Contribution from the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and AT&T Bell Laboratories, Murray Hill, New Jersey 07974

EXAFS Studies of the Nitrogenase Iron Protein from *Azotobacter vinelandii*

Paul A. Lindahl,[†‡] Boon-Keng Teo,[§] and William H. Orme-Johnson*[†]

Received May 27, 1987

We have recently shown that the [4Fe-4S]$^+$ cluster of the iron protein can exist in $S = {}^1/_2$, $S = {}^3/_2$, and probably $S = {}^5/_2$ spin states, depending on the solvent and on the occupancy of the protein's nucleotide binding sites. We have now examined the structure of the cluster in the reduced (1+) and oxidized (2+) electronic states, the $S = {}^1/_2$ and $S = {}^3/_2$ spin states, and in the MgATP-bound form by using the EXAFS technique. The average calculated Fe–S coordination number for the protein data is 4.0 (5) at a distance of 2.31 (1.5) Å with a Debye–Waller factor of 0.06 (1) Å. The cluster is probably bound to the protein via four sulfur (cysteine) ligands. Significant differences in the average Fe–S distance of oxidized synthetic clusters and reduced Fe protein clusters were observed. Neither the MgATP-induced conformational change nor the $S = {}^1/_2$ to $S = {}^3/_2$ spin-state conversion requires changes in the average Fe–S bond length of more than about 0.02 Å. The average calculated Fe–Fe coordination number for the protein data is 2.4 (8), at a distance of 2.73 (3) Å. The Debye–Waller factor for the Fe–Fe term of the Fe protein data (0.10 (2) Å) is larger than that for the model cluster data (0.08 Å), and this dissimilarity results in an underestimate of the Fe–Fe coordination number in the Fe protein cluster.

Introduction

Nitrogenase, the enzyme responsible for biological nitrogen fixation, is made up of two proteins: the molybdenum–iron protein and the iron (Fe) protein.[4-6] The Fe protein is a dimer of identical subunits containing a single [4Fe-4S] cluster apparently bound between the two subunits.[7] The cluster is present in the paramagnetic, 1+ core oxidation state in the reduced protein and in the diamagnetic, 2+ state in the oxidized protein. The reduced cluster exhibits a $g = 1.94$ type EPR signal as well as features around $g = 5.8$ and 5.1.[1,2,8,9] Approximately 40% of the clusters have $S = {}^1/_2$ spin states, and these yield the $g = 1.94$ signals. The remaining clusters have $S = {}^3/_2$ ground states, which give rise to the low-field EPR signals. The proportion of clusters in each spin state can be altered by the addition of certain reagents to Fe protein solutions. About 85% of the clusters have spin $S = {}^3/_2$ in samples containing 0.5 M urea, while about 90% have spin $S = {}^1/_2$ in samples containing either 15% HMPA or 50% ethylene glycol.[1]

There are two MgATP binding sites per protein, the occupancy of which causes a protein-conformational change and altered cluster properties. The cluster midpoint potential is lowered,[10,11] the cluster iron becomes susceptible to chelators,[12,13] the CD and MCD spectra change,[14] and the ^{57}Fe quadrupole splittings of the cluster decrease,[1] all as a result of MgATP binding. Crystals of the Fe protein are unstable in solutions of MgATP.[15] The proportions and types of spin states present also change when MgATP binds; the $g = 1.94$ signal changes from rhombic to axial symmetry,[16-18] while the low-field features change shape and intensity as well.[2] It also appears that a minor proportion of clusters have $S = {}^5/_2$ ground states in the MgATP-bound form.[2] The extent to which this protein conformational change affects the Fe protein cluster structure is not known.

The structural changes required to switch one spin ground state for another in [4Fe-4S]$^+$ clusters are not well established, though it appears that a minor perturbation, such as a change in the orientation of the external cluster ligands, may be sufficient. Recently, the cluster $(Et_4N)_3[Fe_4S_4(SC_6H_{11})_4]$ has been found to exhibit an $S = {}^3/_2$ ground state with EPR, Mössbauer, and magnetization characteristics essentially identical with those of the Fe protein $S = {}^3/_2$ cluster.[19] This indicates that no gross structural changes are required for such spin-state transitions. The X-ray crystal structure of a similar cluster with an $S > {}^1/_2$ spin ground state[20] exhibits average Fe–S and Fe–Fe bond lengths essentially the same as in $S = {}^1/_2$ clusters. Structural data on the Fe protein cluster in its different spin-state forms are required to determine whether these analogies can be extended to the Fe protein cluster.

The external ligands used to bind the Fe protein cluster are not known, though they are assumed to be cysteine sulfurs.[7] This idea is supported by evidence that 10 cysteine residues per protein have been evolutionally conserved.[21,22] However, given the unusual magnetic properties of the cluster in this protein and the fact the spacing of the cysteine residues in the protein is atypical of those in other [4Fe-4S]-containing proteins,[23] there is room for doubt.

[†] Massachusetts Institute of Technology.
[‡] Present address: Gray Freshwater Biological Institute, University of Minnesota, Navarre, MN 55392.
[§] AT&T Bell Laboratories. Present address: Department of Chemistry, University of Illinois, Chicago, IL 60607.

