Direct observation of bis-sulfur ligation to the heme of Bacterioferritin

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Abstract: X-ray absorption spectroscopy was used to examine the ligation of the heme of *Azotobacter vinelandii* bacterioferritin scrupulously cleaned of non-heme iron. We find that the iron of the protoporphyrin IX of the protein has two axial sulfur ligands at 2.35Å, with four nitrogen ligands at 1.97Å. This result confirms the previous suggestion of Cheesman *et al.* (*Nature* **1990**, *346*, 771-773, *Biochem. J.* **1992**, *286*, 361-367), on the basis of less direct spectroscopic techniques, that the heme of bacterioferritin is ligated by a pair of methionine ligands. To date, this mode of coordination is unknown in any other heme protein.

Introduction

The ferritins are a class of iron-storage proteins, found in both eukaryotic and pro-karyotic organisms, that safely store iron until it is needed for cellular metabolism (1). They are large proteins, with 24 subunits each of Mr approximately 18,000 defining a rhombic dodecahe-dral shell that can enclose up to 4000 iron atoms in an oxide/hydroxide/phosphate core. The bacterioferritins are distinguished by possessing a protoporphyrin IX heme of remarkably low oxidation-reduction midpoint potential, the value of which depends upon the presence and size of the iron core (2-4). Cheesman *et al.* have compared bacterioferritin near infra red magnetic circular dichroism (MCD) and electron paramagnetic resonance (EPR) spectra with model com-pounds, (5,6) and have suggested that the bacterioferritin heme has bis-methionine ligation. As such ligation is unprecedented, and as EPR *g*-values of ferric hemes are sensitive to many fac-tors, including, for example, ring orientation of any histidine ligands, we have investigated the ligands of the heme iron of bacterioferritin more directly. Using Extended X-ray Absorption Fine Structure (EXAFS), we find that the heme does indeed possess two sulfur ligands, at a dis-tance of 2.35Å from the iron. Taken together, the EXAFS, EPR and MCD results provide com-pelling evidence for a bis-methionine coordinated heme.

Experimental Section

Bacterioferritin was prepared from *Azotobacter vinelandii* as previously described (3,7). The iron core was removed by thioglycollic acid dialysis followed by anaerobic treatment with 1mM bipyridyl and reduced methyl viologen for 30 min., and gel filtration (4,8). Proton induced X-ray emission, inductively coupled plasma emission spectrometry and optical spec-troscopy indicated 12.7 iron atoms, and 12.3 hemes, respectively, per 24 subunits. Microcou-lometry showed that 13.1 electrons were required to fully reduce the hemes. These results are

consistent with the presence of iron only in the hemes. Samples were prepared in 25mM N-tris-[Hydroxymethyl]methyl-2-aminoethanesulfonate, pH 7.5, with 20% glycerol, and frozen in lucite cuvettes with thin mylar windows. X-ray absorption spectroscopy was done at the Stanford Synchrotron Radiation Laboratory on beam-line SB07-3 using a Si(220) double crystal monochromator, with an upstream vertical aperture of 1mm. Six thirty minute scans were accumulated while the samples were maintained at a temperature close to 5K in an Oxford Instruments CF1204 flowing liquid helium cryostat. X-ray absorption spectra were measured as fluorescence excitation spectra using a Canberra 13-element detector (9), and the absorption of an iron foil standard was measured simultaneously by transmittance. The EXAFS oscillations were quantitatively analyzed as previously described (10), using theoretical curve-wave phase and amplitude functions calculated from the program *feff* of Rehr and colleagues (11). The X-ray energy was calibrated with respect to the first inflection of the iron metal foil, which was assumed to be 7111.3 eV.

