Mutagenesis Studies of the FeSII Protein of *Azotobacter vinelandii*: Roles of Histidine and Lysine Residues in the Protection of Nitrogenase from Oxygen Damage[†]

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ABSTRACT: The *Azotobacter* FeSII protein, also known as the Shethna protein, forms a protective complex with nitrogenase during periods when nitrogenase is exposed to oxygen. One possible mechanism for its action is an oxidation state-dependent conformational interaction with nitrogenase whereby the FeSII protein dissociates from the MoFe and Fe proteins of nitrogenase under reducing conditions. Herein we report the construction and characterization of five site-directed mutants of the FeSII protein (H12Q, H55Q, K14A, K15A, and the double mutant K14A/K15A) which were individually purified after being individually overexpressed in *Escherichia coli*. These mutant FeSII proteins maintain native-like assembly and orientation of the 2Fe−2S center on the basis of EPR and NMR spectroscopic characterization and their redox midpoint potentials, which are within 25 mV of that of the wild type protein. The abilities of the individual mutant proteins to protect nitrogenase were assessed by determining the remaining nitrogenase activities after adding each pure version back to extracts from an FeSII deletion strain, and then exposing the mixture to oxygen. In these assays, the H12Q mutant functioned as well as the wild type protein. However, mutation of His55, a few residues away from a cluster-liganding cysteine, results in much less efficient protection of nitrogenase. These results are consistent with pH titrations in both oxidation states, which show that His12 is insensitive to 2Fe−2S cluster oxidation state. His55's pK is weakly responsive to oxidation state, and the pK increase of 0.16 pH unit upon 2Fe−2S cluster oxidation is indicative of ionization of another group between His55 and the 2Fe−2S cluster, which could modulate the FeSII protein's affinity for nitrogenase in a redox state-dependent manner. Both K14A and K15A mutant FeSII proteins partially lost their ability to protect nitrogenase, but the lysine double mutant lost almost all its protective ability. The nitrogenase component proteins in an *Azotobacter* strain bearing the double lysine mutation (in the chromosome) were degraded much more rapidly in vivo than those in the wild type strain under carbon substrate-limited conditions. These results indicate that the two lysines may have an important role in FeSII function, perhaps in the initial steps of recognizing the nitrogenase component proteins.

Biological nitrogen fixation represents one of the most important ways to make N available for the growth of plants. Ability to fix nitrogen has been found in a wide range of microbes, including obligate aerobes, obligate and facultative anaerobes, and oxygenic photosynthetic microorganisms. One of the common features of nitrogenases found in these microorganisms is that the enzymes are rapidly and irreversibly inhibited by oxygen (*1*). However, as oxygen is essential for the survival of aerobes and some diazotrophs photoevolve oxygen, effective mechanisms for protecting nitrogenases from oxygen damage are critical for the diazotrophic growth of aerobic and oxygenic photosynthetic microorganisms.

Nitrogen-fixing microorganisms have evolved a variety of physiological and biochemical strategies for protecting nitrogenases from irreversible oxygen damage (*1*). *Azotobacter vinelandii* is an obligate aerobic nitrogen-fixing bacterium, and much of our knowledge about nitrogenases has been derived from studies of the component proteins from this Gram-negative soil microorganism (*2*). Although both of the purified nitrogenase components, the Fe protein and the MoFe protein, are inactivated in the presence of oxygen, this soil bacterium is capable of diazotrophic growth under fully aerobic growth conditions.

Two distinct strategies for avoiding oxygen inactivation of nitrogenase are known to be utilized by *A. vinelandii*. During exponential growth, membrane-bound terminal oxidases vigorously consume oxygen so that its concentration

Table 1: Bacterial Strains and Plasmids

	description	origin or ref
strain		
A. vinelandii		
CA	wild type	*40*
CA Δ*fesII*::KIXX1	*fesII* gene deleted, with the *kan*[R] cassette inserted in the same orientation as *fesII*	*7*
DJ	wild type, easily transformable	D. Dean
E. coli		
DH5α	cloning host	Gibco-BRL
W3110	protein expression strain	lab stock
plasmid		
pUC19	Amp^r; cloning vector	Clonetech
pFU1	pSKII+, containing the 3.85kb *Sal*I fragment surrounding the *A. vinelandii fesII* gene	*7*
pKS-SaBx1	pSKII+, containing the 1.5 kb *Sal*I−*Bst*XI fragment of pFU1	this work
pUC-SaBx1	pUC19, containing the 1.5 kb *Sal*I−*Bst*XI fragment of pKS-SaBx1	this work
pK14A	Amp^r; pUC-SaBx1 containing the *fesII* gene in which Lys14 was replaced by alanine	this work
pK15A	Amp^r; pUC-SaBx1 containing the *fesII* gene in which Lys15 was replaced by alanine	this work
pKK14,15AA	Amp^r; pUC-SaBx1 containing the *fesII* gene in which Lys14 and -15 were each replaced by alanines	this work
pH12Q	Amp^r; pUC-SaBx1 containing the *fesII* gene in which His12 was replaced by glutamine	this work
pH55Q	Amp^r; pUC-SaBx1 containing the *fesII* gene in which His15 was replaced by glutamine	this work

in the cytoplasm is maintained at a level compatible with nitrogen fixation. In fact, *Azotobacter* species possess the highest respiration rate among all known bacteria. The significance of this "respiratory protection" mechanism is clearly demonstrated in mutants deficient in the cytochrome *d* complex; such mutants are incapable of diazotrophic growth under fully aerobic conditions (*3*). When the respiratory protection mechanism is inadequate as under substrate-limiting conditions, another mechanism termed "conformational protection" stabilizes the nitrogenase enzyme complex. Conformational protection involves a small 2Fe−2S protein (FeSII), which was initially discovered by Shethna and colleagues (*4*). Under high-oxygen tension conditions, this protein binds to the MoFe protein and Fe protein of nitrogenase to form an oxygen-stable complex which does not fix nitrogen (*5*). Once the oxygen concentration decreases to a level favorable for nitrogenase function, nitrogen fixation resumes upon dissociation of the FeSII protein from the MoFe protein and Fe protein. Although the oxidation state-dependent "conformational protection" mediated by the FeSII protein has been known for almost 30 years, its mechanisms at the molecular level remain unclear. As the *fesII* structural gene was recently cloned and sequenced and the protein was successfully overexpressed in *Escherichia coli* (*6, 7*), we further investigated the basis of the oxidation state-dependent interactions between the nitrogenase components and the FeSII protein. In this study, we address the roles of residues we predict could be involved in the protective function, by a site-directed mutagenesis approach.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *A. vinelandii* was grown aerobically (shaking in baffled flasks at 220 rpm) at 30 °C in Burk's sucrose medium (BS; *8*), usually supplemented with 25 mM ammonium acetate as the N source. For the growth of strain CAΔfesII (the source of the nitrogenase-containing extracts for the protection assays), the BS was not supplemented with N. *E. coli* strains bearing plasmids were grown at 37 °C in LB medium containing appropriate concentrations of antibiotics (*9*).

