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Vanadium *K*-edge X-ray-absorption spectroscopy of the functioning and thionine-oxidized forms of the VFe-protein of the vanadium nitrogenase from *Azotobacter chroococcum*

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Vanadium *K*-edge X-ray-absorption spectra were collected for samples of thionine-oxidized, super-reduced (during enzyme turnover) and dithionite-reduced VFe-protein of the vanadium nitrogenase of *Azotobacter chroococcum* (Ac1*). Both the e.x.a.f.s and the x.a.n.e.s. (X-ray-absorption near-edge structure) are consistent with the vanadium being present as part of a VFeS cluster; the environment of the vanadium is not changed significantly in different oxidation states of the protein. The vanadium atom is bound to three oxygen (or nitrogen), three sulphur and three iron atoms at 0.215(3), 0.231(3) and 0.275(3) nm respectively.

INTRODUCTION

In recent years three genetically distinct nitrogenase systems have been identified in *Azotobacter* (Bishop *et al.*, 1982, 1986; Joerger *et al.*, 1986; Robson, 1986). In addition to the well-characterized molybdenum nitrogenase system, both *Azotobacter vinelandii* (Hales *et al.*, 1986*a*,*b*) and *Azotobacter chroococcum* (Robson *et al.*, 1986; Eady *et al.*, 1987) have a vanadium containing nitrogenase, and *A. vinelandii* also has a system that apparently contains only iron (Chisnell *et al.*, 1988). E.p.r./m.c.d. (Morningstar & Hales, 1987; Morningstar *et al.*, 1987) studies on the VFe-proteins of vanadium nitrogenase indicate that these proteins contain similar redox centres to those of the MoFe-proteins of molybdenum nitrogenase. Furthermore, an iron–vanadium cofactor (FeVaco), analogous to the iron–molybdenum cofactor (FeMoco) that is the putative active site of molybdenum nitrogenase (Orme-Johnson, 1985; Eady, 1986), has been extracted from the VFe-protein of *A. chroococcum* (Ac1*) (Smith *et al.*, 1988). The present results and our earlier vanadium *K*-edge X-ray-absorption study of dithionite-reduced Ac1* (Arber *et al.*, 1987) demonstrate that vanadium is present as a VFeS cluster. A similar cluster arrangement has been found for the closely related system of *Azotobacter vinelandii* (George *et al.*, 1988).

MATERIALS AND METHODS

Ac1* was isolated and assayed as previously reported (Robson *et al.*, 1986; Eady *et al.*, 1987). Samples were prepared in an anaerobic glove-box under N_2 (less than 1 p.p.m. O_2). Ac1* was in 50 mM-Hepes buffer, pH 7.4, containing 100 mM-NaCl and 2 mM-$Na_2S_2O_4$, and concentrated to 165 mg/ml by ultrafiltration on a Minicon concentrator (Amicon). The specific activity of the Ac1* used was 1486 nmol of H_2 evolved/min per mg of protein. Thionine-oxidized protein was prepared without significant loss of activity by incubation with excess solid thionine for 5 min before freezing. E.p.r. spectroscopy showed the absence of the $g = 5.6$ feature associated with dithionite-reduced protein. Comparison with e.p.r. data from redox titrations (D. J. Lowe, personal communication) indicated that the redox potential of the solution was 0 ± 50 mV versus the normal hydrogen electrode. To prepare samples of Ac1* under turnover conditions 0.4 ml of Ac1* solution was cooled to 6 °C and added to a freeze-dried reaction mixture (0.2 ml) containing MgATP and an ATP-regenerating system (Eady *et al.*, 1972). After thorough mixing, 20 μmol of $Na_2S_2O_4$ was added. Enzyme turnover was initiated by the addition of Ac2* solution (0.2 ml, pre-cooled to 6 °C, containing 30 mg of protein). The final concentrations in the mixture were: $MgCl_2$ (27 mM), ATP (18 mM), phosphocreatine (50 mM), $Na_2S_2O_4$ (35 mM), Hepes buffer (50 mM) and Tris buffer (16 mM), with 200 μg of creatine kinase. The reaction mixture was transferred to a pre-cooled e.p.r. tube and an e.x.a.f.s. sample cell, and frozen in light petroleum (b.p. 60–80 °C) at −50 °C, 2.75 min after the addition of Ac2*. E.p.r. measurements indicated that Ac1* was >75% 'super-reduced' under these conditions. Samples were stored in liquid N_2 after being loaded anaerobically into cells.

