and kinetic results in the presence of various salts. We also thank Gail Feldman for typing the manuscript. We also deeply appreciate the assistance of George Rose in estimating the separation distance of side chains in dihydrofolate reductase.

Registry No. DHFR, 9002-03-3; Leu, 61-90-5; Glu, 56-86-0; Arg, 74-79-3; Gln, 56-85-9; Lys, 56-87-1.

REFERENCES

Ackers, G. K., & Smith, F. R. (1985) *Annu. Rev. Biochem. 54*, 597.

Amdur, I., & Hammes, G. G. (1966) *Chemical Kinetics*, McGraw-Hill, New York.

Baccanari, D., Phillips, A., Smith, S., Sinski, D., & Burchall, J. (1975) *Biochemistry 14*, 5267.

Baccanari, D., Averett, D., Briggs, C., & Burchall, J. (1977) *Biochemistry 16*, 3566.

Beasty, A. M., Hurle, M. R., Manz, J. T., Stackhouse, T., Onuffer, J. J., & Matthews, C. R. (1986) *Biochemistry 25*, 2965.

Becktel, W. J., Schellman, J. A., & Baase, W. A. (1987) *Biophys. J. 51*, 11a.

Bolin, J. T., Filman, D. J., Matthews, D. A., Hamlin, R. C., & Kraut, J. (1982) *J. Biol. Chem. 257*, 13650.

Cupo, J. F., & Pace, C. N. (1983) *Biochemistry 22*, 2654.

Hecht, M. H., Sturtevant, J. M., & Sauer, R. T. (1986) *Proteins: Struct., Funct., Genet. 1*, 43.

Hillcoat, B. L., Nixon, D. F., & Blakely, R. L. (1967) *Anal. Biochem. 21*, 178.

Howell, E. E., Villafranca, J. E., Warren, M. S., Oatley, S. J., & Kraut, J. (1986) *Science (Washington, D.C.) 231*, 1123.

Hurle, M. R., Tweedy, N. B., & Matthews, C. R. (1986) *Biochemistry 25*, 6356.

Hyde, C. C., Ahmed, S. A., Padlem, E. A., Miles, E. W., & Davies, D. R. (1988) *J. Biol. Chem. 263*, 17857.

Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1.

Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459.

Matthews, C. R. (1987) *Methods Enzymol. 154*, 498.

Pace, C. N., & Grimsley, G. R. (1988) *Biochemistry 27*, 3242.

Perry, K. M., Onuffer, J. J., Touchette, N. A., Herndon, C. S., Gittelman, M. S., Matthews, C. R., Chen, J. T., Mayer, R. J., Taira, K., Benkovic, S. J., Howell, E. E., & Kraut, J. (1987) *Biochemistry 26*, 2674.

Richardson, J. S. (1981) *Adv. Protein Chem. 34*, 168.

Robson, B., & Pain, R. H. (1976) *Biochem. J. 155*, 331.

Rogers, N. K., Moore, G. R., & Sternberg, M. J. E. (1985) *J. Mol. Biol. 182*, 613.

Russell, A. J., Thomas, P. G., & Fersht, A. R. (1987) *J. Mol. Biol. 193*, 803.

Schellman, J. A. (1978) *Biopolymers 17*, 1305.

Shortle, D., & Meeker, A. K. (1986) *Proteins: Struct., Funct., Genet. 1*, 81.

Shortle, D., Meeker, A. K., & Frieire, E. (1988) *Biochemistry 27*, 4761.

Smith, D. R., & Calvo, J. M. (1980) *Nucleic Acids Res. 8*, 2255.

Statistical Analysis Systems Institute Inc. (1985) *SAS User's Guide: Statistics*, Version 5 ed., p 575, Cary, NC.

Stone, S. R., & Morrison, J. F. (1984) *Biochemistry 23*, 2753.

Touchette, N. A., Perry, K. M., & Matthews, C. R. (1986) *Biochemistry 25*, 5445.

Wong, K.-P., & Tanford, C. (1973) *J. Biol. Chem. 248*, 8518.

Vanadium K-Edge X-ray Absorption Spectroscopy of Bromoperoxidase from *Ascophyllum nodosum*[†]

J. M. Arber,[‡,§] E. de Boer,[∥] C. D. Garner,[‡] S. S. Hasnain,[*,§] and R. Wever[∥]

Department of Chemistry, University of Manchester, Manchester M13 9PL, U.K., Daresbury Laboratory, Warrington WA4 4AD, U.K., and Laboratory of Biochemistry, University of Amsterdam, 1000 HD Amsterdam, The Netherlands

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ABSTRACT: Bromoperoxidase from *Ascophyllum nodusum* was the first vanadium-containing enzyme to be isolated. X-ray absorption spectra have now been collected in order to investigate the coordination of vanadium in the native, native plus bromide, native plus hydrogen peroxide, and dithionite-reduced forms of the enzyme. The edge and X-ray absorption near-edge structures show that, in the four samples studied, it is only on reduction of the native enzyme that the metal site is substantially altered. In addition, these data are consistent with the presence of vanadium(IV) in the reduced enzyme and vanadium(V) in the other samples. Extended X-ray absorption fine structure data confirm that there are structural changes at the metal site on reduction of the native enzyme, notably a lengthening of the average inner-shell distance, and the presence of terminal oxygen together with histidine and oxygen-donating residues.

