The "Nitrogenase-Protective" FeSII Protein of *Azotobacter vinelandii*: Overexpression, Characterization, and Crystallization†

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ABSTRACT: The *Azotobacter vinelandii* FeSII protein confers conformational protection to nitrogenase by binding to the MoFe and Fe proteins under periods of oxidative stress to create an inactive but O_2-stabilized tripartite complex. In this work the FeSII protein has been overexpressed in *Escherichia coli*, and the recombinant protein has been purified to homogeneity, crystallized, and characterized in terms of its functional, spectroscopic, and redox properties. The recombinant protein is a homodimer and is expressed as a holoprotein with one $[2Fe\text{-}2S]^{2+,+}$ cluster in each subunit. It is shown to be functional in reconstituting an O_2-stable nitrogenase complex *in vitro*. Spectroscopic studies using the combination of UV−visible absorption, CD, and variable temperature MCD, EPR, and resonance Raman indicate that the $[2Fe\text{-}2S]^{2+,+}$ cluster is coordinated exclusively by cysteine residues. The arrangement of coordinating cysteines in the primary sequence and the EPR properties of the $[2Fe\text{-}2S]^{+}$ cluster (g = 2.04, 1.95, 1.88) are very similar to those of chloroplast ferredoxins. However, the variable-temperature MCD, resonance Raman, and redox properties (E_m = −262 ± 10 mV based on dye-mediated EPR redox titrations) are more characteristic of hydroxylase-type ferredoxins such as adrenodoxin. In contrast to chloroplast-type ferredoxins, the vibrational properties of the $[2Fe\text{-}2S]^{2+,+}$ cluster in the FeSII protein indicate that none of the cysteinyl Fe−S−C−C dihedral angles are close to 180° and that the cluster is not exposed to solvent. Preliminary X-ray diffraction analysis indicates that the protein crystallizes in an orthorhombic space group with unit cell dimensions a = 135 Å, b = 135 Å, and c = 38 Å and that there are at least two dimers per asymmetric unit.

The ATP requirements of nitrogen fixation necessitate that oxygen-utilizing respiratory processes, coupled to energy-conserving reactions, occur in most diazotrophic microorganisms. Such contact with oxygen is exacerbated by the so-called "oxygen problem", i.e., the enzyme complex responsible for the reduction of molecular dinitrogen to ammonia is extremely sensitive to oxygen inactivation. Thus nitrogen-fixing bacteria have evolved a number of physiological mechanisms that "protect" their nitrogenases from rapid and irreversible inactivation by oxygen. These include a wide range of regulatory and biochemical adaptations which allow the diverse species of nitrogen-fixing bacteria to utilize gaseous dinitrogen as their primary source of nitrogen, while growing in habitats which contain oxygen levels ranging from strictly anaerobic to fully aerobic levels [see Gallon (1992) for a recent review].

Much of our current understanding of the biochemistry and enzymology of nitrogenase has come from studies on *Azotobacter vinelandii*, a strictly aerobic Gram-negative soil bacterium, which is capable of diazotrophic growth under fully aerobic growth conditions [see Dean and Jacobson (1992) or Kim and Rees (1994) for reviews of nitrogenase]. Two distinct mechanisms are known to be utilized by *A. vinelandii* to enable O_2-dependent growth and efficient nitrogen fixation under fully aerobic growth conditions (Gallon, 1992; Yates, 1988). The physiological mechanism known as "respiratory protection" involves a remarkably high respiratory level mediated through a cytochrome *d* terminal oxidase complex. This respiratory activity essentially renders the inside of the cell microaerobic such that nitrogenase can function unaffected by oxygen inhibition. Another mechanism, known as "conformational protection", involves the noncovalent binding of an iron−sulfur protein (FeSII protein;[1] Shethna et al., 1968) to the MoFe- and Fe-protein components of nitrogenase under periods of high oxygen tension; this causes the nitrogenase components to be in a catalytically inactive state but a state in which they are also protected from irreversible oxygen-mediated inactivation (Scherings et al., 1979; Laane et al., 1983; Wang et al., 1985). The "oxygen-stable nitrogenase complex", composed of all three components, is an isolable molecular complex (Bulen & LeComte, 1972; Robson, 1979). When O_2 levels are favorable for nitrogenase function, the complex presumably dissociates and the N_2-fixing process can proceed without need for *de novo* nitrogenase synthesis. Nitrogenase activity in cell-free extracts of mutant strains lacking the FeSII protein is much more sensitive to oxygen-mediated inactivation as

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[1] Abbreviations: FeSII protein, *Azotobacter vinelandii* iron−sulfur protein II; (VT)MCD, (variable-temperature) magnetic circular dichroism; RR, resonance Raman; DMS, dimethyl suberimidate; Fd(s), ferredoxin(s); NHE, normal hydrogen electrode; S^b, bridging or inorganic S; S^t, terminal or cysteinyl S; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PEG, poly(ethylene glycol).

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compared to extracts from the wild type (Moshiri et al., 1994). Also, in whole cells of the mutants, the MoFe- and Fe-protein components of nitrogenase are rapidly degraded (as compared with the wild-type cells) when the cells are incubated under conditions where the respiratory protection system is diminished (i.e., during carbon substrate limitation).

As the gene encoding the FeSII protein has been cloned, we wished to overexpress the protein in a heterologous system to facilitate further analyses of the protein. Here we describe the efficient purification of large amounts of homogeneous FeSII protein from *Escherichia coli*. The purified recombinant protein has been characterized functionally by its ability to reconstitute an oxygen-stable nitrogenase complex *in vitro* as well as spectroscopically (UV−visible absorption, CD, and variable-temperature magnetic CD (VTMCD), EPR, and resonance Raman (RR)). The results facilitate comparison of the electronic, magnetic, vibrational, and redox properties of the [2Fe-2S] cluster in the FeSII protein with those of other well-characterized [2Fe-2S] proteins [see Fu et al. (1994) and Holden et al. (1994) for reviews]. We also report on the generation of diffraction-quality crystals of the protein suitable for structural analyses.