X-ray-absorption spectra were recorded in fluorescence mode on e.x.a.f.s. station 7C at the Photon Factory operating at 2.5 GeV and an average current of 200 mA; about ten scans were recorded and averaged for each sample. A sagittal-focusing double-crystal Si(111) monochromator was used together with a 200 mm-long

Abbreviations used: x.a.n.e.s., X-ray-absorption near-edge structure; m.c.d., magnetic circular dichroism; FeMoco, iron–molybdenum cofactor; FeVaco, iron–vanadium cofactor; Ac1* and Ac2*, vanadium–iron- and iron-proteins of the vanadium nitrogenase of *Azotobacter chroococcum* respectively.

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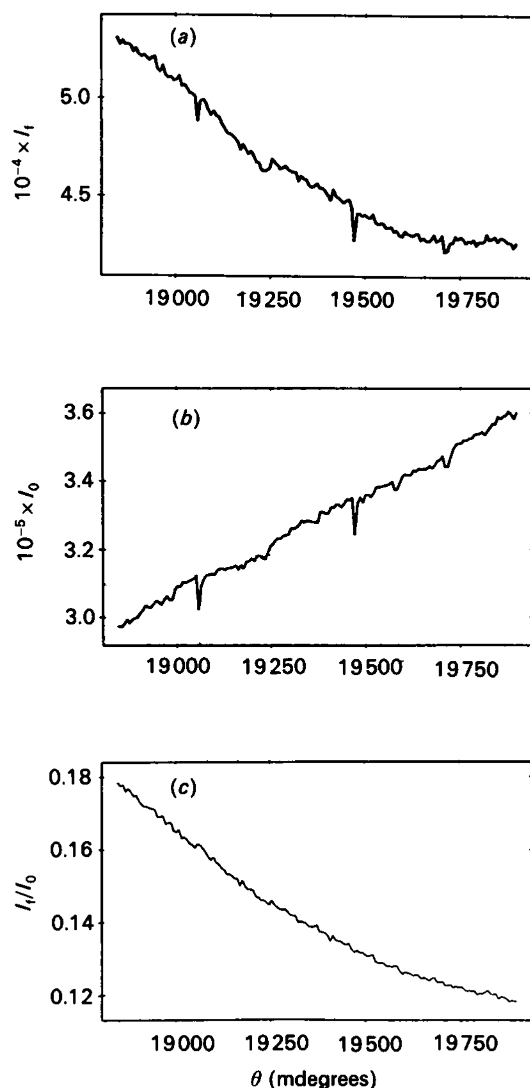

Fig. 1. Section of a scan of dithionite-reduced Ac1*: (*a*) fluorescent intensity (I_f); (*b*) incident intensity (I_0); (*c*) I_f/I_0

fused quartz mirror placed at an angle of approx. 8 mrad to decrease harmonic contamination. In addition, the careful alignment of the sagittal-focusing crystal resulted in an excellent normalization of the crystal glitches (see Fig. 1), which is frequently a serious problem in the study of metalloproteins by X-ray-absorption spectroscopy. Our initial study (Arber *et al.*, 1987) was limited in data range, primarily because of the presence of glitches. Non-normalization of crystal glitches results from the misalignment of the crystals with respect to the optic axis (S. S. Hasnain, C. Morrel, B. R. Dobson & M. Hart, unpublished work). The incident intensity was measured by using a windowless ionization chamber, and a Lytle cell, filled with 100% Ar and masked with a titanium foil, monitored the fluorescent intensity. During data collection, an average sample temperature of 80 K was maintained by use of a liquid-N_2 cryostat.

Data analysis was accomplished via the single-scattering curved-wave method of e.x.a.f.s. calculation, and phase-shifts were derived from calculations *ab initio* as described previously (Lee & Pendry, 1975; Perutz *et al.*, 1982; Gurman *et al.*, 1984).

RESULTS AND DISCUSSION

Edge and x.a.n.e.s. regions

Fig. 2 shows the vanadium *K*-absorption edge and x.a.n.e.s. of both the thionine-oxidized and the dithionite-reduced forms of Ac1*, together with a single scan for the protein during enzyme turnover in the presence of MgATP and Ac2*. The structure of the edge and x.a.n.e.s. are similar for all samples, namely a weak pre-edge feature and edge inflexion and a 'doublet' in the x.a.n.e.s., and also resemble those previously observed for the dithionite-reduced protein (Arber *et al.*, 1987). This overall similarity implies that no major changes are occurring in the local environment of the vanadium.