Bromoperoxidase from the marine brown alga *Ascophyllum nodosum* was recently shown to be a vanadium-containing enzyme (de Boer et al., 1986a), in contrast to the more commonly recognized hemoproteins which are found in most haloperoxidases (Morrison & Schonbaum, 1976). This was the first demonstration of the existence of an enzyme with vanadium at the active site. Subsequently, vanadium-containing bromperoxidases have been isolated from other marine algal species (de Boer et al., 1986b) and vanadium nitrogenases identified (Hales et al., 1986; Robson et al., 1986). Steady-state kinetic studies on vanadium-containing bromoperoxidases have suggested the existence of a bromoperoxidase–bromide inhibitory complex and a bromoperoxidase–hydrogen peroxide intermediate (de Boer et al., submitted for publication). The

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[‡] University of Manchester.

[§] Daresbury Laboratory.

[∥] University of Amsterdam.

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Table I: Vanadium K-Absorption Edge Data for Bromoperoxidase

bromoperoxidase sample	ΔPEF[a] (eV) (±0.02)	ΔE[b] (eV) (±0.4)	I[c] (%) (±5)	$\nu_{1/2}$[d] (eV) (±0.3)	A[e]	η[f]
native	4.0	8.0	63	1.8	1.13 ± 0.21	2.52 ± 0.14
native + H_2O_2	4.0	8.1	59	1.7	1.00 ± 0.20	2.52 ± 0.14
native + Br^-	3.9	7.8	75	1.8	1.35 ± 0.24	2.45 ± 0.14
reduced	3.1	5.7	34	2.4	0.82 ± 0.16	1.91 ± 0.13

[a] Shift in energy of preedge feature relative to the energy of the preedge feature of a vanadium foil spectrum (≡5456.0 eV). [b] Shift in edge position measured as the energy at half the edge height after normalization and calibration against a vanadium foil spectrum, where edge energy is equal to 5463.5 eV. [c] Intensity, i.e., percentage height of the preedge feature relative to the normalized edge height. [d] Width of the preedge feature at half-maximum height. [e] $A = I\nu_{1/2}$. [f] Coordination charge derived from energy of preedge feature (see Figure 3).

presence of such an intermediate is supported by the ability of vanadium(V) compounds to form stable peroxo–vanadium complexes with hydrogen peroxide (Mimoun et al., 1983). EPR[1] studies have shown (de Boer et al., 1986a, 1988a) that vanadium in native bromoperoxidase is present in the +5 oxidation state since no signal is observed for the native enzyme, but upon reduction an apparently axial EPR signal is formed with g values and hyperfine splittings characteristic of a vanadyl cation (VO^{2+}) (de Boer et al., 1986a, 1988). In addition, it has been suggested that vanadium(IV) is not involved in catalytic turnover, and a model has been proposed in which the vanadium(V) of the native enzyme serves only to bind both hydrogen peroxide and bromide. A ^{51}V NMR study of native bromoperoxidase (Rehder et al., 1987; Vilter & Rehder, 1987) has led to the suggestion that vanadium is coordinated by 6–8 oxygens. We recently reported X-ray absorption spectra for the VFe protein from the nitrogenase of *Azotobacter chroococcum* (Arber et al., 1987). This technique has now been applied to the native, native plus bromide, native plus hydrogen peroxide, and dithionite-reduced forms of the vanadium-containing bromoperoxidase from *A. nodosum*, and we present here vanadium K-edge X-ray absorption spectra for this enzyme.

MATERIALS AND METHODS

Bromoperoxidase was prepared according to Wever et al. (1985) as modified by de Boer et al. (1986a). Enzyme (2.7 mM) which contained 2.7 mM vanadium was dissolved in 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 100 mM Na_2SO_4, and 10% glycerol, pH 6.0. After addition of sodium dithionite, hydrogen peroxide, and potassium bromide to the appropriate samples, all samples were frozen and stored in liquid nitrogen. Brominating activity was measured before and after the EXAFS experiments; X-ray irradiation had no effect on enzymic activity.

X-ray absorption spectra for the enzyme samples were recorded in fluorescence mode on EXAFS station 8.1 at the Daresbury Synchrotron Radiation Source operating at 2 GeV and an average current of 130 mA, a single NaI scintillation detector with a titanium foil being used to record the fluorescent intensity. A slitless double-crystal Si(111) monochromator was employed, thus minimizing harmonic contamination (van der Hoek et al., 1986), the novel design of the monochromator (Dobson et al., 1986) allowing high-resolution data to be collected. Energy resolution of the monochromator is 1 eV at the vanadium K-edge. As noted previously (Arber et al., 1987), crystal glitches limited the data range to approximately 400 eV beyond the edge. Samples were maintained at ~80 K during data collection by use of a liquid nitrogen cryostat. For each bromperoxidase sample ~15 scans were averaged. Vanadium K-edge X-ray absorption data were also collected for a number of model compounds. These data were collected on powdered samples at room temperature in transmission mode and a single scan was considered sufficient.

