Circular Dichroism and X-ray Spectroscopies of Azotobacter vinelandii Nitrogenase Iron Protein

MgATP AND MgADP INDUCED PROTEIN CONFORMATIONAL CHANGES AFFECTING THE [4Fe-4S] CLUSTER AND CHARACTERIZATION OF A [2Fe-2S] FORM*

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Nucleotide interactions with nitrogenase are a central part of the mechanism of nitrogen reduction. Previous studies have suggested that MgATP or MgADP binding to the nitrogenase iron protein (Fe protein) induce protein conformational changes that control component protein docking, interprotein electron transfer, and substrate reduction. In the present study, we have investigated the effects of MgATP or MgADP binding to the Azotobacter vinelandii nitrogenase Fe protein on the properties of the [4Fe-4S] cluster using circular dichroism (CD) and x-ray absorption spectroscopies. Previous CD and magnetic CD studies on nitrogenase Fe protein suggested that binding of either MgATP or MgADP to the Fe protein resulted in identical changes in the CD spectrum arising from transitions of the [4Fe-4S]2+ cluster. We present evidence that MgADP or MgATP binding to the oxidized nitrogenase Fe protein results in distinctly different CD spectra, suggesting distinct changes in the environment of the [4Fe-4S] cluster. The present results are consistent with previous studies such as chelation assays, electron paramagnetic resonance, and NMR, which suggested that MgADP or MgATP binding to the nitrogenase Fe protein induced different conformational changes. The CD spectrum of a [2Fe-2S]2+ form of the nitrogenase Fe protein was also investigated to address the possibility that the MgATP- or MgADP-induced changes in the CD spectrum of the native enzyme were the result of a partial conversion from a [4Fe-4S] cluster to a [2Fe-2S] cluster. No evidence was found for a contribution of a [2Fe-2S]2+ cluster to the CD spectrum of oxidized Fe protein in the absence or presence of nucleotides. A novel two-electron reduction of the [2Fe-2S]2+ cluster in Fe protein was apparent from absorption, CD, and electron paramagnetic resonance data. Fe K-edge x-ray absorption spectra of the oxidized Fe protein revealed no changes in the structure of the [4Fe-4S] cluster upon MgATP binding to the Fe protein. The present results reveal that MgATP or MgADP Binding to the oxidized state of the Fe protein result in different conformational changes in the environment around the [4Fe-4S] cluster.

Nitrogenase catalyzes the biological reduction of nitrogen according to the overall reaction.

\[ \text{N}_2 + 8 \text{H}^+ + 16 \text{MgATP} + 8 \text{e}^- \rightarrow 2\text{NH}_3 + \text{H}_2 + 16\text{MgADP} + 16\text{P} \]

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tors upon Fe protein binding MgATP (13, 14). MgADP binding to the Fe protein does not result in an increased rate of chelation. Likewise, MgATP binding to the reduced Fe protein has been shown to result in changes in the lineshape of the EPR spectrum of the [4Fe-4S] cluster, which converts from rhombic to axial forms (15, 16). MgADP binding to the Fe protein, however, does not result in changes in the EPR spectrum. These studies suggested that MgATP binding to the Fe protein results in significant changes in the environment of the [4Fe-4S] cluster, but provided little information about any MgADP-induced changes. It has been found that MgATP binding to the Fe protein lowers the redox potential of the Z°/A° couple of the [4Fe-4S] cluster from −310 to −430 mV (versus the standard hydrogen electrode) (15, 17). MgADP binding to the Fe protein results in the same lowering of the reduction potential, clearly suggesting that MgADP binding does affect the properties of the [4Fe-4S] cluster. While these results demonstrated an effect on the [4Fe-4S] cluster by both MgADP and MgATP binding, little information exists to distinguish between MgATP- and MgADP-induced changes in the [4Fe-4S] cluster. Proton NMR spectra of the reduced Fe proteins from Clostridium pasteurianum (18) and Azotobacter vinelandii (19) revealed a series of isotropically shifted proton resonances arising from cysteinyl protons, which are ligands to the paramagnetic [4Fe-4S]1+ cluster. The chemical shifts of these protons were sensitive to MgATP or MgADP binding to the Fe protein, providing evidence for differences in the cluster environment upon binding of either nucleotide.

