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X-ray-absorption and electron-paramagnetic-resonance spectroscopic studies of the environment of molybdenum in high-pH and low-pH forms of *Escherichia coli* nitrate reductase

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Previous e.p.r. work [George, Bray, Morpeth & Boxer (1985) Biochem. J. **227**, 925–931] has provided evidence for a pH- and anion-dependent transition in the structure of the Mo(V) centre of *Escherichia coli* nitrate reductase, with the low-pH form bearing both an anion and probably a hydroxy-group ligand. Initial e.x.a.f.s. measurements [Cramer, Solomonson, Adams & Mortenson (1984) J. Am. Chem. Soc. **106**, 1467–1471] demonstrated the presence of sulphur (or chloride) ligands in the Mo(IV) and Mo(VI) oxidation states, as well as a variable number of terminal oxo (Mo=O) groups. To synthesize the e.p.r. and e.x.a.f.s. results better, we have conducted new e.p.r. experiments and complementary e.x.a.f.s. measurements under redox and buffer conditions designed to give homogeneous molybdenum species. In contrast with results on other molybdoenzymes, attempts to substitute the enzyme with ^{17}O by dissolving in isotopically enriched water revealed only very weak hyperfine coupling to ^{17}O. The significance of this finding is discussed. Experiments with different buffers indicated that buffer ions (e.g. Hepes) could replace the Cl^- ligand in the low-pH Mo(V) enzyme form, with only a small change in e.p.r. parameters. E.x.a.f.s. studies of the oxidized and the fully reduced enzyme were consistent with the e.p.r. work in indicating a pH- and anion-dependent change in structure. However, in certain cases non-stoichiometric numbers of Mo=O interactions were determined, complicating the interpretation of the e.x.a.f.s. Uniquely for a molybdenum cofactor enzyme, a substantial proportion of the molecules in a number of enzyme samples appeared to contain no oxo groups. No evidence was found in our samples for the distant 'heavy' ligand atom reported in the previous e.x.a.f.s. study. The nature of the high-pH–low-pH transition is briefly discussed.

INTRODUCTION

The dissimilatory nitrate reductase of *Eschericia coli* is the terminal enzyme in the respiratory chain of the bacterium grown anaerobically in the presence of nitrate (Adams & Mortenson, 1985). It is a molybdoenzyme (Bray, 1986, 1988), containing in addition to the pteridine molybdenum cofactor (Cramer & Stiefel, 1985), iron–sulphur clusters and a cytochrome *b* unit (Forget, 1974; Clegg, 1976; Johnson *et al.*, 1985). The molybdenum centre is thought to be the site of nitrate reduction and has been the subject of much study both by e.p.r. (Vincent & Bray, 1978; Vincent, 1979; George *et al.*, 1985) and by e.x.a.f.s. (Cramer *et al.*, 1984) spectroscopic techniques. The metal can exist as Mo(VI), Mo(V) or Mo(IV), with oxidation–reduction potentials reported to be $+220 \pm 20$ mV and $+180 \pm 20$ mV for Mo(VI)–Mo(V) and Mo(V)–Mo(IV) respectively (Vincent, 1979).

Vincent & Bray (1978) used e.p.r. spectroscopy to show that the Mo(V) form of the enzyme exhibits a pH-dependent equilibrium between high-pH and low-pH forms, the latter possessing a strongly coupled exchangeable proton not apparent in the high-pH species. It was assumed in this early work that the relation between the

high-pH and low-pH forms was a simple one in which the proton whose pK_a influences the interconversion was the coupled exchangeable proton of the low-pH species. More recently it has been shown that, as is the case for the closely related enzyme sulphite oxidase (Bray *et al.*, 1983), conversion from the high-pH into the low-pH form requires (George *et al.*, 1985) binding of an anion to the molybdenum site. Furthermore, studies by the latter workers on enzyme exchanged into ^2H_2O showed that one or more exchangeable protons contribute to unresolved hyperfine coupling in the high-pH e.p.r. signal. Anion binding in the low-pH form appears to have relatively little specificity; although nitrate binds with the greatest affinity, a wide variety of other anions (chloride, fluoride, nitrite, chlorate and bromate) can also bind to the molybdenum site (George *et al.*, 1985).

The previous e.x.a.f.s. studies on nitrate reductase (Cramer *et al.*, 1984) were performed before anion binding to the enzyme was known to occur, and under conditions that make comparison with the e.p.r. data difficult. In order to understand the effects of anion binding on the structure of the molybdenum site, we have re-examined nitrate reductase by e.x.a.f.s. and e.p.r.

spectroscopy, under conditions of controlled anion binding and with precautions to control the oxidation state.

MATERIALS AND METHODS

Sample preparation

Nitrate reductase was prepared in its intact form by the method of Morpeth & Boxer (1985), and was routinely characterized by activity measurements (Kemp *et al.*, 1975; Morpeth & Boxer, 1985), colorimetric molybdenum analysis with toluene-3,4-dithiol (Hart *et al.*, 1970) and e.p.r. spectroscopy. Typical preparations of the enzyme had specific activities of 17 μmol of NO_3^- reduced/min per nmol of Mo and contained about 20% of total molybdenum as Mo(V) (estimated by e.p.r.) and unknown amounts of Mo(IV) and Mo(VI).