MATERIALS AND METHODS

Overexpression of the A. vinelandii FeSII Protein in E. coli. Starting from plasmid pFU1, a 600 bp *Pst*I−*Sph*I restriction fragment [corresponding to positions 196−796 in the sequenced region; see Moshiri et al. (1994)] was obtained after a complete *Pst*I digestion, followed by a partial *Sph*I digestion (this fragment contains an internal *Sph*I site). The fragment was initially subcloned into the *Pst*I−*Sph*I sites within the polylinker of plasmid pUC19 and, after verification, subcloned as a *Pst*I−*Hin*dIII restriction fragment out of pUC19, into the *Pst*I−*Hin*dIII sites within the polylinker of plasmid pBluescript SKII(+), yielding plasmid p*Av*FeSII (ampicillin resistant). The insert in plasmid p*Av*FeSII was partially sequenced to verify the correctness of the coding region. Plasmid p*Av*FeSII was transformed into competent cells prepared from a number of different strains of *E. coli* (Ausubel et al., 1989). These included HB101, JM83, DH5α, and XL-1 Blue (all from laboratory stocks), as well as strain W3110 (from B. Bachman, *E. coli* Genetic Stock Center, Yale University). Of the tested strains, transformants of strain W3110 were found to be superior in terms of both total cell yield and the expressed level of FeSII protein. A number of minimal and rich media were subsequently evaluated for their effect on the levels of expressed FeSII protein (Ausubel et al., 1989). A phosphate-buffered tryptone−yeast extract medium (TYP), containing (per liter) 5 g of Na_2HPO_4, 16 g of tryptone (Difco), and 10 g of yeast extract (Difco), pH 7.4, supplemented with 150 mg of ampicillin, was found to be most suitable. The addition of 50 μM $FeSO_4$ was found to further increase the yield of FeSII protein. For protein purification, a single colony of p*Av*FeSII/W3110 was used to inoculate 10 mL of TYP+Fe. After overnight growth at 37 °C, this was used to inoculate 750 mL of prewarmed TYP + Fe (in 2 L baffled flasks). The culture was grown for 16−20 h at 37 °C on a gyrotory shaker at 220 rpm. The cells were harvested by centrifugation, washed once with ice-cold 50 mM Na-HEPES buffer, pH 7.4, containing 5 mM $MgCl_2$, and used immediately for subsequent purification.

Purification of the FeSII Protein. All the steps for the purification were carried out at 0−4 °C. The washed cells from 750 mL cultures were resuspended in 20 mL of 50 mM Na-HEPES buffer, pH 7.4, containing 5 mM $MgCl_2$ and 1 mM phenylmethanesulfonyl fluoride. The cells were broken by two passages through a French pressure cell. Sodium chloride and poly(ethylene glycol) 8000 (both as powders) were added to the whole mixture to a final concentration of 0.4% and 11%, respectively. After gentle mixing over a 45 min period, the whole mixture was centrifuged for 15 min at 12000 rpm in a Sorvall-type SS-34 rotor. The clear, dark red supernatant was removed and loaded onto an approximately 2.5 × 7.0 cm (25 mL bed volume) DEAE-Sepharose column (Pharmacia), equilibrated with 10 mM Na-HEPES buffer, pH 7.4, containing 1 mM $MgCl_2$. The red-colored FeSII protein adsorbed to the column. The column was subsequently washed with 50 mL of 10 mM Na-HEPES buffer, pH 7.4, containing 1 mM $MgCl_2$. The FeSII protein was eluted by further washing of the column with 50 mM Na-HEPES buffer, pH 7.4, containing 5 mM $MgCl_2$. Light red fractions containing the FeSII protein (total volume of approximately 90−120 mL) were pooled, and the pH of the combined fractions was lowered to 5−5.2 by the dropwise addition of 1 M acetic acid, with rapid stirring. The sample was then concentrated using an Amicon ultrafiltration unit with a YM-30 membrane, operating under nitrogen pressure. After concentration down to approximately 5 mL, the sample was diluted approximately 5-fold with water and reconcentrated. The dark red sample was finally loaded on an approximately 0.8 × 4.5 cm (4 mL bed volume) S-Sepharose column (Pharmacia), equilibrated with 50 mM sodium acetate buffer, pH 5.2. The FeSII protein was bound and, after washing of the column with 20 mL of 50 mM sodium acetate buffer, pH 5.2, containing 50 mM NaCl, was eluted by further washing of the column with the same buffer, containing 80 mM NaCl. The light red fractions containing the FeSII protein (total volume of 40−60 mL) were combined and concentrated, as before. After concentration to approximately 30−50 mg of protein/mL, pure glycerol was added to the protein sample to a final concentration of 10% (v/v), the sample was quick-frozen in 200−300 μL aliquots, and kept at −70 °C. The whole purification, starting from the harvesting of the cells to the end, could be accomplished in approximately 12 h. The N-terminal amino acid sequencing of the recombinant FeSII protein was performed at the Johns Hopkins Protein/Peptide Operations and Core Facility. Protein concentrations were determined using the BCA protein assay kit (Pierce), with bovine serum albumin as standard. The iron content was determined using the *o*-phenanthroline method (Fortune & Mellon, 1938), using ferrous ammonium sulfate solutions as standards.

Cross-Linking Reactions. Solutions of the purified FeSII protein, in 50 mM Na-HEPES and 5 mM $MgCl_2$, pH 7.8, at different protein concentrations, were treated with 10 mM dimethyl suberimidate (DMS, Pierce), added from a freshly prepared 100 mM solution (in water, pH adjusted to 7.8 with NaOH). The reaction was allowed to proceed at room temperature for indicated times and quenched with the addition of an equal volume of 2× SDS sample buffer, followed by boiling for 5 min.

FeSII Protein "Add Back" Experiments. A. vinelandii mutant strain CA-Δ*fesII*::KIXX1 was grown in nitrogen-

fixing liquid cultures, and cell-free extracts were prepared by the osmotic lysis method of Shah et al. (1972). Cell-free extracts were prepared in argon-sparged buffers, but sodium dithionite was omitted (Moshiri et al., 1994). Cell-free extracts were kept under argon, on ice, and were used within 12 h after preparation. The oxygen inactivation experiments, with or without added FeSII protein, were performed as follows: 0−10 μL aliquots of FeSII protein, in 25 mM Na-HEPES and 5 mM $MgCl_2$, pH 7.4, were transferred to a 0.5 mL Eppendorf tube. After being capped with a tight butyl stopper, the tube was sparged with argon (through needles) for 2 min. A 240 μL aliquot of CA-Δ*fesII*::KIXX1 cell-free extract was added by a gas-tight syringe. After being sparged for an additional minute, the sample was allowed to incubate at 30 °C for 5 min. An 80 μL aliquot was removed by a gas-tight syringe and immediately assayed for nitrogenase activity by the acetylene reduction assay. The tube containing the remainder of the sample (160 μL) was decapped, and 40 μL of 100% oxygen-sparged Na-HEPES buffer (concentration of oxygen assumed to be 1.25 mM) was added. The sample was kept at room temperature for different periods of time (typically 10 min), and 100 μL aliquots were assayed for nitrogenase activity. For each experiment, the remaining nitrogenase activity (after 10 min of exposure to 250 μM oxygen) was normalized relative to the activity of the unexposed control. The activity of unexposed samples containing different concentrations of added FeSII protein was within 10% of the unexposed cell-free extract with no added FeSII protein. Gas chromatography (to quantitate ethylene formation in the acetylene reduction assay) was done in triplicate and averaged. Other details of oxygen inactivation and nitrogenase activity measurements by the acetylene reduction assay were as described previously (Moshiri et al., 1994).

Spectroscopic Characterization of the FeSII Protein. The sample concentrations given in the figure legends and used in quantifying spectroscopic results are based on protein concentrations and are expressed per dimer (two [2Fe-2S] clusters per dimer, M_r = 13 140 for the monomer; Moshiri et al., 1994). UV−visible absorption spectra were recorded on a Shimadzu UV3101PC spectrophotometer. 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 CuEDTA as the standard and the procedures outlined by Aasa and Vänngård (1975). Spectral simulations were performed using the Simfonia software package (Bruker Instruments).