Circular dichroism spectroscopy in the visible wavelength region is a useful way to monitor the type and environment of Fe-S clusters in proteins (20–24). The circular dichroism spectrum of proteins containing Fe-S clusters is more structurally, leading to the conclusion that the [4Fe-4S] cluster environment of these proteins is highly conserved. These studies further demonstrated that the oxidized Fe protein CD spectrum changed upon binding MgATP or MgADP. Both MgADP and MgATP binding were found to result in essentially the same CD spectrum (22, 26, 28).

In order to define the function of MgATP binding and hydrolysis in the nitrogenase mechanism in conjunction with studies using site-specifically altered Fe proteins (6–8, 29, 30), we have re-examined the CD spectra of nitrogenase Fe protein in the absence or presence of MgATP or MgADP. In accord with the earlier studies, we find a distinctive CD spectrum for the oxidized Fe protein and changes in this spectrum upon binding of nucleotides. However, unlike the earlier CD studies, we have found that MgATP or MgADP binding to the Fe protein result in very different CD spectra. These results clearly show that MgATP or MgADP binding to the Fe protein are communicated as different changes in the environment of the [4Fe-4S] cluster and demonstrate that CD is a sensitive way to monitor these changes. Resonance Raman studies of the [4Fe-4S]2+ cluster of the Fe protein (31) have suggested that MgATP binding to the Fe protein results in different resonance bands that could be modeled by the partial conversion of the Fe protein [4Fe-4S] cluster to a [2Fe-2S] cluster. Since proteins containing [2Fe-2S] clusters (with four Cys ligands) generally have more intense CD spectra relative to proteins containing [4Fe-4S] clusters, it was suggested that the change in the CD spectrum of the Fe protein observed upon binding MgATP was the result of the partial conversion to a [2Fe-2S] cluster (31). To test this possibility, we have prepared a [2Fe-2S] cluster form of the Fe protein using a published procedure (32) and examined it by absorption, CD, and EPR spectroscopy. CD spectra of plant/algae type [2Fe-2S] cluster containing proteins, unlike those exhibited by other types of protein bound Fe-S clusters, are relatively insensitive to protein environment (20). The [2Fe-2S] cluster containing Fe protein exhibited CD spectra very similar to the classical plant/algae ferredoxin [2Fe-2S] type, which does not resemble the nucleotide-bound spectra of the [4Fe-4S] form of the Fe protein. Therefore, we can preclude any contribution of a [2Fe-2S]2+ cluster to the observed CD spectrum of the [4Fe-4S] cluster form of the Fe protein in the absence or presence of MgATP or MgADP. Fe K-edge XAS studies of the [4Fe-4S]2+ cluster of the oxidized Fe protein were also performed and suggested no major structural changes in the cluster upon Fe protein binding MgATP. Finally, a novel two-electron reduction of the [2Fe-2S]2+ cluster in the Fe protein by dithionite was inferred from dithionite titration experiments using absorption, CD, and EPR spectroscopies.

MATERIALS AND METHODS

Nitrogenase Proteins—Nitrogenase iron protein (Fe protein) and molybdenum-iron protein (MoFe protein) were purified anaerobically from A. vinelandii as described previously (29). Specific activities for the nitrogenase proteins were 2400 nmol of acetylene reduced-min−1·(mg of MoFe protein)−1 and 2200 nmol of acetylene reduced-min−1·(mg of Fe protein)−1. All proteins were homogeneous as determined by Coomassie staining of SDS-polyacrylamide gels (6). Protein concentrations were determined by a modified Biuret method with bovine serum albumin as standard (33). Molar absorption coefficients used for Fe protein samples were 13.3 mm−1·cm−1 at 400 nm for oxidized Fe protein (8) and 10 mm−1·cm−1 at 400 nm for the oxidized [2Fe-2S] cluster form of Fe protein (32). The molecular mass for A. vinelandii Fe protein was taken to be 64,000 Da.

