Selenol binds to iron in nitrogenase iron–molybdenum cofactor: An extended x-ray absorption fine structure study

(*Azotobacter vinelandii*/GNXAS)

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ABSTRACT   The biological N_2-fixation reaction is catalyzed by the enzyme nitrogenase. The metal cluster active site of this enzyme, the iron–molybdenum cofactor (FeMoco), can be studied either while bound within the MoFe protein component of nitrogenase or after it has been extracted into *N*-methylformamide. The two species are similar but not identical. For example, the addition of thiophenol or selenophenol to isolated FeMoco causes its rather broad $S = 3/2$ electron paramagnetic resonance signal to sharpen and more closely approach the signal exhibited by protein-bound FeMoco. The nature of this thiol/selenol binding site has been investigated by using Se-K edge extended x-ray absorption fine structure (EXAFS) to study selenophenol ligated to FeMoco, and the results are reported here. EXAFS data analysis at the ligand Se-K edge was performed with a set of software, GNXAS, that provides for direct calculation of the theoretical EXAFS signals and least-squares fits to the experimental data. Data analysis results show definitively that the selenol (and by inference thiol) binds to Fe at a distance of 2.4 Å. In contrast, unacceptable fits are obtained with either Mo or S as the liganded atom (instead of Fe). These results provide quantitative details about an exchangeable thiol/selenol binding site on FeMoco in its isolated, solution state and establish an Fe atom as the site of this reaction. Furthermore, the utility of ligand-based EXAFS as a probe of coordination in polynuclear metal clusters is demonstrated.

The biological N_2-fixation reaction is catalyzed by the enzyme nitrogenase. The active site of this enzyme is a metal cluster of stoichiometry $Mo:Fe_7:S_{8-9}^{2-}$:homocitrate, which is commonly designated as the iron–molybdenum cofactor (FeMoco) (1–3). FeMoco can be studied either when it is inside the MoFe protein component of nitrogenase or after it has been extracted into the solvent *N*-methylformamide (NMF) (1, 4, 5). The two species are similar but not identical (4, 5). In 1978, Rawlings *et al.* (6) reported that the addition of thiophenol to isolated FeMoco caused its $S = 3/2$ electron paramagnetic resonance (EPR) signal to sharpen and resemble closely the signal exhibited by protein-bound FeMoco. Subsequently, quantitative EPR (7) and ^{19}F NMR [using p-$CF_3C_6H_4S^-$ and p-$CF_3C_6H_4Se^-$ ($PhSe^-$) as the reporter ligands (8, 9)] have demonstrated that the thiol binds specifically and reversibly to a single site on FeMoco. These data, combined with sequence comparisons (10), suggested strongly that the α-subunit residue, αCys-275, might be the single cysteine ligand to FeMoco in the MoFe protein. The ligation of FeMoco by a cysteinyl was further supported by

site-directed mutagenesis studies (11–13). This prediction was confirmed by recent x-ray crystallographic results (14–16), which showed that αCys-275 is a ligand of one of the Fe atoms in FeMoco.

FeMoco contains seven Fe atoms but only one Mo atom (4, 5, 14, 15). The first definitive structural evidence that Mo and Fe were involved in an unprecedented polynuclear metal cluster came from extended x-ray absorption fine structure (EXAFS) analysis at the Mo-K edge (17, 18). X-ray absorption spectroscopy was also used to probe for chemical reactivity (19) and electronic structural changes at the Mo site (20), but little evidence of any significant effects or direct involvement of Mo was observed in these studies. Fe-K edge EXAFS was used to confirm the results obtained from the Mo-K studies and provided evidence for longer-range interactions in FeMoco (21–23).

Insight into the complete structure of FeMoco has come from the work of Rees and collaborators (14, 15). In this seminal study, they determined a model for protein-bound FeMoco derived from the protein crystallography electron density map, currently at a resolution of 2.2 Å. This model reveals a structure for FeMoco that consists of two subclusters (FeS_3Fe_3 and Fe_3S_3Mo), which are bridged by two S atoms and one additional ligand whose identity has not yet been definitively established as published but that currently is believed to be S. The only endogenous protein ligand that coordinates FeMoco through S is αCys-275, which binds to a single Fe atom at the terminus of the FeS_3Fe_3 subcluster. The only additional ligands to FeMoco in the current model (14, 15) are αHis-442 and homocitrate, both of which bind to Mo to complete its hexacoordination.

