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

By Jie Chen, Jason Christiansen, Nino Campobasso, Jeffrey T. Bolin, Roland C. Tittsworth, Brian J. Hales, John J. Rehr, and Stephen P. Cramer*

The enzyme nitrogenase catalyzes the reduction of dinitrogen to ammonia.[1] Substrate conversion is thought to occur at the M clusters, which are two MoFe₂S₈ clusters embedded in the α subunits of the α₂β₂ MoFe protein (M, = 220,000).[2] Each M cluster presumably receives electrons from a companion P cluster, an Fe₃S₄ 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 Cpl and Avi 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 fewer than 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, greater 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.[6] 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 Avl, a single crystal of Cpl, and the capped-prismane model complex (Li(NH₄)₂)[Fe₂S₈Cl₂(Mo(CO)₅)₂][7] 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–O–O interaction. Fitting the EXAFS yields distances within 0.05 Å of the values obtained by X-ray crystallography[8] (Table 1).

The Fourier transform of the Mo EXAFS spectrum of Avl 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,[7,8] 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 ripples. 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 cosθ[9], where θ is the angle between the photon polarization vector E and the metal–metal vector.[10] Although there are four Mo atoms in the unit cell, their long axes tend to lie near the b* plane, as shown in Figure 1. Furthermore, the longer Mo–Fe vectors form an angle of only about 15° (cos 15° = 0.93) with the approximately threefold symmetry axes.[11] Thus, by orienting a crystal with the E vector parallel to the

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In the model obtained from X-ray diffraction, the long Fe–Fe distances average to 3.73 Å with a 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 Cpl (resolution 2.3 Å) from an ongoing X-ray diffraction study. The comparison was done by refining our model as a rigid body against the Cpl diffraction data using the program TNT. 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 Cpl 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 Cpl 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 (\( \text{EL}_3\text{N}_2\text{Fe}_{50}\text{S}_5\text{Cl}_3\text{Mo(CO)}_3\)).

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<tr>
<td>Mo–O</td>
<td>N</td>
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<tr>
<td>Mo–S</td>
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<tr>
<td>Mo–Fe</td>
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<td>3.0</td>
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<tr>
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<tr>
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<td>2.29</td>
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<td>2.8</td>
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<td>Fe–Fe’</td>
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<td>2.62</td>
<td>0.087</td>
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<td>Fe–Fe’</td>
<td>1.3</td>
<td>2.75</td>
<td>0.074</td>
<td>1.0</td>
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[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 mm 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. [d] Crystallographic value ref. [e] Total standard deviation mm = mm + σ and mm. [f] Statistical standard deviation.
De Novo Design of a Novel Heterodinuclear Three-Helix Bundle Metalloprotein**

By M. Reza Ghadiri† and Martin A. Case

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 RuIII 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 metalloproteins3 One such design, a three-helix bundle structure4 was the starting point in the present study for rational modification into a heterodinuclear RuIII,CuII metalloprotein. Our strategy is illustrated in Figure 1. As in our prior 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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