EPR redox titrations were performed at ambient temperature (25−27 °C) in a glove box under anaerobic conditions (<5 ppm O_2) using a 200 mM PIPES buffer, pH 7.0. Mediator dyes were added, each to a concentration of ca. 50 μM, in order to cover the desired range of redox potentials, i.e., methyl viologen, benzyl viologen, neutral red, safranin, phenosafranin, anthroquinone-1,5-disulfonate, indigodisulfonate, methylene blue, 1,2-naphthoquinone, duroquinone, and 1,2-naphthoquinone-4-sulfonate. Samples were first reduced completely by the addition of excess sodium dithionite, followed by oxidative titration with potassium ferricyanide. After equilibration at the desired potential, a 0.2 mL aliquot was transferred to a calibrated EPR tube and immediately frozen in liquid nitrogen. Potentials were measured with a platinum working electrode and a saturated calomel reference electrode and are reported relative to the normal hydrogen electrode (NHE).

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 the signal-to-noise ratios 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 Coherent Innova 200-K2 krypton ion laser were used for excitation, and plasma lines were removed using a Pellin Broca Prism premonochromator. Scattering was collected from the surface of a frozen 10 μL droplet using a custom-designed anaerobic sample cell (Drozdzewski & Johnson, 1988) attached to the cold finger of an Air Products Displex Model CSA-202E closed cycle refrigerator. This enables the samples to be cooled to 17 K, which facilitated improved spectral resolution and prevented laser-induced sample degradation.

Crystallization of the FeSII Protein. Tubes containing aliquots of frozen, concentrated FeSII protein were thawed and, after being diluted 10−15-fold with water, were concentrated approximately 10-fold by centrifugation (at 4 °C) in a Centricon-30 concentrator (Amicon). The FeSII protein was retained by the membrane. The dilution/concentration cycle was repeated three more times to desalt the pure FeSII protein, which remained soluble at high concentrations (40−60 mg/mL). The final sample was clarified by a 5 min microcentrifuge spin and kept on ice and used within 2 h. Hanging drops of 10−15 μL volume were set up on silanized glass coverslips by standard protocols (McPherson, 1982). Typically, equal volumes of the desalted FeSII protein (at different protein concentrations) and the desired precipitant solution were used, and vapor-phase equilibration was achieved at room temperature against an undiluted reservoir of the same precipitant solution. Poly(ethylene glycol)s were from Fluka ("MicroSelect" grade). Other chemicals and reagents were of the highest purity available.

RESULTS AND DISCUSSION

Overexpression in E. coli and Purification of the A. vinelandii FeSII Holoprotein. In the course of the cloning and sequencing of the structural gene for the *A. vinelandii* FeSII protein (Moshiri et al., 1994), we noticed that *E. coli* strains containing certain subclones of the genomic region encompassing the structural gene had a distinctive reddish color. When extracts of these cells were probed with affinity-purified antibodies prepared against the FeSII protein, the FeSII protein (at its correct size) was evident. Further optimization aimed at increasing the level of expressed protein yielded plasmid p*Avf*eSII (see Materials and Meth-

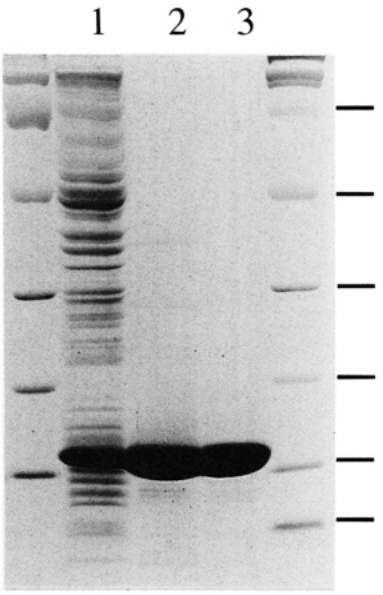

FIGURE 1: SDS−PAGE of the FeSII protein at different stages of purification from *E. coli* pA*v*FeSII/W3110 (15% acrylamide gel). Lanes 1−3 contain 30 μg of total protein. The numbers in parentheses indicate the total protein yield from cells from 1 L of culture. The two outside lanes are molecular weight standards: from the bottom, aprotinin, M_r = 6500; lysozyme, M_r = 14 400; trypsin inhibitor, M_r = 21 500; carbonic anhydrase, M_r = 31 000; ovalbumin, M_r = 45 000; serum albumin, M_r = 66 200. Lane 1: French pressure cell lysate (1205 mg of protein). Lane 2: pooled DEAE-Sepharose fractions (103 mg of protein). Lane 3: pooled S-Sepharose fractions (77 mg of protein).

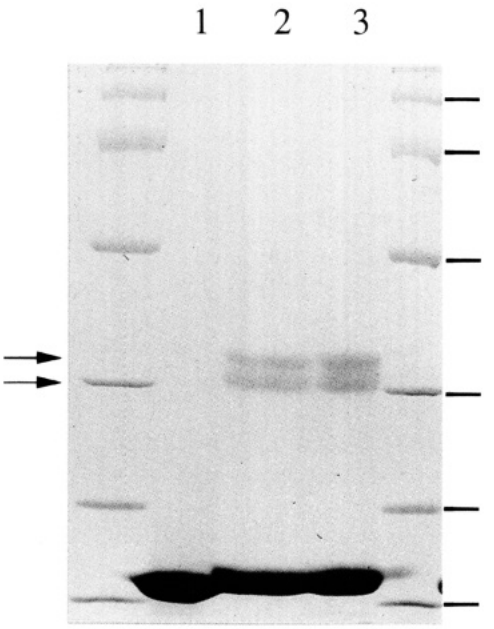

FIGURE 2: SDS−PAGE of the products of the dimethyl suberimidate cross-linking of purified FeSII protein (12.5% acrylamide gel). Lanes 1−3 contain 30 μg of total protein. The lanes at the outside show molecular weight standards: from the bottom, lysozyme, M_r = 14 400; trypsin inhibitor, M_r = 21 500; carbonic anhydrase, M_r = 31 000; ovalbumin, M_r = 45 000; serum albumin, M_r = 66 200; phosphorylase B, M_r = 97 400. Lane 1: not treated with DMS. Lane 2: reaction products after 15 min of cross-linking when [DMS] = 10 mM and [FeSII] = 19 μM. Lane 3: reaction products after 15 min of cross-linking when [DMS] = 10 mM and [FeSII] = 190 μM. Arrows indicate the bands corresponding to cross-linked products.

ods), and the overexpressed FeSII protein was found to accumulate as the soluble holoprotein, with stoichiometric levels of [2Fe-2S] clusters. Thus, *in vitro* unfolding/refolding and the chemical reassembly of its metallocluster were unnecessary, in contrast to some other [2Fe-2S] proteins (Jacobson et al., 1993; Holden et al., 1994).