(1) Lindahl, P. A.; Day, E. P.; Kent, T. A.; Orme-Johnson, W. H.; Münck, E. *J. Biol. Chem.* **1985**, *260*, 11160.
(2) Lindahl, P. A.; Gorelick, N. J.; Münck, E.; Orme-Johnson, W. H. submitted for publication in *J. Biol. Chem.*
(3) Abbreviations: EXAFS, extended X-ray absorption fine structure spectroscopy; FT, Fourier transform; FABM, fine adjustment based on model method; DW, Debye–Waller factor; CN, coordination number; EPR, electron paramagnetic resonance; CD, circular dichroism; MCD, magnetic circular dichroism; ATP, adenosine triphosphate; Av2, iron protein from *Azotobacter vinelandii*; Av2/native, Av2 in the native and reduced state; Av2/ATP, reduced Av2 in the presence of 5 mM MgATP; Av2/urea, reduced Av2 in small amounts (e.g. 0.5 M) of urea; HMPA, hexamethylphosphoramide; Av2/HMPA, reduced Av2 in 15% HMPA.
(4) Orme-Johnson, W. H. *Annu. Rev. Biophys. Chem.* **1985**, *14*, 419.
(5) Burgess, B. K. In *Advances in Nitrogen Fixation Research*; Veeger, C., Newton, W. E., Eds.; Nijhoff/Junk: The Hague, The Netherlands, 1984; p 103.
(6) Thorneley, R. N. F.; Lowe, D. J. in *Metal Ions in Biology*; Spiro, T., Ed.; Wiley: New York, 1985; Vol. 7, Chapter 5.
(7) Hausinger, R. P.; Howard, J. B. *J. Biol. Chem.* **1983**, *258*, 13486.
(8) Hagen, W. R.; Eady, R.; Dunham, W. R.; Haaker, H. *FEBS Lett.* **1985**, *189*, 250.
(9) Watt, G. D.; McDonald, J. W. *Biochemistry* **1985**, *24*, 7226.
(10) Zumft, W. G.; Mortenson, L. E.; Palmer, G. *Eur. J. Biochem.* **1974**, *46*, 525.
(11) Morgan, T. V.; Prince, R. C.; Mortenson, L. E. *FEBS Lett.* **1986**, *206*, 4.
(12) Walker, G. A.; Mortenson, L. E. *Biochemistry* **1974**, *13*, 2382.
(13) Ljones, T.; Burris, R. H. *Biochemistry*, **1978**, *17*, 1866.
(14) Stephens, P. J.; McKenna, C. S.; Smith, B. E.; Nguyen, H. T.; McKenna, M.-C.; Thomson, A. J.; Devlin, F.; Jones, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 2585.
(15) Rees, D. C.; Howard, J. B. *J. Biol. Chem.* **1983**, *258*, 12733.
(16) Orme-Johnson, W. H.; Hamilton, W. D.; Ljones, T.; Tso, M.-Y. W.; Burris, R. H.; Shah, V. K.; Brill, W. J. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 3142.
(17) Smith, B. E.; Lowe, D. J.; Bray, R. C. *Biochem. J.* **1973**, *135*, 331.
(18) Zumft, W. G.; Palmer, G.; Mortenson, L. E. *Biochim. Biophys. Acta* **1973**, *292*, 413.
(19) Carney, M. J.; Holm, R. H.; Papaefthymiou, G. C.; Frankel, R. B. *J. Am. Chem. Soc.* **1986**, *108*, 3519.
(20) Berg, J. M.; Hodgson, K. O.; Holm, R. H. *J. Am. Chem. Soc.* **1979**, *101*, 4586.
(21) Sundaresan, V.; Ausubel, F. M. *J. Biol. Chem.* **1981**, *256*, 2808.
(22) Hausinger, R. P.; Howard, J. B. *J. Biol. Chem.* **1982**, *257*, 2483.