Results and Discussion

Figure 1 shows the iron K-edge absorption spectrum of *A. vinelandii* bacterioferritin. The spectra of horse-heart myoglobin azide, and horse heart cytochrome c are also shown for comparison. As discussed above, it has been suggested that bacterioferritin contains iron coordinated by four pyrole nitrogens and two methionine sulfurs. Cytochrome c is known to contain iron coordinated to five nitrogens and a single methionine sulfur, while myoglobin azide contains iron coordinated by six nitrogens. The small feature at about 7112eV is the formally dipole forbidden *1s→3d* transition, which gains small intensity from being quadrupole allowed, or from gaining dipole allowed character by mixing with *p*-orbitals in non centrosymmetric environments. The intensity of the *1s→3d* peak can be used as an indicator of the coordination state (12); being most intense for five coordinate hemes, and least intense for

six (an increase in intensity corresponding to a decrease in molecular symmetry and increased *p*-orbital mixing). The weak presence of the *1s→3d* feature in Figure 1 confirms that all three proteins contain six coordinate low-spin ferric iron. There are, however, clear differences between the different absorption edges. In particular, there is a subtle shift of the edge to lower energy with increasing sulfur coordination, which is to be expected from the greater covalency of the Fe-S bond, compared to the Fe-N bond.

Figure 2 shows the Fourier transform of the EXAFS oscillations of the heme of bacterioferritin collected to k=14.2Å^{-1}. There are two pronounced features in the first shell, due to Fe-N (or O) and Fe-S (or Cl) . The former, at a distance of 1.97Å, is undoubtedly dominated by contributions from the pyrole nitrogens, while the latter, at 2.35Å, probably originates from axial ligands to the iron. The pronounced features at about 3 and 4Å originate from the outer shell carbons of the pyrole rings (13).

Figures 1 and 2 are certainly consistent with the bis-methionine ligation proposed by Cheesman *et al.* (5,6), but in order to more accurately address the number of sulfur ligands to the iron, we have Fourier filtered the EXAFS oscillations with a 0.3Å width half-Gaussian window function from R=1.3-2.8Å to exclude all but the first shell interactions. We then performed best integer fits (see Table 1) on the filtered data, with the results illustrated in Figure 3, and tabulated in Table 1. The best fit to the data is 4 Fe-N interactions (from the tetrapyrole) and 2 Fe-S interactions from the axial ligands. The Fe-N distance is consistent with low-spin Fe^{3+}, with the iron atom being close to the plane of the heme. The Fe-S distance is appropriate for bis-methionine ligation. Although there are no other reports of biological systems with bis-methionine heme coordination, there are well characterized low molecular weight model compounds. Mashico *et al.* (14) have synthesized bis(tetrahydrothiophene)(*meso*-tetraphenylporphinato)iron(II) and bis(pentamethylene sulfide)(*meso*-tetraphenylporphinato) iron(III). Both possess iron with thioether coordination similar to that proposed for the heme of

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bacterioferritin. The similarity of the model compound Fe-S and Fe-N bond-lengths to those of *A. vinelandii* bacterioferritin is quite striking; for the iron(III) compound the average model Fe-S distance is 2.34Å compared to 2.35Å for the protein, and the model Fe-N distance is 1.98Å, compared to 1.97Å in the protein. In contrast to bis-thioether hemes, there are few examples of low-spin heme model compounds possessing bis-thiolate coordination. A bis-benzenethiolato low-spin spin ferric heme has been reported (15), possessing Fe-S bond-lengths of 2.27Å and 2.43Å. Our EXAFS results indicate that this type of ligation is not present in *A. vinelandii* bacterioferritin, as Fe-S bonds lengths differing by more than 0.12Å, should be resolved in the EXAFS analysis. In agreement with this, attempts to fit the data starting with two different Fe-S bond lengths converged to a fit with a single value of 2.35Å. Taken together, the EXAFS presented here, and the EPR and MCD results (5,6) provide very strong evidence that the heme iron in *A. vinelandii* bacterioferritin possesses bis-methionine ligation.