Samples for e.x.a.f.s., at a final molybdenum concentration of about 0.6 mM, were exposed to air at 5 °C until less than about 5% of the molybdenum was present as Mo(V). Negligible losses of activity occurred during this process. The high-pH form was prepared in 0.3 M-sodium carbonate buffer, pH 10.2 (note that carbonate does not affect the e.p.r. spectrum and so presumably does not bind to molybdenum), and the low-pH/chloride form in Pipes/NaOH buffer, pH 6.8, in the presence of 0.3 M-KCl. Examination by e.p.r. indicated that in both samples essentially all of the residual Mo(V) was in the desired form. Samples required in the reduced [Mo(IV)] form were treated anaerobically with $Na_2S_2O_4$ at a final concentration of 10 mM for 10 min at 20–25 °C. This has been shown for numerous enzyme samples to cause complete disappearance of Mo(V) e.p.r. signals.

For experiments in ^{17}O-enriched water (from Monsanto Research Corp., Miamisberg, OH, U.S.A.; enrichment 52%), highly concentrated enzyme was diluted with enriched buffer and incubated for 2 h at 5 °C.

Preparation of ^{17}O-enriched $NaNO_3$

A 20 μl portion of 70% (w/w) HNO_3 was added to 100 μl of ^{17}O-enriched water (as above). The sample was heated in a sealed tube at 110–120 °C for 16 days (Anbar & Guttman, 1961) and, after cooling, neutralized by the addition of a stoichiometric quantity of solid Na_2CO_3. The final isotopic enrichment of the nitrate was checked by m.s. of thermally generated NO_2.

E.p.r. spectroscopy

Spectra were recorded for enzyme either as prepared or after addition of small amounts of dithionite to maximize Mo(V) signals (George *et al.*, 1985). Spectra of samples frozen in 3 mm- or 4 mm-internal-diameter silica tubes were recorded on a Varian E9 instrument linked to a computer and visual display system. Recording conditions were similar to those used by George *et al.* (1985). E.p.r. spectral simulations were performed as described by these workers.

X-ray-absorption data collection

X-ray-absorption spectra were collected at the Stanford Synchrotron Radiation Laboratory with the storage ring SPEAR, operating in dedicated mode at 3.0 GeV and with 30–70 mA ring current, on beam lines VII-3 and VI-2, with Si[2,2,0] crystal monochromators. The spectrometer was calibrated with an Mo foil standard, the spectrum of which was recorded simultaneously with

the data. The position of the first inflexion was taken as 20003.9 eV. The X-ray fluorescence-excitation spectrum was measured with an array of NaI scintillation detectors with Zr filters of appropriate thickness (Cramer & Scott, 1981; Cramer *et al.*, 1981). For each sample four to ten scans of 20 min duration were averaged. During data collection, samples were held at 90 or 4 K in an Oxford Instruments CF204 liquid-helium flow cryostat.

X-ray-absorption data analysis

The e.x.a.f.s. spectra, $\chi(k)$, were analysed quantitatively by using the following approximate expression (Cramer *et al.*, 1981):

$$\chi(k) \simeq \sum_b \frac{N_b A_{ab}(k)}{k R_{ab}^2} \cdot e^{-2\sigma_{ab}^2 k^2} \cdot \sin[2k R_{ab} + \alpha_{ab}(k)]$$

where k is the photoelectron wavenumber, N_b is the number of b-type atoms interacting with the absorber atom a, at a distance R_{ab}, and σ_{ab}^2 is the mean square deviation of R_{ab}. Parameterized empirical total phase shift $\alpha_{ab}(k)$ and total amplitude $A_{ab}(k)$ functions were used in all fits (Cramer, 1981). A fixed value of E_0, the threshold energy, of 20025 eV was used throughout.

RESULTS

E.p.r. studies: isotopic enrichment with ^{17}O

Enrichment with the spin-$\frac{5}{2}$ ^{17}O nucleus has been widely used in e.p.r. studies of the ligation of molybdenum in molybdoenzymes (see e.g., Bray & Gutteridge, 1982; Cramer, 1983). The effect of producing the high-pH Mo(V) e.p.r. signal with nitrate reductase exchanged into ^{17}O-enriched water is shown in Fig. 1. Only a slight broadening of the lineshape is observed, from a correspondingly small hyperfine coupling [$A_{av.}(^{17}O) = 0.238$ mT]. Neither ^{17}O-enriched NO_3^- nor ^{17}O-enriched water had any observable effect upon the e.p.r. lineshape of the low-pH nitrate signal (not illustrated). However, the small size of the coupling in the high-pH signal leads us to anticipate a similarly small coupling for the low-pH signal that could easily have been missed. Any broadening that was present in our spectra did not exceed that in the high-pH signal. In other experiments, allowing the enzyme to turn over ^{17}O-enriched NO_3^- (in ^{17}O-enriched or in unenriched water) did not produce any detectable changes in the low-pH nitrate e.p.r. signal. Significantly better signal-to-noise ratios or studies at lower microwave frequencies would be required for further work on ^{17}O effects. *E. coli* nitrate reductase thus appears to be unique among molybdenum cofactor enzymes in not showing resolved ^{17}O hyperfine couplings in its Mo(V) e.p.r. signals (Cramer *et al.*, 1979b; Gutteridge *et al.*, 1980; Gutteridge & Bray, 1980; Bray & Gutteridge, 1982; George & Bray, 1988).