Circular Dichroism Spectroscopy—Oxidized Fe protein samples (Fe proteinox) were prepared in an argon- or nitrogen-filled anaerobic glovebox (Vacuum Atmospheres, CA). 20 mg of dithionite-reduced Fe protein (Fe proteinredu) were passed down a Sephadex G-25 column (0.7 × 10 cm) equilibrated with oxygen-free 100 mM Tris buffer, pH 8.0, to remove the dithionite. The Fe protein containing fraction was oxidized by the addition of 20 µl of 20 mM indigo disulfonate (IDS) made in 50 mM Tris buffer, pH 8.0, and allowed to react for 5 min. IDS (oxidized and reduced) was removed from the oxidized Fe protein (Fe proteinox) by passage through a Dowex 1® column (0.5 × 5 cm) equilibrated with 100 mM Tris buffer, pH 8.0. The Fe proteinox fraction was diluted to a total of 4 ml with 100 mM Tris buffer, pH 8.0, and 2 ml was placed into each of two quartz cuvettes (1 cm pathlength) fitted with a butyl septum to maintain anaerobility. Circular dichroism spectra were recorded on an Aviv model 62DS spectropolarimeter with a 1-nm spectral bandwidth, and data were collected every 1 nm at 25°C. Background spectra were recorded for each cuvette with buffer alone and were subtracted from all recorded spectra. MgATP or MgADP were added to the indicated final concentrations with gas tight syringes from stock solutions containing 25 mM ATP or ADP and 30 mM MgCl2 made in 1 M Tris buffer, pH 8.0. For spectra recorded at pH 7.4, all manipulations were the same as above, except that the buffer was 25 mM HEPES, pH 7.4, with 0.1 mg/ml dithiothreitol and 2 mM MgCl2. In all cases, the presence of the oxidized Fe protein was confirmed before and after CD spectra were taken from the visible region absorption spectrum. Fe proteins assayed for acetylene reduction activity after CD spectra were taken retained greater than 95% of their original activity. In all cases, CD spectra were recorded with Fe protein samples from several independent preparations.

Preparation of the [2Fe-2S] Form of Fe Protein—The oxidized [2Fe-2S] form of A. vinelandii Fe protein was prepared by partial chelation of iron from the [4Fe-4S] cluster by a,a′-dipryridyl essentially as described previously (32). 20 mg of Fe proteinredu was oxidized by the addition of 25 mM IDS until a green color remained. The IDS was removed from oxidized Fe protein by passage through a Dowex 1®...
column equilibrated with 100 mM Tris buffer, pH 8.0. The Fe protein sample was diluted to 1 ml with 100 mM Tris buffer, pH 8.0. To the oxidized Fe protein sample, MgATP was added to a final concentration of 5 mM followed by the addition of \( \alpha,\alpha'\text{-dipirydyl} \) to 5 mM. The sample was allowed to react for 10 min at 25 °C. The oxidized, [2 Fe-2 S] form of the Fe protein was separated from smaller material by passage through a Sephadex® G-25 column (0.7 × 10 cm) equilibrated with 100 mM Tris buffer, pH 8.0. The [2 Fe-2 S] Fe protein sample was diluted to 4 ml with 100 mM Tris buffer, pH 8.0, and 2 ml were transferred into each of two quartz cuvettes (1 cm) with butyl stoppers. CD spectra were recorded as described above. The [2 Fe-2 S] Fe protein was quantified from its absorption spectrum with an absorption coefficient of 10 mM \( \text{cm}^{-1} \) at 400 nm (32). Absorption spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer. Native Fe protein was found to contain 3.5 ± 0.2 Fe protein, while the [2 Fe-2 S] form of Fe protein contained 1.7 ± 0.2 Fe protein using a technique described previously (6). As previously reported, the [2 Fe-2 S] Fe protein was unable to support the reduction of \( \text{C}_{6} \text{H}_{4} \) or proteins (32).