Data analysis was accomplished via the curved-wave method for EXAFS calculation, and phase shifts were derived from ab initio calculations (Lee & Pendry, 1975; Perutz et al., 1982; Gurman et al., 1984, 1986). Multiple scattering from outer-shell atoms was included in a manner described elsewhere (Strange et al., 1987). Atoms of an imidazole group were refined initially as a rigid group, thus minimizing the number of free parameters. An overall E_0 was used, as this has been found to be sufficient for cases where all the backscattering atoms are of a similar nature. Refinement of the Debye–Waller factor was done in the last stage of refinement. Similarly, N values have been held constant and have not been treated as free parameters. Thus in the final three-shell model, we have refined distances of each shell and a single overall E_0, giving a total of four free parameters. Debye–Waller factors have been refined as constrained parameters. In addition to the least-squares refinement, both statistical (Joyner et al., 1987) and visual inspection of the agreement between the theoretical and experimental EXAFS and their Fourier transforms was used to assess the coorectness of the model. All data analysis has been performed on the raw EXAFS in k space. Direct fitting of multishell data in k space avoids the complexities and pitfalls of Fourier filtering (Lytle et al., 1988). Fourier filtering has only been used as an aid to define the nature of the inner shell while the final refinement was done on the raw data. Further details of the analysis procedure are described elsewhere (Perutz et al., 1982; Strange et al., 1987; Hasnain, 1987).

RESULTS AND DISCUSSION

Figure 1 shows the vanadium K-edge and X-ray absorption near-edge structure (XANES) for the four samples studied. The profile and position of the edge and XANES for the native, bromide, and peroxide samples are very similar; the data for the reduced enzyme differ considerably. This implies that the environment of the vanadium is altered considerably on reduction but no major changes take place on addition of bromide or peroxide to the native enzyme. Position and intensity data for the edge and preedge feature are summarized in Table I for the four enzyme samples. It should be noted that at pH 6.0 the equilibrium between bromoperoxidase and bromide and the enzyme–bromide complex requires ~700 mM bromide (de Boer & Wever, 1988). In the sample studied here ~250 mM bromide was present. Thus, less than 50% of the preparation was enzyme–bromide complex. Similarly, on addition of hydrogen peroxide to native bromoperoxidase there is a release of oxygen. Although all samples were rapidly frozen in liquid nitrogen, such a reaction may reduce the probability of observing an enzyme–peroxide intermediate. Despite these experimental difficulties a major change in the vanadium environment upon addition of bromide or hydrogen peroxide can be ruled out as such a rearrangement would result in a different component both in XANES and also in the

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[1] Abbreviations: EPR, electron paramagnetic resonance; ESE, electron spin echo; EXAFS, extended X-ray absorption fine structure; XANES, X-ray absorption near-edge structure.

Table II: Vanadium K-Absorption Edge Data for Some V(IV) and V(V) Compounds

vanadium compd (inner-shell coordination)	ref	ΔPEF[a] (eV) (±0.1)	ΔE[b] (eV) (±0.2)	I[c] (%) (±3)	$\nu_{1/2}$[d] (±0.2)	A[e]	$\bar{R}$[f] (Å)	η[g]
A: NH_4VO_3 [V(V)–4 O]	*h*	4.1	6.9	85	3.1	2.64 ± 0.19	1.72	2.80
B: V_2O_5 [V(V)–5 O]	*i*	4.3	6.9	64	2.9	1.86 ± 0.15	1.83	2.80
C: $NH_4[VO_2(dipicolinate)]H_2O$ [V(V)–4 O, 1 N]	*j*	4.1	6.5	78	2.1	1.64 ± 0.17	1.86	2.64
D: VO(pentane-2,4-dionate)$_2$ [V(IV)–5 O]	*k*	3.4	5.6	55	2.3	1.27 ± 0.13	1.89	2.24
E: VO(2-methyl-8-quinolinolate)$_2$ [V(IV)–3 O, 2 N]	*l*	3.0	4.7	54	2.4	1.30 ± 0.13	1.90	1.97
F: VO[*N*,*N*′-ethylenebis(salicylideneaminate)] [V(IV)–3 O, 2 N]	*m*	3.1	5.1	45	2.5	1.13 ± 0.12	1.91	1.97
G: $K_3[VO_2(oxalate)_2]$ [V(V)–6 O]	*n*	4.7	6.6	54	2.3	1.24 ± 0.13	1.95	2.80
H: (tmed)VO(malonate)$_2$$H_2O$ [V(IV)–6 O]	*o*	3.8	5.3	40	2.4	0.96 ± 0.11	1.97	2.24
I: VO[*N*-(2-pyridylmethyl)iminodiacetate] [V(IV)–4 O, 2 N]	*p*	3.4	5.0	39	2.4	0.94 ± 0.11	2.01	2.02
J: [VOCl(vinyl-imid)$_4$]Cl [V(IV)–O, 4 N, Cl]	*q*	3.6	4.0	38	2.2	0.86 ± 0.10	2.09	1.71
K: $K_3[VO(O_2)(ox)_2]$ [V(V)–7 O]	*n*	4.4	7.4	44	1.9	0.84 ± 0.10	1.96	2.80
L: $VO(O_2)$(bipy)(pyridine-2-carboxylate) [V(V)–4 O, 3 N]	*r*	4.3	6.5	46	1.8	0.83 ± 0.11	1.99	2.45

