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Registry No. 1, 3965-56-8; **2**, 30213-19-5; **3**, 5059-76-7; (±)-**4**, 88211-17-0; **5**, 88211-18-1; **6**, 88211-19-2; **7**, 88269-11-8; **8**, 51788-41-1; (±)-**9**, 88211-20-5; **10**, 88211-21-6; **10** (ethylene thioketal), 88211-28-3; **11**, 88211-22-7; **12**, 88269-12-9; **13**, 10531-02-9; **14**, 61597-60-2; **15**, 61597-61-3; (+)-**19**, 88269-13-0; (+)-**20**, 88211-23-8; (−)-**21**, 88211-24-9; (±)-**24**, 88211-25-0; (±)-**25**, 88314-72-1; alcohol dehydrogenase, 9031-72-5; norbornadiene, 121-46-0; norbornene, 498-66-8; cyclohexanol, 108-93-0; (±)-*cis*-1-bromo-3,5-bis((benzyloxy)methyl)cyclohexene, 88211-26-1; (±)-*cis*-1-acetoxy-3,5-bis((benzyloxy)methyl)cyclohexene, 88211-27-2; *cis*-1,3-bis((benzyloxy)methyl)cyclohexane, 88211-29-4; (±)-*cis*-1-bromo-3,5-bis(acetoxymethyl)cyclohexene, 88211-30-7; (±)-*cis*-3,5-bis(acetoxymethyl)cyclohexene, 88211-31-8; (±)-*cis*-1-acetoxy-3,5-bis(acetoxymethyl)cyclohexene, 88211-32-9.

Molybdenum Sites of *Escherichia coli* and *Chlorella vulgaris* Nitrate Reductase: A Comparison by EXAFS

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Abstract: The molybdenum sites in two different types of nitrate reductase, assimilatory enzyme from *Chlorella vulgaris* and dissimilatory enzyme from *Escherichia coli*, have been investigated and compared by using X-ray absorption edge and EXAFS spectroscopy. The molybdenum environment in *Chlorella* nitrate reductase was found to strongly resemble that in hepatic sulfite oxidase. In the oxidized state of the *Chlorella* enzyme the molybdenum has two terminal oxygens at 1.71 ± 0.03 Å as well as two or three sulfurs at 2.44 ± 0.03 Å. Additional undetected ligands may be present in this and the other structures reported. A single terminal oxygen at 1.67 ± 0.03 Å and a set of sulfurs at 2.37 ± 0.03 Å are found upon full NADH reduction. Data are also presented on dithionite-reduced and cyanide-inhibited forms of the *Chlorella* enzyme. In contrast with all other non-nitrogenase molybdenum enzymes, the *E. coli* nitrate reductase molybdenum appears devoid of oxo groups when fully reduced, although an oxo species appears upon oxidation by nitrate. The reduced *E. coli* enzyme shows evidence for two or three sulfurs at 2.36 ± 0.03 Å and one or two nitrogens and/or oxygens at 2.10 ± 0.03 Å. Furthermore, a feature consistent with a bridged Mo–X interaction at about 2.8 Å is observed. Mechanistic models are proposed that incorporate these results.

Nitrate reductase, sulfite oxidase, xanthine oxidase, and formate dehydrogenase contain a common molybdenum cofactor, "Mo-co", and yet they display significantly different chemical and physical properties.[2] In an effort to understand the structural basis for this diversity, we have undertaken a systematic X-ray absorption study of molybdenum-containing proteins.[3,4] This paper reports results of EXAFS experiments on nitrate reductase from two different sources—*Chlorella vulgaris*[5] and *Escherichia coli*.[6] Nitrate reductase from *Chlorella* is a homotetramer with a molecular weight of about 360 000,[7] which contains one molybdenum, one heme, and one FAD prosthetic group per subunit. It is an assimilatory enzyme which reduces nitrate to nitrite, which ultimately is reduced to ammonia for incorporation of the nitrogen into amino acids. In contrast, *E. coli* nitrate reductase is a dissimiliatory enzyme which uses nitrate as a terminal electron acceptor in the absence of O_2. This enzyme is a heterodimer of molecular weight 200 000 containing one molybdenum, 16 irons, and 16 acid-labile sulfurs.[6] The latter extrude as four Fe_4S_4 clusters (Adams, M. W. W.; Mortenson, L. E., unpublished results). The EXAFS experiments described in this paper indicate that the molybdenum site of *Chlorella* nitrate reductase is quite similar to that previously observed for sulfite oxidase,[4] whereas the molybdenum site of *E. coli* nitrate reductase exhibits properties different from any molybdenum enzyme yet examined.