E.p.r. studies: effect of Cl$^-$ on the low-pH–high-pH Mo(V) transition

For the X-ray spectroscopic work described in subsequent sections, chloride was chosen to produce the low-pH/anion-bound species, since it is a likely contaminant and because, unlike, e.g., bromate, it is not a substrate, so that studies of both oxidized and reduced enzyme forms can readily be carried out in its presence. [We did not use the e.x.a.f.s.-detectable anion, bromide, since the earlier finding by George *et al.* (1985) that bromide complexes with the enzyme to give small

g-value

2.00 1.99 1.98 1.97 1.96 1.95

(a)

(b)

(c)

(d)

(e)

334 336 338 340 342

Field (mT)
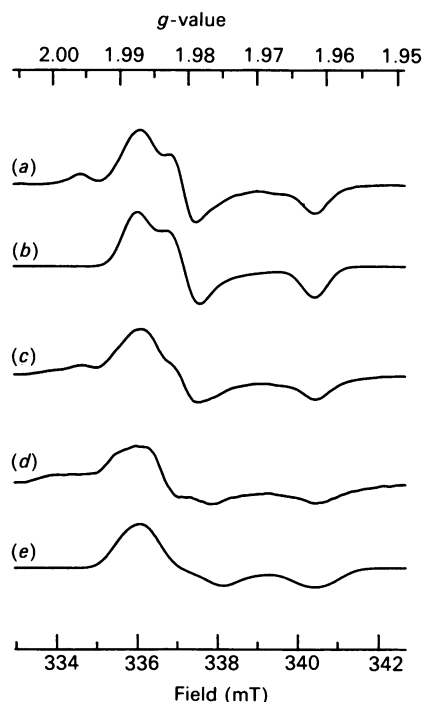
Fig. 1. Effect of ^{17}O substitution on the high-pH Mo(V) e.p.r. signal of nitrate reductase

(a) shows the signal of the control (unenriched) sample, (c) that in ^{17}O-enriched water (approx. 50 % enriched) and (d) is the difference spectrum corresponding to 100 % enrichment and one coupled oxygen atom. (b) and (e) are simulations of (a) and (d) respectively, with the following parameters (cf. George et al., 1985): $g_{x,y,z}$ 1.9878, 1.9809 and 1.9617; $A(^{17}O)_{x,z,y}$ 0.196, 0.319 and 0.200 mT; half-linewidths, 0.365, 0.350 and 0.410 mT (these values allow for unresolved ^{1}H hyperfine coupling); microwave frequency 9.348 GHz.
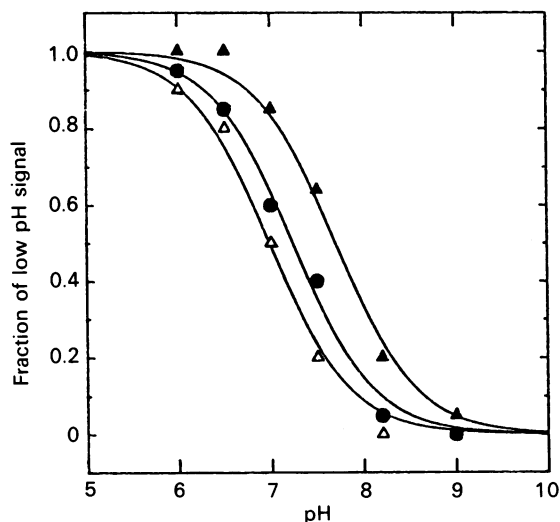

Fig. 2. Effect of pH and of chloride concentration on the high-pH/low-pH e.p.r. transition for nitrate reductase

Concentrations of chloride (as KCl) were 300 mM (▲), 5 mM (●) and approx. 0.2 mM (estimated concentration of contaminating chloride) (△). The fraction of the Mo(V) signal in the low-pH form is expressed on the basis of integrated signal intensities.

amounts of a well-defined e.p.r. spectrum could not be reproduced; it is possible that there was a field calibration error in the original work.] In order properly to characterize the conditions required to form the Mo(V) low-pH/chloride species, and to investigate the nature of the reaction giving the high-pH species, we performed pH titrations in the presence of different concentrations of chloride, as shown in Fig. 2. It can be seen that, although the trend is similar, the relation between pH and Cl⁻ binding is not quantitatively the simple one found for sulphite oxidase by Bray et al. (1983), in which a unit increase in p[Cl⁻] caused a unit decrease in the apparent pK_a for the high-pH-low-pH transition.