[a] Shift in energy of preedge feature relative to the energy of the preedge feature of a vanadium foil spectrum (≡5456.0 eV). [b] Shift in edge position measured as the energy at half the edge height after normalization and calibration against a vanadium foil spectrum, where edge energy is equal to 5463.5 eV. [c] Intensity, i.e., percentage height of the preedge feature relative to the normalized edge height. [d] Width of the preedge feature at half-maximum height. [e] $A = I\nu_{1/2}$. [f] Average inner-shell distance. [g] Coordination charge (see Figure 3 caption). [h] Hawthorne & Calvo, 1977. [i] Bachmann et al., 1961. [j] Nuber et al., 1978. [k] Dodge et al., 1961. [l] Shiro & Fernando, 1971. [m] Riley et al., 1986. [n] Stomberg, 1986. [o] Pajunen & Pajunen, 1980. [p] Ooi et al., 1979. [q] Calviou et al., unpublished results. [r] Sventivanyi & Stomberg, 1983.

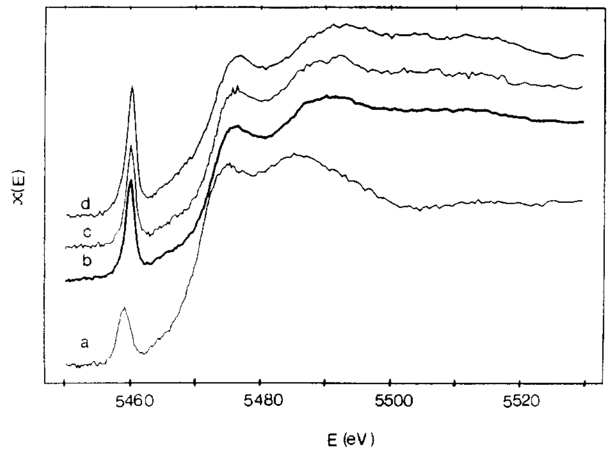

FIGURE 1: Vanadium K X-ray absorption edge and near-edge structure of bromoperoxidase: (a) dithionite-reduced bromperoxidase, (b) native bromoperoxidase, (c) native bromoperoxidase plus 10 mM hydrogen peroxide, and (d) native bromoperoxidase plus 250 mM potassium bromide.

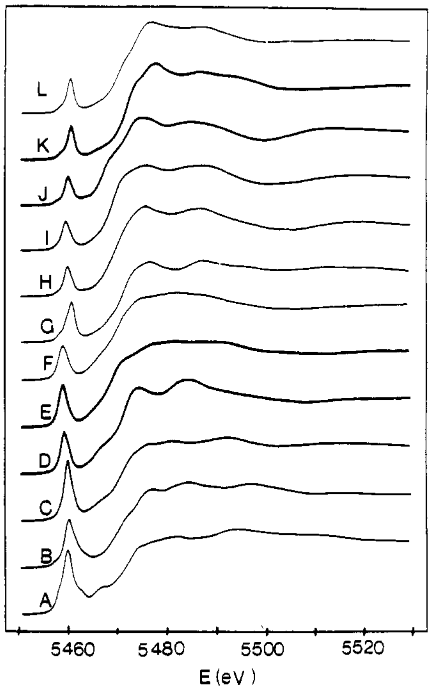

FIGURE 2: Vanadium K X-ray absorption edge and near-edge structure of some vanadium(IV) and vanadium(V) compounds (see Table II for details).

EXAFS region. However, we cannot rule out some subtle rearrangement at the vanadium site.

Previous studies (Wong et al., 1984; Hallmeier et al., 1986) have demonstrated the wealth of information available from the vanadium K-edge region. In particular, the energy of the edge features has been correlated with oxidation state or, more generally, coordination charge, and the area under the preedge feature has been correlated with inner-shell coordination distance and geometry. Figure 2 shows the vanadium K-edge profiles of several vanadium(IV) and vanadium(V) systems; relevant parameters are given in Table II. These data confirm the previously observed trend of increasing energy of the preedge feature and edge with increasing coordination charge (Wong et al., 1984; Hallmeier et al., 1986). For example, comparison of the data for V_2O_5 (B) and VO(acac)$_2$ (D) shows that a change in oxidation state from vanadium(V) to vanadium(IV) results in smaller edge and preedge shifts. Similarly, the data for (tmed)VO(mal)$_2$$H_2O$ (H) and VO(pmida) (I) demonstrate the reduction in the shift observed when oxygen is replaced by (less electronegative) nitrogen. These data are summarized in Figure 3 where the shifts in the position of the preedge feature (relative to a vanadium foil) are plotted versus coordination charge (η, see Figure 3 caption). Least-squares analysis of the data presented in Figure 3 produces the line shown, and application of the parameters derived to the data for bromoperoxidase provides estimates of the coordination charge for each sample as shown in Table I. (The well-defined nature of the preedge peak makes its use preferable to that of the edge.) These values are consistent with a change in oxidation state from vanadium(V) in the native, bromide, and peroxide samples to vanadium(IV) in the reduced enzyme, as implied by EPR studies (de Boer et al., 1986a, 1988a), and with oxygen/nitrogen inner-shell coordination (compare Figure 2 and Table II), as suggested by a recent electron spin-echo (ESE) study (de Boer et al., 1988b).