Experimental Section

Sample Preparation. ***Chlorella vulgaris*** nitrate reductase was purified as described previously[8] to an A_{413}/A_{280} ratio of better than 0.55 and concentrated in 80 mM pH 7.6 potassium phosphate buffer with an Amicon YM-10 membrane to 34 mg/mL. The molybdenum concentration in the sample was 0.31 mM.[9] The initial NADH:nitrate reductase activity, measured spectrophotometrically,[9] was typically about 80 μmol NADH oxidized per min per mg of protein. NADH-reduced enzyme was prepared by addition of sufficient 0.2 M NADH to generate a 20-fold molar excess of NADH in a deoxygenated sample; cyanide-inactivated (reversible) enzyme was generated by addition of 10 μL of 1.0 M KCN to a 0.3 mL of NADH-reduced sample.[10] Dithionite-reduced enzyme was prepared by addition of a few grains of sodium dithionite to an oxidized sample. After preparation, all samples were frozen in 20 × 10 × 1.5 mm Lucite cuvettes. Enzyme activity was checked after data collection and was typically better than 95% of its initial value.

E. coli nitrate reductase was isolated as described[6] and assayed using reduced methylviologen as an electron donor.[6,11] The specific activity of all preparations was 78–80 μmol of NO_3^- reduced per min per mg of protein. Samples were concentrated to 180 mg/mL in 50 mM pH 7.0 potassium phosphate buffer by Amicon filtration with an XM-100 membrane; the molybdenum concentration was 0.7 mM. Reduced samples were prepared by anaerobic incubation with 5 mM sodium dithionite or H_2/hydrogenase/methylviologen.[6] Ferricyanide-oxidized enzyme was prepared by addition of potassium ferricyanide to a final concentration of 9 mM (under argon), while air-oxidized enzyme was prepared by exposing a concentrated sample to air for several hours with occasional stirring. Nitrate-oxidized enzyme was prepared by treatment of a dithionite-reduced sample with excess nitrate. All samples retained greater than 90% of their nitrate reductase activity after irradiation. The model

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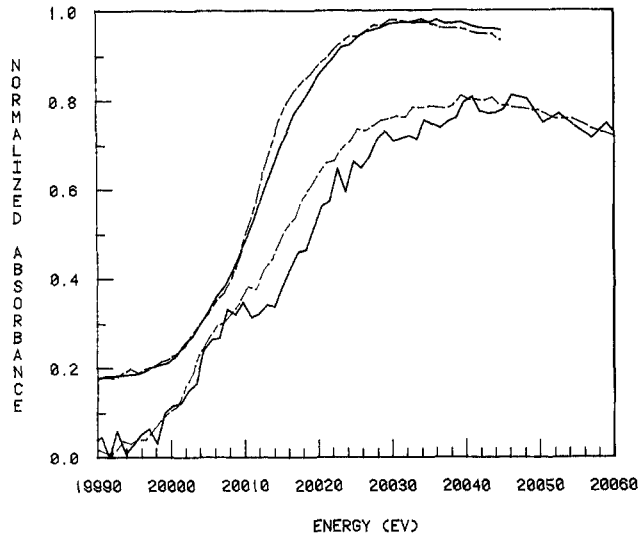

Figure 1. The molybdenum K absorption edges of nitrate reductase. Top: ferricyanide-oxidized (—) vs. dithionite-reduced (---) *E. coli* nitrate reductase. Bottom: oxidized (—) vs. NADH-reduced (---) *Chlorella vulgaris* nitrate reductase.

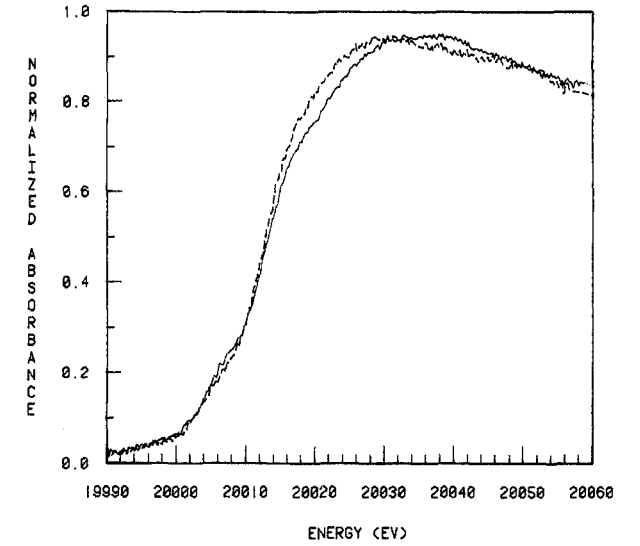

Figure 2. A comparison of the molybdenum K absorption edges of dithionite-reduced (---) and nitrate-oxidized (—) *E. coli* nitrate reductase.