0020-1669/87/1326-3912$01.50/0

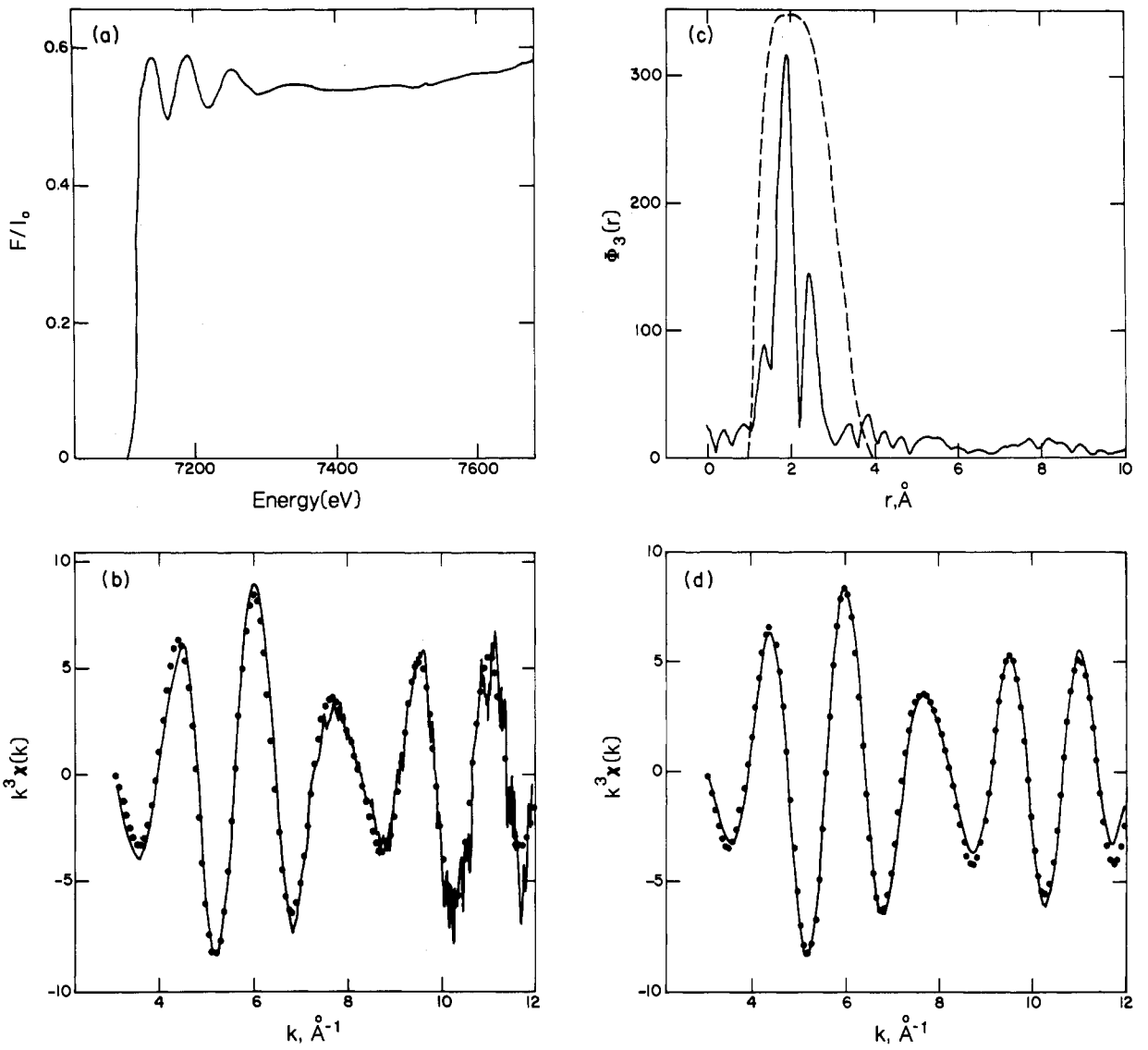

Figure 1. EXAFS data analysis: (a) averaged X-ray absorption spectra of Av2/native, preparation A; (b) background-removed and Fourier-filtered (O) data, with a small region of data at 10.2 Å^{-1} removed; (c) FT of the background-removed data, with a broken line showing the filter window used; (d) Fourier-filtered data and theoretical fit (O). The FABM-refined parameters used for the fit are listed in Table I.

Thus, it is important to determine the nature of the ligands that bind the cluster to this protein.

We have used the EXAFS technique to probe the structure of the Fe protein cluster. We have examined the reduced, oxidized, $S = {}^1/_2$ state, $S = {}^3/_2$ state, and the MgATP-bound form of the Fe protein. In this paper, we report that the Fe protein EXAFS data, from all states studied, fit properly only to Fe–S and Fe–Fe terms and fit to distances and coordination numbers similar to those found in [4Fe-4S] clusters with four external sulfur ligands. We observe significant differences in the Fe–S bond lengths in oxidized and reduced cluster spectra. The $S = {}^3/_2$, $S = {}^1/_2$ spin-state conversion or the MgATP-induced conformational change does not affect the structure of the [4Fe-4S] cluster within the accuracy of the technique.

Experimental Section

The *Azotobacter vinelandii* Fe protein was purified, characterized, and manipulated into various states as described.[1] Three Fe protein preparations were used in the EXAFS study, with specific activities of 2000 (preparation A), 1430 (preparation B), and 1700 (preparation C) nmol of C_2H_4 min^{-1} mg^{-1}. EXAFS data on two different Av2/native samples (preparation A and C), an Av2/oxidized sample (preparation B), Av2/urea and Av2/HMPA samples (preparation C), and an Av2/ATP sample (preparation C) were obtained. Preparation A was ca. 85% pure according to SDS-gel electrophoresis, and contained 3.3 irons/protein molecule. The protein was concentrated in 50 mM Tris pH 7.5, 40 mM sodium chloride, 8% glycerol, and mM sodium dithionite to 1.8 mM protein. (Although this sample contains glycerol, we will refer to it as Av2/native.) Under these conditions, quantitation of the characteristic rhombic $g = 1.94$ signal yielded 0.61 spin/4Fe atoms. The sample lost 4% activity during the EXAFS experiment. The enzymatically oxidized sample showed no significant EPR signal after oxidation. The sample lost 20% activity during the oxidation process and another 25% during the EXAFS experiment. (The Fe protein is extremely oxygen sensitive, and so it is difficult to oxidize and perform experiments on oxidized samples without incurring a loss of activity.) The protein from preparation C contained 2.9 Fe atoms/protein molecule, and had a $g = 1.94$ signal, which integrated to 0.51 spin/4Fe atoms. The protein was in a buffer of 50 mM Tris pH 7.5 plus 2 mM dithionite. The $g = 1.94$ EPR signal from the sample containing 5 mM MgATP showed a rhombic-to-axial symmetry conversion. The spectrum of this sample had a prominent feature at $g = 4.8$, but there was little intensity at $g = 4.3$. We believe that a $g = 4.3$ feature is characteristic of Av2/ATP, so this sample may not have been fully converted.[2] The sample containing 0.6 M urea had EPR signals at 5.8 and 5.1, as expected. Quantitation of the $g = 1.94$ signal from this sample yielded 0.07 spin/4Fe atoms. The sample containing 15% HMPA had a g = 1.94 signal, which integrated to 1.1 spins/4Fe atoms.