The intensity of the pre-edge feature (approx. $7 \pm 0.4\%$ of the edge height) implies distorted octahedral co-ordination of the vanadium in all the systems studied (Wong *et al.*, 1984). Furthermore, the size of the 'molecular cage', i.e. the distance from the absorbing atom to its nearest neighbours for a given co-ordination geometry, has been linked to the intensity of this feature (Kutzler *et al.*, 1980; Wong *et al.*, 1984), and thus the lack of any significant difference in its intensity in the thionine-oxidized, dithionite-reduced and super-reduced (during enzyme turnover) forms suggests little change in the first shell co-ordination distance or atom type. Slight differences in the resolution of the pre-edge feature from the edge are discussed below. The positions of the pre-edge feature and edge are listed in Table 1, the small shifts observed being within the estimated experimental errors (± 1 eV). As reported previously (Arber *et al.*, 1987), the position of these features relative to vanadium metal indicates an oxidation state of between V(II) and V(IV).

As noted above, the x.a.n.e.s. regions in all the samples studied are essentially of the same form (see Fig. 2). However, close inspection reveals slight differences in the relative intensities of the two maxima at approx. 5475 and 5486 eV. Thus from the dithionite-reduced to the thionine-oxidized form (Figs. 2*b* and 2*c*) the maximum at approx. 5486 eV has a greater intensity relative to that at 5475 eV. The x.a.n.e.s. profile for the 'super-reduced' sample (Fig. 2*a*) also resembles that of the dithionite-reduced sample (Fig. 2*b*), but the poor quality of the former data precludes further comment. The resolution of the pre-edge feature from the edge shows a similar variation, this feature being better separated from the

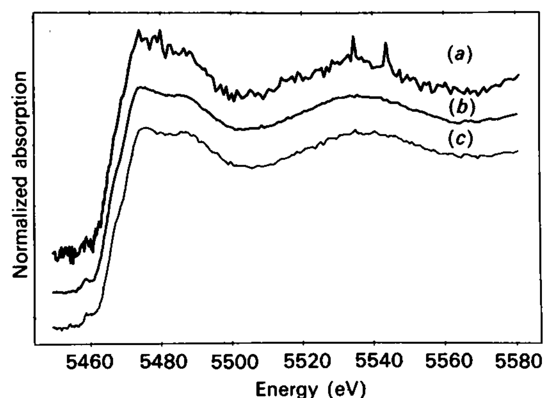

Fig. 2. Vanadium *K*-edge and x.a.n.e.s. of (*a*) super-reduced Ac1* (*b*) dithionite-reduced Ac1* and (*c*) thionine-oxidized Ac1*

Table 1. Position of pre-edge feature and edge in the vanadium *K*-edge X-ray-absorption spectra of Ac1* systems

Spectra were calibrated by comparison with vanadium-foil spectra, and edge position being taken as the point at half the normalized edge height. An internal energy calibration was not used during data collection and the estimated error is approx. ±1 eV.

System	Energy (eV)	
	Pre-edge	Edge
Super-reduced Ac1*	5459	5466
Dithionite-reduced Ac1*	5459 5460†	5466 5467†
Thionine-oxidized Ac1*	5459	5466

† Data from Arber *et al.* (1987).

edge in the data for the reduced and super-reduced forms than for the thionine-oxidized form. The small differences in the x.a.n.e.s. and pre-edge feature may indicate some slight variation in the vanadium environments and, bearing in mind the overall uniformity of the edge features noted above and the similarity of the e.x.a.f.s. (see below), angular rather than radial differences are implied. It is also noteworthy that, of the three samples studied, the edge and x.a.n.e.s of the thionine-oxidized sample most closely resemble the same data for a VFe_3S_4 cubane-like cluster, $[NMe_4][VFe_3S_4Cl_3(DMF)_3]$ (where DMF represents *NN*-dimethylformamide), suggested as a possible model for the vanadium environment of these centres (Kovacs & Holm, 1986).

E.x.a.f.s.