Effects of buffer ions on the e.p.r. spectra

One possible explanation of the different titration results for the two enzymes is that binding of buffer ions influences the equilibria for nitrate reductase. We investigated the effects of different buffers on the e.p.r. spectrum, and found that buffers containing sulphonic acid groups (specifically Mes, Hepes and Pipes) gave a slight but significant increase in g_x with respect to the chloride complex (Fig. 3). This result suggests that these buffers do indeed bind to the molybdenum site, presumably via their sulphonic acid moieties. Despite these complications, it is clear from Fig. 2 that an essentially homogeneous sample [at least for the e.p.r.-active Mo(V) oxidation state] can readily be made by a simple adjustment of conditions.

2.03 2.02 2.01 2.00 1.99 1.98 1.97 1.96 1.95 1.94

g-value
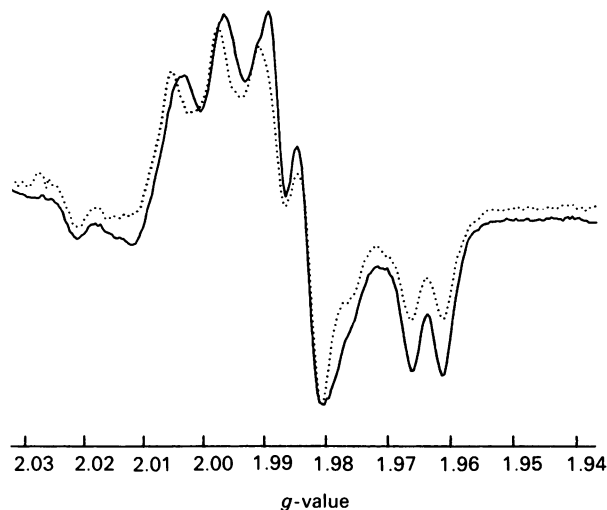
Fig. 3. Comparison of the low-pH e.p.r. spectrum of nitrate reductase in Hepes/NaOH buffer with that in the presence of an excess of Cl⁻ ions

·····, Difference spectrum for a sample of the enzyme in 50 mM-Hepes/NaOH buffer, pH 7.0, after subtraction of the accompanying high-pH signal. ——, Spectrum of the same sample, after addition of 300 mM-NaCl. In this case the signal was essentially all in the low-pH form and no high-pH signal was subtracted. Measurement of the g_x region of the spectrum in this and a number of comparable experiments gave, in Hepes/NaOH buffer alone, g_x 2.0017 and $A(^{1}H)_x$ 1.34 mT, and, with excess chloride, g_x 2.0003 and $A(^{1}H)_x$ 1.18 mT. Comparable effects were obtained if Pipes/NaOH or Mes/NaOH buffer was substituted for Hepes/NaOH buffer.
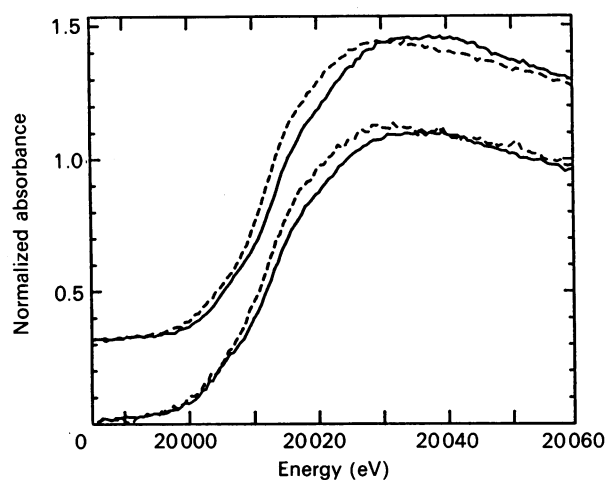
Fig. 4. Molybdenum X-ray-absorption edges of nitrate reductase

The upper and lower pairs of traces show the edges of low-pH and high-pH nitrate reductase samples respectively for oxidized (———) and reduced (------) samples. In this and in the other Figures the high-pH samples were run at a temperature of 90 K and the low-pH samples at 4.2 K.

Fig. 5. Fourier transforms of nitrate reductase e.x.a.f.s.

The upper and lower pairs of traces show the Fourier transforms of oxidized and reduced nitrate reductase respectively under high-pH (------) and low-pH (———) conditions. The transform k range used was 4–15 Å^{-1} in all cases.