compounds $(NEt_4)_2[(SC_6H_5)_2FeS_2MoS_2]$ and $(NEt_4)_2[Cl_2FeS_2MoS_2FeCl_2]$ were prepared by literature methods.[12]

Data Collection. The spectra were recorded at the Stanford Synchrotron Radiation Laboratory on several occasions using bending magnet or wiggler beam lines and Si [2,2,0] crystal monochromators. During data collection the samples were kept frozen at −5 to −15 °C. A fluorescence detection apparatus[13] using zirconium filters permitted collection of a reasonable spectrum in about 8 h.

Data Analysis. The spectra were processed and interpreted by use of previously described procedures and functions.[4,14,15] The compound $(NEt_4)_2[(SC_6H_5)_2FeS_2MoS_2]$ was used to extract a Mo–Fe phase shift, while $(NEt_4)_2[Cl_2FeS_2MoS_2FeCl_2]$ was used to obtain an amplitude correction factor for the theoretical Fe amplitude.[15]

Results

Edges. The molybdenum K-edge regions for oxidized and reduced forms of *E. coli* and *Chlorella* nitrate reductase are compared in Figure 1. The edges for the *Chlorella* enzyme appear quite similar to those previously observed for sulfite oxidase.[16] Thus, the edge for the oxidized enzyme exhibits a pronounced low-energy shoulder characteristic of compounds with terminal oxo or sulfido groups.[3a,17] Upon reduction of the enzyme with NADH the edge moves several volts to lower energy, and the intensity of the bound-state transition apparently diminishes. In contrast with the data for *Chlorella* nitrate reductase, the edge for the ferricyanide-oxidized *E. coli* enzyme exhibits an extremely weak bound-state transition. Reduction of the enzyme produces only a small shift to lower energy, and still a very weak bound-state transition. In fact, the clearest indication of an oxo-type shoulder for the *E. coli* enzyme was found in the nitrate-oxidized edge, as illustrated in Figure 2. Since the nitrate-oxidized sample represents a mixture of species,[6,18] caution is required in interpreting this result. In summary, the overall shape and redox behavior of the edge region is quite different for the two nitrate reductase enzymes, qualitatively indicating a structural dissimilarity in the molybdenum centers.

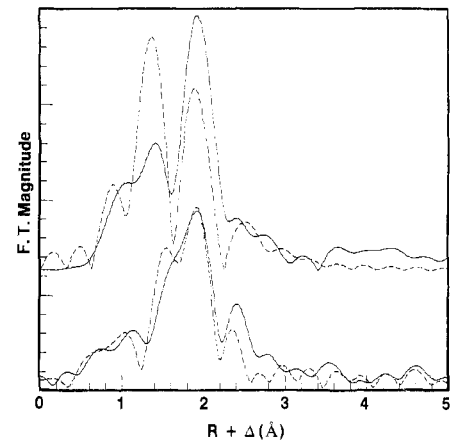

Figure 3. EXAFS Fourier transforms for nitrate reductase molybdenum. Top: oxidized *Chlorella vulgaris* nitrate reductase (---) vs. NADH-reduced nitrate reductase (—). Bottom: "oxidized" *E. coli* nitrate reductase (---) vs. "reduced" *E. coli* nitrate reductase (—). Transform range: k = 4–14 Å^{-1}, k^3 weighting.