In addition to the change in position of the preedge peak on reduction of bromoperoxidase, Figure 1 shows the dramatic change in the profile of this feature. The height of the peak is approximately halved, and it is significantly broadened (Table I). The origin of this absorption feature is a 1s to 3d transition, i.e., a dipole-forbidden transition (Shulman et al., 1976). Therefore, in perfect octahedral systems no transition is observed, but as the symmetry is lowered and ligand p and metal 3d orbitals overlap, its intensity increases. However, it is not possible to directly relate the intensity of the preedge

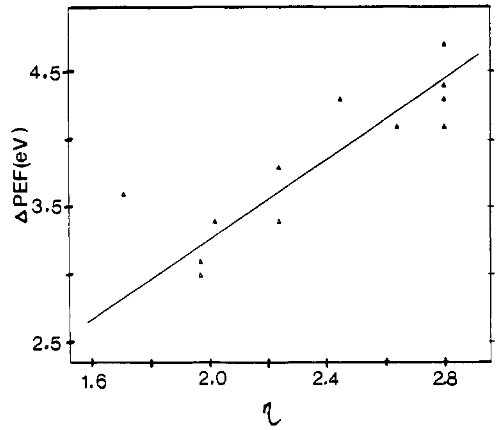

FIGURE 3: Plot of energy shift of preedge feature (ΔPEF) vs coordination charge (η) for the vanadium(IV) and -(V) compounds shown in Table II and Figure 2. The coordination charge was calculated as by Wong et al. (1984); i.e., the single-bond ionicity was calculated by using Pauling electronegativities (Cotton & Wilkinson, 1972) and the coordination charge calculated as the product of the vanadium oxidation state (Z) and the ionicity (I) ($\eta = ZI$), where $I = 1 - \exp[-{}^1/_4(\chi_A - \chi_B)^2]$ and χ_A and χ_B are the electronegativities of the surrounding atoms and the central atom. Where necessary, a weighted average electronegativity was used. Least-squares analysis of the data produces the following parameters: gradient 1.48 and intercept 0.29. (The data for [VOCl(vinyl-imid)$_4$]Cl are plotted but were not included in the least-squares analysis. The deviaton of this compound from the general trend is presently not understood.)

feature to a particular geometry as it is also affected by the average inner-shell coordination distance. This is known as the molecular cage size effect (Kutzler et al., 1980). For example, compounds B–F (Figure 2 and Table II) are all distorted square pyramidal. As the average inner-shell distance ($\bar{R}$) increases, the area under the preedge feature (A) decreases. Thus, the loss of intensity in the preedge feature on reduction of bromoperoxidase is consistent with a decrease in coordination number for vanadium and/or a slightly longer average first shell distance. Despite the limited quantitative information extracted from this part of the edge, the qualitative observation of the change in the preedge feature on reduction coupled with the change in the XANES (Figure 1) indicates that the vanadium environment is substantially altered.

The changes observed in the edge and XANES regions on reduction of the active (i.e., native) enzyme (see above) are further reflected in the very different EXAFS of the two samples (Figure 4). The EXAFS amplitude is decreased and the frequency increased on reduction of the native enzyme (Figure 4), supporting the idea deduced above that a significant change has taken place in the vanadium environment.

In the reduced sample (Figure 4a) the dominant contribution is from light atom backscattering at ~2 Å. A smaller but clearly resolved peak is observed in the Fourier transform at ~1.5 Å. The transform also indicates that other light atom backscattering contributions occur at ~3.0 and 4.3 Å. To define the nature of inner coordination sphere, analysis of Fourier filtered data (k^3 weighted, 0.8–2.5 Å) has been carried out in addition to the simulation on the raw data. Such an analysis shows that the introduction of a short (~1.6-Å) oxygen backscattering contribution (in addition to four or five other light atoms at ~1.96 Å) decreases the fit index (Joyner et al., 1987) by ~80%. Further splitting of the remaining inner-shell ligands is supported by the improved simulation (fit index decreases by more than 15%) when, in addition to the short oxygen, backscattering from two or three oxygens at ~1.90 Å and two oxygens/nitrogens at ~2.05 Å is included. The differences between the split shell five and six coordinate models, and also between those employing oxygen and nitrogen backscattering as opposed to oxygen only, are insignificant (fit index varies by ~3%).

