the hypothesis that the function of the transient cluster is to provide iron and sulfide necessary for the formation of the Fe-S cores of the nitrogenase metalloclusters. The role of the permanent [2Fe-2S] clusters contained within NifU is not yet known. However, the observed release of the transient cluster on reduction indicates that the role of the permanent clusters could be to provide reducing equivalents for that process. In this context we note that both irons contained in the NifU-bound transient cluster are in the ferric oxidation state and that previous work has shown that chemical reconstitution of Fe-S cluster-containing proteins is most effective when ferrous ions are used in the reconstitution system (2). Thus, reduction of the transient cluster might be important not only for its release but also for placing irons destined for nitrogenase metallocluster core formation in the appropriate oxidation state. It is also possible that the permanent $[2Fe\text{-}2S]^{2+,+}$ clusters could have a redox function in the acquisition of iron for initial binding at the mononuclear sites. There is no *a priori* reason why the permanent clusters could not participate in all of these functions.

Genes encoding homologs to NifU and NifS are also located within the genomes of a wide variety of non-nitrogen fixing organisms (14). We have designated these as *isc* genes to indicate the proposed role of their products in the housekeeping function of general Fe-S cluster assembly. In line with this proposal the *isc* genes have been found to be essential for *A. vinelandii* viability (14). There is also mounting biochemical and genetic evidence from other laboratories that the *isc* gene products are involved in Fe-S cluster assembly in both prokaryotic (15) and eukaryotic (16–19) organisms. A comparison of the organization of the NifU protein and its proposed housekeeping counterpart, designated IscU, is relevant to the work described here. For example, the IscU protein is considerably truncated when compared with NifU, bearing sequence identity only to the N-terminal third of NifU. This portion of NifU corresponds to the NifU-1 fragment described in the present work. The NifU Cys^{35}, Cys^{62}, and Cys^{106} residues contained within this segment are also strictly conserved in all *iscU* gene products identified so far. In fact, the IscU primary sequence is among the most conserved sequence motifs in nature (20). The IscU protein does not contain a sequence corresponding to the permanent $[2Fe\text{-}2S]^{2+,+}$ cluster-binding domain present in NifU. However, there is another gene contained within the *isc* gene cluster whose product does harbor weak primary sequence identity when compared with the $[2Fe\text{-}2S]^{2+,+}$ cluster-binding region of NifU. This small ferredoxin has been purified and shown to contain a $[2Fe\text{-}2S]^{2+,+}$ cluster that is nearly identical in its spectroscopic and electronic properties when compared with the [2Fe-2S] clusters contained within as-isolated NifU (21, 22). Thus, a function analogous to that provided by the NifU permanent cluster might also be duplicated by this ferredoxin. It is interesting that a bacterial ferritin-associated $[2Fe\text{-}2S]^{2+,+}$ ferredoxin having the same spatial arrangement of cluster-coordinating cysteines, as well as the same spectroscopic and electronic properties as the *isc*-specific ferredoxin, has also been identified in *E. coli* (23, 24). This observation has led to speculation that a function of the bacterial ferritin-associated ferredoxin could involve the release of iron from ferritin for Fe-S cluster assembly, a suggestion also in line with a possible role for the permanent clusters contained within NifU.

Although it is not yet known whether a [2Fe-2S] cluster can be assembled on IscU, both IscU and IscS from *A. vinelandii* have been recombinantly produced and isolated. IscS exhibits an L-cysteine desulfurase activity (14) similar to that demonstrated for NifS (4). Also, in preliminary work, IscS and IscU have been found to form a macromolecular complex similar to that described for NifS and NifU (our unpublished results). Despite these similarities the assembly of Fe-S clusters catalyzed by the Isc system might be considerably more complex than we have found so far for the Nif system. For example, there are heat-shock-cognate (Hsc) proteins encoded within bacterial genomes that have been suggested to have chaperone functions involving either the formation of Fe-S clusters or their insertion into various target proteins (13). Also, in *Saccharomyces cerevisiae*, certain Hsc proteins have already been implicated in the physiological assembly of Fe-S clusters (16, 18, 25). In contrast, there are no known *nif*-specific gene products homologous to the Hsc family of proteins.

Finally, there remain a number of important gaps in our understanding of the mobilization of the iron and sulfur required for maturation of Fe-S cluster-containing proteins. Although the current work demonstrates the *in vitro* ability of NifU to assemble a transient [2Fe-2S] cluster in the presence of NifS, whether this system functions by directly donating a 2Fe-2S unit for *in vivo* cluster assembly still needs to be determined. If Fe-S clusters are assembled in this way, then there are fundamental issues concerning how the transient cluster is delivered to the target protein and how the 2Fe-2S units might be assembled into higher-order clusters. In addition, it is not yet known how the NifS-bound persulfide is physiologically released for assembly of the transient cluster. We believe that the Nif system will continue to provide a model for the biochemical-genetic approach to address these issues.

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