Reduction of the [2 Fe-2 S]\(^{2+} \) Fe protein was accomplished by the addition of aliquots of a calibrated dithionite (\( \text{Na}_{2} \text{S}_{2} \text{O}_{4} \)) solution in 50 mM Tris buffer, pH 8.0. The dithionite concentration of the stock solution was quantified by titration with redox dyes (34). An aliquot of the dithionite solution was added to an anaerobic solution of methylene blue or potassium ferricyanide in 50 mM Tris buffer, pH 8.0. From the change in the absorption spectra of the dye and the known absorption coefficients (22.8 mM \( \text{cm}^{-1} \) at 600 nm for methylene blue and 1.0 mM \( \text{cm}^{-1} \) at 420 nm for potassium ferricyanide), the dithionite concentration was determined to be 2.3 mM (34).

Electron Paramagnetic Resonance Spectroscopy—EPR spectra were recorded in a Bruker model ESP 300 spectrometer equipped with an Air Products LTR3 liquid helium cryostat. The [4Fe-4S]\(^{2+} \) cluster form of the Fe protein contained 2 mM dithionite and a 10-fold molar excess of MgATP and was frozen under argon as described previously (6). The [2 Fe-2 S] cluster form of the Fe protein was prepared as described above, and a 6-fold molar excess of dithionite was added prior to freezing the sample. X-ray Absorption Spectroscopy of Fe protein—Oxidized Fe protein for XAS was prepared as follows. 29 mg of dithionite-reduced Fe protein was passed through a Sephadex® G-25 column (0.7 × 10 cm) equilibrated with 100 mM Tris buffer, pH 8.0. The Fe protein fraction was oxidized by the addition of an excess of a 25 mM IDS solution. The oxidized Fe protein was separated from IDS by passage through a Dowex 1 × 8 column (0.5 × 5 cm) equilibrated with 100 mM Tris buffer, pH 8.0. The oxidized Fe protein fraction was concentrated to 230 \( \mu \)l with a Centricon®-30 in an anaerobic glove box. A 75-\( \mu \)l aliquot of this sample was loaded directly into a sample holder and frozen in liquid nitrogen. A second sample was made to 5 mM in MgATP prior to freezing. Sample holders were 20 × 2 mm slots cut in a 1-mm-thick piece of aluminum and covered on both sides with 0.025-mm-thick Mylar® film. During data collection at the National Synchrotron Light Source beam line X9b, the sample was maintained in an evacuated chamber at about 100 °C. Spectra were recorded from 3500 to 500 nm with a 2-mm slit. Data analysis using amplitude and phase functions derived from FEFF 5.05 (or 6) calculations (35, 36) was as described previously (37) except that the least-squares residual reported (38) for estimating parameter uncertainties now follow recommendations of the International Workshops on Standards and Criteria in XAFS (38). New amplitude and phase functions were derived for Fe-S and Fe-Fe interactions using the crystallographic coordinates of [\( \text{ICH}_{3} \text{N}_{2} \text{Fe}_{5} \text{S}_{6} \text{S(OH)}_{3} \)] (39). Fitting EXAFS of this model complex, iron metal, and low and room temperature samples of FeS\(_{4}\)CNET\(_{3}\) revealed that the FEFF-generated functions for Fe-S and Fe-Fe overestimate the amplitude of the EXAFS, but good fits (with \( n \) within ±20% of the true value and \( r \) within ±0.1 Å of crystallography) can be obtained by using empirical amplitude reduction factors of 0.75 and 0.55, respectively, and \( E_{\text{b}} = 7122 \text{ eV} \). For plots and weighting, \( k \) is calculated using 7125 eV (37). Disorder factors, \( \Delta \alpha^{2} \), are relative to the disorder calculated by FEFF using Debye temperature of 100 K.