Cheesman *et al.* (5) have discussed how only four amino acids have side chains with sufficient ligand field strengths to maintain hemes in the low-spin state; histidine, lysine, methionine and cysteine. To date, only a few of the potential combinations have been found. Bis-histidine [*e.g.* mammalian cytochrome b_5 (16)], histidine-methionine [*e.g.* cytochrome *c* (17)] and histidine-lysine [*e.g.* cytochrome *f* (18)] have all been described. Bacterioferritin is so far the only known case of bis-methionine ligation.

Whether all bacterioferritins possess hemes with bis-methionine coordination remains to be determined. Cheesman *et al.* (5,6) have shown that the bacterioferritins from *A. vinelandii, Escherichia coli* and *Pseudomonas aeruginosa* display quite similar EPR and MCD spectra, suggesting that at least these share such coordination. While there have been speculations, based on sequence information, upon the identity of putatively coordinating methionines (5,6,19), it is not yet clear whether this should focus on potential ligands within or between

subunits. Bacterioferritin contains 24 identical subunits. If a single amino acid ligand is supplied by each subunit then this allows a maximum of 12 hemes, in apparent agreement with previous (3-6) and present analytical data. In contrast, Kadir and Moore (20) suggest a maximum of 24 hemes for *Ps. aeruginosa* bacterioferritin, but it is not yet clear whether all share bis-methionine coordination. Mammalian ferritin does not contain heme as typically prepared, although it can be made to bind up to approximately 16 hemes per 24 subunits (21). Moore *et al.* (22) have recently suggested that these hemes are bound with *bis*-histidine axial ligation between subunits, and it is conceivable that such ligation might account for the extra binding sites in *Ps. aeruginosa* bacterioferritin.

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TABLE 1

Results of best-integer fits to the EXAFS oscillations from the heme iron of bacterioferritin.

Scatterer	Number	Distance (Å)	σ^2 (Å^2)	ΔE_0 (eV)
Fe-N	4	1.971 (0.012)	.00594 (.00077)	-12.65 (2.29)
Fe-S	2	2.350 (0.010)	.00575 (.00071)	-2.51 (1.57)

The best integer fit was found by constraining the total coordination number to be six, which is expected for a low-spin ligand field, and fitting all possible combinations of Fe-N and Fe-S coordination numbers. The interatomic distances, the Debye-Waller factors (σ^2, the mean square deviation in interatomic distance), and ΔE_0 values (the shift in threshold energy) were refined during the fits. The values in parentheses are the 95% confidence limits determined by the curve-fitting algorithm, although the systematic error (statistical bias) arising from inaccuracy of the EXAFS phase and amplitude functions will add to this to yield an accuracy of about 0.02Å. The error function, F, which was minimized in the curve-fitting is defined as,

$$F = \frac{\sum \left(\chi_{obsd} - \chi_{calc} \right) k^6}{\sum \chi_{obsd} k^6}$$

, where the summations are over all data points. During the best-intger fits F varied between 0.342 for an Fe-S coordination number of one, to 0.211 for an Fe-S coordination number of two, to 0.341 for an Fe-S coordination number of three. The possibility of a five coordinate heme was also tested, and rejected because of a poor fit ($F = 0.33$) with a chemically unreasonable (negative) value for σ^2 for the Fe-S interaction. The differing values obtained for ΔE_0 should not cause errors of more than 0.02Å in the fits.

Figure Legends

Figure 1. Iron K-edge X-ray absorption spectra of *A. vinelandii* apobacterioferritin (solid line) compared with horse ferri-cytochrome c (dotted line) and horse ferric myoglobin azide (dashed line).

Figure 2. Iron K-edge EXAFS Fourier transform of *A. vinelandii* apobacterioferritin. The transform was calculated over the k-range $1\text{-}14.2\text{Å}^{-1}$ and has been phase corrected for Fe-N back-scatterers.

Figure 3. Iron K-edge EXAFS spectrum (solid line) and best fit (broken line) of filtered data (filtered with a 0.3Å width Gaussian window over 1.3-2.8Å), using the parameters in Table 1.