X-ray-absorption edge spectra

The molybdenum X-ray K-edge spectra for high-pH and low-pH samples in both oxidized and reduced forms are shown in Fig. 4. None of the data exhibit the well-resolved $1s \rightarrow 4d$ bound-state transitions that are typical of dioxo-molybdenum compounds or dioxo-molybdenum enzymes such as sulphite oxidase (Cramer et al., 1981), assimilatory nitrate reductase (Cramer et al., 1984) or desulpho xanthine dehydrogenase (Cramer et al., 1981). This result is similar to that observed previously (Cramer et al., 1984), and suggests the presence of at most a single Mo=O interaction. The primary inflexion points of the reduced edges are shifted to lower energy by 1–2 eV relative to the oxidized spectra. Shifts in this direction are expected for a lower oxidation state of molybdenum in the reduced enzyme forms, although the displacement is significantly smaller than the 3–4 eV shift observed for sulphite oxidase or xanthine dehydrogenase (Cramer et al., 1979a, 1981).

E.x.a.f.s. analysis of high-pH samples

The high-pH oxidized and reduced forms exhibit essentially a single e.x.a.f.s. Fourier-transform peak (Fig. 5), assignable primarily to Mo–S interactions, without a well-resolved Mo=O component. The position of this major transform peak is shifted slightly to lower R under reducing conditions. Curve-fitting analysis of the e.x.a.f.s. (Fig. 6 and Table 1) indicates a change in Mo–S bond length from 2.41 to 2.34 Å (i.e. 0.241 to 0.234 nm). However, neither the high-pH oxidized nor the high-pH reduced data can be fitted well with Mo–S interactions alone. Additional Mo–O or Mo–N (nitrogen and oxygen are indistinguishable by e.x.a.f.s.) components, as well as a small Mo=O component, are required to fit the data (Table 1). With the σ value constrained to the chemically reasonable value of 0.045 Å, it was found that approx. 0.5 Mo=O interactions improved the fit significantly for both the oxidized and the reduced data (Table 1). Although e.x.a.f.s. amplitudes have often been notoriously inaccurate, Mo=O interactions have been quantified
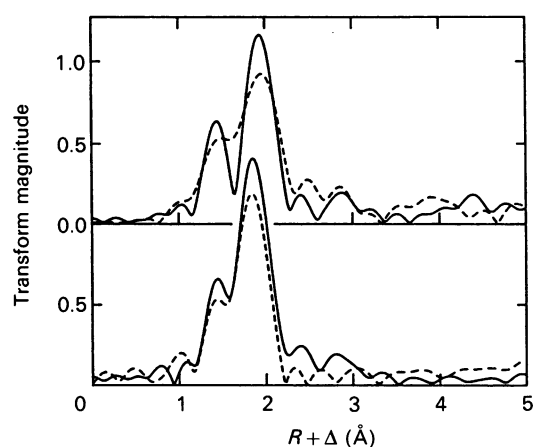
quite well (Cramer, 1981; Cramer et al., 1981). This sub-integral result thus suggests a mixture of oxo-free and oxo-bearing sites, despite our efforts to produce homogeneous samples.

E.x.a.f.s. analysis of low-pH/chloride samples

The oxidized low-pH/chloride species shows two well-resolved peaks, assignable primarily to Mo=O and Mo–S/Cl bond lengths. By using a σ value of 0.045 Å, 0.9 Mo=O interactions per Mo were calculated. This is consistent with a homogeneous mono-oxo Mo(VI) species, but a mixture of oxo-free and di- or tri-oxo species cannot immediately be excluded. The average Mo–S/Cl distance derived from curve-fitting was 2.39 Å. On reduction, the Mo=O feature is diminished, and the transform is dominated by a single peak, as for the high-pH forms. Curve-fitting found 0.5 Mo=O at 1.71 Å, as well as 4–5 S (Cl) at an average distance of 2.34 Å.

Comparison of low-pH/chloride and high-pH samples

A comparison for oxidized and reduced samples of the low-pH/chloride and the high-pH Fourier transforms (Fig. 5) shows an increase in the amplitude of the Mo–S feature in the presence of chloride. Additionally, the apparent Mo–S co-ordination number obtained in the e.x.a.f.s. curve-fitting is increased from 3–4 in the high-pH data to 4–5 in the low-pH/chloride data (Table 1). For the oxidized samples, the optimized value for σ also increased (despite the lower temperature used for the latter sample). These results are consistent with a chloride ligand to molybdenum in the low-pH Mo(VI) and Mo(IV) forms of the enzyme. Analogy with the Mo(V) forms of the enzyme (George et al., 1985) leads us to expect the presence of such Mo–Cl bonds in the low-pH/chloride forms of the enzyme. Chloride and sulphur ligands are not readily distinguishable by e.x.a.f.s., and, provided that the bond lengths are similar, chloride ligation should be reflected as an increase in the apparent Mo–S co-ordination number. Small differences between Mo–S and Mo–Cl bond lengths would have the effect of increasing the Debye–Waller factor in the fit.
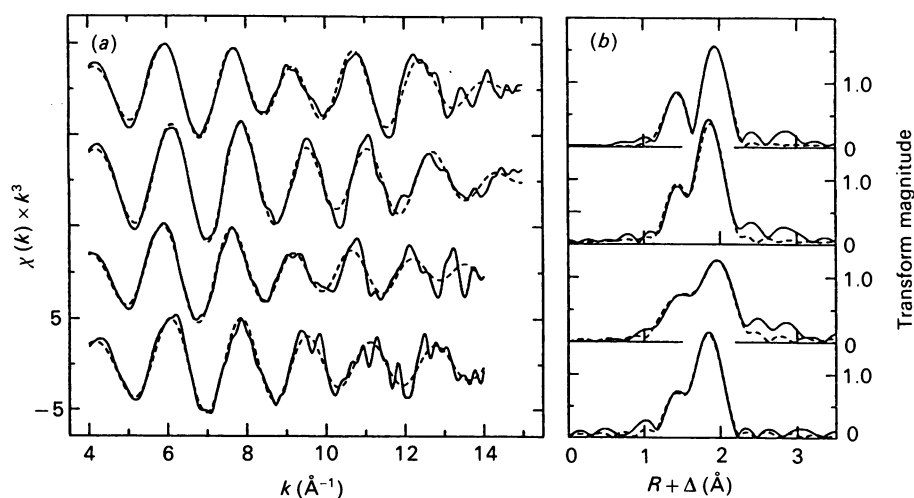
Fig. 6. E.x.a.f.s. and Fourier transforms of nitrate reductase

