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Refinement of a Model for the Nitrogenase Mo–Fe Cluster Using Single-Crystal Mo and Fe EXAFS**

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The enzyme nitrogenase catalyzes the reduction of dinitrogen to ammonia.[1] Substrate conversion is thought to occur at the M clusters, which are two $MoFe_7S_9$ clusters embedded in the α subunits of the $\alpha_2\beta_2$ MoFe protein (M_r = 220000).[2] Each M cluster presumably receives electrons from a companion P cluster, an Fe_8S_8 structure located at the interface between the α and β subunit. Accurate dimensions for these clusters are important for synthetic modeling, for theoretical studies, and for the observation of possible structural changes during the catalytic cycle. The structures for MoFe proteins Cp1 and Av1 from *C. pasteurianum*[3] and *A. vinelandii*[4] respectively, are being determined by X-ray diffraction with increasing accuracy, and models for the structures of the M cluster and P cluster have been presented. However, given the limited resolution of the diffraction data (2.2 Å), it is likely that the root mean square error in the atomic positions of these models is on the order of 0.2–0.3 Å, and errors in the interatomic distances are somewhat larger. Since changes of metal–metal distances of even 0.1 Å are chemically significant, more accurate cluster dimensions are important. Now that the construction of the cluster frameworks has been established by the X-ray diffraction experiments, greatly improved metric information can be obtained from solution and especially single-crystal EXAFS spectra.

We report here the first single-crystal Fe and Mo EXAFS spectra of nitrogenase at low temperature. A new Mo–Fe component at approximately 5 Å was observed along with seven other distances between metal atoms and neighboring atoms. These data were combined with that from previous solution Fe EXAFS[5] and a new solution Mo EXAFS to refine the current model for the M cluster. The model is compared with a current electron density map for the protein from *C. pasteurianum* having a 2.3 Å resolution.[3] Although the spectroscopic results are qualitatively in complete agreement with the Kim and Rees model for the M cluster, some average distances differ by roughly 0.2 Å. The EXAFS results also suggest less variation in individual Fe–Fe and Mo–Fe distances than seen in the Kim and Rees model; in other words, the Mo–Fe cluster is more symmetrical.

Fourier transforms of the Mo and Fe K edge EXAFS for a solution of Av1, a single crystal of Cp1, and the capped-prismane model complex $(Et_4N)_3[Fe_6S_6Cl_6\{Mo(CO)_3\}_2]$[6] are shown in Figure 1. The transform of the model complex shows the expected first-shell Mo–C, Mo–S, and Mo–Fe distances (2.0, 2.6, and 2.9 Å, respectively) as well as a long-range Mo–Fe interaction at 4.28 Å. Buried under the shorter Mo–Fe component is a multiple-scattering contribution from a Mo-C-O interaction. Fitting the EXAFS yields distances within 0.05 Å of the values obtained by X-ray crystallography[6] (Table 1).

The Fourier transform of the Mo EXAFS spectrum of Av1 in solution exhibits two main peaks, which fit (Fig. 1) as Mo–S and Mo–Fe interactions at 2.37 and 2.70 Å, along with an unresolved Mo–O,N component at 2.20 Å (Table 1). The Mo–S and Mo–Fe distances, like those from previous EXAFS studies,[7–9] are clearly shorter than the corresponding distances in both the capped-prismane model and the respective average distances of 2.46 and 2.92 Å in the Kim and Rees model of the M cluster. As shown in Figure 1, the Mo EXAFS spectrum generated from the distances obtained by X-ray diffraction has different frequencies and beats (because of different distances) and damps out more rapidly (because of the greater spread in the distances between Mo and neighboring atoms). In the 3–5 Å region of the Fourier transform, a number of smaller peaks are observed, which may arise from interactions of C atoms of homocitrate and histidine ligand with Mo, as well as Fourier transform truncation ripple. We do not interpret these features at this time. There is also a modest peak at approximately 5 Å, where a second Mo–Fe interaction is expected. However, the feature is quite weak, and numerous other interactions might occur at such a distance.