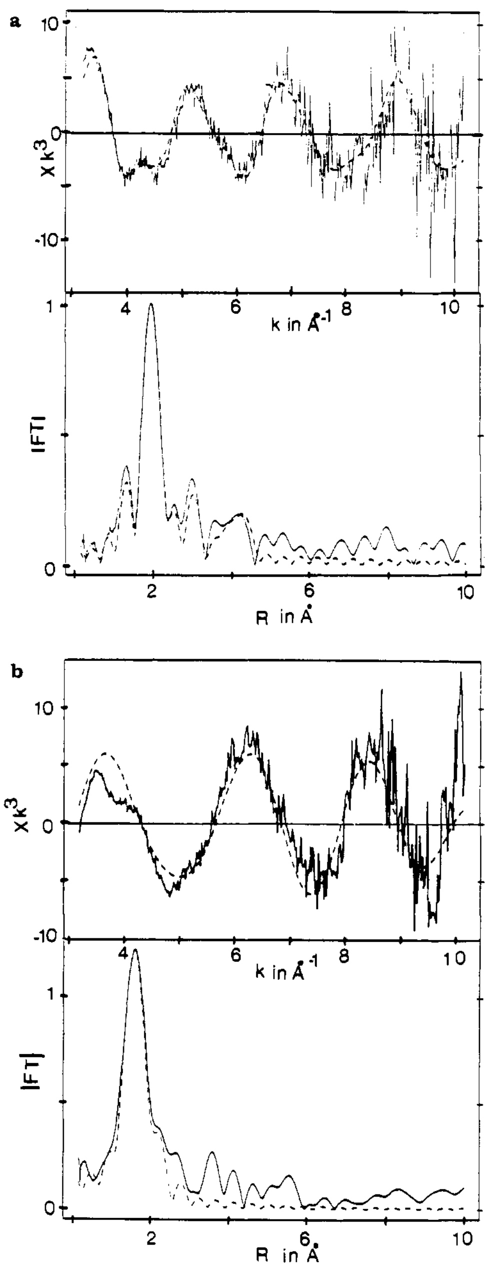

FIGURE 4: EXAFS (χk^3) data and Fourier transform for (a) dithionite-reduced bromoperoxidase and (b) native bromoperoxidase. (Solid line represents experimental data; broken line, simulation using the appropriate parameters from Table III.)

Simulations using only single scattering calculations can reproduce the EXAFS reasonably well but are only able to do so by moving the outer-shell contribution in to ~4.0 Å, clearly too short compared to the distance indicated by the Fourier transform Figure 4a). This effect has been observed previously (Blackburn et al., 1983, 1984, 1987; Strange et al., 1987) and shown to result from the multiple scattering effect from the outer atoms of groups such as imidazole. Thus, the shortening of the contribution at ~4.3 Å together with the observation of backscattering from light atoms at ~2.0 and ~3.0 Å is strong evidence for histidine coordination to vanadium in reduced bromoperoxidase, consistent with ESE data (de Boer et al., 1988b).

Inclusion of multiple scattering effects from imidazole results in an improvement in the simulation, notably in the low-k

Table III: Parameters Used To Simulate the EXAFS of Bromoperoxidase[a]

bromoperoxidase sample	atom[b]	N	R (Å)	$2\sigma^2$ (Å^2)[c]
reduced	O	1.0	1.63 (3)	0.002
	O	3.0	1.91 (3)	0.007
	N[d]	2.0	2.11 (3)	0.006
	C	2.0	3.03 (5)	0.009
	C	2.0	3.06 (5)	0.009
	C	2.0	4.34 (5)	0.020
	C	2.0	4.42 (5)	0.020
native	O	1.0	1.61 (3)	0.001
	O	3.0	1.72 (3)	0.011
	N	2.0	2.11 (3)	0.002

[a] Estimated errors are given in parentheses. [b] EXAFS cannot distinguish elements of adjacent atomic number, e.g., C, N, O. [c] Debye–Waller parameter. [d] The angles used in the simulation of the multiple scattering contribution from histidine were as follows: V–N–C (~3.0 Å) 126 ± 3° and V–N–C (~4.4 Å) 166 ± 4°. Atoms from the imidazole group were refined as a single unit in order to minimize the number of free parameters.

region, using the parameters given in Table III. Two imidazoles at 2.11 (5) Å, together with contributions from one oxygen at 1.63 (3) Å and three oxygens at 1.91 (3) Å, suggest a distorted octahedral geometry in reduced bromoperoxidase. This is not unusual for vanadium(IV) compounds with oxygen/nitrogen coordination (Table II), which generally have one short oxygen (~1.6 Å) and four or five other light atoms at ~2 Å, and is consistent with the above remarks concerning the edge data. Additionally, the short vanadium–oxygen distance may correspond to that which dominates the ligand field in EPR data (de Boer et al., 1986a, 1988a); the presence of nitrogen equatorially ligated to a vanadyl cation has been deduced from ESE studies (de Boer et al., 1988b). The distances derived for the imidazole groups are similar to those found in [VOCl(vinyl-imid)$_4$]Cl (L. J. Calviou, W. Clegg, D. Collison, C. D. Garner, and J. M. Arber, unpublished results). The EXAFS of this compound will be presented elsewhere (Calviou et al., unpublished results), and the data, in agreement with XRD data, give an average vanadium–nitrogen distance of 2.12 Å with further backscattering from imidazole carbons and nitrogens at ~3.1 and ~4.3 Å.