**Fig. 1.** Circular dichroism spectra of oxidized A. vinelandii Fe protein in the absence or presence of MgATP or MgADP. CD spectra of IDS oxidized nitrogenase Fe protein (Fe protein\(_{\text{ox}} \)) in 100 mM Tris buffer, pH 8.0, were recorded as described under “Materials and Methods.” Trace 1, Fe protein\(_{\text{ox}} \); trace 2, Fe protein\(_{\text{ox}} \) with 1 mM MgATP; trace 3, Fe protein\(_{\text{ox}} \) with 1 mM MgADP. The molar absorption coefficient \( (\Delta \alpha) \) is plotted against the wavelength. All spectra were baseline subtracted.

**RESULTS AND DISCUSSION**

Circular Dichroism Spectra of Oxidized Nitrogenase Fe protein in the Absence or Presence of MgATP or MgADP—The [4Fe-4S]\(^{2+} \) cluster in nitrogenase Fe protein is bound between and at the top of the two identical subunits, with Cys-97 and Cys-132 providing the ligands (12). The one electron redox couple of the cluster between the \( 1^{+} \) and \( 2^{+} \) states is well characterized with a reduction potential of −310 mV (15, 17). Reduction to an all ferrous state has also been recently reported (41). Previous near UV/visible and near IR CD studies of the reduced Fe protein demonstrated a weak, somewhat featureless CD arising from electronic transitions from the [4Fe-4S]\(^{2+} \) cluster (21, 22). The CD showed little change when the Fe protein bound MgATP or MgADP. It was concluded that either CD was not sensitive to changes around the [4Fe-4S]\(^{2+} \) cluster in the reduced state or that the reduced state of the Fe protein did not undergo significant conformational changes upon binding nucleotides. In contrast, the one electron oxidized state of the Fe protein was found to give a well structured CD spectrum (22). This spectrum was similar in intensity, but unique in form, to the CD observed for other proteins containing [4Fe-4S]\(^{2+} \) clusters, which are known to be very protein environment-dependent (20, 24). Both enzymatically oxidized and thionine-oxidized Fe proteins gave similar CD spectra. Upon the addition of MgATP or MgADP to the oxidized Fe protein, a significant change in the CD Spectrum was observed leading to a more intense and more structured spectrum. However, the effects of MgATP or MgADP binding were found to be virtually identical, suggesting identical structural changes in the environment of the [4Fe-4S]\(^{2+} \) cluster (21, 22).

We have measured a CD spectrum for indigo disulfonate-oxidized Fe protein from A. vinelandii (Fig. 1, trace 1), which is essentially identical to that previously reported for thionine oxidized Fe protein (21, 22). The addition of MgADP to a final concentration of 1 mM resulted in a significant change in the CD spectrum (Fig. 1, trace 3), consistent with previously observed CD spectra (21, 22). Titration of the oxidized Fe protein with MgADP revealed a conversion from the oxidized Fe protein CD to the MgADP-bound form CD over a MgADP concentration range from 0 to 150 \( \mu \)M (Fig. 2A). The change in the CD spectrum was saturated by 150 \( \mu \)M MgADP and did not change further when the MgADP concentration was increased to 1 mM.
The results of the present study, therefore, reveal that MgATP or MgADP binding to the Fe protein result in distinct conformational changes in the [4Fe-4S] cluster, which can be monitored by CD spectroscopy.

Circular Dichroism Spectra of a [2Fe-2S] Form of Fe Protein—Resonance Raman spectroscopy has recently been used to investigate the Fe–S stretching modes of the [4Fe-4S] cluster of oxidized Fe protein from *C. pasteurianum* (31). The Raman results were interpreted based on the presence of a cubane [4Fe-4S] cluster. The addition of MgATP to the oxidized Fe protein did not affect the frequencies of the Raman bands, suggesting a lack of structural perturbation of the cluster. The effects of MgADP binding to the Fe protein on the Raman
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oxidized protein. One band (391 cm⁻¹) spectrum were not examined. Two additional Raman bands spectrum of oxidized nitrogenase Fe protein and preparation of the oxidized [2Fe-2S] form of the Fe protein were as described under "Materials and Methods." Oxidized Fe protein in the absence (trace 1) or presence of 1 mM MgADP (trace 2) or 1 mM MgATP (trace 3) or the oxidized [2Fe-2S] form of Fe protein in the absence of nucleotides (trace 4).