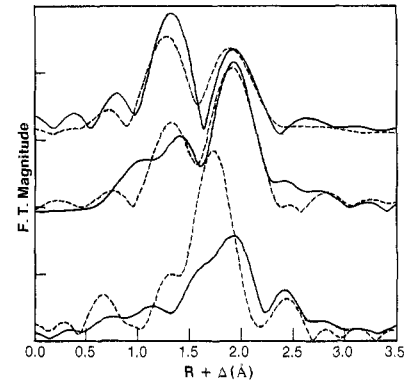

Figure 4. Comparison of nitrate reductase EXAFS transforms with sulfite oxidase and a Mo–Fe model compound. Top: oxidized *Chlorella* nitrate reductase (—) vs. oxidized chicken liver sulfite oxidase (---). Middle: reduced *Chlorella* nitrate reductase (—) vs. reduced chicken liver sulfite oxidase (---). Bottom: reduced *E. coli* nitrate reductase (—) vs. $(NEt_4)_2[S_2MoS_2Fe(SC_6H_5)_2]$ (---). Transform range: top and middle, k = 4–12 Å^{-1}; bottom, k = 4–14 Å^{-1}, k^3 weighting for all transforms.

EXAFS Transforms. Figure 3 shows the Fourier-transformed EXAFS data for oxidized and reduced samples of *E. coli* and

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Table I. Nitrate Reductase Curve-Fitting Results

sample	Mo-O no.	Mo-O R, Å	Mo-O σ, Å	Mo-S no.	Mo-S R, Å	Mo-S σ, Å	Mo-X no.	Mo-X type	Mo-X R, Å	Mo-X σ, Å	residual[a]
oxidized *Chlorella*[b] nitrate reductase	2	1.71	0.036	2	2.44	0.064					0.749
	2	1.72	0.034	3	2.44	0.084					0.733
NADH-reduced *Chlorella*[c] nitrate reductase	1	1.67	0.058	2	2.37	0.035					0.879
	1	1.67	0.062	4	2.37	0.069					0.765
	1	1.67	0.060	3	2.37	0.056					0.705
	1	1.67	0.060	3	2.38	0.054	1	O, N	2.07	0.085	0.658
	0.8	1.68	0.04	3.1	2.37	0.055	0.6	O, N	2.07	0.06	0.598[d]
$S_2O_4^{2-}$-reduced *Chlorella*[c] nitrate reductase	1	1.71	0.069	3	2.38	0.046					0.694
	1	1.71	0.070	4	2.38	0.060					0.678
	1	1.72	0.07	4	2.38	0.058	1	O, N	2.05	0.056	0.580
	1	1.71	0.069	3	2.39	0.044	1	O, N	2.10	0.067	0.534
CN^--inactivated *Chlorella*[b] nitrate reductase	1	1.68	0.025	3	2.38	0.057					0.417
"reduced" *E. coli*[b] nitrate reductase	2	2.15	0.128	3	2.34	0.075					0.756
	1	2.09	0.031	2	2.36	0.045					0.745
	2	2.10	0.081	2	2.35	0.054					0.729
	2	2.10	0.071	2	2.36	0.054	1	Fe	2.79	0.080	0.534
	2	2.11	0.080	2	2.35	0.058	1	Mo	2.98	0.057	0.477
"NO^-_3-oxidized" *E. coli*[b,d] nitrate reductase	0.8	1.66	0.04	2.1	2.42	0.06	2.0	O, N	2.15	0.06	0.469

[a] Defined as $[(\Sigma k^6(\chi_{obsd} - \chi_{calcd})^2)/N]^{1/2}$, where χ_{obsd} is observed EXAFS and χ_{calcd} is calculated EXAFS, and N is the number of data points. [b] 4–12 Å^{-1}. [c] 4–14 Å^{-1}. [d] σ held fixed.

Chlorella nitrate reductase. On the basis of previous model compound studies, peaks assignable to Mo–O and Mo–S interactions are clearly evident in the *Chlorella* data. The spectra of the *Chlorella* enzyme strongly resemble previous sulfite oxidase results.[4] This can be seen by direct comparison of the tranforms in Figure 4.

The spectra of *E. coli* nitrate reductase are unlike any previously recorded protein or model compound patterns. A peak in the Mo–S region is observed, but there is no well-resolved Mo=O feature. Instead, a shoulder or peak is observed between the Mo=O and Mo—S regions. Furthermore, a reasonably strong peak is seen beyond the Mo—S feature in the reduced *E. coli* spectrum. Correction for a typical phase shift yields a Mo–X interaction at 2.6–2.8 Å. The identity of X is uncertain, although phosphorus, iron, or molybdenum are chemically reasonable. A comparison of *reduced E. coli* nitrate reductase with a model compound having a single sulfur-bridged Mo–Fe interaction is given in Figure 4. The first coordination sphere features for *oxidized E. coli* nitrate reductase also show clear Mo–O(N) and Mo–S features. However, there was some variability in the longer distance Mo–X peak, perhaps reflecting the existence of several forms of oxidized enzyme.