We have used single-crystal EXAFS to enhance this signal at 5 Å and confirm the Mo–Fe assignment. The strength of specific metal–neighbor EXAFS components varies by $\cos^2\theta$, where θ is the angle between the photon polarization vector **E** and the metal–metal vector.[9] Although there are four M centers in the unit cell, their long axes tend to lie near the *bc** plane, as shown in Figure 1. Furthermore, the longer Mo–Fe vectors form an angle of only about 15° ($\cos^2 15° = 0.93$) with the approximately threefold symmetry axes.[3] Thus, by orienting a crystal with the **E** vector parallel to the

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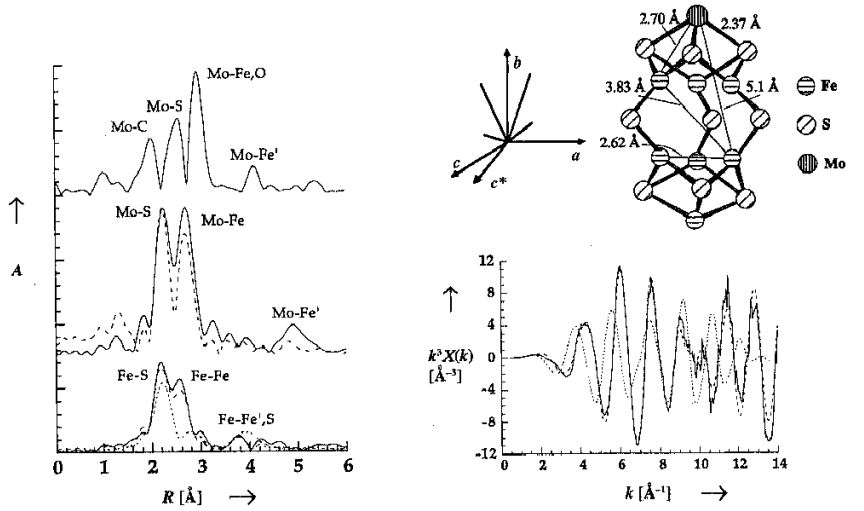

Fig. 1. Left: phase-corrected Fourier transforms (top) of the k^3 Mo EXAFS spectrum for $(Et_4N)_3[Fe_6S_6Cl_6\{Mo(CO)_3\}_2]$; Fourier transforms of the Mo (middle) and Fe EXAFS spectra (bottom) for the MoFe proteins Av1 (solution, – – –) and Cp1 (crystal, ——). Right: top: approximate orientation of the threefold symmetry axes in the M clusters of the unit cell of the MoFe protein and an assignment of EXAFS distances in the M cluster; bottom: experimental (——) and fitted (– – –) EXAFS spectra of the Mo K edge for the MoFe protein in solution. The Mo EXAFS spectrum (right bottom: dotted line) and Fourier transform of the Fe EXAFS spectrum (left bottom: dotted line) generated from crystallographic models having 2.2 Å resolution are also shown for comparison. A = amplitude.

b axis in the bc^* plane, the amplitude of the interaction at approximately 5 Å should be enhanced. In fact, a nearly twofold increase is observed (Table 1 and Fig. 1). Fourier-filtering and fitting this peak yields a good fit for several Mo–Fe interactions at 5.1 Å, close to the value of 5.2 Å obtained by X-ray crystallography. The Mo–Fe interaction at 2.7 Å also gets stronger in this orientation, while the intensity of the Mo–S signal is isotropic, consistent with three Mo–S bonds at nearly right angles to each other in each M center.

Solution EXAFS spectra of the Fe K edge reveal Fe–Fe components at 2.63 and 3.75 Å. These values change little with orientation in the single-crystal spectra, and similar Fe–Fe distances were found for the isolated FeMo cofactor.[10] The crystallographic model can be divided into two groups of Fe–Fe distances averaging 2.87 and 3.73 Å (averaging over both P and M clusters). However, the 2.87 Å group has a root mean square deviation (σ) of 0.22 Å, which does not include thermal disorder. Using the average crystallographic coordination number, the EXAFS simulation finds $\sigma = 0.09$ Å, including thermal disorder. As shown in Figure 1, larger disorder in the group with the short Fe–Fe distances would result in drastically damped Fe–Fe EXAFS. We conclude that most of the short Fe–Fe distances lie within 0.1 Å of the average distance of 2.62 Å. This is significantly shorter than the range 2.69–2.85 Å seen in Fe–S clusters with four coordinate Fe.[6, 11]