Subcloning of the A. vinelandii fesII Gene and Site-Directed Mutagenesis. Plasmid pKS-SaBx1 was constructed by subcloning an approximately 1.5 kb *Sal*I−*Bst*XI fragment of pFU1 (*6*), after blunting the *Bst*XI site using the Klenow fragment of DNA polymerase, into pBluescript KSII(+) (Stratagene, Inc.) previously digested with *Sal*I and *Sma*I. The *fesII* structural gene and its flanking DNA were subsequently subcloned out of pKS-SaBx1 and into pUC19 as a *Sal*I−*Bam*HI fragment, to create pUC-SaBx1. Plasmid pUC-SaBx1 was the starting plasmid for creating all the site-directed mutants of the FeSII protein described in this work. Mutagenesis was carried out using the Transformer site-directed mutagenesis kit (Clonetech, Inc.). Mutagenic oligonucleotides (25−28 bp) were designed on the basis of the sequence of the *fesII* gene to create the desired mutants. The mutations discussed in this work are K14A, K15A, the double mutant (K14A/K15A), H12Q, and H55Q. In the case of the double mutant K14A/K15A and H55Q, the mutations were designed to create new restriction sites (*Eag*I and *Pvu*II, respectively), which were used for the initial screening. All mutated genes were subsequently sequenced to confirm the presence of the desired mutation, and the absence of unwanted substitutions.

Expression and Purification of Mutant FeSII Proteins from E. coli. The new derivatives of plasmid pUC-SaBx1 were found to express low levels of the FeSII protein in *E. coli*. Digestion of these plasmids with *Pst*I and subsequent recircularization removed approximately 600 bp of the sequence upstream of the *fesII* structural gene, positioning the gene so as to greatly increase the yield of protein expressed from the *lac* promoter pUC19. The level of protein expressed from these constructs was comparable to that reported previously for the wild type FeSII protein from plasmid pAvFeSII (*7*). Plasmids conferring the mutant *fesII* structural gene were electroporated (at 25 000 V for 5 s in 2 mm gap cuvettes) into competent cells of *E. coli* strain W3110. Previous work showed that strain W3110 was a particularly high-yield host (*7*). Colonies bearing plasmids were screened on LB-Amp (0.2 mg/mL) plates, and a single colony was used to inoculate 5 mL of LB-Amp medium. For protein overexpression in *E. coli*, the 5 mL culture was used to inoculate 800 mL of phosphate-buffered tryptone yeast extract medium [TYP, 5 mg/mL Na_2HPO_4, 16 mg/mL tryptone, and 10 mg/mL yeast extract (pH 7.4)] supplemented

with 0.2 mg/mL ampicillin and 50 μM FeSO$_4$. The cells were grown at 37 °C overnight (14−16 h).

Cells from 800 mL of culture were harvested by centrifugation (6100g for 20 min at 4 °C) and washed twice with 50 mM Na/HEPES buffer (pH 7.4) containing 5 mM MgCl$_2$. Cells exhibiting good expression of FeSII protein had a dark pink color. Crude extracts were prepared by passing cells through a French pressure cell (1120 kg/cm^2) two times. Sodium chloride and polyethylene glycol 8000 were added to the above mixture to final concentrations of 0.4 and 11%, respectively, and the whole mixture was gently stirred for 30 min at 4 °C. After centrifugation (15000g for 15 min at 4 °C), the clear pink supernatant was collected and loaded onto a DEAE-Sepharose column (5.5 cm × 2 cm) pre-equilibrated with 10 mM Na/HEPES buffer (pH 7.4) containing 1 mM MgCl$_2$. The column was further washed with approximately 300 mL of the same buffer, and the FeSII protein was then eluted with 50 mM Na/HEPES buffer (pH 7.4) containing 5 mM MgCl$_2$. The pink fractions were pooled together (approximately 80−150 mL) and concentrated to approximately 5 mL using an Amicon ultrafiltration unit with a YM-10 membrane under nitrogen pressure (50 psi). After 10-fold dilution with 50 mM sodium acetate buffer (pH 5.2), the sample was concentrated down to 5 mL. The concentrated sample was then loaded onto an S-Sepharose column (5 cm × 1.5 cm) which had been pre-equilibrated with 50 mM sodium acetate buffer (pH 5.2). The column was washed with 200 mL of the same buffer, and the pink FeSII protein was eluted by adding NaCl (final concentration of 50 mM) to the washing buffer. After concentration using the YM-10 membrane (to approximately 20 mg/mL), the FeSII protein was stored in a sterile solution of 10% glycerol (at −70 °C) in volumes of less than 300 μL. The protein purification progress was monitored by SDS−polyacrylamide gel electrophoresis.

EPR Spectroscopy and Redox Titrations of the Mutant FeSII Protein. UV−visible absorption spectra were recorded on a Shimadzu UV3101PC spectrophotometer. The samples were prepared in 50 mM sodium acetate buffer (pH 5.2), and protein concentrations (0.15 mM) were based on a dimer molecular mass of 26 300 Da (see ref 7). 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. EPR-monitored redox titrations were performed at ambient temperature (25−27 °C) in a glovebox under anaerobic conditions (<5 ppm O$_2$) using 200 mM PIPES buffer (pH 7.0). Mediator dyes were added, each to a concentration of ca. 50 μM, to cover the desired range of redox potentials, i.e., methyl viologen, benzyl viologen, neutral red, safranin, phenosafranin, anthroquinone-1,5-disulfonate, indigo disulfonate, methylene blue, 1,2-naphthoquinone, duroquinone, and 1,2-naphthoquinone-4-sulfonate. Samples were first reduced completely by 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).