In Figure 5, Fourier transforms for other forms of nitrate reductase are presented. Comparison of the transforms for dithionite-reduced and cyanide-inactivated *Chlorella* enzyme shows that the two spectra have nearly identical Mo–S peaks, but in the CN^- spectrum the Mo=O feature is better resolved and a weak peak at 2.8 Å (including phase shift) appears. This would corrspond to a true distance of 3.0–3.2 Å, and this feature might therefore represent a Mo–C–N interaction. The nitrate-oxidized *E. coli* spectrum in Figure 5 is significant primarily for the appearance of a short distance Mo=O peak, which had been absent in the data for other forms of *E. coli* nitrate reductase.

EXAFS Curve Fitting. The EXAFS spectra were quantitatively analyzed by a curve-fitting procedure using the following approximate expression:

$$\chi(k) \simeq \sum_b (N_b/kR_{ab}^2)A_{ab}(k)\exp(-2\sigma_{ab}^2k^2)\sin(2kR_{ab} + \alpha_{ab}(k))$$

In this expression N_b is the number of type b neighbors at distance R_{ab} from the type a X-ray absorber, in this case molybdenum. $A_{ab}(k)$ is the total amplitude function, k is the photoelectron wave vector, σ_{ab}^2 is the mean square deviation of R_{ab}, and $\alpha_{ab}(k)$ is the total phase shift. The functions $A_{ab}(k)$ and $\alpha_{ab}(k)$ are either assumed transferable from model compounds[14] or the modifications required to use theoretical functions are assumed to be transferable.[15] In either case, the variables to be optimized are N_b, R_{ab}, and σ_{ab}^2. Since the coordination number and Debye–Waller factor are highly correlated, in the following fits either N was constrained to integer values, then optimizing R and σ^2, or σ^2 was constrained to reasonable values, in which case N and

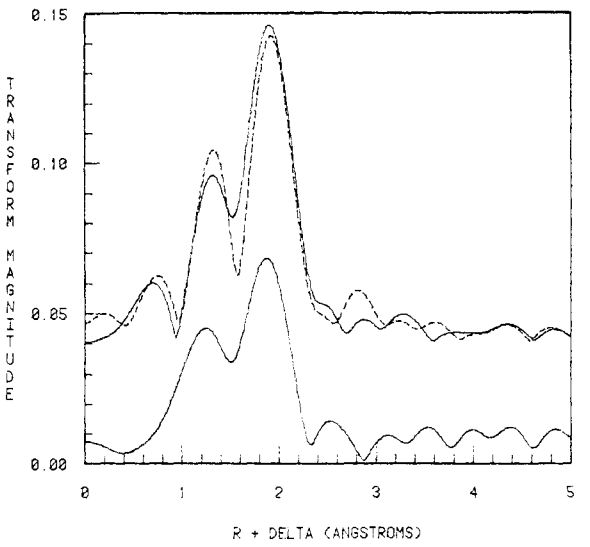

Figure 5. EXAFS Fourier transforms for additional forms of nitrate reductase. Top: dithionite-reduced *Chlorella* enzyme (—) vs. cyanide-inactivated *Chlorella* enzyme (- -). Bottom: nitrate-oxidized *E. coli* enzyme. Transform range: top, k = 4–14 Å^{-1}; bottom, k = 4–12 Å^{-1}; both k^3 weighting.

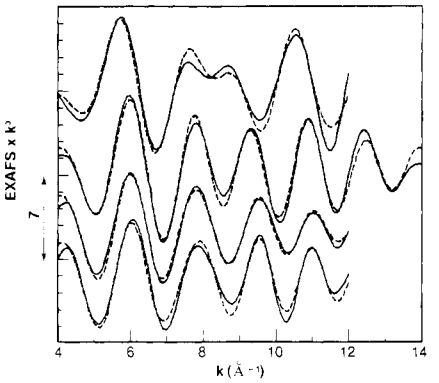

Figure 6. Filtered EXAFS (—) and best fits (- -) for nitrate reductase. Top to bottom: oxidized *Chlorella*, reduced *Chlorella*, oxidized *E. coli*, reduced *E. coli*. The *E. coli* fits shown used Mo–Fe as the long-distance interaction.