The conditions for the purification of the FeSII holoprotein were based in part on the published purification scheme from nitrogen-fixing *A. vinelandii* (Scherings et al., 1977), with modifications which significantly improved the iron content and the homogeneity of the purified protein. The purification protocol did not include any precautions to exclude oxygen. Cycles of reduction/oxidation (by treatment with sodium dithionite and air, respectively) which were used in the previously described purification of the protein from *A. vinelandii* and were noted to reduce the iron content of the protein (Scherings et al., 1979) were omitted. The addition of 11% PEG-8000 to *E. coli* cell-free extracts aided in the precipitation of nucleic acids and other proteins and also allowed for the binding of the FeSII protein to DEAE-Sepharose at pH 7.4 (in 50 mM Na-HEPES + 5 mM $MgCl_2$). Further washing of the column with the same buffer (without PEG-8000) eluted the FeSII protein at a purity of greater than 95% (Figure 1, lane 3). Cation-exchange chromatography on S-Sepharose, at pH 5.2, yielded protein which was pure, as judged both by SDS−PAGE (Figure 1, lane 4) and by N-terminal amino acid analysis. Using our overexpression system and purification scheme, we can routinely purify 75−100 mg of FeSII holoprotein/L of *E. coli* culture. The protein was also sufficiently homogeneous to be crystallized (see below). The N-terminal methionine was absent in the protein purified from *E. coli*, as for the protein purified from *A. vinelandii* (Moshiri et al., 1994).

Characterization of the Recombinant FeSII Protein. Colorimetric iron analyses indicated 1.9 ± 0.3 Fe/subunit (average of four iron and protein determinations). This is in accord with analytical data for the best preparations of native FeSII proteins from both *A. vinelandii* (Scherings et al., 1983) and *Azotobacter chroococcum* (Robson, 1979), which indicated one [2Fe-2S] cluster per 13−14-kDa subunit. The FeSII protein purified from both *A. vinelandii* and *A. chroococcum* was shown to be a dimer in solution, based on native gel filtration chromatography (Scherings et al., 1983; Robson, 1979). The recombinant FeSII protein purified from *E. coli* also has a mobility in gel filtration chromatography consistent with it being a dimer (data not shown). Treatment of the purified protein with the homobifunctional cross-linking reagent DMS (Wong, 1991), followed by SDS−PAGE, revealed the formation of two distinct species, with apparent M_r values of 31 500 and 32 500 from the cross-linking treatment (Figure 2). Both cross-linked species were able to be formed, at similar levels, over a protein concentration range of over 20-fold (from 15 to 300 μM, calculated on the basis of the dimer molecular weight of 26 300). It is likely that the two different cross-linked species each correspond to different intramolecular dimers, which have different mobilities in SDS−PAGE due to different branched structures. Both species were also formed when total cell-free extracts of either non-nitrogen-fixing or nitrogen-fixing cells of *A. vinelandii* were treated with DMS, and the cross-linked species were detected by immunoblotting (data not shown). These results are all consistent with the FeSII protein being a homodimer both *in vitro* and *in vivo*.

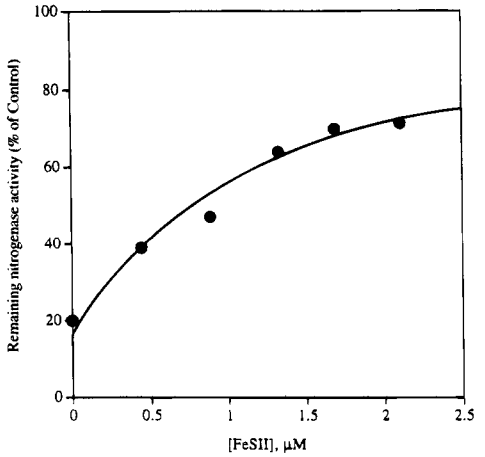

FIGURE 3: Concentration-dependent activity of purified FeSII protein in protecting total nitrogenase activity from oxygen inactivation in cell-free extracts of the FeSII deletion mutant, CA-Δ*fesII*::KIXX1. The values shown correspond to the nitrogenase activity remaining after 10 min of exposure of samples to 250 μM oxygen, normalized relative to the activity of an unexposed duplicate sample. The nitrogenase specific activity of unexposed cell-free extracts was 41.0 ± 4 nmol of ethylene produced min^{-1} (mg of protein)$^{-1}$, and total protein concentration was 5.8 mg/mL (the contribution of the FeSII protein to the total protein concentration was less than 2%). For more details, see Materials and Methods.

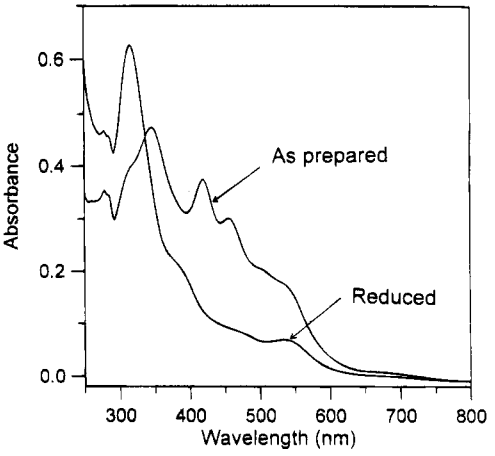

FIGURE 4: Room temperature UV−visible absorption spectra of oxidized (as prepared) and reduced recombinant FeSII protein. The protein concentration was 0.14 mM, calculated for the dimer, and the buffering medium was 50 mM sodium acetate with 50 mM NaCl and 50% (v/v) glycerol (pH 5.2). Reduction was accomplished by anaerobic addition of a single grain of solid dithionite, and the intense band at 314 nm arises from excess dithionite. The cuvette path length was 1.0 mm.

Biological Activity of the Recombinant FeSII Protein. Thus far we have been unable to determine any direct catalytic activity for the FeSII protein other than its only known activity in protecting the Fe protein and MoFe protein of nitrogenase from irreversible oxygen inactivation. Our assay for the oxygen protective activity of the FeSII protein is based on utilizing cell-free extracts of an *A. vinelandii* mutant strain bearing a deletion of the FeSII structural gene and performing "add back" experiments using purified recombinant FeSII protein. As shown in Figure 3, the FeSII protein purified from *E. coli* is capable of protecting the total nitrogenase activity of cell-free extracts of strain CA-Δ*fesII*::KIXX1, in a concentration-dependent manner. The protection reaction has a dependence not only on [FeSII] but also on [MoFe protein] and [Fe protein]. This is to be expected for a higher order reaction. Although we have been unable to obtain precise association constants for the binding of all the three components in the protection reaction, from the data shown in Figure 3, we would estimate that, under these conditions, the concentration of the FeSII protein required for half-maximal protection of the MoFe and Fe protein is approximately 1.0 μM. No significant inhibitory activity of the FeSII protein on total nitrogenase activity was noted throughout the entire tested concentration range. Even at the highest tested concentrations of the FeSII protein, no protection was observed if the reaction was performed in the presence of added 150 mM NaCl, a condition known to dissociate the complex (Haaker & Veeger, 1977). The "oxygen-stable complex" is by no means *absolutely* stable toward oxygen inactivation, neither *in vitro* (Scherings et al., 1983; Laane et al., 1983; Wang et al., 1985) nor *in vivo* (Moshiri et al., 1994). Its rate of inactivation, however, is approximately 5−20 times slower than that of the nitrogenase components in the absence of the FeSII protein.