Figure 1

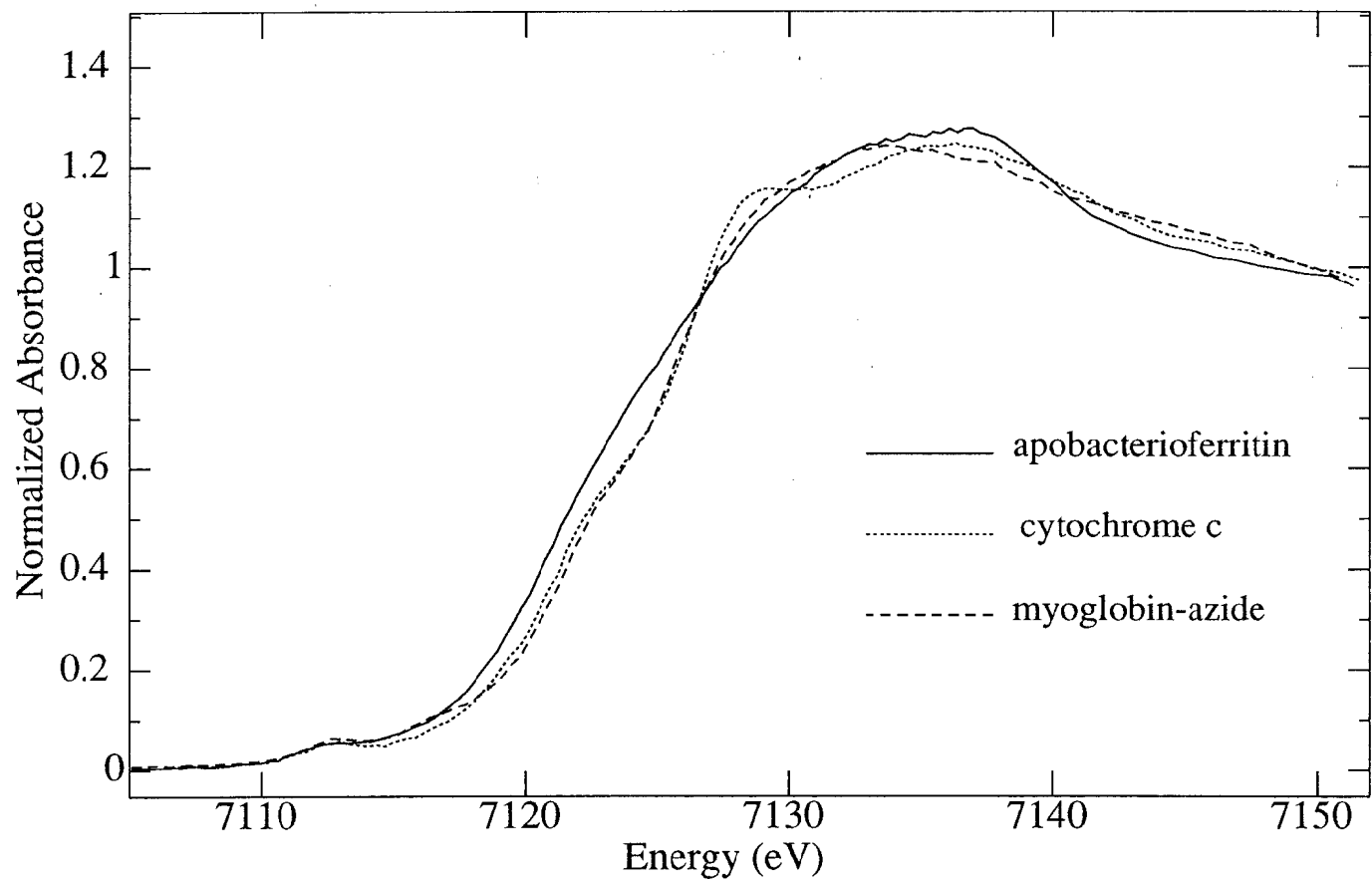