Structural Changes Induced by Catalytic Turnover at the Molybdenum Site of *Arabidopsis* Nitrate Reductase

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Assimilatory nitrate reductases catalyze the reduction of nitrate to nitrite, which is the first and rate-limiting step of nitrogen assimilation in algae, fungi, and higher plants.[1] The nitrate reductase from the thale cress, *Arabidopsis thaliana*, is a dimer, with each of the ~103000 molecular weight monomers containing one molybdenum associated with a single pterin dithiolene cofactor, a flavin adenine dinucleotide cofactor, and a cytochrome *b*-type heme. During the catalytic cycle, reducing equivalents in the form of NADH enter the enzyme at the flavin site and are subsequently transferred by intramolecular electron transfer via the heme to the molybdenum center, where the two-electron reduction of nitrate takes place:

$$enzyme-Mo^{IV} + NO_3^- + 2H^+ \rightarrow$$
$$enzyme-Mo^{VI} + NO_2^- + H_2O$$
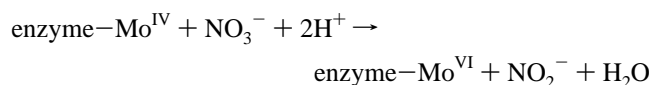
The assimilatory nitrate reductases are classified as members of the sulfite oxidase family of molybdenum enzymes.[1,2] The active-site structure of sulfite oxidase has been the subject of much study[3−6] and the oxidized enzyme possesses two Mo=O ligands, two Mo−S from the cofactor dithiolene, plus one Mo−S from a conserved cysteine residue.[4,6,7] The amino acid sequences of the molybdenum cofactor binding domain of assimilatory nitrate reductases and sulfite oxidases are very similar.[1,2] Because of this, closely related molybdenum active site structures have been widely assumed, with two Mo=O, plus dithiolene and cysteine (Cys-191) sulfur donors to molybdenum.[8] We report herein an extended X-ray absorption fine structure (EXAFS) study of the molybdenum site of *Arabidopsis* nitrate reductase, and show

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(8) The assimilatory nitrate reductase from *Chlorella vulgaris* has previously been studied by EXAFS spectroscopy [Cramer, S. P.; Solomonson, L. P.; Adams, M. W. W.; Mortenson, L. E. *J. Am. Chem. Soc.* **1984**, *106*, 1467−1471]. This study indicated an oxidized active site possessing two Mo=O and between two and three Mo−S ligands, and a reduced active site with a single Mo=O, about three Mo−S ligands and probably one Mo−O ligand.
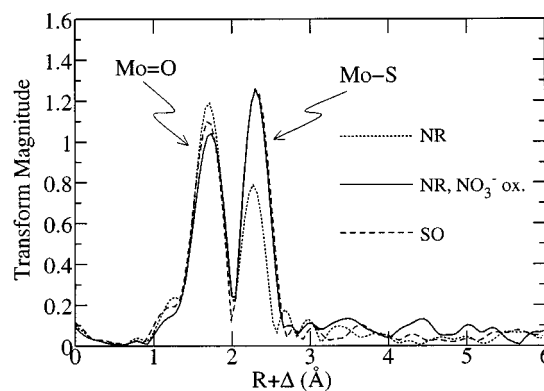
Figure 1. Molybdenum K-edge EXAFS Fourier transforms (phase-corrected for sulfur backscattering) of oxidized *Arabidopsis* nitrate reductase, compared with that of oxidized human sulfite oxidase.[4] The transforms were computed using identical *k*-ranges of 2−14 Å^{-1}.

that, unlike sulfite oxidase, the enzyme undergoes changes in Mo−S coordination during catalytic turnover.

In this paper we present the Mo K-edge EXAFS of *Arabidopsis* nitrate reductase in three different forms: oxidized as-isolated, reduced, and oxidized after catalytic turnover of excess nitrate (nitrate-oxidized).[9] Figure 1 compares the EXAFS Fourier transforms of oxidized as-isolated nitrate reductase and nitrate-oxidized nitrate reductase[9] with that of oxidized sulfite oxidase. The transforms show that the EXAFS of all three species is dominated by two major interactions, giving rise to the peaks at $R + \Delta \approx 1.8$ and 2.3 Å. These are attributable to Mo=O and Mo−S interactions at about 1.7 and 2.4 Å, respectively.[13] For all three Fourier transforms the Mo=O peak has similar intensity. However, while the transform of nitrate-oxidized enzyme is almost identical with that of oxidized sulfite oxidase,[4] the as-isolated oxidized nitrate reductase shows a significantly less intense Mo−S peak. Figure 2 shows the EXAFS data, the results of curve-fitting, plus the corresponding EXAFS Fourier transforms of nitrate reductase in oxidized as-isolated, dithionite reduced, and nitrate-oxidized forms. The curve-fitting analysis indicates two Mo=O and two ~2.4 Å Mo−S ligands for as-isolated enzyme, and two Mo=O and three Mo−S for the nitrate-oxidized sample. Gel filtration of the nitrate-oxidized sample restores the as-isolated oxidized enzyme (the EXAFS is identical), and the above results are reproducible with repeated cycles of reduction and reoxidation, indicating that redox-conditioning[14] is not occurring. Furthermore, addition of excess nitrite or nitrate to oxidized as-isolated enzyme