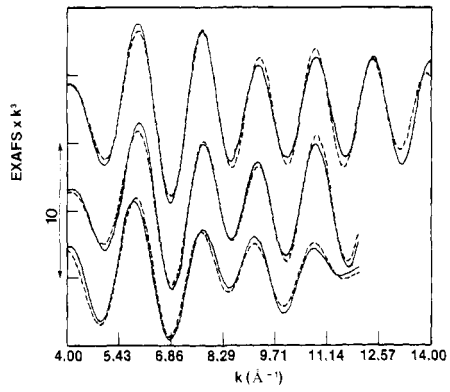

Figure 7. Filtered EXAFs (—) and best fits (- - -) for additional forms of nitrate reductase. Top to bottom: dithionite-reduced *Chlorella*, cyanide-inactivated *Chlorella*, nitrate-oxidized *E. coli*.

R were refined. Comparisons of the observed and calculated EXAFS are shown in Figures 6 and 7, and the numerical results are summarized in Table I.

Oxidized Chlorella Nitrate Reductase. The EXAFS for oxidized *Chlorella* nitrate reductase could be adequately reproduced by using two components, a short Mo=O interaction at 1.72 Å and a longer Mo—S interaction at 2.44 Å. Unless otherwise stated all distances are considered accurate to ±0.03 Å, on the basis of experience with systematic errors in curve-fitting model compounds of known structure. The statistical errors obtained from the fits themselves were typically less than 0.01 Å. The best integer estimate for the number of oxo groups was clearly two, whereas the inclusion of either two or three sulfurs yielded fits of almost equal quality. The σ obtained using three sulfurs, 0.084 Å, is larger than one would expect for thermal motion alone.[4] Hence, the data are compatible with either two Mo–S bonds of nearly equal length or three Mo–S bonds with some static spread in the Mo–S distance. Furthermore, since chlorine and phosphorus are indistinguishable from sulfur in the current analysis, any combination of two or three P, S, or Cl ligands at an average of 244 Å is consistent with the data.

NADH-Reduced *Chlorella* Nitrate Reductase. Curve-fitting analysis predicted only a single terminal oxygen at 1.67 Å, as well as three or four sulfurs at 2.38 Å. Again, the ambiguity, in coordination number derives primarily from its close correlation with the Debye–Waller factor. The same ambiguity about P, S, or Cl also applies. As with sulfite oxidase, inclusion of an additional oxygen or nitrogen ligand at an intermediate distance improved the fit; in this case the Mo–O′(N) distance was found to be 2.07 Å. Since there was some concern that the observed Mo=O species might be in equilibrium with a Mo—OH group, a final fit was done with a fixed σ and variable coordination numbers. The calculated value, 0.8 Mo=O, indicates that this is certainly the dominant form.

Dithionite-Reduced *Chlorella* Reductase. This spectrum was quite similar to the NADH-reduced enzyme. A single oxo group remains, with Mo=O bond length of 1.71 Å. The Mo—S and Mo—O′(N) distances are also similar to the NADH-reduces values. There is some evidence in the literature that dithionite can reversibly inhibit enzymes such as sulfite oxidase or nitrate reductase by "overreduction".[19] From the current results it appears that reduction of the molybdenum stops at Mo(IV), since there are no known oxo compounds of Mo(III) or of lower molybdenum oxidation states.

Cyanide-Inactivated *Chlorella* Nitrate Reductase. A good fit to this spectrum was obtained by using three sulfurs at 2.38 Å and one terminal oxygen at 1.69 Å. It was not possible to obtain an unambiguous value for a Mo–C interaction, perhaps because $Mo(CO)_6$ was an inappropriate model compound. Nevertheless, it is clear that the oxo group is retained during CN^- binding and that the average Mo–S bond lengths are virtually unchanged from those in the other reduced forms of the enzyme.

Oxidized *E. Coli* Nitrate Reductase. So that a better signal-to-noise ratio for the spectrum of oxidized *E. coli* nitrate reductase could be obtained, the ferricyanide-oxidized and air-oxidized spectra were averaged. Since there is a time dependence to the ferricyanide activation process and since air-oxidized samples may still contain some Mo(V), the spectrum obtained probably represents several different molybdenum species, and the spectra should be interpreted with caution. Nevertheless, the most important point is that a dioxomolybdenum species, such as found in the oxidized *Chlorella* enzyme, oxidized sulfite oxidase, or oxidized cyanolyzed xanthine oxidase, was not found in oxidized *E. coli* nitrate reductase. Of course, it is still possible that other conditions of pH, anion concentration, or oxidant could be used to generate such a species. Preliminary fitting analysis indicated at least two oxygen (and/or nitrogen) donor ligands at an average distance of 2.10 Å, as well as two sulfurs at 2.37 Å.