To determine if the formation of a [2Fe-2S] cluster could account for the CD spectrum recorded for oxidized Fe protein, we prepared the [2Fe-2S] form of A. vinelandii Fe protein and measured the CD spectrum. Fig. 4 shows the CD spectrum of the oxidized, [2Fe-2S] form of the Fe protein (trace 4) compared with CD spectra recorded for the [4Fe-4S]²⁺ form of Fe protein in the absence or presence of MgATP or MgADP. It is clear from these results that the CD spectra observed for the [4Fe-4S]²⁺ states of the Fe protein, either with or without nucleotides, are not the result of a conversion to a [2Fe-2S]²⁺ cluster but would rather be consistent with changes in the environment of the [4Fe-4S] cluster upon Fe protein binding nucleotides. The CD spectra observed for the [2Fe-2S] form is very similar to the CD spectra exhibited by plant/algal type [2Fe-2S] clusters containing ferredoxins (20, 24), confirming the [2Fe-2S] cluster in this form of the Fe protein is of this type.

The [2Fe-2S] cluster form of the Fe protein was confirmed by its absorption spectra, which also exhibit features characteristic of plant/algal ferredoxin type [2Fe-2S] clusters (20, 24) and by total iron analysis (6). The [4Fe-4S] cluster containing Fe protein was found to contain 3.5 ± 0.2 Fe/protein, while the [2Fe-2S]-form was found to contain 1.7 ± 0.2 Fe/protein. We found that addition of MgATP or MgADP to the [2Fe-2S] form of the Fe protein did not result in any noticeable changes in the CD spectrum. It is likely, however, as noted above, that the CD spectra of the [2Fe-2S] form will be relatively insensitive to the same kinds of protein conformational changes to which the [4Fe-4S] cluster form is sensitive. We also examined CD spectra for the reduced state of the [2Fe-2S] form of the nitrogenase Fe protein. The CD spectrum of dithionite reduced [2Fe-2S] cluster containing Fe protein was not of the plant/algal ferredoxin type, and was generally quite featureless. The absorption, furthermore, was bleached on addition of dithionite by approximately 80 percent at 400 nm, about twice the bleaching typical of one electron reduction of a plant/algal type [2Fe-2S] cluster (20). Titration of this protein with dithionite (Fig. 5) revealed two isosbestic points (at 420 and 520 nm) in the CD, suggesting conversion from the oxidized state to a single reduced state. The titration was observed to be complete upon the addition of one molar equivalent of dithionite. Since dithionite is a two-electron reductant, this suggested that complete reduction of the nitrogenase Fe protein [2Fe-2S] cluster required the addition of two electrons without a significant population of a one-electron reduced intermediate. This reduction stoichiometry was independently determined by titration of the Fe protein [2Fe-2S] cluster by absorption spectroscopy (Fig. 6). The fully reduced [2Fe-2S] form of the Fe protein could be reoxidized to the original absorption spectrum, confirming the reversibility of the redox reaction.