Clostridium pasteurianum ferredoxin was purified[24] to an A_{400}/A_{280} absorbance ratio of 0.78 and was used as a protein standard known to

(23) Tanaka, M.; Haniu, M.; Yasunobu, K. T.; Mortenson, L. E. *J. Biol. Chem.* **1977**, *252*, 7093.

(24) Rabinowitz, J. *Methods Enzymol.* **1972**, *24*, 431.

contain $[4Fe\text{-}4S]^{2+}$ clusters in the oxidized state. The oxidized synthetic $[4Fe\text{-}4S]^{2+}$ cluster $(Et_4N)_2[Fe_4S_4(SPh)_4]$ and its reduced counterpart were prepared and analyzed as described.[25,26] EXAFS data for the model clusters were obtained from samples of concentrations similar to those of the protein samples (4 mM) and concentrations significantly higher than (30 mM) those of the protein samples.

The iron EXAFS data were collected on three separate occasions on the C1 line at the CHESS (Cornell High Energy Synchrotron Source) facility at Cornell University. The monochrometer was detuned by 30–50% for the rejection of harmonic reflections. The beam size was masked to 1–3 mm high and 18–25 mm wide. The intensity of the incident beam (I_0) was measured with an ion chamber. The frozen sample was located at an angle of 45° to the beam, in an helium atmosphere, in a cryostat[27] maintained at about 150 K throughout the data collection procedure. The fluorescent X-rays passed through a manganese filter located on the inside of the soller slit assembly of the fluorescent ion chamber detector.[27] The beam energy was varied from 7.09 to 7.9 KeV in 2–4-eV increments. Typical scan times were 9–13 min. The ratio of the fluorescent intensity to the incident intensity was plotted against beam energy.

Spectra were added to improve the signal to noise ratio. Twenty spectra of Av2/native (preparation A), seven spectra of Av2/native (preparation C), nine spectra of Av2/HMPA, 15 spectra of Av2/urea, 12 spectra of Av2/ATP, eight spectra of Av2/oxidized, two spectra of 30 mM oxidized synthetic cluster (run along with preparation A), six spectra of 4 mM oxidized cluster (run along with preparation C), and two reduced synthetic cluster spectra (run along with preparation B) were averaged separately for analysis.

The data were truncated at 7680 eV. The background was removed via a cubic spline function with three sections. The EXAFS data were weighted by k^3, and truncated at $k = 3$ and $k = 12$ Å^{-1}. A filter window between 0.9 and 3.8 (±0.1) Å isolated the significant Fourier transform (FT) peaks, which were then back-transformed into k-dependent data. The best-fit distances and coordination number values were determined by fitting the Fourier-filtered data to theoretical phase and amplitude functions of Teo and Lee.[28] Minor adjustments of the calculated best-fit values were made by using the fine adjustment based on models (FABM) method.[29] Characteristic values were determined from the oxidized synthetic cluster EXAFS and X-ray crystal structure results.[30] The average of the calculated Fe–S and Fe–Fe distances in the oxidized synthetic clusters were set equal to the crystallographically obtained values by adjusting the threshold energy parameter, ΔE_0, in the data analysis. This ΔE_0 was subsequently used in the analysis of the protein data.

Errors in the Fe–S and Fe–Fe distances and Debye–Waller (DW) factors were estimated by varying the appropriate term in the fit to the Fourier-filtered data. For the Fe–S terms, the range required to double the χ^2 of the fit was taken to be twice the uncertainty in the parameters. For the Fe–Fe terms, the range required to increase the χ^2 by 1.75 times was used. Errors in the coordination numbers were estimated as described in ref[31] and as follows. The appropriate term in the fit was systematically varied and the range required for the fit to extend beyond the noise in the background-removed data was visually estimated. A similar technique is used to estimate the number of possible Fe–O or Fe–N bonds that could be bound to the cluster iron. This method utilizes the noise in the actual data to gauge the uncertainty of the fit. Both methods gave similar results for the Fe–S coordination number errors, but those of the Fe–Fe term were significantly larger when estimated by the former method. This is due to the dissimilar DW factors in the protein and model cluster data.

Results and Discussion

The averaged X-ray absorption spectra of Av2/native, the corresponding background-removed EXAFS data, the FT of the background-removed data, and the fit to the data are shown in Figure 1 and illustrate the procedure used in analysis. Two major peaks are present in the FT. These were isolated, back-transformed into k-dependent data, and fit to Fe–S and Fe–Fe terms. The results from the other Fe protein states are generally the same. The background-removed data and the fits to the Fourier-filtered data from the other samples are shown in Figure 2. The average calculated Fe–S coordination numbers are 4.0 for the protein samples, at distances of 2.31 Å with DW factors of 0.06 Å. The average calculated Fe–Fe coordination numbers are 2.4 for the protein samples, at distances of 2.73 Å with DW factors of 0.10 Å. The parameters for each data set and associated uncertainties are listed in Table I.