From calculations on the concentration of FeSII protein in cell-free extracts of wild-type *A. vinelandii* [based on the data in Dingler et al. (1988) and Moshiri et al. (1994)], the biological activity of the recombinant FeSII protein purified from *E. coli* is nearly identical to the activity of the FeSII protein present endogenously in *A. vinelandii*, within the limits of our determinations. Incremental additions of the purified FeSII protein to the wild-type cell-free extracts could in fact further increase the oxygen stability of these extracts, as would be expected. Comparing our "specific activity" results with those from previous studies is further complicated by the subtle but significant variations that have been used to measure the oxygen stability of a reconstituted complex. For example, the different studies have monitored oxygen protection at different oxygen concentrations, with different purified preparations, some in the presence and some in the absence of endogenous dithionite, and at different ionic strengths [see Laane et al. (1983), Robson (1979), and Wang et al. (1985)]. On the basis of our recalculation of some of the data in these previous studies, the concentration of FeSII protein needed for half-maximal protection in these studies is well within the same order of magnitude as our results with the FeSII protein purified from *E. coli*. The differences in oxygen stability reported by Wang et al. (1985) and our results are most likely due to the relatively high ionic strengths employed by Wang et al. during complex reconstitution (G. Watt, personal communications).

Spectroscopic and Redox Characterization of the FeSII Protein [2Fe-2S] Cluster. UV−visible absorption spectra of the as prepared and dithionite-reduced recombinant FeSII protein are shown in Figure 4. The spectra are very similar to those reported previously for native FeSII proteins (DerVartanian et al., 1969; Scherings et al., 1977; Robson, 1979) except for higher Fe-S cluster to protein ratios, as evidenced by A_{418}/A_{280} values (A_{418}/A_{280} = 1.1 for recombinant compared to values between 0.8−1.0 for native FeSII proteins). This is in accord with the higher iron content of our recombinant FeSII protein as compared with the proteins purified directly from *Azotobacter*. The spectra and the molar extinction coefficients, i.e., ϵ_{344} = 16 200 M^{-1} cm^{-1}, ϵ_{418} = 12 900 M^{-1} cm^{-1}, ϵ_{460} = 10 600 M^{-1} cm^{-1}, and ϵ_{550} (shoulder) = 5900 M^{-1} cm^{-1} for the oxidized and ϵ_{540} = 3000 M^{-1} cm^{-1} for the reduced (based on protein concentration and M_r = 13 140), are indicative of a single, dithionite-

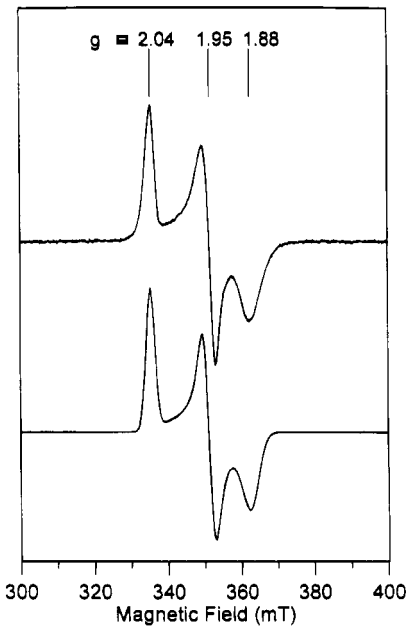

FIGURE 5: X-band EPR spectra of dithionite-reduced recombinant FeSII protein. The sample is described in Figure 4. Conditions of measurement: microwave frequency, 9.46 GHz; modulation amplitude, 0.6 mT; microwave power, 1 mW; temperature, 40 K. The lower spectrum is a simulated spectrum with $g = 2.039$, 1.946, and 1.885 with line widths of 2.43, 2.79, and 4.47 mT, respectively.

reducible $[2Fe\text{-}2S]^{2+,+}$ cluster in each subunit. (A summary of the UV−visible absorption properties of [2Fe-2S] centers in a wide range of biological environments can be found in Dailey et al. (1994).) The UV−visible CD spectra of as prepared and dithionite-reduced recombinant FeSII protein (not shown) were indistinguishable from those reported for native *A. vinelandii* FeSII protein (DerVartanian et al., 1969; Scherings et al., 1977).

The EPR properties of reduced recombinant FeSII protein are very similar to those reported by DerVartanian et al. (1969) and Scherings et al. (1977) for the native protein and were unaffected by the addition of 50% (v/v) glycerol (necessary as a glassing agent for low-temperature MCD studies). The spectrum consists of a rhombic $S = {}^1/_2$ species, $g = 2.04$, 1.95, 1.88, that is readily observed without significant broadening at temperatures up to 100 K; see Figure 5. As noted previously, these EPR properties are remarkably similar to those of chloroplast-type [2Fe-2S] Fds (typically $g = 2.05$, 1.96, 1.88), and the average g-value ($g_{av} = 1.96$) together with the g-value anisotropy is indicative of biscysteinyl ligation at the Fe^{2+} site of the localized valence $[2Fe\text{-}2S]^+$ cluster (Bertrand et al., 1985; Werth et al., 1990). While previous workers have reported sub-stoichiometric quantitation (60%) for this resonance based on Fe and protein concentrations of native FeSII (Scherings et al., 1983), such unexplained complications were not apparent in our work with the recombinant protein, and the EPR signal consistently accounted for 0.95 ± 0.05 spins/13-kDa subunit. Taken together, the absorption data, Fe analyses, and quantitative EPR results provide unambiguous evidence for one $[2Fe\text{-}2S]^{2+,+}$ cluster in each subunit of the homodimer.

VTMCD and RR studies used in conjunction with EPR studies provide a means of assessing the ligation at both the reducible and nonreducible Fe sites and facilitate detailed physicochemical comparisons with other classes of biological

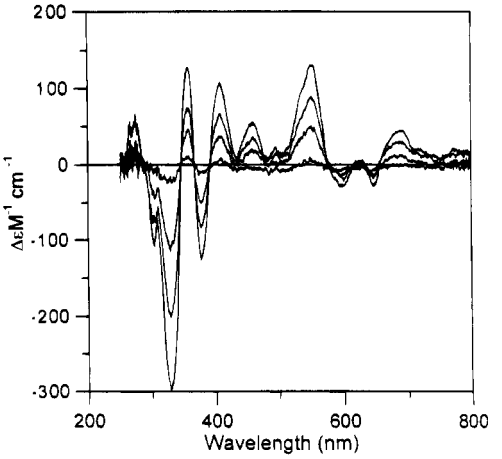

FIGURE 6: VTMCD spectra of dithionite-reduced recombinant FeSII protein. The sample is described in Figure 4. MCD spectra were recorded in 1-mm cuvettes with a magnetic field of 4.5 T at 1.60, 4.22, 8.0, and 45 K. All MCD bands increase in intensity with decreasing temperature.