For isolated FeMoco, ^{19}F NMR experiments that compared FeMoco to model complexes have provided indirect evidence for thiol binding to Fe (8, 9), although there is no specific evidence that it binds to the same Fe atom as αCys-275. Indirect evidence for thiol binding at Fe was also provided by the observation from EXAFS at the Mo-K edge. In that study, it was shown that changes in ligation, which amounted to an increase in S coordination number at Mo of <1 when FeMoco was treated with thiophenol or selenol, did not originate from added thiol/selenol. If selenol were present, it would have been readily detectable because of its much stronger EXAFS backscattering behavior (18).

In the work described below, we present a series of experiments that examine ligand binding to isolated FeMoco.

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Selenium (substituted for S in benzenethiol) was used as a "reporter" ligand by examining the Se-K edge EXAFS. In the case of uncoordinated selenol, one would see only carbon and solvent (or perhaps counterion if there were a contact ion pair) contributing to the EXAFS signal. If (and only when) strong selenol binding occurred to FeMoco (as established by the NMR studies referenced above), a very significant perturbation to the Se EXAFS would be expected. The EXAFS could then be interpreted in terms of the nature and metric details of the bound complex. This EXAFS study, as presented below, provides unequivocal evidence that the thiol binding site on isolated FeMoco is to an Fe atom and not to Mo. A very preliminary account of this work, using a less complete EXAFS analysis, has appeared (24, 25).

MATERIALS AND METHODS

Sample Preparations. FeMoco was isolated and concentrated as published (26) to give 1.91 ± 0.07 mM Mo, with a specific activity of 234 ± 12 nmol of C_2H_4 formed per min per ng atom of Mo, after reconstitution of a FeMoco-deficient MoFe protein from a mutant *Azotobacter vinelandii* strain. This activity remained within 10% after the experiment. Semireduced FeMoco was produced by addition of 10 electron equivalents of a neutralized, aqueous solution of $Na_2S_2O_4$ (Eastman) just prior to data collection. p-$C_6H_4CF_3SeH$ was synthesized from p-$C_6H_4CF_3Br$ by reaction of elemental Se with the corresponding aryl Grignard reagent. The FeMoco sample investigated here had 0.8 equivalent of $PhSe^-$ per FeMoco (based on Mo) added as an NMF solution (8, 9). ^{19}F NMR studies have shown that under the conditions used for preparing these samples the analogous thiolate is present in its ionized form due to the high basicity of the NMF (9). Model compounds for x-ray absorption spectroscopy (XAS) analysis using empirical parameters were dibenzoselenophene ($C_{12}H_8Se$) (27, 28), $(NH_4)_2Se(S_2O_3)_2\cdot1.5H_2O$ (29, 30), and $(Me_4N)_2[Fe_4Se_4(SPh)_4]$ (31).

Data Collection. XAS data (models and FeMoco) were acquired at the Stanford Synchrotron Radiation Laboratory (dedicated conditions, 3 GeV, 30–80 mA) on unfocused beam line 4-2, using a double-crystal Si(220) monochromator. Internal energy calibration was performed by assigning the inflection point of elemental Se to 12,658 eV. Model compound data were collected in transmission mode using nitrogen-filled ionization chambers. The solids were diluted to the appropriate concentration using naphthalene, which also provided a uniform, compressible packing agent. Data for FeMoco in frozen NMF solution were measured by fluorescence methodology using an array of eight NaI detectors. The samples were kept under anaerobic conditions and cooled to −116°C to −148°C during measurements.

Data Analysis. The data were analyzed by two approaches. The GNXAS programs provide for *ab initio* modeling of EXAFS spectra. The approach utilizes Hedin–Lundqvist exchange and correlation potentials, handles single- and multiple-scattering signals with proper treatment of correlated Debye–Waller factors, and fits directly the experimental spectra (32, 33). This approach has been thoroughly tested on simple compounds of known structure (34, 35). In a study more directly relevant to polynuclear metal clusters in biological systems, GNXAS has been recently used to analyze both Mo and Fe EXAFS data from $MoFe_4S_6[P(C_2H_5)_3]_4Cl$ (36). Errors in first coordination shell distances within 3 Å of the absorbing atom were found to be 0.01–0.02 Å and sensitivity to coordination numbers about one atom in four. For the fits reported here using the same GNXAS package, the Se–C and Se–X distances were varied along with their Debye–Waller factors, while the coordination numbers were fixed at integer values. A more complete description of the GNXAS approach can be found elsewhere (32, 33).