The average Fe–S distance in the reduced Fe protein cluster (2.31 (1.5) Å) is somewhat longer than that in the oxidized

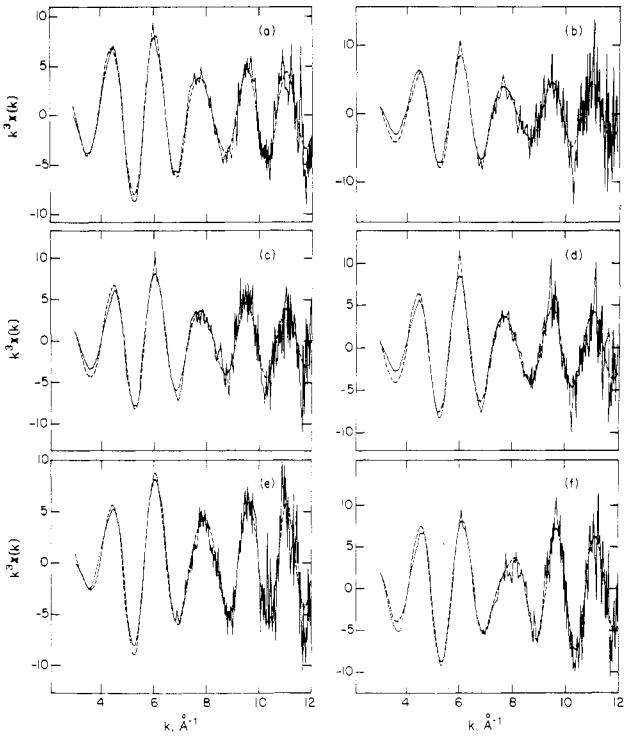

Figure 2. Background-removed EXAFS data (—) and fits (--): (a) Av2/native, preparation C; (b) Av2/HMPA; (c) Av2/ATP; (d) Av2/urea; (e) Av2/oxidized; (f) $(Et_4N)_2[Fe_4S_4(SPh)_4]$, 4 mM.

Table I. Fe Protein EXAFS Results[a]

	iron–sulfur term			iron–iron term		
compd	CN	R, Å	DW, Å	CN	R, Å	DW, Å
Reduced Clusters						
Av2/native (prep A)	4.2	2.32	0.061	2.6	2.75	0.09
Av2/native (prep C)	3.8	2.30	0.060	2.1	2.72	0.12
Av2/urea	3.9	2.31	0.055	2.2	2.72	0.12
Av2/HMPA	4.0	2.31	0.058	2.4	2.73	0.10
Av2/ATP	3.9	2.30	0.066	2.1	2.72	0.11
$(Et_4N)_3[Fe_4S_4(SPh)_4]$	4.1	2.30	0.065	2.9	2.75	0.08
Oxidized Clusters						
Av2/oxidized	3.8	2.30	0.066	2.4	2.74	0.09
$(Et_4N)_2[Fe_4S_4(SPh)_4]$ (30 mM)	3.5	2.29	0.052	2.6	2.75	0.09
$(Et_4N)_2[Fe_4S_4(SPh)_4]$ (4 mM)	4.4	2.27	0.057	3.4	2.73	0.08

[a] FABM-refined EXAFS structural parameters, coordination numbers (CN), distances (R), and Debye-Waller (DW) factors were obtained from two-term fits to the Fourier-filtered data using Fe–S and Fe–Fe theoretical functions. The characteristic values used in the FABM refinement of the best-fit values were the average of those obtained from the unrestricted fits of both $(Et_4N)_2[Fe_4S_4(SPh)_4]$ filtered data sets. The values used for σ^*, S^*, and $\Delta E_0{}^*$ were 0.055 Å, 0.498, and 6.94 eV for the Fe–S term, and 0.068 Å, 0.352, and 5.25 eV for the Fe–Fe term. The estimated errors of the CN, R, and DW factors are ±0.5, ±0.015 Å, and ±0.01 Å for Fe–S and ±0.8, ±0.03 Å, and ±0.02 Å for Fe–Fe.

(25) Averill, B. A.; Herskovitz, T.; Holm, R. H.; Ibers, J. A. *J. Am. Chem. Soc.* **1973**, *95*, 3523.
(26) Cambray, J.; Lane, R. W.; Wedd, A. G.; Johnson, R. W.; Holm, R. H. *Inorg. Chem.* **1977**, *16*, 2565.
(27) The EXAFS Co. Seattle, WA 98168.
(28) Teo, B. K.; Lee, P. S. *J. Am. Chem. Soc.* **1979**, *101*, 2815.
(29) Teo, B. K.; Antonio, M. R.; Averill, B. A. *J. Am. Chem. Soc.* **1983**, *105*, 3751.
(30) Que, L.; Bobrik, M. A.; Ibers, J. A.; Holm, R. H. *J. Am. Chem. Soc.* **1974**, *96*, 416.
(31) Antonio, M. R., Ph.D. Thesis, Michigan State University, East Lansing, MI, 1983.