NMR Spectroscopy and pH Titrations of His12 and His55. All NMR spectra were recorded at 303 K on a Bruker AMX 300 spectrometer. Samples contained 0.5 mL of unbuffered 0.5−2 mM FeSII protein in ^{2}H$_2$O and were observed using (a) a 90° pulse-observe sequence preceded by low-power water presaturation to eliminate the signal of residual ^{1}HO^{2}H, (b) a spin echo incorporating two 20 ms delays to suppress most protein resonances and emphasize the slowly relaxing His H2 and H4 protons as well as the pH indicator signals (Figure S1 in the Supporting Information), or (c) a WEFT sequence (*10*) incorporating a 15 ms relaxation delay, a 180° pulse, and a 35 ms delay prior to a 90° pulse and 25 ms of digitization, to suppress slowly relaxing resonances (*11*). The purified protein was transferred into ^{2}H$_2$O by repeated concentration in a Centricon instrument and redilution with ^{2}H$_2$O.

NMR samples used for pH titrations were supplemented with NaCl and internal pH indicators and DSS at final concentrations of 20 mM NaCl, 1 mM acetate, 1 mM cacodylate, 1 mM 2,4-dimethylimidazole, and 0.2 mM DSS. For titrations in the oxidized state, samples were simply titrated with 50 mM NaOD, collecting a spin echo spectrum of FeSII at each pH point, and determining the internal pH by comparison of the chemical shifts of the pH indicators with chemical shift versus pH calibration curves obtained earlier using a pH electrode to measure the pH of a solution which was also sampled by NMR. The standard curves were obtained using D$_2$O solutions, and pH meter readings were not corrected for isotope effects. All chemical shifts were measured relative to internal DSS at 0 ppm. To obtain a pH titration curve of the reduced state, it was necessary to prepare several oxidized samples at different pHs, degas them by repeated evacuation and equilibration with Ar, reduce them with dithionite, and flame-seal them or seal them with a stopcock and septum seal. The actual pH of these samples was determined by NMR from the chemical shifts of the pH indicators, obtained in the same spin echo spectrum used to measure the chemical shifts of the His H2 protons. His H2 chemical shifts were plotted versus pH, and the data were fit with the Henderson−Hasselbalch equation with allowance for cooperativity:

$$\frac{\delta_A - \delta_{obs}}{\delta_A - \delta_B} = \frac{K^n}{K^n + 10^{-n(pH)}}$$

where δ_A and δ_B are the chemical shifts of the acid and base forms (obtained from the fit), respectively, δ_{obs} is the observed chemical shift at a given pH, K is the acid dissociation constant obtained from the fit, and n is the Hill coefficient obtained from the fit.

The H2 resonances of His12 and -55 were collectively identified as H2 protons on the basis of their characteristic chemical shifts near 8.5 ppm (*12*) and their relatively slow relaxation which permitted their selective observation in a 20 ms spin echo spectrum (Figure S1 in the Supporting Information). The two His H2 protons were assigned to His12 and -55, respectively, on the basis of the comparisons of the wild type NMR spectrum with the spectra of the H55Q and H12Q mutants, in which only the resonance of His12 or His55 was present, respectively (Figure S2 in the Supporting Information).

Construction of an A. vinelandii Double Mutant Strain Containing the FeSII K14A/K15A Mutation in the Chromosomal FeSII Locus. Recombination of the FeSII K14A/K15A

double mutation into the *A. vinelandii fesII* locus was achieved by congression using a selectable rifampicin resistance marker, essentially as described previously (*6*, *13*). Strain DJ which has an increased transformation efficiency was used for this construct exactly as described previously for the FeSII deletion strain, CA-ΔfesII::KIXX1 (*6*). Competent cells of DJ-Δ*fesII*::KIXX1 were transformed with a mixture of plasmid pUC-SaBx1-FeSII K14A/K15A (linearized with *Sal*I and *Bam*HI) and plasmid pDB303 (from D. Dean, linearized with *Eco*RI), at a ratio of 100:1. Transformants were grown on BSN with 5 μg/mL rifampicin agar plates. Colonies were transferred to nitrocellulose disks and lysed by brief incubation in a solution of 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 1% SDS, and the lysate on the filter was washed with water to remove bacterial debris. Blots were subsequently probed with affinity-purified rabbit anti-FeSII antibodies, followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Boehringer-Mannheim), and developed essentially as described previously (*6*). Colonies expressing FeSII immunoreactive material were subsequently purified on BSN plates with 5 μg/mL rifampicin. Three out of four colonies expressing FeSII immunoreactive proteins were found to be sensitive to kanamycin at 5 μg/mL, indicating that the double mutant FeSII K14A/K15A gene had replaced the genomic Δ*fesII*::KIXX1 region. All three strains were also found to contain a new *Eag*I site on genomic Southern blots probed with the insert from pUC-SaBx1 (data not shown). One strain, DJ-FeSII, containing the double mutation K14A/K15A was used for subsequent characterization.

Assessment of Nitrogenase Protection Ability. A. vinelandii deletion strain CA-Δ*fesII*::KIXXI was grown in a Burk's sucrose medium and harvested in midlog phase (OD_{585} of 0.6−0.8). Crude extracts were prepared under 100% argon by an osmotic lysis method (*14*) with the following minor modification that increased the lysis efficiency. Na/HEPES (25 mM, pH 7.4) was used instead of Tris buffer, and 710−1180 μm glass beads (Sigma) were used. Protease inhibitors leupeptin (final concentration of 5 μg/mL) and phenylmethanesulfonyl fluoride (PMSF; final concentration of 35 μg/mL) were added before the cell breakage. All buffers were thoroughly sparged with Ar before use, and all procedures were performed under a stream of Ar. Aliquots or fractions after cell rupture were transferred by use of Ar-sparged syringes and serum-stoppered vials or centrifuge tubes. Centrifuge tubes with rubber O-rings were used to minimize exposure of crude extracts to oxygen. Cell membranes were removed from the crude extracts by ultracentrifugation (140000*g* for 30 min at 4 °C). This step was necessary to limit the respiratory activities of membrane fractions in crude extracts. Aliquots of wild type or mutant FeSII proteins (2 μM final concentration in the assay mixture) were added in a 10 mL glass bottle and sparged with argon for 5 min. An aliquot of the CA-ΔfesII:KIXXI crude extract (final protein concentration of 1−2 mg/mL) was added and sparged with Ar for an additional 2 min. The bottles containing samples were capped with gastight rubber stoppers, sparged with Ar again, and incubated in a 30 °C water bath for 5 min. After addition of $^1/_4$ volume of 100% oxygen-sparged Na/HEPES (25 mM, pH 7.4) containing 5 mM $MgCl_2$, the bottles were opened to air and incubated at room temperature for up to 30 min. Samples (100 μL) were withdrawn at indicated time

intervals using a glass syringe, and each sample (three replicates of each assay condition were used) was immediately injected into a nitrogenase assay vial (see below).