In the model obtained from X-ray diffraction, the long Fe–Fe distances average to 3.73 Å with a σ of 0.44 Å. Most of the diagonal interactions among the Fe atoms of the M cluster lie near 3.85 Å (see Fig. 1), and this is no doubt the group that the EXAFS fits find with an average distance of 3.72 Å. Although there are numerous other long Fe–Fe vectors in the range 3.0–4.4 Å, the EXAFS analysis cannot deal with a large number of minor components.

Altogether, the single-crystal Mo and Fe EXAFS studies provide eight predictions for metal–neighbor distances. Combining all the values from EXAFS and using the geometry of the M cluster proposed by Kim and Rees allows a refined model to be constructed, as shown in Figure 1. We have compared our refined model with an unbiased electron density map of Cp1 (resolution 2.3 Å) from an ongoing X-ray diffraction study.[3] The comparison was done by refining our model as a rigid body against the Cp1 diffraction data using the program TNT.[12] This process gives a very good fit to the diffraction data, as shown by Figure 2, and electron density values at the atomic positions. The mean interpolated electron density values (on an arbitrary scale) in the electron density map of Cp1 for the seven Fe and nine S atoms in the refined EXAFS model are 1886 (σ = 182) and 969 (141). For comparison, the values for the current crystallographic model for Cp1 which has been refined to $R = 0.17$ against data with 2.3 Å resolution, are 1903 (191) and 928

Table 1. Comparison of interatomic distances found by X-ray diffraction and EXAFS for nitrogenase MoFe protein and the model complex $(Et_4N)_3[Fe_6S_6Cl_6\{Mo(CO)_3\}_2]$.

Absorbing–backscattering pair	MoFe protein Av1 (solution) EXAFS [a]			Cp1 (crystal) EXAFS [b]		Diff. Av1 [c]		$(Et_4N)_3[Fe_6S_6Cl_6\{Mo(CO)_3\}_2]$ EXAFS [a]			Diff. [d]
	N	R [Å]	$\sigma_{tot.}$ [e]	N'	R [Å]	R [Å]	$\sigma_{stat.}$ [f]	N	R [Å]	$\sigma_{tot.}$ [e]	R [Å]
Mo-O,N	3.0	2.21	0.075	3.9	2.20	2.24	0.012	3.0	1.97	0.048	2.020
Mo-S	3.0	2.35	0.043	3.0	2.39	2.46	0.041	3.0	2.60	0.044	2.582
Mo-Fe	3.0	2.69	0.048	3.9	2.70	2.91	0.066	3.0	2.95	0.070	2.930
Mo-Fe'	3.0	5.09	0.066	5.8	5.10	5.22	0.095	3.0	4.28	0.073	4.241
Fe-S	2.9	2.29	0.071	2.8	2.27	2.34	0.070				
Fe-Fe	3.2	2.62	0.087	3.4	2.61	2.87	0.22				
Fe-Mo	0.2	2.74	0.035	0.4	2.71	2.91	0.066				
Fe-Fe'	1.3	3.75	0.074	1.0	3.68	3.73	0.44				

[a] The appropriate coordination number N was derived from the Kim and Rees model, averaging over both P and M clusters. [b] The relative amplitude N' was obtained by fixing $\sigma_{tot.}$ at the value derived from the solution fit and optimizing N'. This is not a coordination number, since the appropriate polarization effects have not been taken into account. [c] Crystallographic value ref. [4]. [d] Crystallographic value ref. [6]. [e] Total standard deviation $\sigma_{tot.} = \sigma_{stat.} + \sigma_{thermal}$. [f] Statistical standard deviation.