Fig. 3 shows the vanadium *K*-edge e.x.a.f.s. data for dithionite-reduced and thionine-oxidized Ac1* (Figs. 3*a* and 3*b* respectively) together with their Fourier transforms and simulations employing the parameters presented in Table 2. The two sets of data resemble each other closely, and are also very like the previously published e.x.a.f.s. data for the dithionite-reduced protein (Arber *et al.*, 1987). Hence it is not surprising that the data can, in both cases, be successfully simulated with back-scattering contributions from oxygen, sulphur and iron. For both forms of the protein, the distance of each shell from vanadium (Table 2) is in good agreement with that previously derived from a more limited range of e.x.a.f.s. data for the dithionite-reduced protein (Arber *et al.*, 1987). Each of these dimensions is essentially the same as the corresponding value in $[NMe_4][VFe_3S_4Cl_3(DMF)_3]$ (Kovacs & Holm, 1986). Therefore the VFeS framework is presumed to involve V(S)(S)Fe rhombs and, as noted earlier (Arber *et al.*, 1988), the bond angles involved mean that multiple scattering effects are not important.

The major differences between these simulations and

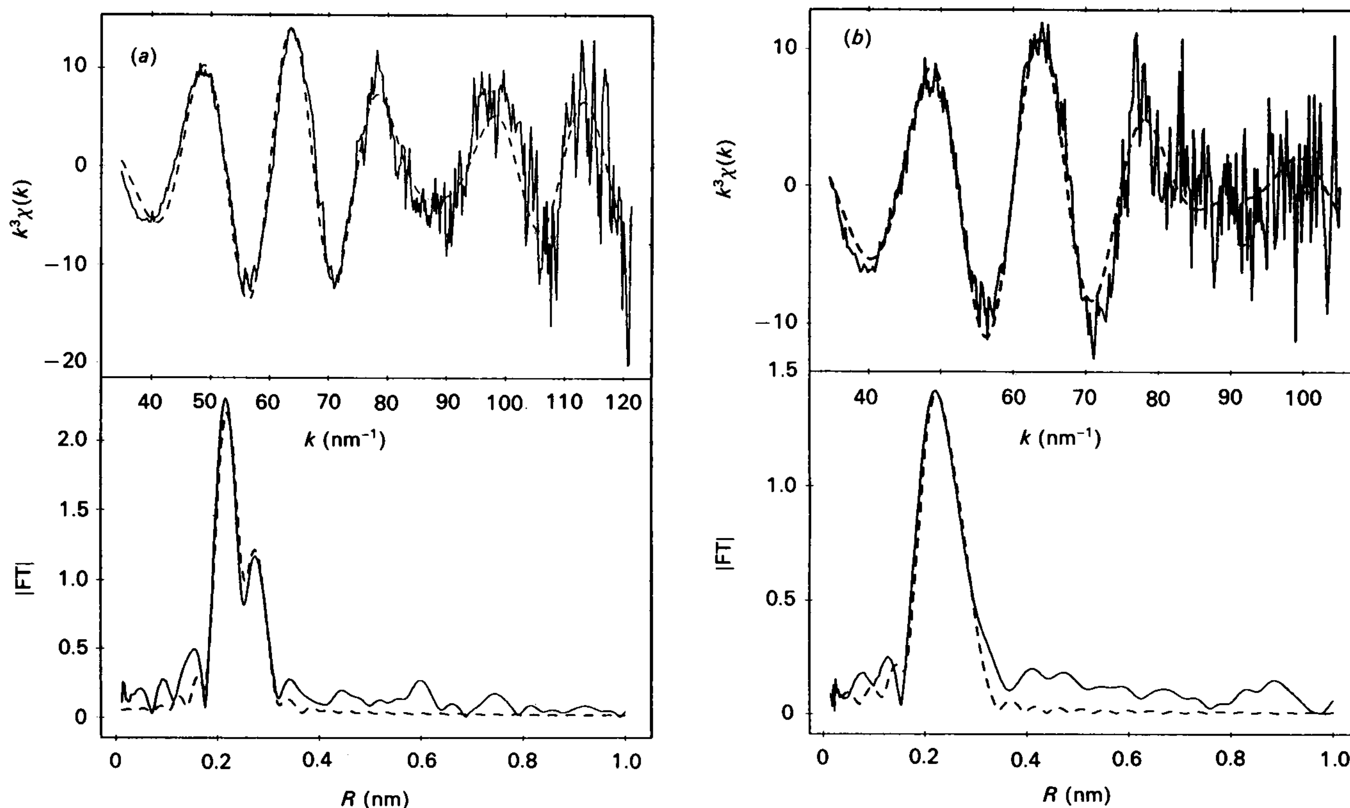

Fig. 3. Vanadium *K*-edge e.x.a.f.s. ($\times k^3$) and Fourier transform of (*a*) dithionite-reduced Ac1* and (*b*) thionine-oxidized Ac1*

Continuous lines represent experimental data; broken lines represent theory using the parameters presented in Table 2. N.B. The resolution in the Fourier transforms shown here is lower than that published by George *et al.* (1988), owing to the use of a broadening function that serves to minimize the truncation error.