(a) E.x.a.f.s. spectra (———) and best fits (------) of nitrate reductase. The curves are for (top to bottom) low-pH oxidized, low-pH reduced, high-pH oxidized and high-pH reduced forms. All data were smoothed by convolution with a Gaussian window of width 0.15 Å^{-1}. (b) Fourier-transforms of the data and best fits. The parameters used to calculate the best-fit curves are given in Table 1.

Table 1. Nitrate reductase e.x.a.f.s.: summary of curve-fitting results

The number (N) and distance (R) of each type of ligand atom is given, with the Debye–Waller factor (σ). The data and the best fits are illustrated in Fig. 6. Residuals are given as the fit error function, defined as $[\Sigma(\chi_o - \chi_c)^2 k^6]/n$, where χ_o and χ_c are the observed and calculated e.x.a.f.s. respectively, and the summation is over all n points within the k range fitted (k is the photoelectron wavenumber). The Mo–S/Cl and Mo=O interactions have an approximate accuracy of ± 0.02 Å for R [uncertainity arising primarily from non-transferability of $\alpha(k)$], and $\pm 20\%$ for N and σ. For the Mo–O/N interaction (which gives the weakest e.x.a.f.s.) correlation with the intense Mo–O/S e.x.a.f.s. in the curve-fitting process results in a lower accuracy for R of ± 0.1 Å.

	Mo–S/Cl			Mo=O			Mo–O/N			
Sample	N	R (Å)	σ (Å)	N	R (Å)	σ (Å)	N	R (Å)	σ (Å)	Residual
Low-pH oxidized	4	2.392	0.072	1	1.726	0.042				0.984*
	4	2.391	0.072	1	1.730	0.053	1	2.003	0.043	0.460*
Low-pH reduced	4	2.338	0.065				1	2.100	0.094	0.796*
	4	2.337	0.066	0.5	1.707	0.040	1	2.116	0.089	0.537*
	5	2.330	0.076	0.5	1.709	0.052	1	2.243	0.071	0.514*‡
High-pH oxidized	3	2.414	0.065				1	2.138	0.047	0.982†
	3	2.414	0.063	0.5	1.727	0.025	1	2.134	0.030	0.712†
High-pH reduced	3	2.338	0.070				1	2.167	0.047	0.858†
	3	2.339	0.070	0.5	1.713	0.049	1	2.160	0.051	0.767†
	4	2.331	0.088	0.5	1.719	0.058	1	2.195	0.087	0.695†

* The fit range was $k = 4$–15 Å^{-1}.

† The fit range was $k = 4$–14 Å^{-1}.

‡ Although the improvement in the residual is small (4%) it should be noted that about 70% of the value given is due to high-frequency spectral noise, which constitutes a background that is unaffected by curve-fitting. Because of this, quite small changes in residual can be significant.

E.x.a.f.s. features from longer-distance interactions

The Fourier transforms of the high-pH reduced form of nitrate reductase and the reduced sample of the earlier study are compared in Fig. 7, with the long-distance-interaction transform peak indicated by the arrow. The long-distance heavy atom ('Mo–X') interaction reported previously (Cramer *et al.*, 1984) is not observed in the spectra of the current samples. The Mo–S interaction is also stronger in the current data, presumably because of

the use of liquid-N_2 or liquid-He temperature as opposed to barely frozen samples.

DISCUSSION

E.p.r. and e.x.a.f.s.