Nitrogenase Assay. The activity of nitrogenase in the crude extract was assayed by the acetylene reduction method described by Shah et al. (*14*) with minor modification. The reaction was conducted in 12 mL glass vials containing 1 mL of reaction mixture: 2.5 nmol of ATP, 30 nmol of creatine phosphate, 50 units of creatine phosphokinase (Sigma C-3755), 10 nmol of dithionite, 50 nmol of Na/HEPES buffer (pH 7.4) and 5 μM $MgCl_2$, and about 2 mg of crude extract protein. The mixture was prepared anaerobically under Ar, and the vials were capped by use of gastight rubber serum stoppers. Acetylene was added to a final concentration of 10%. After preincubation of the assay mixture at 30 °C for 5 min, reactions were started by the addition of anaerobic crude extracts using gastight syringes. The reaction mixture was incubated while it was shaken at 30 °C for 15 min (*6*, *14*). Reactions were stopped by the addition of 30 μL of 30% trichloroacetic acid (TCA). Ethylene was quantitated using a Shimadzu GC-mini2 gas chromatograph equipped with a Porapak-N column (*6*). Two replicate quantitations of ethylene were performed on each assay vial.

Immunological Methods. Antibodies to the FeSII protein, Fe protein, and the α or β subunits of the MoFe proteins were prepared as described previously (*6*). Purified *A. vinelandii* MoFe protein and Fe protein as well as anti-MoFe and anti-Fe protein whole sera were generously provided by V. Shah of the University of Wisconsin (Madison, WI). For Western blotting experiments, cells collected from 100 μL of culture were resuspended in the SDS−PAGE sample buffer and lysed by heating in a boiling water bath for 10 min. SDS−PAGE and subsequent transfer onto nitrocellulose membranes were performed using established protocols (*9*). After electrophoretic transfer onto nitrocellulose, blots were incubated at room temperature with antibodies diluted 1:1000 in 5% nonfat dry milk in 10 mM Tris-HCl, 150 mM NaCl, and 0.01% Tween-20 (pH 7.4) for 2 h. Following washing, blots were incubated with alkaline phosphatase-conjugated anti-rabbit secondary antibody at a dilution of 1:2000 and immunoreactive bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium according to the manufacturer's instructions.

Analysis and Material. Bacterial growth was monitored by measuring the optical density at 585 nm. An OD_{585} of 1.0 corresponds to a viable cell concentration of 6.0×10^8 cells/mL. Protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce) using bovine serum albumin as a standard. Molecular biology reagents and enzymes were purchased from New England Biolabs, Inc. (Beverly, MA), or other commercial providers. All gases were purchased from Specialty Gases, Welder's Supply Co. (Baltimore, MD).

RESULTS

Overexpression, Purification, and Characterization of Mutant FeSII Proteins from E. coli. After removal of approximately 600 bp of DNA sequence upstream of the fesII structural gene, the gene was expressed from the *lac* promoter in the pUC19 polylinker. The level of protein expressed from

FIGURE 1: SDS−PAGE analysis of purified mutant FeSII proteins. A 15% acrylamide gel was used in this experiment and stained with Coomassie blue. Each lane contained approximately 20 μg of protein. Lanes 2−7 contained H55Q, H12Q, the double mutant (K14A/K15A), K15A, K14A, and wild type FeSII proteins, respectively, purified by use of both anion exchange and cation exchange columns. Lane 8 contained the FeSII fraction from the DEAE-sepharose column only. Lane 9 contained cell-free crude extracts. Lanes 1 and 10 contained molecular mass standards (M_r, from the bottom): aprotinin (6500 Da), lysozyme (14 400 Da), trypsin inhibitor (21 500 Da), carbonic anhydrase (31 000 Da), ovalbumin (45 000 Da), and serum albumin (66 200 Da).
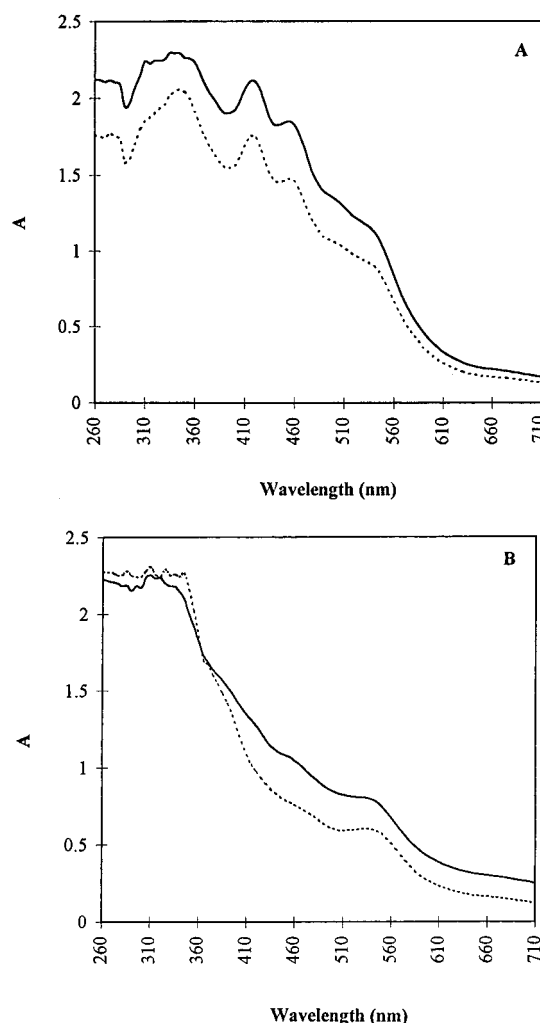

FIGURE 2: UV−visible absorption spectra of oxidized (as prepared, A) and dithionite-reduced (B) FeSII H55Q (dotted lines) and wild type FeSII (solid lines) proteins. Spectra of the other four mutant proteins (H12Q, K14A, K15A, and the double lysine mutant) were very similar to that of H55Q. The spectra were recorded at room temperature, and the protein samples (final concentration of 0.15 mM) were prepared in 50 mM sodium acetate (pH 5.2). Reduction was accomplished by addition (under argon) of solid dithionite (final concentration of 5 mM).

these constructs was comparable to that reported previously for the wild type FeSII protein from plasmid pAvFeSII (7). Previous work showed that *E. coli* strain W3110 is superior to other host strains in overexpressing the FeSII protein, so W3110 was selected as the host strain to express all of the mutant FeSII proteins. To increase the expression efficiency, cells bearing plasmids containing the *fesII* gene were grown in a rich medium (see Materials and Methods) supplemented with ampicillin and $FeSO_4$ (7). By using anion exchange (DEAE-Sepharose) and cation exchange (S-Sepharose) columns, the FeSII proteins were purified to near homogeneity (see Figure 1). The identity of the expressed FeSII protein was further confirmed by Western blotting using the purified anti-FeSII protein antibody (data not shown). Typically, the FeSII protein accounted for approximately 3−5% of the total protein in *E. coli* cells.