For comparison, data were also analyzed by a more conventional approach using empirically derived phase and amplitude parameters (37, 38). Phase and amplitude parameters were derived as described (37, 38) from the model compounds listed (see above). However, a model suitable for extracting reliable Se–Mo parameters could not be found. In the fits using the empirical parameters, the distance and coordination number were allowed to vary. Results from C–Se–X fits to these EXAFS data using the empirical Se–C and Se–Fe amplitude and phase parameters gave results similar (1.5 C atoms at 1.92 Å and 1.4 Fe atoms at 2.42 Å from Se) to those found for the GNXAS study whose results are described in more detail in the next section. Additional data were measured and analyzed for a reduced sample with a $PhSe^-$/FeMoco ratio of 2.4 and for two oxidized samples with a ratio of 0.8 and 2.4, respectively (data not shown). The fit results were 1.2–1.5 C at 1.92–1.94 Å and 0.67–1.15 Fe at 2.42–2.44 Å. C–Se–S fits gave chemically unreasonable results, whereas fits including Mo could not be tested because of a lack of suitable model compounds. For this reason, the empirical parameter fits will not be considered further except to point out that they are entirely consistent with the GNXAS results discussed below.

RESULTS AND DISCUSSION

The experiment described here uses ligand-based EXAFS (from the Se in $PhSe^-$) to directly probe ligand binding to FeMoco. The Se EXAFS will always reflect the presence of the covalently bound phenyl group, with the main contribution deriving from the covalently bound C atom. There is also evidence in the EXAFS data (from the Fourier transforms at higher R) of weak contributions from the more distant C atoms in the phenyl rings as well, although this is not important for the results described here (since they make a constant contribution to all the Se EXAFS). However, if the Se atom were liganded to a metal (in particular to Mo or Fe in FeMoco), this would make a very strong and easily quantifiable contribution to the EXAFS. Such an interaction is referred to below as C–Se–X. Another, more unlikely, possibility could be the formation of a mixed –S–Se– bond, and this possibility is also examined.

As mentioned above, analysis of the Se EXAFS data using an empirical approach was not possible because of the lack of suitable model compounds for determination of Se–Mo phase and amplitude parameters. This is a not infrequently encountered problem because empirical phase and amplitude determination depends on having structurally characterized models with well-defined and separated coordination shells for the appropriate absorber–scatterer pairs (Se–Fe, Se–Mo, and Se–S in this case). This limitation can be circumvented if reliable phase and amplitude parameters can be obtained from theoretical calculations. Recent advances in theory have been described by several groups and have given rise to quite reliable means to calculate phases and amplitudes (39–42). We have developed in parallel and used a set of programs called GNXAS (32–36), which provide for an integrated approach to EXAFS data analysis. This includes direct calculation of the theoretical signals, experimental background removal, handling of multiple scattering pathways, and correlated Debye–Waller factors and least-squares fits to the experimental data in which selected parameters (typically distance and Debye–Waller factor as in the study reported here) can be varied.

The results of the GNXAS analysis and fits for C–Se–X (X = Fe, Mo, or S) are summarized in Table 1. In Fig. 1, Fourier transforms for the experimental data are compared with Fourier transforms of the best fits for the Se to C + Fe and C + Mo cases. The difference signal is also shown. Particularly apparent in this difference signal is the much higher

Table 1.   Results for Se-K edge GNXAS EXAFS fits

C–Se–X*	Se–C Å	Se–C σ^2, Å^2	Se–X Å	Se–X σ^2, Å^2	$R^\dagger$
C–Se–Fe	1.90	0.0022	2.41	0.0022	0.9
C–Se–Fe$_2$	1.92	0.0046	2.41	0.0079	3.1
C–Se–Mo	1.90	0.0010	2.25	0.0060	3.3
C–Se–S	1.98	0.008	2.18	0.010	110

*Coordination number for Se fixed at 1 X and 1 PhSe⁻ group; Debye–Waller factors floated.

†Least-squares residual × 10^6. For definition of R, see ref. 36.

residual for the C + Mo fit compared with the C + Fe fit. The disagreement is so significant that other parameters such as background variables are affected, leading, for example, to slight differences in the Fourier transform of the experimental data (Fig. 1). The actual EXAFS data compared with best fit and difference signal are shown in Fig. 2. It is apparent from inspection of these results that the best fit is obtained for the C–Se–Fe model. This fit, with coordination number of C and Fe fixed at 1, gives very reasonable bond distances and Debye–Waller factors for both Se–C and Se–Fe when compared to those for the X = Mo case (where coordination numbers were also fixed at 1).