A major problem in any spectroscopic study is that of sample homogeneity. The Mo(V) e.p.r. signals observed in nitrate reductase, and in molybdenum enzymes
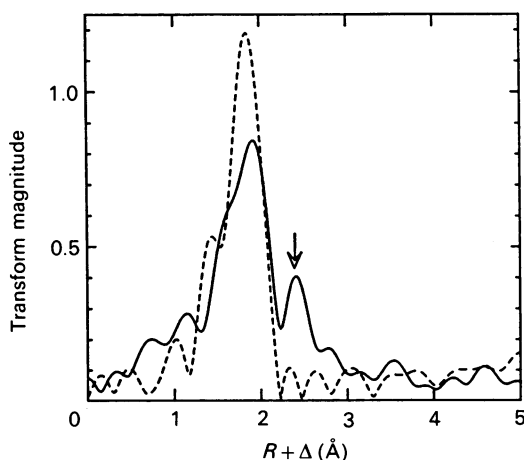
Fig. 7. Comparison of the e.x.a.f.s. Fourier transforms for the reduced high-pH sample (------) and the reduced sample reported by Cramer et al. (1984) (——)

The long-distance interaction indicated by the arrow is discussed in the text.

generally, have narrow linewidths, enabling complex mixtures of species to be characterized, with structural information forthcoming on individual species (Bray, 1984, 1988). The e.x.a.f.s. technique for studying ligation has relatively low distance resolution (approx. 0.11 Å in our case), which is often insufficient for resolving different components of mixtures. Hence, in the case of a mixture, it will generally yield average bond lengths and co-ordination numbers. For this reason, and because nitrate reductase does not yield 100% Mo(V) species, we have used e.x.a.f.s. to characterize the behaviour of the fully oxidized [Mo(VI)] or fully reduced [Mo(IV)] species. We used the e.p.r. signals of the approximately 5% of residual Mo(V) as an index of sample homogeneity. Of course, this method presumes that the Mo(IV) or Mo(VI) species that constitute the bulk of the sample follow the same equilibria as the Mo(V) species monitored by e.p.r., which unfortunately is not necessarily correct. Furthermore, there is always the possibility of additional forms of molybdenum that may be redox-inactive or e.p.r.-silent or both.

In the following sections, discussion of the e.p.r. results precedes that of the e.x.a.f.s.

Structural conclusions: Mo(V) species

Spectroscopic information on the structures of the molybdenum site in the high-pH and low-pH forms of the Mo(V) state of nitrate reductase consists solely of e.p.r. data for the enzyme and relevant model compounds (Cleland et al., 1987; Dowerah et al., 1987). Though ^{95}Mo couplings for the nitrate reductase species have not yet been properly evaluated (cf. Vincent & Bray, 1978), nevertheless e.p.r. parameters (George et al., 1985) for both the low-pH and high-pH species seem consistent, from the model compound work, with mono-oxo-molybdenum structures, even if non-oxo structures, for which little model compound data are available (cf. Stiefel, 1977), are not rigorously excluded.

Accepting mono-oxo structures, we consider first the high-pH Mo(V) species. As discussed above, we observed line broadening from ^{17}O. Our value for the hyperfine

coupling constant [$A(^{17}O)_{av.} = 0.238$ mT], though lower than that in other molybdoenzymes (see, e.g., Bray & Gutteridge, 1982; Morpeth et al., 1984), is consistent with an Mo=^{17}O structure. Possibly the weakly coupled proton detectable in the e.p.r. spectrum (George et al., 1985) is present as an Mo–NHR ligand, though direct evidence is lacking.

For the low-pH species, the model compound work (Dowerah et al., 1987) makes it likely that there is, in addition to the oxo group, as long predicted (cf. Bray, 1980), a hydroxy ligand, this being the source of the strongly coupled proton of the e.p.r. spectrum (George et al., 1985). The well-resolved ^{19}F coupling (George et al., 1985) observed in the presence of fluoride is direct evidence for an anion (in this case F$^-$) as an additional ligand in the low-pH species. The effects of Hepes and other buffer ions on the spectrum give further confirmation of the presence of an anion ligand. Our negative findings on the low-pH species when ^{17}O-substitution was used were based on spectra recorded at relatively poor signal-to-noise ratios, and further work is needed. Coupling to ^{17}O in both the oxo grouping and the hydroxy ligand might have been expected (cf. Dowerah et al., 1987). Failure to detect ^{17}O coupling certainly does not argue against an oxo ligand, since the small coupling expected for an axial oxo group could easily have been missed. Failure to detect coupling from a hydroxy-group oxygen atom on the other hand is more problematical, and might argue for –NH rather than –OH in this enzyme species. We also failed to detect ^{17}O coupling from enriched nitrate when this was the anion ligand (this complex is thought to be analogous to the Michaelis complex; George et al., 1985). However, since couplings to ^{17}O are expected (cf. Froese, 1966; Goodman & Raynor, 1970) to be an order of magnitude smaller than those to ^{19}F, our negative result does not undermine the conclusion (George et al., 1985) that all the low-pH Mo(V) anion complexes of nitrate reductase have similar structures.