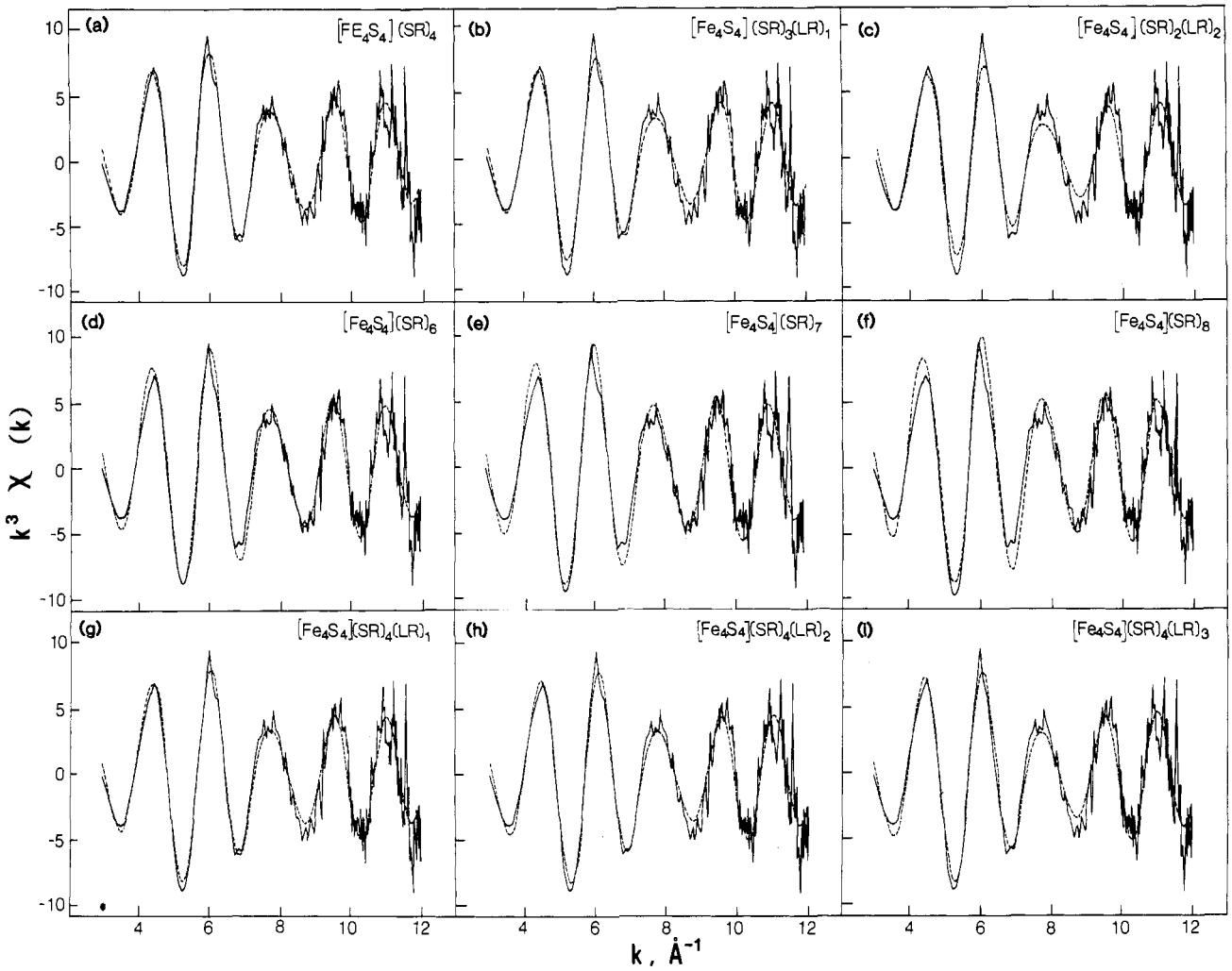

Figure 3. Examination of Fe–S coordination number uncertainties and the possibility of non-cysteinyl ligands. Theoretical fits of the type $[Fe_4S_4](SR)_x(LR)_{4-x}$, $[Fe_4S_4](SR)_{4+x}$, and $[Fe_4S_4](SR)_4(LR)_x$, where x = 0–4 and L = oxygen or nitrogen, were constructed to address the possibility of atypical coordination to the Fe protein cluster. These fits (– –) are shown along with the background-removed Av2/native, preparation C data (—): (a) $[Fe_4S_4](SR)_4$; (b) $[Fe_4S_4](SR)_3(LR)$; (c) $[Fe_4S_4](SR)_2(LR)_2$; (d) $[Fe_4S_4](SR)_6$; (e) $[Fe_4S_4](SR)_7$; (f) $[Fe_4S_4](SR)_8$; (g) $[Fe_4S_4](SR)_4(LR)$; (h) $[Fe_4S_4](SR)_4(LR)_2$; (i) $[Fe_4S_4](SR)_4(LR)_3$. The parameters used for the Fe–L term (1.90 Å, ΔE_0 = 11.4 eV, S^* = 0.31, σ^* = 0.0 Å), were obtained from an $[Fe_4S_4](OPh)_4$ model compound.[32] The fits originated from the best fit to the data. Fe–S coordination number changes utilized the scaling reduction factor in Table I.