(9) *Arabidopsis* nitrate reductase was expressed in *Pichia* and purified as previously described[10] except that crude extracts were subjected to a 1.5−12.5% poly(ethylene glycol) (MW 8000) precipitation, and the resulting pellet was resuspended and bound to 5′AMP Sepharose, with elution by NADH. Specific activities of enzyme preparations were as previously reported.[10] EXAFS samples were prepared at a final concentration of approximately 0.3 mM Mo in 25 mM MOPS buffer at pH 7.0, with no added chloride. Enzyme was reduced by anaerobic addition of 10 mM dithionite and 40 *μ*M methyl viologen. Nitrate-oxidized samples were generated by adding 20 mM nitrate to reduced enzyme and equilibrating for 10 min at 4 °C. Data acquisition was carried out on SSRL beamline 7−3 as previously described,[5] using a sample temperature of 10 K. Between 10 and 16 35-min scans were accumulated for each sample. The EXAFS oscillations χ(*k*) were quantitatively analyzed using the EXAFSPAK suite of computer programs[11] employing ab initio theoretical phase and amplitude functions generated with the program FEFF version 7.02.[12] No smoothing or related manipulation was performed upon any of the data.
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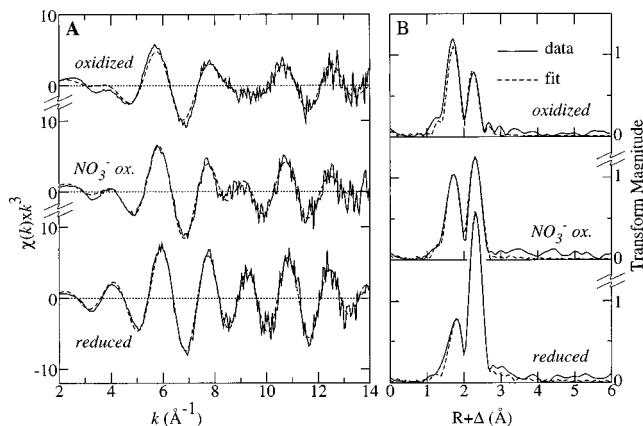(13) George, G. N. *JBIC* **1997**, *2*, 790−796.

Figure 2. Molybdenum K-edge EXAFS and EXAFS Fourier transforms of *Arabidopsis* nitrate reductase. The solid lines show experimental data, while the broken lines show the best fit. Part A shows the EXAFS oscillations and part B shows the corresponding EXAFS Fourier transforms, phase-corrected for sulfur backscattering. The best fit for oxidized, as-isolated enzyme was obtained with 2 Mo=O at 1.733(3) Å, $\sigma^2 = 0.0028$-(2) Å^2, 2 Mo−S at 2.404(5) Å, $\sigma^2 = 0.0039(3)$ Å^2, 1 Mo−S at 2.573(9) Å, $\sigma^2 = 0.0043(3)$ Å^2, for nitrate-oxidized enzyme 2 Mo=O at 1.714(3) Å, $\sigma^2 = 0.0032(2)$ Å^2, 3 Mo−S at 2.403(4) Å, $\sigma^2 = 0.0049(2)$ Å^2, and for dithionite reduced enzyme 1 Mo=O at 1.714(4) Å, $\sigma^2 = 0.0013(3)$ Å^2, 3 Mo−S at 2.398(3) Å, $\sigma^2 = 0.0022(1)$ Å^2, 1 Mo−O at 2.18(2) Å, $\sigma^2 = 0.0049(2)$ Å^2. As discussed in the text, the presence of a 2.57 Å Mo−S in oxidized as-isolated enzyme is only tentatively established. The σ^2 values are the Debye−Waller factors.[13] The values in parentheses are the estimated standard deviations obtained from the diagonal elements of the covariance matrix; we note that these precisions will be smaller than the accuracies which are typically estimated as ±0.02 Å for bond lengths and ±20% for coordination numbers and Debye−Waller factors.