These same trends are reflected quantitatively in the fit residuals reported in Table 1, where the C–Se–Fe best fit is 3-fold lower than any other fit. Fits were also tried for the C + S case to test for the possible formation of a Se–S bond. The C + S fit was very significantly worse (data not shown) than either the C + Fe or C + Mo fits with a residual >100-fold greater. A model, which included two Fe atoms at the same distances such as would exist if Se bridged two Fe atoms, was also evaluated, but these fits (also not shown) were also significantly worse, being comparable to the C + Mo fits in quality.

These results provide further direct evidence for, and quantitative details about, an exchangeable thiol/selenol binding site in FeMoco. The site is clearly established, from the EXAFS results presented here as being Fe, under the conditions of these experiments in NMF solution. These results are consistent with those of our previously published K edge study at Mo (18), in which no contribution by Se to the Mo-K EXAFS was seen, as well as with a preliminary report on Fe-K edge studies (43), in which the presence of Se was indicated in the form of 0.2–0.3 Se/Fe at a distance range of 2.35–2.4 Å. EXAFS analysis on solutions gives the average environment of all the absorbing species and thus, from
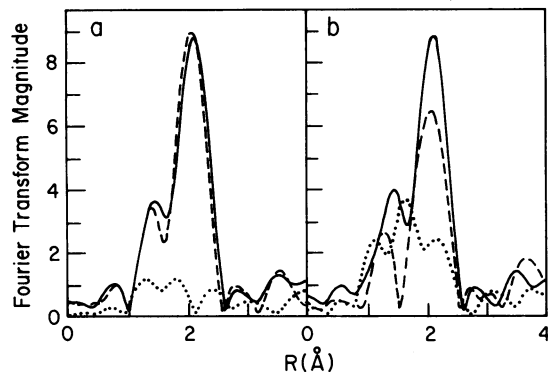

FIG. 1.   Fourier transforms (k range, 2.9–12.2 Å^{-1}) of the Se-K edge EXAFS data for FeMoco + PhSe⁻. Transform of the experimental data (——) is compared with that of the best theoretical fit (– – –), and the Fourier transform of the difference between the data and the fit is shown (· · ·) for C + Fe fit (*a*) and for C + Mo fit (*b*). Note the very good agreement of the fit with the data for C + Fe, while the C + Mo fit shows poor agreement over the whole first shell region.
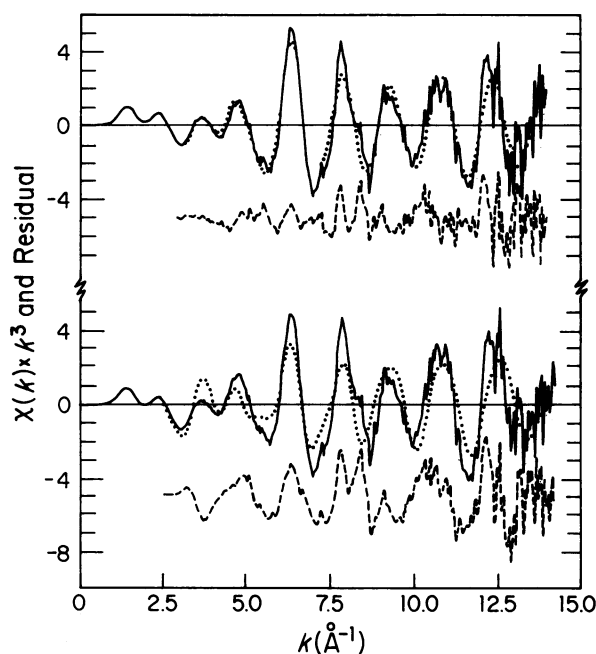

FIG. 2.   Comparison of experimental EXAFS data (——) with results of the best theoretical fit (· · ·) for C + Fe (upper) and C + Mo (lower) with fits over a range of k = 2.5–14.2 Å^{-1}. Below each spectrum is plotted the difference (residual, – – –) between experimental and theoretical signals. In addition to illustrating the very good fit for C + Fe (upper), the theoretical parameters fit well over the full k range, especially at $k < 4$ Å^{-1}, where empirical parameters become increasingly unreliable.

the present data, it is not possible to associate this site with a specific Fe atom in the structural model proposed for FeMoco from crystallography (14–16). It could be either at the terminal Fe (ligated in the protein by αCys-275) or at one (or more) of the six core Fe atoms in either of the two subclusters. In either case, it is interesting and important to observe that these results further substantiate an increasing body of evidence that Fe may play a significant role in the chemistry of FeMoco. The results also demonstrate the utility of ligand-based EXAFS as a probe of binding sites in complex polynuclear metal clusters.

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