Like the wild type protein, the purified mutant proteins had a pink color, suggesting that a 2Fe−2S cluster was properly assembled. The UV−visible absorption spectra of all of the oxidized mutant FeSII proteins (as prepared) were very similar to those of the native and wild type recombinant FeSII protein, and had absorption bands centered around 330, 418, and 460 nm, with a broad shoulder centered around 550 nm (Figure 2A). The A_{418}/A_{280} values were 1.0 ± 0.1 for all five mutant proteins, and for the wild type recombinant protein. Upon dithionite reduction, the visible absorption was bleached and a broad band at approximately 540 nm appeared (Figure 2B). Such absorption characteristics indicate the presence of a dithionite-reducible 2Fe−2S cluster in all five mutant proteins (7, 15−17). Moreover, the EPR spectra of each of the reduced mutant proteins were unchanged compared to those of the wild type ($g = 2.04, 1.95$, and

1.88), indicating no significant change in the environment of the reduced cluster.

The NMR spectra of paramagnetically shifted resonances of wild type FeSII are shown in Figure 6. In the oxidized state, we observe broad features between 50 and 20 ppm due to several overlapping resonances, similar to the spectra of plant type and vertebrate ferredoxins in the oxidized state (18−20). Any changes in ligand chemical shifts upon His mutagenesis are small relative to the large resonance line widths. In the reduced state, we observe four strongly paramagnetically shifted resonances between 120 and 85 ppm, as well as six sharper shifted resonances between 40 and 10 ppm in a spectrum that resembles that of plant type ferredoxins, but not vertebrate ferredoxins (20). We do not observe significant changes in the chemical shifts of these resonances, assigned to the β and α protons of the ligand Cys (21), upon mutagenesis of either His in the reduced state. Thus, our results indicate that the electronic state and geometry of the 2Fe−2S cluster and ligands are similar to
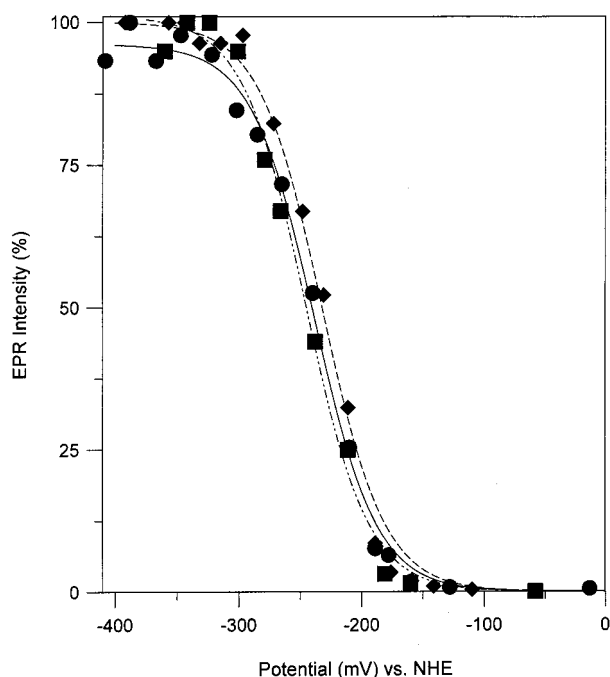
FIGURE 3: EPR-monitored dye-mediated redox titration for recombinant variant forms of the *A. vinelandii* FeSII protein (pH 7.0): H12Q (●), solid line being the best fit to a one-electron Nernst plot, $E_m = -239$ mV; H55Q (◆), dashed line being the best fit to a one-electron Nernst plot, $E_m = -239$ mV; and K14A/K15A (■), dotted−dashed line being the best fit to a one-electron Nernst plot, $E_m = -245$ mV. EPR intensity was assessed by the peak-to-trough intensity of the $g = 1.94$ component measured at 30 K with a 10 mW microwave power, and the midpoint potentials are vs NHE. The experimental protocol is described in Materials and Methods.
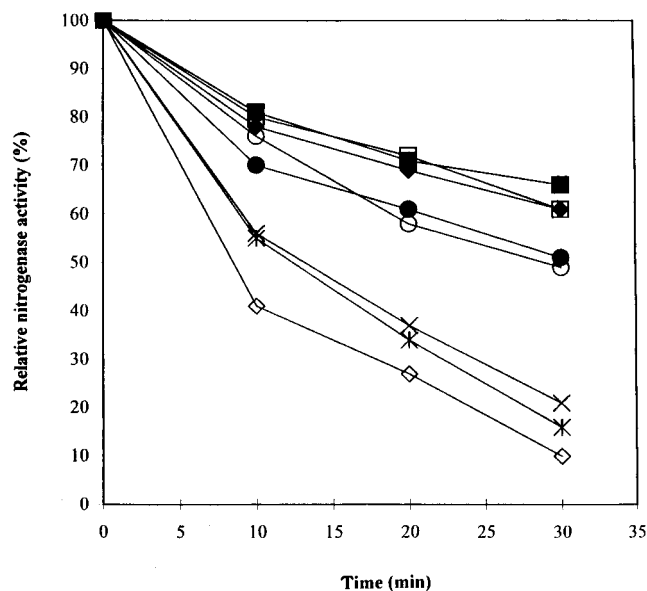

FIGURE 4: Effects of lysine or histidine mutations on the ability of the FeSII protein to protect nitrogenase from oxygen inactivation. Cellular crude extract preparation and "add back" experiments were carried out as described in Materials and Methods. Nitrogenase activity was measued after exposure of the extract to oxygen, and the results were calculated as the percentage of the original nitrogenase activities. The original nitrogenase activities for the *A. vinelandii* wild type strain and the *fesII* deletion strain were 50.1 ± 5 and 42.5 ± 3 nmol of ethylene produced min⁻¹ mg of protein⁻¹, respectively. All the values were obtained from the average of three individual experiments with duplicate measurements. The standard error coefficients were less than 10%: (◆) crude extracts from the wild type strain (no addition) and (◇) crude extracts from the *fesII* deletion strain (no addition). The other points depict data from the crude extract of the deletion strain containing the following added FeSII protein versions at zero time: (■) wild type FeSII, (×) K14A/K15A double mutant, (○) K14A, (●) K15A, (□) H12Q, and (∗) H55Q.

those of plant type ferredoxins, and that they are essentially the same in both His mutant proteins and in wild type FeSII.