synthetic cluster (2.28 (1.5) Å). Although these differences approach the limiting accuracy of the technique, they are outside of the calculated uncertainty and outside of the noise in the background-removed data. Moreover, the differences in the average Fe–S bond length of the oxidized and the reduced cluster data (0.02–0.03 Å) are close to that observed by X-ray crystallographic studies for the oxidized[30] and reduced[32] models (0.025 Å). Thus, this difference is probably due to different cluster oxidation states:[33] the 1+ core oxidation state for the reduced cluster and the 2+ state for the oxidized cluster. Smaller differences that are within the uncertainty of the data are found in the reduced synthetic cluster and the oxidized protein fits. The desired oxidation state for these samples may not have been completely attained. An alternative explanation is that the longer Fe–S bonds in the Fe protein result from the cluster being held between the two Fe protein subunits, and that the Fe–S bond lengthening due to oxidation state changes in [4Fe-4S] clusters correspond to smaller changes than we observed here.

It is possible that oxygen or nitrogen ligands (EXAFS from nitrogen and oxygen ligands are indistinguishable) bind to the cluster, either substituting for sulfurs according to the formula $[Fe_4S_4](SR)_x(LR)_{4-x}$ or binding in addition to sulfur, in the form $[Fe_4S_4](SR)_4(LR)_x$. We simulated spectra of these types and compared the simulations with the background-removed data in order to estimate the number of such ligands that could fit within the noise of the data. If we assume each iron in the cluster to be four coordinate, only a simulation with a single oxygen-for-sulfur substitution is within the noise of the data (see Figure 3a–c). However, if iron coordination numbers greater than four are considered, even an additional three sulfur (Figure 3d–f) or two oxygen (Figure 3g–i) ligands essentially fit within the noise. Thus, although the best fit is with four external sulfur ligands, we cannot exclude an oxygen-for-sulfur substitution or the binding of up to an additional three sulfur or two oxygen ligands.

Further restrictions can be made by use of the Fe protein Mössbauer data.[1] The four iron sites of $[4Fe\text{-}4S]^{2+}$ clusters with cysteine sulfur external ligands generally have isomer shifts of about 0.44 mm/s. The isomer shift essentially reflects the oxidation state of the cluster and, to a minor extent, the nature of the external ligands. The isomer shift increases by only about 0.04 mm/s when all four thiolate ligands are replaced by phenolates.[34] However, much more dramatic increases, on the order of 0.2–0.3 mm/s are observed when an iron of the cluster becomes five coordinate. An example of this is the activated [4Fe-4S]

(32) Laskowski, E. J.; Frankel, R. B.; Gillum, W. O.; Papaefthymiou, G. C.; Renaud, J.; Ibers, J. A.; Holm, R. H. *J. Am. Chem. Soc.* **1978**, *100*, 5322.

(33) Teo, B. K.; Shulman, R. G.; Brain, G. S.; Meixner, A. E.; *J. Am. Chem. Soc.* **1979**, *101*, 5624.

(34) Cleland, W. E.; Holtman, D. A.; Sabat, M.; Ibers, J. A.; DeFotis, G. C.; Averill, B. A. *J. Am. Chem. Soc.* **1983**, *105*, 6021.

cluster in the enzyme aconitase.[35] The isomer shift of the oxidized Fe protein cluster is 0.45 mm/s, typical of $[4Fe\text{-}4S]^{2+}$ clusters with a four-coordinate environment about each iron.

Thus, the Mössbauer results restrict the possible binding arrangements of the cluster to those with four-coordinate iron sites. The EXAFS results further restricts the possibilities to clusters with either four external sulfur ligands or to those with a single oxygen-for-sulfur substitution. Finally, since the Fe protein consists of two identical subunits and it appears that the cluster is bound between them, it is unlikely that unsymmetrical binding arrangements, such as those entailed by a single oxygen-for-sulfur substitution, would occur.

The ability to observe differences in Fe–S distances in these data indicate that similar or greater differences, arising from other phenomena, would be observed if they were actually present. It appears, however, that the $S = {}^1/_2$, $S = {}^3/_2$ spin-state conversion requires a change in the average Fe–S bond length of less than ~0.03 Å. The data of both $S = {}^1/_2$ (Av2/HMPA) and $S = {}^3/_2$ (Av2/urea) states fit to Fe–S and Fe–Fe terms with similar distances and coordination numbers. This indicates that the clusters in both spin states are of the same type, with no major cluster rearrangement and no major change in the average Fe–S bond length. In addition, there is no indication that additional ligands are coordinating to the cluster in order to effect spin-state conversion. Of course, small changes in the coordination environment, such as an oxygen ligand binding to one of the iron atoms of the cluster, may have gone undetected. However, invoking minor coordination changes in the cluster to effect these spin-state changes seem unnecessary at present, given the evidence (see Introduction) that similar changes in the magnetic properties of other $[4Fe\text{-}4S]^+$ clusters are caused by only minor changes in cluster structure. We suspect that minor changes in bond lengths, but possibly more substantial changes in bond angles, are required for the Fe protein spin-state changes. Similar conclusions can be drawn for the effect of the MgATP-induced conformational change on the cluster structure, since there were no noticeable changes in the EXAFS data of the MgATP-bound protein; the cluster structure probably changes very little.