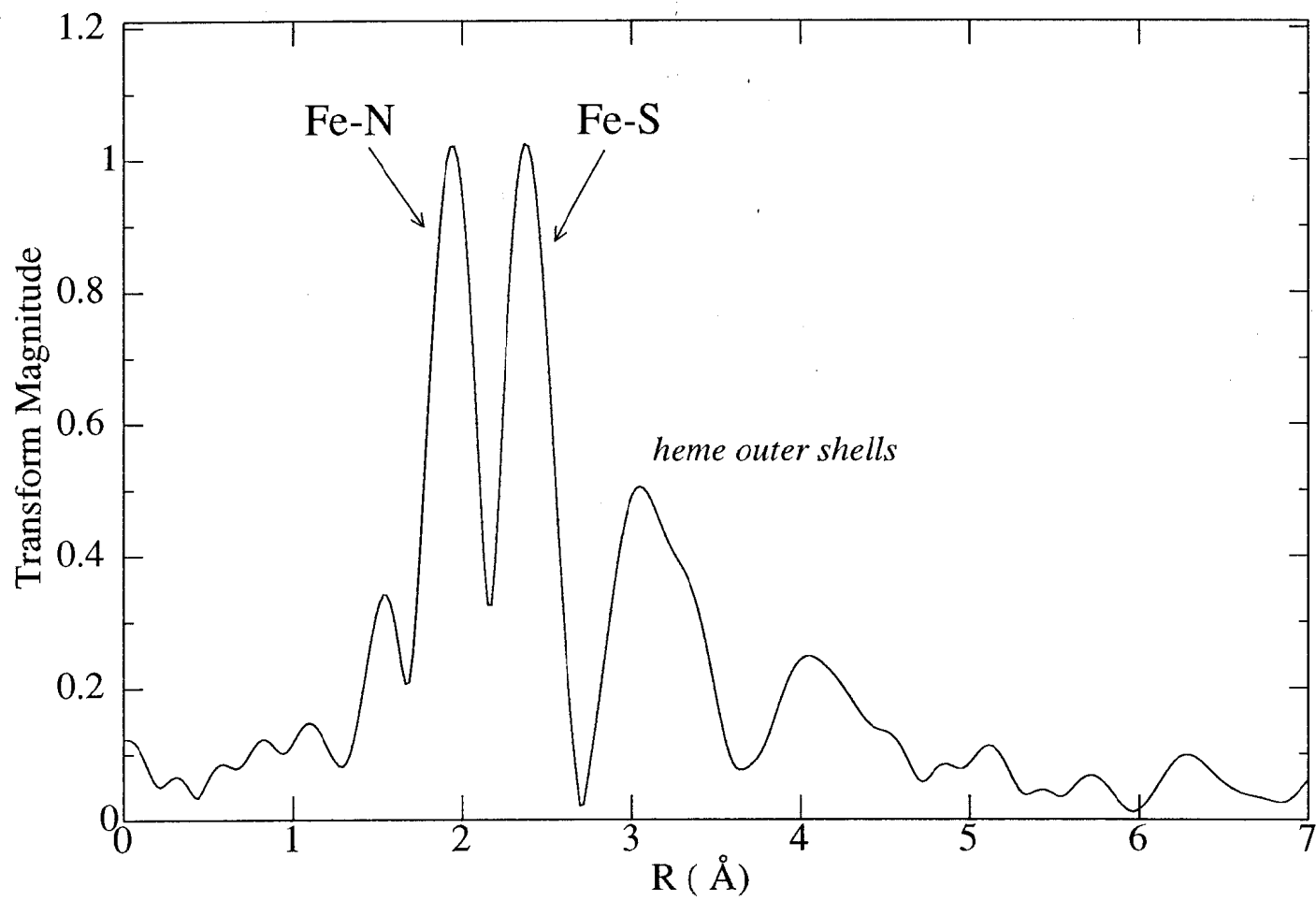