Recently, Im et al. (42) revealed the first reported case of a two-electron reduction of a [2Fe-2S] cluster in a ferredoxin using a novel Cr(II) macrocycle as reductant. At 400 nm, the absorption spectrum of the ferredoxin is 80% bleached relative to the oxidized state. This bleaching and the lineshape of the two-electron reduced absorption spectrum are virtually identical to the oxidized state. This bleaching and the lineshape of the two-electron reduced absorption spectrum are virtually identical to the oxidized state. This bleaching and the lineshape of the two-electron reduced absorption spectrum are virtually identical to the oxidized state. This bleaching and the lineshape of the two-electron reduced absorption spectrum are virtually identical to the oxidized state.
dized [2Fe-2S] cluster Fe protein was EPR silent near \( g \approx 2 \), and exhibited only an isotropic adventitious Fe(III) signal at \( g = 4.3 \) (data not shown). This is consistent with the presence of only [2Fe-2S]\(^{2+}\) and [4Fe-4S]\(^{2+}\) clusters. [2Fe-2S] cluster Fe protein treated with a 0.5 mol eq of dithionite was EPR silent, with the exception of a small residual \( g = 4.3 \) signal (data not shown). [2Fe-2S] cluster Fe protein reduced by 6 equivalents of dithionite exhibited a small amount of an axial EPR signal at \( g \approx 2 \) (Fig. 7), with apparent \( g \) values identical to those of the MgATP-bound state of the reduced [4Fe-4S] cluster form of Fe protein.
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Fourier filtering of X-ray (without windowing) employed ranges of k = 1.0 to 13.0 Å⁻¹ and r = 1.0 to 2.7 Å. The filtered EXAFS was fit for k = 2.2 to 13.0 Å, using e.s.d. of filtered determined by linear interpolation of (k, 2(k)). Numbers in parentheses indicate ± uncertainty in last digit determined by the range of values allowing fits with χ² < χ²(min) + 1.0 (38).

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**Table I**

| Fe protein ox | Fe protein ox + MgATP | [ICH₃N]₂[Fe₅S₅(SH)₄]²⁻ |
|---------------|-----------------------|------------------------|
| Fe K-edge XAFS |                      |                        |
| S @ r (Å)     | 2.278(5)              | 2.278(5)               |
| ⁴Fe    (Å)²  | 0.0010(6)             | 0.0012(6)              |
| n Fe, n = a | 3.7(9)                | 3.2(8)                 |
| @ r (Å)      | 2.722(7)              | 2.720(7)               |
| ⁴Fe    (Å)²  | 0.00021(19)           | 0.00015(19)            |
| L (min)      | 2.5                   | 2.1                    |
| c          | 1.0                   | 0.88                    |
| d          | 1.0 (38)              |                        |

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a X-ray crystal structure shows four Fe-S bonds averaging 2.280 Å and three Fe-Fe distances per iron 2.736 Å (39).

b The relatively large uncertainty ranges for the average number of Fe-Fe scattering interactions per iron are due to correlation coefficients of >0.9 between nFe and Δσ²(Fe).

c Uncertainty range spans nFe = 1.9 to 4.4.

d Minimized least square residual (38).

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Cluster has also been used to monitor possible changes in the cluster structure. Previous studies on the reduced Fe protein [4Fe-4S]¹ cluster have revealed that no structural changes within the cluster could be detected upon the addition of MgATP (44). The oxidized Fe protein [4Fe-4S]²⁻ cluster was not previously examined. We have now examined the Fe K-edge X-ray absorption spectra of the IDS oxidized Fe protein in the absence or presence of MgATP and can detect no changes in either the edge or EXAFS spectra (Figs. 8 and 9). The EXAFS spectra can be best fitted using four sulfur and three iron scatterers around each iron atom (as in [4Fe-4S] clusters), but they are fit poorly if only one Fe-Fe scatterer is assumed (as would be the case if [2Fe-2S] clusters were formed) (Fig. 9). Parameters from least-squares fits are within estimated error for both forms of the protein, with refined bond distances essentially identical to those obtained from data for a [4Fe-4S] model compound (Table I). These results from x-ray spectroscopy indicate that the iron is in the [4Fe-4S] cluster form in samples of oxidized Fe protein with and without added MgATP, and so rule out extensive formation of a [2Fe-2S] cluster induced by addition of MgATP.

In summary, we have demonstrated unique CD spectra arising from the [4Fe-4S]²⁻ cluster upon nitrogenase Fe protein binding either MgATP or MgADP. These results suggest that each nucleotide communicates different conformational changes to the environment of the cluster. We provide direct evidence that the observed CD changes in the Fe protein upon binding nucleotides are not the result of a conversion of the [4Fe-4S] cluster to a [2Fe-2S] cluster. The Fe K-edge XAS results support this conclusion and suggest that nucleotides do not result in major changes in the cluster structure but rather in changes in the environment around the cluster.

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