Comparison with previous e.x.a.f.s. results

Despite the very close similarity of the Mo(V) e.p.r. signals (Johnson et al., 1985; George et al. 1985), there are some differences in the molybdenum e.x.a.f.s, and hence in the structure near molybdenum between the preparations of the enzyme used here, and in the earlier work of Cramer et al. (1984). We noted above that the 'Mo–X' interaction at about 2.8 Å seen in the previous data was not observed in the current spectra. One possible explanation is that this feature represents an Mo–O–P interaction, due to the binding of phosphate from the buffer. The possibility also exists that the heat-treatment method of purification used for the previous work (Cramer et al., 1984) might somehow modify the local structure of the molybdenum domain in e.p.r.-silent states. Finally, given the finite signal-to-noise of the data, there is always the possibility that the small e.x.a.f.s. feature was an experimental artifact. Although the exact identity of this peak remains uncertain, its absence in the current data indicates it is not essential for catalytic activity.

Structural conclusions: Mo(IV) and Mo(VI) states

As is indicated above, the e.x.a.f.s. results are consistent with the e.p.r. data in indicating pH- and anion-de-

pendent structural changes in the enzyme, involving the uptake of an anion ligand in the low-pH form. Detailed interpretation of the e.x.a.f.s. spectra of the reduced [Mo(IV)] and oxidized [principally Mo(VI)] samples is made difficult by the observation of non-stoichiometric amounts of terminal Mo=O species. Two alternative analyses are plausible at this stage. One interpretation starts with the e.p.r. evidence favouring an oxo ligand in the high-pH Mo(V) species, as discussed above. It explains the low Mo=O anomalies in terms of sample inhomogeneity, with, despite the extensive exposure to air, a substantial proportion of the molybdenum in all samples being in an e.p.r.-silent oxo-free form. Mo(IV) and Mo(V) compounds are not always readily air-oxidizable: one Mo(V) species from xanthine oxidase (Lowe *et al.*, 1976) is highly resistant to oxidation. Further work with other types of oxidants might be useful in this regard. We note, however, that previous work both with ferricyanide-oxidized and with nitrate-oxidized enzyme (Cramer *et al.*, 1984) did not appear to produce the expected dioxo-Mo(VI) species. If, for the low-pH samples, the presumed alternative Mo species was half the sample, and free of Mo=O groups, then the observed change for the total Mo would be consistent with transition of the active species between dioxo-Mo(VI) and mono-oxo-Mo(IV). Such a change has been observed previously for sulphite oxidase (Cramer *et al.*, 1981), *Chlorella* nitrate reductase (Cramer *et al.*, 1984) and desulpho xanthine oxidase and dehydrogenase (Cramer *et al.*, 1981; Turner *et al.*, 1989; S. P. Cramer & G. N. George, unpublished work). Consistent with the decrease in oxo content on reduction, the average −SR bond distance (Table 1) also decreased on reduction, this being in part due to a less-crowded co-ordination sphere. A comparable decrease in the average −SR bond length on reduction is also seen for the high-pH sample. This suggests there might be some decrease of oxo content here too on reduction [e.g. partial reduction of a 50% content of a mono-oxo Mo(VI) species], even though this is not apparent from the curve-fitting.

A second, very different, interpretation arises if it is assumed that the low-pH oxidized sample represents a single homogeneous mono-oxo-Mo(VI) species, with partial transformation of this, at high-pH values or on reduction, to a non-oxo species. Mono-oxo-Mo(VI) structures, though less common than those with dioxo or trioxo coordination, are well known in the literature (Mennemann & Mattes, 1977; Holzbach *et al.*, 1981; Young *et al.*, 1983; Bristow *et al.*, 1985; Gheller *et al.*, 1988). Structures tend to be hepta-co-ordinate, with a strongly bound oxo group, Mo=O bond lengths ranging from 1.68 to 1.70 Å (Mennemann & Mattes, 1977; Bristow *et al.*, 1985). The bond length of the Mo=O group in low-pH oxidized nitrate reductase (1.73 ± 0.02 Å) is slightly long for a mono-oxo-Mo(VI) species (Mayer, 1988).

Whichever interpretation is accepted, a substantial proportion of the molecules in three out of four of our samples appear to lack oxo groups. Such molecules represent a unique example among molybdenum cofactor enzymes of non-oxo molybdoenzyme species.

Concerning the nature of the high-pH/low-pH transition, the observation of an increase in oxo content of oxidized enzyme samples on decreasing the pH value is diametrically opposed to the proposal (Bray *et al.*, 1983) that [for the Mo(V) state] the high-pH–low-pH transition

involves replacement of an oxo group in the high-pH species by a hydroxy group and an anion ligand in the low-pH species. This transition is clearly complex (see also George, 1985; Bray, 1986), involving major changes in the structure of the molybdenum site, and is not yet fully understood. However, the Mo=O bond that is lost on going to the high-pH species would be difficult to break, particularly if the low-pH structure is a mono-oxo one, a process that would require attack by a group with considerable π-electron-donor quality. One possible mechanism might involve an −NH group (of either an amino acid side chain or part of the molybdenum cofactor), which, with increasing pH, could become deprotonated and then possess sufficient π-electron-donor quality to displace the Mo=O group. However, until the cause of the fractional contents of oxo groups in our samples is determined, it may be premature to speculate further.

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