caused no significant changes in the EXAFS. Thus, catalytic turnover causes changes in the Mo−S coordination in the oxidized enzyme, and these can be reversed by gel-filtration, suggesting involvement of a low molecular weight species. No such changes in Mo−S coordination are observed for sulfite oxidase.[4] The present data show no direct evidence for anion coordination to molybdenum,[5] although these might not be detectable by EXAFS because low atomic number scatterers can be elusive.[13] On the other hand, we also note that oxidized sulfite oxidase, which is very similar to nitrate-oxidized nitrate reductase, is not expected to have anion coordination to molybdenum.[4−6]

Inclusion of a third sulfur ligand in the curve-fitting gave a slightly improved fit (the fit-error[15] was reduced by 7%), with a Mo−S bond length of 2.57 Å. Unfortunately, despite the good signal to noise of our data,[16] this long Mo−S cannot be identified definitively because its EXAFS is nearly of opposite phase to that of the 2.40 Å Mo−S, and partial cancellation occurs.[13] This is why no well-defined 2.57 Å Fourier transform peak is observed in Figure 2. Nevertheless, the refined bond length and Debye−Waller factors are chemically reasonable, and are typical of Mo−S coordinated trans to a Mo=O group.[14,17] Thus, the oxidized and nitrate-oxidized active site structures may be simply related by a conformational change around molybdenum, one of three Mo−S ligands being located trans to one Mo=O in the as-isolated enzyme, re-arranging to a cis relationship following catalytic turn-
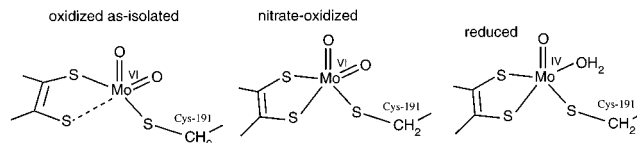

Figure 3. Postulated structures for the active sites of *Arabidopsis* nitrate reductase in oxidized as-isolated, nitrate-oxidized, and reduced forms. While geometric information is not directly available from the present EXAFS analysis, the oxo groups are expected to be cis,[22] as shown. One of the dithiolene Mo−S ligands is presumed to be trans to an Mo=O ligand in the oxidized as-isolated enzyme, although we note that other possibilities exist.

over (Figure 3). Ligand rearrangement from the cis to the trans position has also been observed following oxo-transfer in low molecular weight molybdenum complexes,[18] and oxo group activation by trans effects from thiolate ligation has been suggested to be important in some oxo-transfer reactions,[19] but evidence for trans oxo-thiolate coordination in the enzyme systems has been lacking.[20] Although the exact relevance to the catalytic mechanism remains to be established, the present work provides the first suggestion of such ligation in a molybdenum enzyme system.

The amino acid sequences of the molybdenum domain of nitrate reductase and sulfite oxidase are very similar. The recent crystal structure of sulfite oxidase[6] shows that the enzyme possesses a substrate binding pocket formed by three arginines, two of which are conserved in the active site of *Arabidopsis* nitrate reductase. The third arginine is replaced by a methionine residue (Met 473) in *Arabidopsis* nitrate reductase, but is not conserved among other nitrate reductases. The crystal structure of sulfite oxidase also shows a sulfate in this pocket that is quite distant from Mo (∼4.7 Å for the closest oxygen) thus structurally distinct from the anion complexes observed by Mo(V) EPR[2,3,5] and by EXAFS.[5] As pointed out by Kisker et al.,[6] the different arginine contents of sulfite oxidase and nitrate reductase can be rationalized in that nitrate has to be bound with one oxygen directed toward Mo. Other significant differences between the two enzymes include a tyrosine residue close to molybdenum in sulfite oxidase (Tyr 322), which is an asparagine (Asn 336) in nitrate reductase. This tyrosine residue has been suggested as a candidate for that controlling the transition between high-pH and low-pH Mo(V) sulfite oxidase;[2,3] however, the observation of analogous Mo(V) EPR signals in *Chlorella* nitrate reductase argues against this.[21]

In summary, our Mo K-edge EXAFS studies have provided direct evidence for conformational changes involving Mo−S ligation induced by catalytic turnover of nitrate to nitrite. This quite unexpected result indicates significant differences between the active site structures of sulfite oxidase and nitrate reductase. The nature of this conformational change is most probably due to increased trans effects for one of the Mo−S bonds, although dissociation of a thiolate ligand cannot be excluded.

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(15) For a definition of the EXAFS error function used, and the curve-fitting methods employed see ref 4.

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