[2Fe-2S] proteins [Fu et al., 1992, 1994; Crouse et al., 1994). At least four distinct classes of Fe-type [2Fe-2S] centers (i.e., $g_{av} \approx 1.96$ and $E_m = +70$ to -470 mV) can be identified on the basis of differences in spectroscopic properties, and each has a distinctive and highly conserved arrangement of coordinating cysteine residues in the primary sequence. These are typified by chloroplast-type Fds ($C\text{-}X_4\text{-}C\text{-}X_2\text{-}C\text{-}X_n\text{-}C$), hydroxylase-type Fds ($C\text{-}X_5\text{-}C\text{-}X_2\text{-}C\text{-}X_n\text{-}C$), *Clostridium pasteurianum* [2Fe-2S] Fd ($C\text{-}X_2\text{-}C\text{-}X_n\text{-}C\text{-}X_3\text{-}C$), and the *A. vinelandii* NIFU protein ($C\text{-}X\text{-}C\text{-}X_n\text{-}C\text{-}X_2\text{-}C$) (Fu et al., 1994, and references therein). On the basis of the arrangement of the four cysteines in the primary sequence ($C\text{-}X_4\text{-}C\text{-}X_2\text{-}C\text{-}X_{51}\text{-}C$) and the EPR properties discussed above, the [2Fe-2S] center in FeSII protein is clearly most similar to those found in chloroplast-type Fds. However, as shown below, VTMCD, RR, and redox titrations indicate that the electronic, vibrational, and redox properties in particular have many similarities to those of hydroxylase-type Fds.

VTMCD provides an exquisitely sensitive probe for the electronic structure of reduced $[2Fe\text{-}2S]^+$ centers, and MCD spectra of the reduced FeSII protein recorded at temperatures between 1.6 and 45 K are shown in Figure 6. The spectra comprise numerous positive and negative temperature-dependent MCD bands, and magnetization studies for discrete transitions indicate that all originate from the EPR-detectable $S = {}^1/_2$ ground state (data not shown). The pattern of bands and frequencies are uniquely indicative of a biological $[2Fe\text{-}2S]^+$ cluster, and on the basis of published spectra for each subclass of Fd-type [2Fe-2S] proteins (Fu et al., 1994; Crouse et al., 1994), the spectrum for the FeSII protein is best considered as a hybrid of the chloroplast-type and hydroxylase-type VTMCD spectra. Assignment of the MCD bands of $[2Fe\text{-}2S]^+$ clusters to broad classes of electronic transition has been made on the basis of comparisons with spectra for oxidized and reduced rubredoxins and resonance Raman excitation profiles (Fu et al., 1992, 1994; Crouse et at., 1994). Charge-transfer transitions involving the localized-valence Fe^{2+} and Fe^{3+} sites are assigned to the multiple bands between 250−350 and 350−525 nm, respectively. The positive MCD band in the 530−560 nm region (and the pronounced feature in the corresponding absorption spectrum) is tentatively assigned to a the $Fe^{2+}-Fe^{3+}$ intervalence transition, and the lower energy

bands (<600 nm) are assigned to ligand field transitions. Strong evidence for biscysteinyl coordination at both the Fe^{3+} and Fe^{2+} sites of the localized valence $[2Fe\text{-}2S]^{+}$ cluster comes from the pattern of bands in the charge-transfer region of the reduced FeSII protein, which is very similar to those of structurally characterized chloroplast-type Fds. While the FeSII protein more closely resembles hydroxylase-type Fds in the intervalence and ligand field regions, it is not yet possible to interpret such minor differences in electronic properties in terms of specific structural perturbations.

Resonance Raman provides a more direct and extremely sensitive method for investigating structural variations among biological [2Fe-2S] clusters. Moreover, it is applicable to both the diamagnetic oxidized state and the paramagnetic reduced state (Spiro et al., 1988; Fu et al., 1992). All of the eight predicted Fe−S stretching modes of the $Fe_2S^b_2S^t_4$ unit of $[2Fe\text{-}2S]^{2+}$ centers (S^b and S^t indicate bridging and terminal sulfurs, respectively) have been assigned predominantly to 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, and the assignments have recently been extended to the localized valence $[2Fe\text{-}2S]^{+}$ clusters under idealized C_{2v} symmetry (Han et al., 1989a,b; Fu et al., 1992). The reduced RR spectra are dominated by vibrations associated with the localized valence Fe^{3+} site, and the spectra are remarkably conserved among different subclasses of Fd-type [2Fe-2S] proteins. In contrast, each subclass exhibits characteristic oxidized RR spectra that differ in terms of the relative intensities of bands as a function of excitation wavelength and the frequencies of corresponding Fe-S stretching modes (Han et al., 1989b; Fu et al., 1992, 1994; Crouse et al., 1994). This behavior has been interpreted in terms of minor differences in Fe−S bond strengths and variability in the cysteine Fe−S^t−C_β−C_α dihedral angles that govern the extent of mixing of the Fe−S^t vibrations with the internal bending modes of coordinated cysteines (Han et al., 1989a,b; Fu et al., 1992), i.e., minimal coupling with dihedral angles of 90° or 270° and maximal coupling with dihedral angles of 180°.

RR spectra for the oxidized (as prepared) and dithionite-reduced forms of the FeSII protein at various wavelengths in the range 457−530 nm are shown in Figures 7 and 8, respectively. The spectra were invariant to pH over the range 5.2−10.4. The Fe−S stretching frequencies and enhancement profiles for individual vibrational modes for the oxidized protein are quite distinct from those of chloroplast-type Fds (Han et al., 1989b) but are strikingly similar to those of adrenodoxin; see Table 1 and Han et al. (1989b). Adrenodoxin is one of the best characterized of the hydroxylase-type [2Fe-2S] Fds, and assignments have been effected on the basis of $^{34}S^b$ isotope shifts. The vibrational assignments for the FeSII protein presented in Table 1 have been made by direct analogy. Since the vibrational frequencies for the $[2Fe\text{-}2S]^{2+,+}$ clusters in adrenodoxin can be rationalized without invoking significant coupling between the Fe−S^t and S^t−C_β−C_α cysteinyl bending mode, this implies cysteine Fe−S^t−C_β−C_α dihedral angles close to 90° or 270° (i.e., minimal coupling) in both adrenodoxin and the FeSII protein. The frequency of the predominantly Fe^{3+}−S^t stretching mode of the reduced clusters that becomes enhanced with higher energy excitation (optimally at 457 nm) has been used as a monitor of the dihedral angles

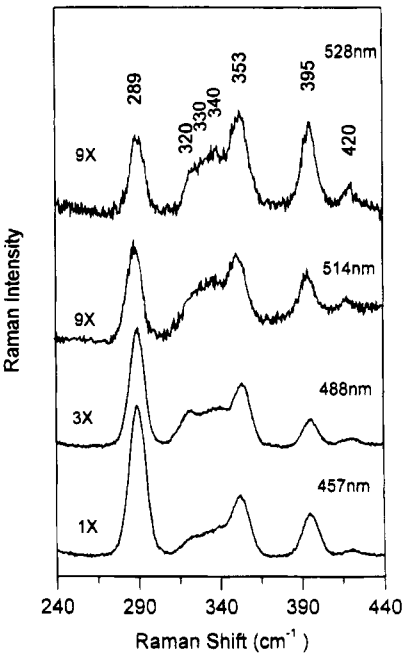

FIGURE 7: Low-temperature resonance Raman spectra of recombinant FeSII protein as prepared. The protein concentration was 0.5 mM (dimer), and the buffering medium was 100 mM sodium acetate with 100 mM NaCl, pH 5.2. The spectra were obtained at 22 K using 457.9-, 488.0-, 514.5-, and 528.7-nm laser excitation and are the sum of 7, 40, 26, and 23 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 multiplication factors refer to relative intensities of the spectra obtained at different wavelengths after being normalized to the intensity of the ice band at 230 cm^{-1}. Bands originating from the frozen buffer solution have been subtracted.