As noted above, the EXAFS of native bromoperoxidase (Figure 4b) is substantially different from that of the reduced form. It is dominated by a short backscattering contribution (~1.7 Å) that lowers the frequency of the EXAFS oscillations compared to reduced bromperoxidase (Figure 4a). However, in spite of such marked differences between the EXAFS of the reduced and native forms close comparison reveals common features. For example, the maximum at ~4 Å^{-1} in native bromoperoxidase is further split on reduction to give two clearly separated features at 3.5 and 4.5 Å^{-1}. Similarly, the slight shoulders at 5.5 and 7.0 Å^{-1} in native bromperoxidase correspond to maxima at these values in the reduced sample. Since the EPR spectra of reduced bromoperoxidase isolated from various organisms are essentially identical (Wever et al., 1988), it is probable that the metal binding site in the reduced form is specific. A first shell analysis of the native bromoperoxidase EXAFS (data k^3 weighted, Fourier filtered 0.8–2.5 Å), assuming a minimum coordination number of six light atoms, shows that allowing the shell to split into two components (only integer coordination numbers were considered) reduces the fit index (Joyner et al., 1987) by ~70%. Further splitting into three components produces a reduction of ~10% in the fit index, the differences between the various three component simulations being of the order of 1%. Thus, the best inner-shell simulation of the data (Figure 4b) employs backscattering from one oxygen at 1.61 Å, three oxygens at 1.72 Å, and two nitrogens at 2.11 Å (Table III). Comparison of these parameters with those derived for the reduced enzyme (Table III) shows that the only significant change is the reduced vanadium–oxygen distance (1.91 to 1.72 Å), consistent with the increased intensity of the preedge peak (see Figure 1). Such short oxygen distances are typical of vanadate (e.g., NH_4VO_3, see Table II) and vanadium(V)–alkoxy systems [V–O ~1.75 Å (Caughlan et al., 1966; Scheidt, 1973; Diamantis et al., 1986)]. It is therefore of interest to note the similarity between the edge and XANES of native bromoperoxidase and vanadate (Figures 1 and 2, respectively) and the previously reported ability of vanadate to reactivate apobromoperoxidase and of phosphate and molybdate (structural analogues of vanadate) to prevent reactivation (de Boer et al., 1986a, 1988a; Wever et al., 1988). The oxygens may be derived from amino acids such as aspartic acid, tyrosine, etc.; the nitrogens, from histidine. The rapid esterification of tyrosine by vanadate has been demonstrated by NMR studies (Tracey & Gresser, 1986) and the presence of a tyrosine–vanadate ester may account for the strong oxygen backscattering in the EXAFS. However, the limited range and quality of the data preclude more detailed analysis, and it is therefore not possible to define the exact nature of the coordinating groups.

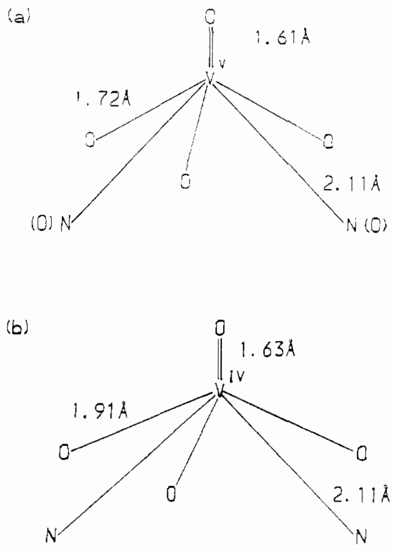

FIGURE 5: Proposed coordination of vanadium in (a) native bromoperoxidase and (b) dithionite-reduced bromoperoxidase.

The data presented here have provided information regarding the vanadium sites in bromoperoxidase under several conditions. The edge and XANES data are consistent with the presence of vanadium coordinated to oxygen/nitrogen either as vanadium(V) (in the native, native plus bromide, and native plus peroxide samples) or vanadium(IV) (in the reduced enzyme). These data further imply that, in the four systems studied, it is only on reduction that the vanadium site is significantly altered. This is confirmed by the EXAFS data which suggest coordination of histidine and oxygen-donating residues such as tyrosine, together with a terminal oxygen, in both native and reduced bromoperoxidase, the average inner-shell distance lengthening on reduction (see Figure 5).

Registry No. V, 7440-62-2; bromoperoxidase, 69279-19-2.

REFERENCES

Arber, J. M., Dobson, B. R., Eady, R. R., Stevens, P., Hasnain, S. S., Garner, C. D., & Smith, B. E. (1987) *Nature* (*London*) *325*, 372–374.

Bachmann, H. G., Ahmed, F. R., & Barnes, W. H. (1961) *Z. Kristallogr. Mineral. 115*, 110–131.

Blackburn, N. J., Hasnain, S. S., Diakun, G. P., Knowles, P. F., Binsted, N., & Garner, C. D. (1983) *Biochem. J. 213*, 765–768.