The Fe–Fe terms of the Fe protein data have significantly larger DW factors (average of 0.10 Å) than the model compound terms (average of 0.08 Å). Such differences diminish the ability of the FABM method to accurately estimate coordination numbers.[29] In the present case, the average calculated Fe–Fe coordination number of 2.4 for the Fe protein data is somewhat lower than the expected number, 3.0. The differences in the DW factors appear to have *lowered* the calculated coordination number of the protein; those spectra with DW factors closer to those of the model compound data had coordination numbers closer to 3. We had thought that clusters in protein may have higher DW factors generally. However, we found that the DW factor in the ferredoxin from *C. pasteurianum*, which contains two [4Fe–4S] clusters, was quite close to the model compound value, and yielded an Fe–Fe coordination number of 3.3.[36] We cannot, at present, determine whether the large DW factors in the Fe protein data result from a distortion in the cluster or are artifacts, since this parameter is difficult to measure accurately.

Acknowledgment. We thank CHESS for providing beam time, Marc Anton Walters for providing and analyzing the synthetic clusters, and Mark Levy for providing the Clostridial ferredoxin. This work was supported by the NSF through Grant PCM 8205764.

Registry No. $(Et_4N)_2[Fe_4S_4(SPh)_4]$, 55663-41-7; $(Et_4N)_3[Fe_4S_4(SPh)_4]$, 63115-82-2; nitrogenase, 9013-04-1.

(35) Emptage, M. H.; Kent, T. A.; Kennedy, M. C.; Beinert, H.; Münck, E. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 4674.

(36) Lindahl, P. A., Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, MA, 1985.

Contribution from the Department of Pharmacology, University of Connecticut Health Center, Farmington, Connecticut 06032

Methanolysis of ((Tosylimino)iodo)benzene

Ronald E. White†

Received February 20, 1987

The hypervalent iodine compound ((tosylimino)iodo)benzene (TosN=IPh) was found to solvolyze in methanol to *p*-toluenesulfonamide and (dimethoxyiodo)benzene, the components of the solution being identified by IR, HPLC, UV, and NMR methods. The solvolysis reaction is a dynamic equilibrium, easily reversible by addition of either product. NMR observation of signals representing TosN=IPh and tosylamine, respectively, at several concentrations of TosN=IPh allowed an estimation of the solvolysis equilibrium constant, $K_s = [TosNH_2][PhI(OMe)_2]/[TosN{=}IPh] = 0.70 \pm 0.10$ M. These results explain the previous observations of several workers that oxidation of substrates with iminoiodinanes catalyzed by metalloenzymes yields an *oxygen* insertion product (alcohol or epoxide) rather than a *nitrogen* insertion product (*N*-alkyl sulfonamide).

Investigations of various redox metalloenzymes have frequently found the use of artificial oxidants to have certain advantages over the use of the natural oxidants for these enzymes. Several hypervalent iodine compounds, including iodosylarenes,[1] (diacetoxyiodo)arenes,[1b] and iminoiodinanes,[2] have been used as artificial oxidants for cytochrome P-450 enzymes. Iodosylbenzene[3] and ((tosylimino)iodo)benzene[4] have also been used in studies of iron bleomycin. Iodosylbenzene[5] and ((tosylimino)iodo)benzene[6] have been favored as oxidants for iron porphyrin based hemeprotein model systems by many workers. Several studies have reported lability of the atom (either oxygen or nitrogen) nominally to be transferred to substrate via the metalloenzyme. For instance, complete incorporation of solvent water into the alcohol product was reported for the iodosylbenzene/P-450 oxidations.[1c,d,2a]

$$PhI{=}O + RH \xrightarrow[H_2{}^*O]{\text{P-450}} PhI + R^*OH \qquad (1)$$

† Present address: The Squibb Institute for Medical Research, Princeton, NJ 08543-4000.

(1) (a) Lichtenberger, F.; Nastainczyk, W.; Ullrich, V. *Biochem. Biophys. Res. Commun.* **1976**, *70*, 939–946. (b) Gustafsson, J.-A.; Rondahl, L.; Bergman, J. *Biochemistry* **1979**, *18*, 865–870. (c) Heimbrook, D. C.; Sligar, S. G. *Biochem. Biophys. Res. Commun.* **1981**, *99*, 530–535. (d) Macdonald, T. L.; Burka, L. T.; Wright, S. T.; Guengerich, F. P. *Biochem. Biophys. Res. Commun.* **1982**, *104*, 620–625.

(2) (a) White, R. E.; McCarthy, M. B. *J. Am. Chem. Soc.* **1984**, *106*, 4922–2926. (b) Svastits, E. W.; Dawson, J. H.; Breslow, R.; Gellman, S. H. *J. Am. Chem. Soc.* **1985**, *107*, 6427–6428.

(3) (a) Murugesan, N.; Ehrenfeld, G. M.; Hecht, S. M. *J. Biol. Chem.* **1982**, *257*, 8600–8603. (b) Padbury, G.; Sligar, S. G. *J. Biol. Chem.* **1985**, *260*, 7820–7823.

0020-1669/87/1326-3916$01.50/0