The midpoint redox potential of 2Fe−2S centers is determined by the asymmetry in the cluster environment and, importantly, solvent exposure and the localization of hydrogen bonds (*22−24*). The midpoint potential values (E_m) for the wild type and each of the mutant proteins were determined through EPR-monitored dye-mediated redox titrations (see Figure 3). The E_m values of the double mutant K14A/K15A and both His-altered versions H12Q and H55Q (−245, −239, and −239 mV, respectively) were all slightly higher than that of the wild type protein (−262 mV; *7*). However, the significance of such small increases (17−23 mV) is questionable since the estimated error in the E_m values is ±10 mV, and the overall conclusion of the spectroscopic and redox studies is that these lysine and histidine mutations do not significantly alter the cluster environment.

Lysine Mutations. A number of factors, such as the oxidation state of proteins, the ionic strength, and the Mg^{2+} concentration, can affect the formation of the oxygen-tolerant three-component nitrogenase complex (*25*). The association of the three individual components was effectively inhibited by high concentrations of NaCl (>150 mM; *1*), indicating that electrostatic interaction is one important factor for the effective interaction of the FeSII protein with the MoFe and Fe proteins. In searching protein databases, we noticed the FeSII protein was highly homologous with FdxD of *Rhodobacter capsulatus* and with FdxH of *Anabaena*. All three of these 2Fe−2S proteins are capable of interacting with

nitrogenase, and they all have a double lysine motif near their N-termini. Indeed, mutagenesis of the lysine residues (lysines 10 and 11) in FdxH revealed the importance of these basic residues in docking to (and subsequent reduction of) nitrogenase (*26*). Therefore, the role of the double lysine motif in the protection function of FeSII protein was tested by mutation of one or both positively charged lysine residues into neutral alanines. The abilities of the mutant proteins to protect nitrogenase were assessed by determining the remaining nitrogenase activity after adding these mutant proteins to the crude extracts of the *A. vinelandii* CA-Δ*fesII* strain and exposing the extract to air for up to 30 min (Figure 4). As reported previously (*6*), nitrogenase in the wild type *A. vinelandii* strain was much more resistant to oxygen inactivation than the enzyme in the *fesII* deletion strain (with 62 and 10% nitrogenase activity remaining after 30 min of exposure to 250 μM oxygen for the wild type and mutant FeSII deletion cell extracts, respectively). Upon replacement of one lysine residue at either position 14 or 15 with alanine, approximately 50% of the original nitrogenase activity was lost after exposure to air for 30 min. Therefore, the FeSII protein with a single lysine mutation is still able to provide some protection to nitrogenase. However, when both lysines were changed to alanine, almost all the protection conferred by the FeSII protein was lost as determined by the 30 min air exposure assay (see Figure 4).
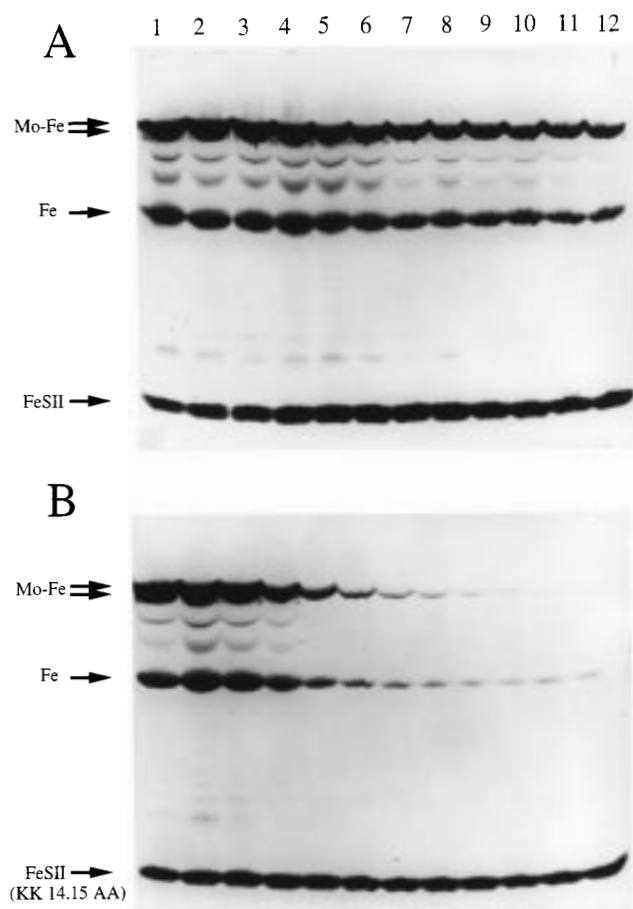
FIGURE 5: Immunoblotting analysis for monitoring the fate of the nitrogenase component proteins. Cell extracts were prepared from *A. vinelandii* wild type strain (A) or the strain containing the FeSII K14A/K15A double mutation in the chromosome (B). Immunoblots were probed with a mixture of affinity-purified antibodies to the α and β subunits of the MoFe protein, the Fe protein, and the FeSII protein. The culture began to enter the stationary phase at sample 4 (for further details of the growth phases, see ref *6*). Samples were taken at 1 h intervals, and subjected to SDS gel electrophoresis. Each lane contained the equivalent of total cell extract from 100 μL of culture.