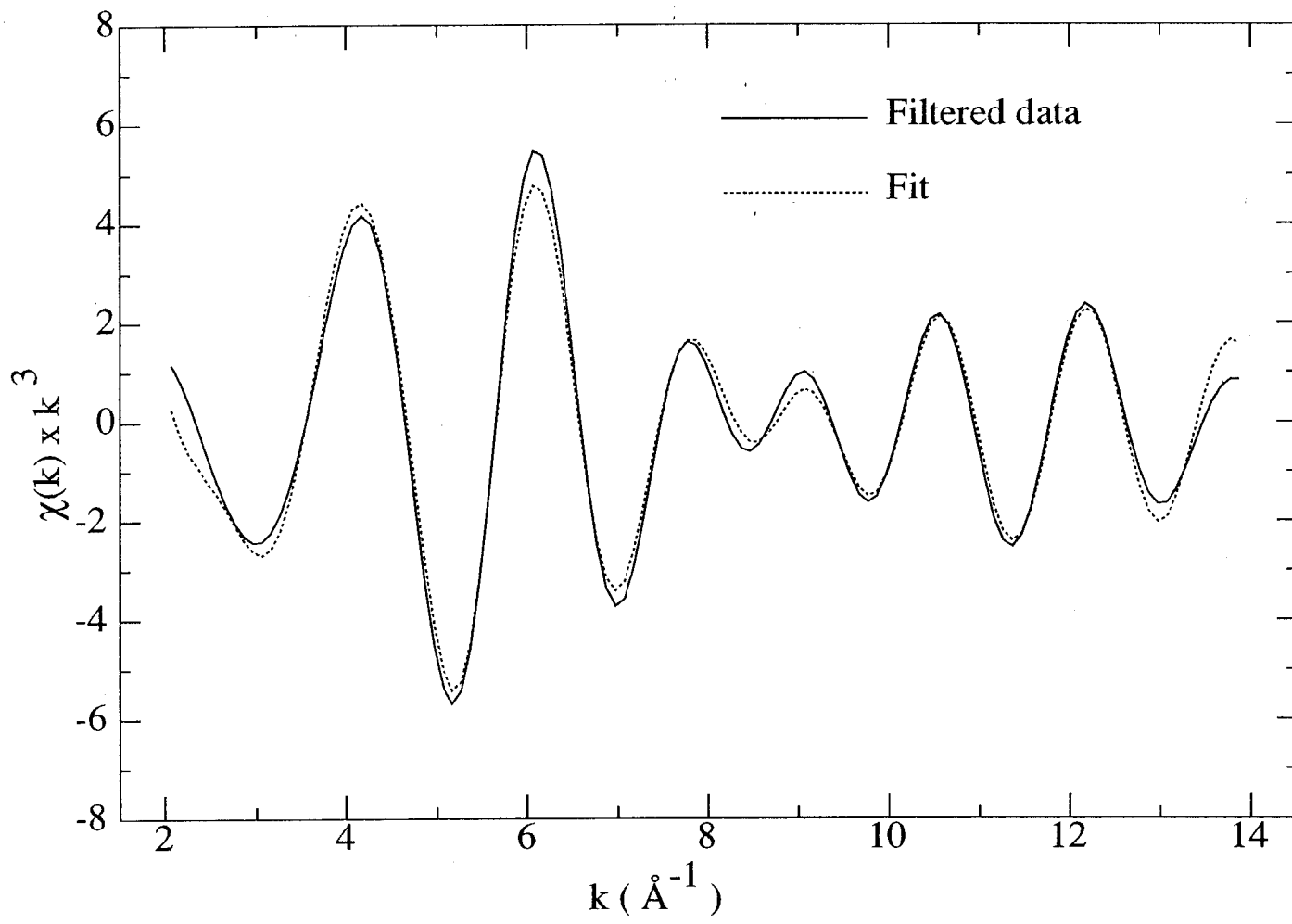Figure 2

Figure 3