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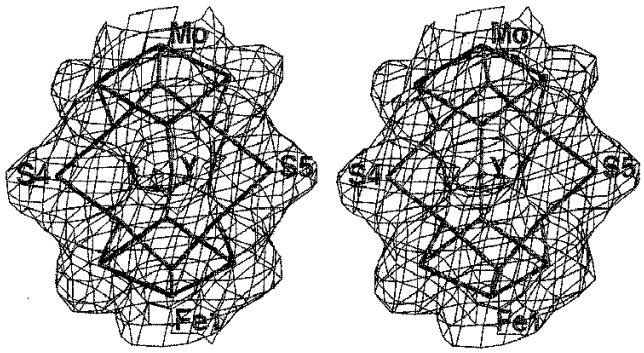

Fig. 2. Comparison of the refined EXAFS model for the M cluster in MoFe protein (red) and electron density map (blue). This stereoplot is oriented so that the Fe-Y-Fe bridge is towards the viewer. The electron density map is from a map with 2.3 Å resolution calculated with experimentally determined phases (model unbiased). The contour level of the map is set at the root mean square value of the electron density in the asymmetric unit.

(174). Clearly, the positions of atoms in our model are in excellent agreement with the electron density map and in fact fit the map as well as those in the crystallographic model.

In conclusion, the structure of the intact MoFe protein has been studied by single-crystal Mo and Fe EXAFS spectroscopy. We observed orientation dependence not only for the "well-known" Mo–Fe interaction at 2.7 Å but also for a new Mo–Fe component at 5.1 Å. Fe–Fe components at 2.6 and 3.7 Å are also stronger in the single-crystal data. Combining the Mo and Fe EXAFS results with crystallographic information allows us to propose a more symmetrical structure for the M cluster with unprecedented precision.

Experimental Procedure

Nitrogenase MoFe protein was purified from *A. vinelandii* at Louisiana State University by methods previously described [13]. Specific activities were 1800 nmol C_2H_2 reduced $min^{-1}mg^{-1}$ protein. MoFe protein crystals were prepared and mounted at Purdue University [3]. The crystals have the space group $P2_1$ with unit cell parameters $a = 69.9$, $b = 151.2$, $c = 121.8$ Å, and $\beta = 110.3°$. The experiments were performed on beamlines 7-3 and 10-2 at SSRL, X10C and X19A at NSLS. The X-ray absorption spectra were measured at 4 K in the fluorescence excitation mode using a 13-element Ge solid-state array detector [14]. The EXAFS fits were calculated using FEFF5 curved-wave multiple-scattering (MS) analysis software [15]. The geometry information was taken from the crystallographic results [4, 6]. In the capped-prismane model compound, multiple scattering is significant for the feature at approximately 3 Å because of the focusing effect arising from three almost linear Mo-C-O (ca. 177°) interactions. However, FEFF5 calculations found that multiple scattering is not significant for the Fe EXAFS in the nitrogenase cluster.

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De Novo Design of a Novel Heterodinuclear Three-Helix Bundle Metalloprotein**

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Proteins, the epitome of molecular form and function, remain the most challenging targets for molecular design. Despite the substantial advances in the design of "small-molecule" models of enzyme active sites,[1] little progress has been made toward the construction of "large-molecule" systems that can mimic both the structural and functional properties of natural proteins.[2] Here we report the design, synthesis, and characterization of a novel three-helix bundle Ru^{II} metalloprotein which is capable of functioning as an efficient type-II copper binding protein.

The foremost problem in the de novo design of functional proteins is the availability of structurally well-defined scaffolds onto which the desired functionalities of the active site may be elaborated. Recently, we have developed a series of metal ion assisted, self-organizing processes for the design and convergent synthesis of topologically predetermined metallopeptides and artificial metalloproteins.[3] One such design, a three-helix bundle structure,[3a] was the starting point in the present study for rational modification into a heterodinuclear $Ru^{II}Cu^{II}$ metalloprotein. Our strategy is illustrated in Figure 1. As in our previous studies, the key design feature in directing the self-assembly process is the incorporation of a bipyridyl (bpy) moiety at the N-terminus of the polypeptide subunit. Metal ion complexation to the bipyridyl moieties is thought to initially sequester three peptide chains to form the putative intermediate shown. The higher coil density and the dramatically increased effective molarity of hydrophobic moieties in the intermediate species strongly bias the system toward formation of a parallel three-helix bundle ensemble. Moreover, it was hypothesized that by placing a second ligand in an appropriate position within each polypeptide sequence, a new composite metal binding site may be formed.

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