It was of interest to determine the physiological effect of the double lysine mutation on nitrogenase protection in vivo. An *A. vinelandii* strain containing the FeSII K14A/K15A mutation in the chromosome was therefore constructed. The protection conferred by the FeSII protein was directly determined by monitoring the stability of the MoFe protein and Fe protein of nitrogenase during substrate-limited growth (Figure 5). Cells were grown in N-free Burk's medium containing limiting (10 mM) sucrose (instead of the usual 60 mM sucrose), and samples were taken at 1 h intervals before and after cultures entered the stationary phase. The amounts of the individual nitrogenase components in vivo were determined by Western blotting using purified antibodies specific to the MoFe protein, Fe protein, or FeSII protein (*7*). In the wild type strain, both nitrogenase components (MoFe and Fe proteins) were clearly present during the active growth (before sample 4) and could still be identified even 8 h after the culture had entered the stationary phase. In contrast, both the MoFe and Fe proteins in the strain containing the FeSII double lysine mutation in the chromosome within the FeSII locus were quickly degraded after cell cultures entered the stationary phase. The double lysine
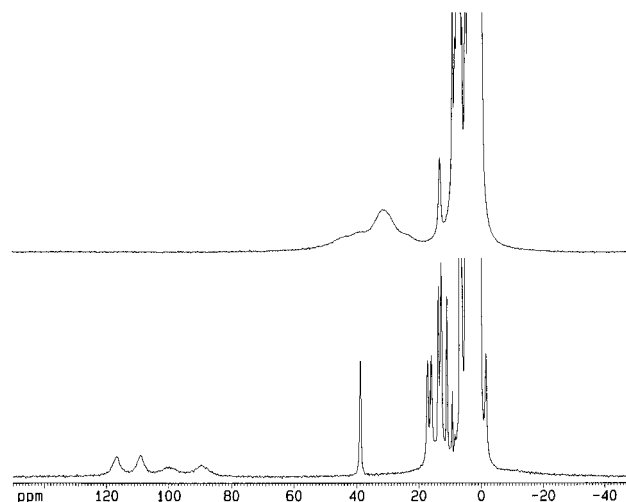

FIGURE 6: Paramagnetically shifted resonances of wild type FeSII in the oxidized (top) and reduced (bottom) states. Spectra were collected at 300 MHz for ^{1}H using a WEFT pulse sequence as described in Materials and Methods. Protein samples were dissolved in ^{2}H$_2$O containing 20 mM NaCl and pH indicators. The sample pHs were 5.23 for the oxidized state and 6.22 for the reduced state.

mutant had a phenotype like the *fesII* deletion strain we previously described (*10*). The nitrogenase component proteins in the lysine mutant or in an FeSII deletion mutant (see Figure 6) are present at near wild type levels when cells are growing.

Histidine Mutations. Because the successful formation of the oxygen-tolerant complex was also dependent on the oxidation state of the three interacting proteins (*27*), electrostatic forces are unlikely to be the sole mechanism responsible for the protein–protein interactions. Histidine residues have been shown to have important roles in protein function, especially in redox proteins (*28, 29*). There are only two histidine residues in the FeSII protein, at positions 12 and 55. When His12 was replaced with glutamine, the mutant protein (H12Q) still functioned as well as the wild type protein in the protection assay (Figure 4). In contrast, only 16% of the starting nitrogenase activity remained after air exposure for 30 min when the H55Q FeSII was the protective component. This result clearly indicates that His55 is a key residue for the protective function of FeSII.

If the ionization state of a residue affects the ability of the FeSII protein to associate with nitrogenase, then if that residue's pK were oxidation state-dependent the residue could mediate oxidation state-dependent FeSII association with nitrogenase. His12 does not appear to be capable of mediating a redox-dependent change in the FeSII protein affinity for nitrogenase since its pK is essentially the same in the reduced state (6.46 ± 0.02) and the oxidized state (6.454 ± 0.008) (Figure 7). The pK of His55 is slightly sensitive to the 2Fe–2S cluster oxidation state, with pKs of 6.24 ± 0.02 and 6.40 ± 0.008 in the reduced and oxidized states, respectively. However, the 0.16 ± 0.02 unit increase upon oxidation is small and is in the opposite direction from that expected for direct electrostatic interaction between His55 and the 2Fe–2S cluster. Finally, the low-pH chemical shift of His55 H2 is estimated to be 0.16 ppm lower in the reduced state than in the oxidized state. Thus, His55 is important for FeSII function and mildly sensitive to the 2Fe–2S cluster and its oxidation state.
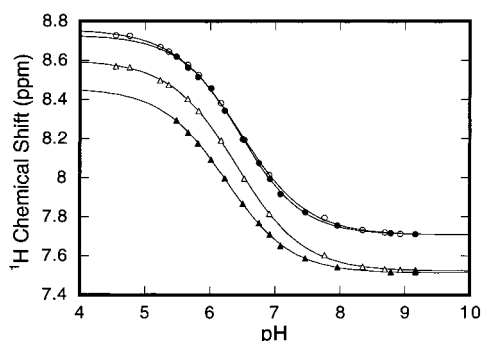
FIGURE 7: pH titrations of His12 (circles) and His55 (triangles) in oxidized (white symbols) and reduced (black symbols) wild type FeSII. The solid curves are fits to the data of the Henderson–Hasselbalch equation using the following parameters: His55 oxidized, $pK = 6.405 \pm 0.008$, $\delta_A = 8.602 \pm 0.005$, $\delta_B = 7.523 \pm 0.003$, and $n = 0.85 \pm 0.01$; His55 reduced, $pK = 6.24 \pm 0.02$, $\delta_A = 8.46 \pm 0.01$, $\delta_B = 7.513 \pm 0.003$, and $n = 0.87 \pm 0.02$; His12 oxidized, $pK = 6.454 \pm 0.008$, $\delta_A = 8.759 \pm 0.004$, $\delta_B = 7.707 \pm 0.003$, and $n = 0.84 \pm 0.01$; and His12 reduced, $pK = 6.46 \pm 0.02$, $\delta_A = 8.73 \pm 0.02$, $\delta_B = 7.709 \pm 0.005$, and $n = 0.91 \pm 0.03$.

DISCUSSION

Although both the MoFe and Fe proteins of nitrogenase in *A. vinelandii* are oxygen-labile (with half-lives of 24 and 2 min when exposed to 10% oxygen, respectively; *30*, *31*), nitrogenase in vivo and in crude extracts is oxygen-tolerant. This observation led to the discovery that a third component, the FeSII protein, could associate with the nitrogenase complex, and confer oxygen stability on the complex (*27*, *30*, *31*). The oxygen-stable nitrogenase complex is formed only under conditions of low ionic strength in the presence of MgCl$_2$, and requires that all three components be present (i.e., the FeSII protein provides little protection to individual MoFe or Fe proteins), and all three be in the oxidized state (*25*, *30*, *32*).