specifically at the nonreducible Fe site (Fu et al., 1992, 1994; Crouse et al., 1994). This mode occurs at 318 cm^{-1} for the $[2Fe\text{-}2S]^{+}$ center in the reduced FeSII protein, which is at the low end of the range established thus far (317−330 cm^{-1}) and again implies minimal coupling. Hence it would appear that the differences in the vibrational properties of the [2Fe-2S] cluster in the FeSII protein compared to chloroplast-type Fds relate to differences in the Fe−S^t−C_β−C_α dihedral angles, with none being close to 180° in this protein. The available X-ray crystal structure data and NMR studies indicate that one of the cysteines coordinating the nonreducible Fe site in chloroplast-type Fds has a dihedral angle close to 180° (Backes et al., 1991; Dugad et al., 1990).

Another important difference in the vibrational properties of the [2Fe-2S] cluster in the FeSII protein compared to other Fd-type [2Fe-2S] proteins lies in the sensitivity of the frequencies to H_2O/D_2O exchange. In all hydroxylase-type and chloroplast-type [2Fe-2S] Fds investigated thus far, significant positive or negative H_2O minus D_2O shifts (ranging from +1.8 to −2.8 cm^{-1}) have been observed upon exchange into the equivalent D_2O buffer solution (Mino et al., 1987; Backes et al., 1991; Fu et al., 1992). These shifts have been attributed to hydrogen bond H/D exchange resulting in weaker or stronger amide N(H/D)−$S^{t/b}$ hydrogen-bonding interactions. Using an analogous H_2O/D_2O exchange protocol [see Fu et al. (1992)], we have been unable to observe any experimentally significant (i.e., > ±0.2 cm^{-1}) H_2O minus D_2O shifts for either the oxidized or reduced FeSII protein. While this result could be interpreted in terms of the complete absence of amide NH−$S^{t/b}$ hydrogen bonds,

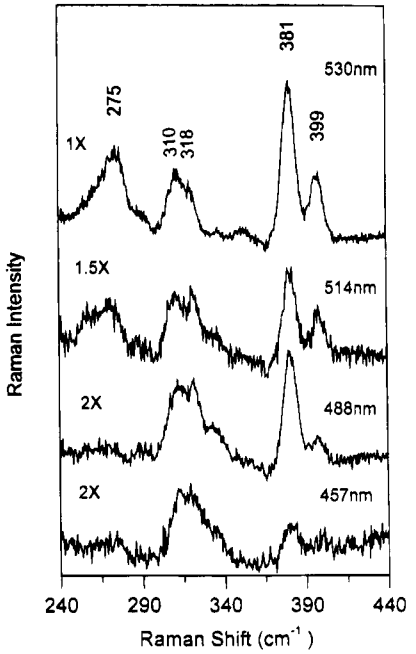

FIGURE 8: Low-temperature resonance Raman spectra of dithionite-reduced recombinant FeSII protein. The protein concentration was 1 mM (dimer) and was in 200 mM CAPS buffer, pH 10.4, and the sample was reduced anaerobically with a single grain of sodium dithionite. The spectra were obtained at 22 K using 457.9-, 488.0-, 514.5-, and 530.9-nm laser excitation and are the sum of 33, 40, 40, and 40 scans, respectively. The data collection parameters and the data manipulation procedures are the same as given in Figure 7.

Table 1: Comparison of the Fe-S Stretching Frequencies and Vibrational Assignments for the $[2Fe\text{-}2S]^{2+,+}$ Clusters in Adrenodoxin and Recombinant FeSII Protein

$[2Fe\text{-}2S]^{2+}$			$[2Fe\text{-}2S]^{+}$		
mode[a] (D_{2h})	adrenodoxin[b] (cm^{-1})	FeSII (cm^{-1})	mode[a] (C_{2v})	adrenodoxin[b] (cm^{-1})	FeSII (cm^{-1})
$B_{2u}{}^{b}$	421	420	B_1	398	399
$A_g{}^{b}$	393	395	A_1	377	381
$B_{3u}{}^{b}$	349	353	A_1	307	310
$B_{1u}{}^{t}$	341	340	B_2	317	318
$B_{2g}{}^{t}$	341	340	B_2	no[c]	no[c]
$A_g{}^{t}$	329	330	A_1	307	310
$B_{1g}{}^{b}$	317	320	B_1	276	275
$B_{3u}{}^{t}$	291	289	A_1	276	275

[a] Symmetry labels under idealized D_{2h} symmetry for the oxidized $Fe_2S^b{}_2S^t{}_4$ unit and under idealized C_{2v} symmetry for the localized-valence reduced $Fe_2S^b{}_2S^t{}_4$ unit and under idealized C_{2v} symmetry for the localized-valence reduced $Fe_2S^b{}_2S^t{}_4$ unit. [b] Assignments taken from Han et al. (1989b) and Fu et al. (1992, 1994). [c] Not observed; normal mode calculations indicate that this mode predominantly involves Fe^{2+}-S^t stretching and therefore is not resonantly enhanced with visible excitation.

the more likely explanation is that the [2Fe-2S] cluster in the FeSII protein is not accessible to solvent.

The O_2-protecting activity of the FeSII protein hinges on the ability of this protein to bind to the Fe and MoFe proteins, forming a tripartite complex that is resistant to oxidative damage. However, the nature of the trigger that facilitates dissociation of the FeSII protein under more anaerobic conditions so that nitrogenase activity can be restored is unknown. An attractive hypothesis is that the binding ability of the FeSII protein is determined by the oxidation state of the $[2Fe\text{-}2S]^{2+,+}$ cluster, in which case the role of the cluster would be as a sensor of the intracellular potential. A similar

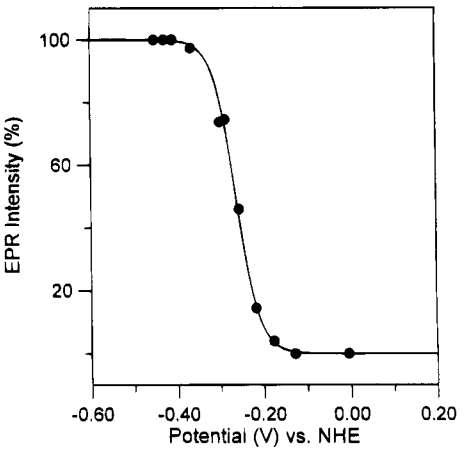

FIGURE 9: EPR-monitored dye-mediated redox titration for recombinant FeSII protein (pH 7.0). EPR intensity was assessed by the peak-to-trough intensity of the $g = 1.94$ component measured at 30 K with 10 mW microwave power. The solid line is best fits to a one-electron Nernst plot with a midpoint potential of −262 mV (*vs* NHE). The experimental protocol is described in Materials and Methods.