Reduced *E. Coli* Nitrate Reductase. The spectra for the dithionite-reduced and the H_2/hydrogenase/methylviologen-reduced *E. coli* nitrate reductase were averaged. Both methods of reducing the enzyme cause the disappearance of the Mo(V) EPR signal, but again, there is no guarantee that precludes a mixture of diamagnetic molybdenum species. The most reasonable assumption is that reduction stops at Mo(IV) in both cases. The curve-fitting analysis indicated one or two oxygens (or nitrogens) at 2.11 Å, as well as two sulfurs at 2.36 Å. The curve fitting cannot rule out a third sulfur donor or chloride ligand, but it is worth noting that when two Mo–S interactions are assumed an optimized σ of 0.054–0.058 Å is obtained, which is the same range of σ obtained when three Mo–S(P,Cl) interactions are assumed for the reduced *Chlorella* sample. One can conclude that either the reduced *E. coli* molybdenum site has a significant spread in its Mo–S(P,Cl) bond lengths or under the conditions employed, the reduced *Chlorella* enzyme molybdenum has an additional ligand at about 2.38 (3) Å not present at the reduced *E. coli* Mo site.

The reduced *E. coli* transform showed strong evidence for a high *R* peak, and a third component was required to adequately fit the data. Either a Mo–Fe distance of 2.80 Å or a Mo–Mo distance of 2.98 Å could be included in a three-component fit. Other assignments such as a Mo–P interaction have not been investigated and cannot yet be excluded.

Nitrate-Oxidized *E. Coli* Nitrate Reductase. Previous EPR studies have indicated that nitrate oxidation of the reduced *E. coli* enzyme yields an EPR signal with an intensity corresponding to 50% Mo(V).[18] The remaining molybdenum might be present as Mo(VI), but it is also conceivable that some of the Mo(V) is coupled to another center. Fitting these data with fixed Debye–Waller factors and variable coordination numbers yields an average Mo environment with 0.8 terminal oxo groups at 1.66 Å, 2.1 sulfurs at 2.42 Å, and 2.0 oxygens and/or nitrogens at 2.15 Å. Since the sample contains a mixture of molybdenum species, it is impossible to extract the structure of the Mo(V) site from these data. Neveretheless, the fact that under certain conditions the *E. coli* nitrate reductase Mo site also exhibits Mo=O bonding is an important result.

Structural Implications. In an attempt to draw structural and mechanistic conclusions about nitrate reductase from the preceding EXAFS results, it is important to recognize that the analysis may not reveal all of the ligands at the molybdenum site. Single ligands with large Debye–Waller factors often make a neglibible contribution to the EXAFS; a prime example is the absence of an observable $Cu–S_{met}$ signal even in single-crystal studies of plastocyanin.[20] It is also difficult to observe Mo–N interactions that fall near the same distance as Mo–S bonds or to see the presence of labile water molecules.[21] Finally, it is impossible to distinguish

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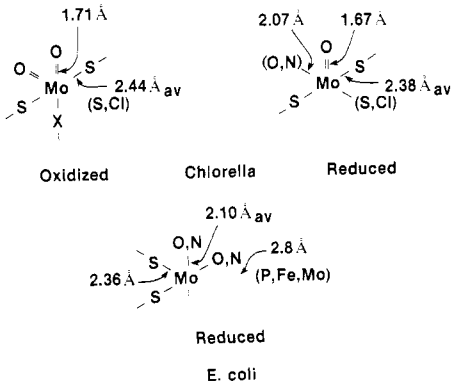
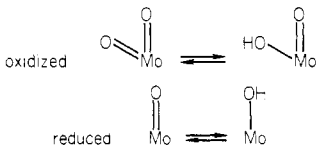


Figure 8. Proposed environments for nitrate reductase molybdenum. Alternate geometries are possible.

Scheme I



between C, N, and O or between P, S, and Cl on the basis of the EXAFS data alone. Despite these limitations, some detailed predictions about the active sites of these enzymes can be made.

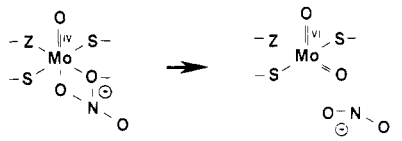
In Scheme I plausible coordination environments for nitrate reductase Mo are depicted. Although the ligands are arranged in an octahedral fashion, it should be emphasized that some very distorted Mo coordination spheres have been observed,[22] and no particular geometry can be deduced from the solution EXAFS alone.