Since the FeSII protein can be disassociated from nitrogenase by the addition of NaCl, it seemed to be reasonable that electrostatic forces might be involved in the formation of the oxygen-stable nitrogenase complex. We noted that FdxH, which is a small cyanobacterial 2Fe–2S ferredoxin serving as a donor of an electron to nitrogenase (*33*, *34*), exhibits sequence similarities to the FeSII protein of *A. vinelandii*. Using site-directed mutagenesis, Schmitz et al. (*26*) determined that lysine residues located on the surface of FdxH were important for the interaction of FdxH with nitrogenase that is required for subsequent electron transfer. Unlike the FdxH from *Anabaena*, the FeSII protein in *A. vinelandii* cannot serve as a donor of an electron to nitrogenase, consistent with its relatively high midpoint redox potential (−262 mV; *7*). Nonetheless, our results show that Lys14 and Lys15 of the FeSII protein (analogous to Lys11 and Lys12 of FdxH) play an important role in the interaction of FeSII with the nitrogenase components. Mutation of these two residues is sufficient to abolish FeSII's protective activity.

His55 in the FeSII protein is only a few residues away from a cluster-liganding cysteine, and mutation of His55 to glutamine nearly destroyed all nitrogenase protective ability. Other examples of histidines that are essential to interactions between Fe−S proteins and their partners include the conserved His56 of adrenodoxin, which was shown to facilitate

electron transfer by coupling reduction of the Fe−S cluster to a change in the binding site (*35*, *36*). Nearby histidine residues may also affect the midpoint redox potential of Fe−S clusters (ref *29*, but see ref *38*). The interaction of histidine with the 2Fe−2S cluster could be partially responsible for the relatively high reduction potential of −290 mV of vertebrate ferredoxins since plant type ferredoxins, which lack histidine residues near the 2Fe−2S cluster, have low (−400 mV) and pH-independent E_m values (*37*). The FeSII protein from *A. vinelandii* has a relatively high (−262 mV) E_m similar to those of vertebrate ferredoxins. However, the placement of ligand cysteines in the vertebrate sequence is completely different from that in FeSII (which resembles plant type ferredoxins in this regard; *7*); the conserved His56 of vertebrate ferredoxins is the immediate neighbor to the third ligand Cys, whereas His55 of FeSII is six residues from the fourth ligand Cys. Therefore, it is unlikely that His55 in FeSII is analogous to His56 of vertebrate ferredoxins, and it is not too surprising that the redox potentials of both the FeSII H12Q and FeSII H55Q mutant proteins were not significantly different from the wild type E_m.

Although the E_m and oxidized state resonance Raman spectra of FeSII resemble those of vertebrate ferredoxins, the reduced state EPR spectrum and paramagnetically shifted NMR spectrum are much more similar to those of bacterial and plant type ferredoxins (*20*) (lacking histidines near the 2Fe−2S cluster), but differ from those of vertebrate ferredoxins (with histidines near the cluster). This corroborates the conclusion of Coughlan et al. (unpublished; see ref *20*) who found that His56 is not responsible for the categorical differences between the NMR spectra of reduced plant type and vertebrate ferredoxins. Thus, our results indicate that the factors producing the NMR spectral differences between plant type and vertebrate ferredoxins are not all the same as those that determine the E_m.

His55's effect on protective activity could indicate this residue's importance by a number of mechanisms. His55 can participate in electrostatic attraction between FeSII and nitrogenase only if it is charged. Thus, its pK would have to be higher than the cytoplasmic pH when FeSII is to bind to nitrogenase. The pKs of 6.24 ± 0.02 and 6.405 ± 0.008 measured for His55 in the reduced and oxidized states, respectively, are relatively low, arguing against a primarily electrostatic role for His55, unless the cytoplasmic pH drops significantly below 6 upon exposure to air. If so, the ambient pH would serve as the direct trigger leading to association between FeSII and nitrogenase, as an indirect result of oxygenation. Despite the reduction in protective ability upon mutation of His55 to Gln, the NMR spectra of paramagnetically shifted resonances, in both oxidation states, indicate that the 2Fe−2S cluster has been only slightly perturbed (at pH 6.0), as does the native-like EPR spectrum of the reduced state and the E_m value that is within 25 mV of the wild type value. Thus, strong conformational coupling between His55 and the 2Fe−2S cluster is also most unlikely.

Nonetheless, His55's NMR line width increases upon oxidation of the 2Fe−2S cluster, indicating that His55 is relatively close to the cluster in space. Although the chemical shift of His55 at high pH is independent of oxidation state, the chemical shift of protonated His55 is slightly different in the two oxidation states, suggesting slightly greater sensitivity to the 2Fe−2S cluster upon protonation. A

protonation state-dependent interaction between His55 and the 2Fe−2S cluster must affect both partners. The resonance Raman spectrum of the oxidized state is pH-independent between pH 5.2 and 10.4 (7), but the positions of the paramagnetically shifted resonances of the reduced state are pH-dependent. Analysis of the pH dependence of the chemical shifts of three relatively sharp and resolved ligand resonances indicates that the reduced 2Fe−2S cluster is sensitive to a pK of approximately 6.3 ± 0.3, as well as a second pK of >9. Thus, the former pK might be that of His55 (6.24 ± 0.02). His55's chemical shift sensitivity to oxidation state when protonated and the sensitivity of His55's pK to oxidation state indicate that the metal center and His55 are weakly interdependent.

Our finding that the pK of His55 changes by only 0.16 pH unit in response to a change in the 2Fe−2S cluster oxidation state argues against a mechanism in which a change in the 2Fe−2S cluster oxidation state results in a change in His55 ionization and thus alteration of the electrostatic interaction between FeSII and nitrogenase. The observed 0.16 unit pK change corresponds to a maximum change in charge of 0.09 per monomer, at pH 6.32. Moreover, the fact that the pK of His55 drops upon reduction of FeSII indicates that His55 is affected by more than just an additional negative charge on the 2Fe−2S cluster, as this would cause His55's pK to increase. Since the minor changes argue against conformational effects, the fact that His55's pK dropped suggests the presence of another ionizable residue which acquires a proton upon reduction of the 2Fe−2S cluster and disfavors protonation of His55. The solution of the crystal structure may suggest what amino acid this might be, and whether it could play a role in complex formation with or protection of nitrogenase.

ACKNOWLEDGMENT

SUPPORTING INFORMATION AVAILABLE

Two figures, one in which the spin echo NMR spectrum used to semiselectively observe histidines and pH indicators is compared with a conventional 90° pulse-observe NMR spectrum and the second in which the NMR spectra of reduced wild type FeSII, H12Q, and H55Q are compared. This material is available free of charge via the Internet at http://pubs.acs.org.

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