role has been recently proposed for the [2Fe-2S] cluster in the *E. coli* superoxide sensing regulatory protein, SoxR (Hidalgo & Demple, 1994; Hidalgo et al., 1995). The redox potential of the FeSII protein is a crucial parameter for future experiments designed to test this hypothesis, but the available data for native FeSII protein are limited to a value based on unpublished observations, $E_m = -225$ mV (Laane et al., 1983), and to room temperature spectropolarimetric titrations, $E_m = -225$ (pH 7.0) and −230 mV (pH 8.0) *vs* NHE (Ke et al., 1974), or −300 mV (Watt, 1979). Hence we undertook dye-mediated EPR-monitored redox titrations (pH 7.0) to determine the midpoint potential of the cluster in recombinant FeSII; see Figure 9. The results are well fit by a one-electron Nernst plot with a midpoint potential of −262 ± 10 mV *vs* NHE. The origin of the discrepancy between the different methods is presently unclear but may be a consequence of freezing the samples for EPR measurements. It is clear, however, that the midpoint potential of the $[2Fe\text{-}2S]^{2+,+}$ cluster in the FeSII protein is substantially higher than in chloroplast-type Fds, $E_m = -350$ to −450 mV but very similar to the values observed for hydroxylase-type Fds, e.g., adrenodoxin, $E_m = -250$ mV, and putidaredoxin, $E_m = -240$ mV. A summary of published midpoint potentials for biological $[2Fe\text{-}2S]^{2+,+}$ clusters can be found in Ackrell et al. (1991).] The factors that determine the midpoint potential of $[2Fe\text{-}2S]^{2+,+}$ are not fully understood, but recent theoretical studies indicate that solvent exposure and the extent and localization of hydrogen bonds are likely to be the major contributing factors for clusters exclusively coordinated by cysteinyl residues (Langen et al., 1992; Jensen et al., 1994).

It is noteworthy that many of the spectroscopic and redox properties of the FeSII protein are very similar to those reported for the FdxD protein from the photosynthetic nitrogen-fixing bacterium *Rhodobacter capsulatus* (Armengaud et al., 1994). Furthermore, the FdxD protein has a 50% sequence identity with the FeSII protein (Moshiri et al., 1994). The function of the FdxD protein in *R. capsulatus*, however, is unknown. It is tempting to speculate that the FdxD protein is a functional homolog of the FeSII protein. It is therefore possible that the FeSII-mediated conforma-

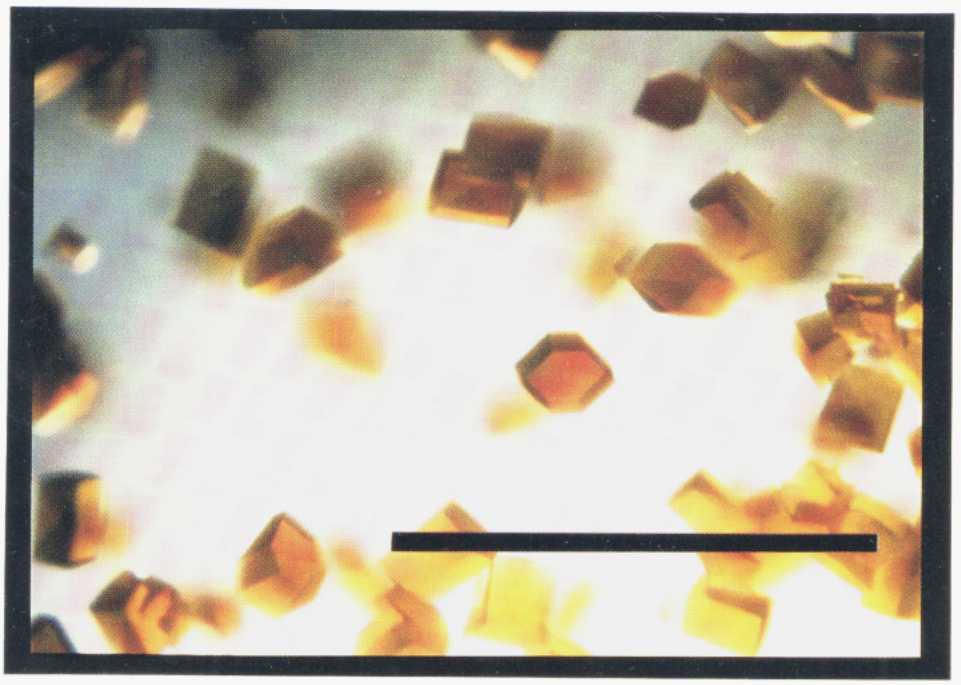

FIGURE 10: Low-magnification photograph of crystals of the FeSII protein. A concentrated desalted solution of FeSII protein (40 mg/mL, 1.5 mM) was mixed 1:1 with a precipitant solution of 100 mM Na-HEPES, 200 mM sodium citrate, and 24% PEG-3000, pH 8.0, with 2% 1-butanol, and hanging drops of this mixture were allowed to vapor-equilibrate against a reservoir containing the precipitant solution for 10−14 days at room temperature. The bar corresponds to 1.0 mm.

tional protection of nitrogenase is not unique to the genus *Azotobacter*, as previously thought (Yates, 1988).

Crystallization of the FeSII Protein. Determining the molecular mechanism of the interactions between the FeSII protein and the MoFe and Fe proteins of nitrogenase will require knowledge of the complete three-dimensional structure of the protein. Initial crystallization trials using the sparse-matrix design (Jancarik & Kim, 1991) showed that the holoprotein was stable in the pH range 4.5−9.5 and that the citrate ion had a significant stabilizing effect. Figure 10 shows crystals grown using the hanging drop method (McPherson, 1982) by mixing equal amounts of concentrated, desalted, FeSII protein with 24% PEG-3000, in 100 mM Na-HEPES and 200 mM sodium citrate, pH 8.0, with 2% 1-butanol, and equilibrating the drops against a reservoir of the same solution at room temperature. The diffraction pattern from crystals of dimensions 0.1−0.3 mm extends to at least 2.5 Å resolution (Cu Kα radiation from a 5 kW rotating anode generator). The pattern has been examined by precession photography as well as by measurement of a partial native data set using the oscillation method. The results of both experiments were consistent with an orthorhombic space group with approximate unit cell dimensions $a = 135$ Å, $b = 135$ Å, and $c = 38$ Å. Assuming a typical crystal density, the crystals are likely to contain at least two dimers per asymmetric unit, and it is therefore possible that the protein crystallizes as a higher oligomer (J. Bolin and M. Becker, personal communications). Attempts to crystallize the FeSII protein purified directly from *A. vinelandii* using similar conditions were unsuccessful, apparently due to the lower iron content and microheterogeneity of the samples.

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