Table 2. Parameters used to simulate the e.x.a.f.s. associated with the vanadium *K*-edge of dithionite-reduced and thionine-oxidized Ac1*

E_0 = 17.45 eV; values in parentheses indicate estimated errors. $2\sigma^2$ is the Debye–Waller parameter.

System	Atom	*N*	*R* (nm)	$2\sigma^2$ (nm^2)
Dithionite-reduced Ac1*	O†	3.0 (±1.0)	0.215 (3)	0.00001 (3)
	S	3.0 (±1.0)	0.231 (3)	0.00014 (4)
	Fe	3.0 (±1.0)	0.275 (3)	0.00013 (4)
Thionine-oxidized Ac1*	O†	3.0 (±1.0)	0.213 (3)	0.00007 (4)
	S	3.0 (±1.0)	0.234 (3)	0.00028 (5)
	Fe	3.0 (±1.0)	0.274 (3)	0.00019 (4)

† O and N are possible first-shell ligands.

those previously reported involve amplitude effects. Thus for both the dithionite-reduced and thionine-oxidized forms the data were best simulated with 3±1 oxygen and 3±1 sulphur atoms, compared with 4±1 and 2±1 respectively, previously suggested for the dithionite-reduced protein. These co-ordination numbers were those that produced the best agreement between experimental and simulated spectra, assuming a total of six ligands at vanadium, consistent with the comments above concerning octahedral co-ordination. For both samples of dithionite-reduced protein studied (i.e. the present and previous work) the total inner-shell (oxygen plus sulphur) back-scattering contributions are almost identical, as are the contributions from the iron shell. The enhanced contribution of the sulphur shell and decreased contribution from the oxygen shell found on least-squares refinement for the dithionite-reduced protein data presented here are attributed to the improved quality of the data and longer data range, which allow some independence between these two in-phase contributions. These factors also allow resolution of the iron from the oxygen and sulphur contributions in the Fourier transform manifested by the peak at approx. 0.27 nm (Fig. 3*a*). Thus this feature is absent from the more limited and poorer quality data for the thionine-oxidized protein (Fig. 3*b*), and refinement of similar back-scattering contributions results in a dominant contribution from oxygen back-scattering (Table 2), although the overall amplitude of the e.x.a.f.s. is lower than for the reduced sample, reflected in the higher Debye–Waller parameters for both the oxygen and the sulphur shells. Higher-quality data are required for the thionine-oxidized form of Ac1* for a definitive interpretation.

Therefore the edge, x.a.n.e.s. and e.x.a.f.s. data presented here for the dithionite-reduced and thionine-oxidized forms of Ac1* are consistent with vanadium being octahedrally co-ordinated in a similar environment to that proposed previously for the reduced protein and present in the synthetic compound $[NMe_4][VFe_3S_4Cl_3(DMF)_3]$. This situation is analogous to that of the MoFe-containing protein of nitrogenases, where X-ray-absorption studies of reduced and dye-oxidized samples showed that molybdenum is present as part of an MoFeS cluster, and that no appreciable differences in the molybdenum *K*-edge e.x.a.f.s. occur upon a change in the oxidation level of the protein (Cramer *et al.*, 1978*a*,*b*). Although electron–nuclear double-resonance studies have clearly demonstrated that the molybdenum nucleus in FeMoco interacts with unpaired electrons on the cluster (Venters *et al.*, 1986; Thomann *et al.*, 1987), e.p.r. studies on ^{95}Mo-enriched protein have shown that the interaction is slight (Smith *et al.*, 1973). Most of the unpaired electron spin is delocalized on to the iron atoms. This probably accounts for the insensitivity of the molybdenum *K*-edge e.x.a.f.s. to changes in the oxidation level of the cluster. Similar effects are probably operable in the case of the VFe cluster in the VFe-protein.

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