Each structure proposed in Figure 8 requires some explanation. For the oxidized *Chlorella* enzyme, it is unclear whether the third sulfur donor or chloride is actually a ligand to molybdenum, or simply in close enough proximity to bind during or after reduction. The significant change upon reduction might reflect simply a redistribution of sulfur ligands, or alternatively, evidence for chloride binding. In the case of the reduced *Chlorella* enzyme, the 1.7-Å feature might represent a hydroxyl group, as might one of the O, N ligands found in the reduced *E. coli* data. Finally, the reduced *E. coli* structure appears to have a significant 2.8-Å interaction with perhaps a bridged iron neighbor, a bridged molybdenum, or even a phosphorus atom.

A major complicating factor, which requires further experimentation, is the possible pH and halide ion dependence of the nitrate reductase data. In particular, the pH dependence of the Mo(V) signals from both *E. coli* and *Chlorella* has revealed a dissociable proton with a pK_a of about 8.2 for *E. coli* and about 9 for *Chlorella* nitrate reductase. Similar equilibria might exist for the Mo(IV) and Mo(VI) species. If such equilibria exist for terminal oxo species, as in Scheme I, it might explain some of the differences observed between the two enzymes. In this case the distinction between the oxo/dioxo and non-oxo/monooxo species might be an extrinsic pH effect rather than intrinsic to the enzyme sites. It is worth noting that the *E. coli* spectra were recorded at pH 7.0, as opposed to pH 7.6 for the *Chlorella* data. Furthermore, no controls were taken to rigorously exclude chloride ions from the buffers. In view of recent EPR work by Bray and co-workers,[23] further studies of halide effects on these spectra are clearly required.

Mechanistic Implications. Despite, or perhaps because of, a previous paucity of structural information about the nitrate reductase molybdenum sites, there has been a wealth of speculation about the mechanism of nitrate reduction.[24] Although each individual proposal has its own pecularities, two broad classes can be distinguished. In one set of mechanisms, electron and proton transfer from molybdenum to substrate are coupled.[25] Another group of mechanisms emphasizes oxo transfer from nitrate to a reduced oxomolybdenum species.

Scheme II. Proposed Mechanism for *Chlorella* Nitrate Reductase



An initial proposal by Garner assumed binding to an oxomolybdenum(V) species, with subsequent electron transfer, loss of NO_2, and disproportionation of the latter to yield NO_2^- and NO_3^-.[26] A subsequent modification using oxo molybdenum(IV) avoids the NO_2 intermediate by invoking a two-electron transfer from Mo(IV) to NO_3^-.[24] In both of these cases it has been stressed that nitrate must be bound in a position cis to the oxo ligand for electron transfer to occur. Combining these oxo transfer hypotheses with the EXAFS results on the *Chlorella* enzyme allows the formulation of a plausible reaction mechanism for this enzyme, as illustrated in Scheme II.

The oxo/dioxo-based mechanism proposed for the *Chlorella* enzyme does not appear compatible with the structural results for *E. coli* nitrate reductase. Although it is tempting to formulate an alternative mechanism based on a Mo–Fe interaction, with coordinated two-electron transfer by both metals to the NO_3^- ion, further studies of the *E. coli* enzyme under controlled conditions are first required.

Summary

This study has unambiguously shown that under certain conditions significant structural differences exist between the assimilatory nitrate reductase from Chlorella *vulgaris* and the dissimilatory enzyme from *E. coli*. The *Chlorella* enzyme has at least two sulfur ligands near the molybdenum site, and shuttles between mono- and dioxo forms. In this respect it strongly resembles sulfite oxidase. In contrast, the *E. coli* enzyme revealed a molybdenum site with at most one oxo ligand. Apart from a minimum of two sulfur donors and at least two oxygen or nitrogen ligands, the *E. coli* nitrate reductase molybdenum has a long-distance interaction with an as yet unidentified neighbor. Although evidence for terminal oxo ligation was seen in the nitrate-oxidized data, it was not possible to generate a pure dioxomolybdenum species.

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Registry No. Mo, 7439-98-7; nitrate reductase, 9013-03-0.

Supplementary Material Available: Listings of the raw data obtained in this study are supplied; a table of parameterized phase shift and amplitude functions is also included (15 pages). Ordering information is given on any current masthead page.

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