Blackburn, N. J., Hasnain, S. S., Binsted, N., Diakun, G. P., Garner, C. D., & Knowles, P. F. (1984) *Biochem. J. 233*, 985–990.

Blackburn, N. J., Strange, R. W., McFadden, L. M., & Hasnain, S. S. (1987) *J. Am. Chem. Soc. 109*, 7162–7170.

Caughlan, C. N., Smith, H. M., & Watenpaugh, K. (1966) *Inorg. Chem. 5*, 2131–2134.

Cotton, F. A., & Wilkinson, G. (1972) in *Advanced Inorganic Chemistry*, Wiley-Interscience, London.

de Boer, E., & Wever, R. (1988) *J. Biol. Chem. 263*, 12326–12332.

de Boer, E., van Kooyk, Y., Tromp, M. G. M., Plat, H., & Wever, R. (1986a) *Biochim. Biophys. Acta 869*, 48–53.

de Boer, E., Tromp, M. G. M., Plat, H., Krenn, G. E., & Wever, R. (1986b) *Biochim. Biophys. Acta 872*, 104–115.

de Boer, E., Boon, K., & Wever, R. (1988a) *Biochemistry 27*, 1629–1635.

de Boer, E., Klaassen, A. A. K., Reijerse, E. K., Collison, D., Garner, C. D., & Wever, R. (1988b) *FEBS Lett. 235*, 93–97.

Diamantis, A. A., Frederiksen, J. M., Salam, Md. A., Snow, M. R., & Tienkink, E. R. T. (1986) *Aust. J. Chem. 39*, 1081–1088.

Dobson, B. R., Hasnain, S. S., Hart, M., van der Hoek, M. J., & van Zuylen, P. (1986) *J. Phys. Colloq. 47, C8*, 121–125.

Dodge, R. P., Templeton, D. H., & Zalkin, A. (1961) *J. Chem. Phys. 35*, 55–67.

Gurman, S. J., Binsted, N., & Ross, I. (1984) *J. Phys. 17*, 143–151.

Gurman, S. J., Binsted, N., & Ross, I. (1986) *J. Phys. 19*, 1845–1861.

Hales, B. J., Case, E. E., Morningstar, J. E., Dzeda, M. F., & Mauterer, L. A. (1986) *Biochemistry 25*, 7251–7255.

Hallmeier, K. H., Szargan, R., Werner, G., Meier, R., & Sheromov, M. A. (1986) *Spectrochim. Acta 42A*, 841–844.

Hasnain, S. S. (1987) *Life Chem. Rep. 4*, 273–331.

Hawthorne, F. C., & Calvo, C. (1977) *J. Solid Chem. 22*, 157–170.

Joyner, R. W., Martin, K. J., & Meehan, P. (1987) *J. Phys. C 20*, 4005–4012.

Kutzler, F. W., Natoli, C. R., Misemer, D. K., Doniach, S., & Hodgson, K. O. (1980) *J. Chem. Phys. 73*, 3274–3288.

Lee, P. A., & Pendry, J. B. (1975) *Phys. Rev. B11*, 2795–2811.

Lytle, F. W., Sayers, D. E., & Stern, E. A. (1988) *Report of International Workshop on Standards and Criteria in X-ray Absorption Spectroscopy*, Brookhaven National Laboratory.

Mimoun, H., Saussine, L., Daire, E., Postel, M., Fischer, J., & Weiss, R. (1983) *J. Am. Chem. Soc. 105*, 3101–3110.

Morrison, M., & Schonbaum, G. R. (1976) *Annu. Rev. Biochem. 45*, 861–888.

Nuber, B., Weiss, J., & Weighardt, K. (1978) *Z. Naturforsch. B 33*, 265.

Ooi, S., Nishizawa, M., Matsumoto, K., Kuroya, H., & Saito, K. (1979) *Bull. Chem. Soc. Jpn. 52*, 452.

Pajunen, A., & Pajunen, S. (1980) *Acta Crystallogr. B 36*, 2425–2428.

Perutz, M. F., Hasnain, S. S., Duke, P. J., Sessler, J. L., & Hahn, J. E. (1982) *Nature (London) 295*, 535–538.

Rehder, D., Vilter, H., Duch, A., Priebsch, W., & Weideman, C. (1987) *Recl Trav. Chim. Pays-Bas 106*, 408.

Riley, P. E., Pecoraro, V. L., Carrano, C. J., Bonadies, J. A., & Raymond, K. N. (1986) *Inorg. Chem. 25*, 154.

Robson, R. L., Eady, R. R., Richardson, T. H., Miller, R. W., Hawkins, M., & Postgate, J. R. (1986) *Nature (London) 322*, 388–390.

Scheidt, W. R. (1973) *Inorg. Chem. 12*, 1758–1761.

Shiro, M., & Fernando, Q. (1971) *Anal. Chem. 43*, 1222.

Shulman, R. G., Yafet, Y., Eisenberger, P., & Blumberg, W. E. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1384–1388.

Stomberg, R. (1986) *Acta Chem. Scand. A 40*, 168–176.

Strange, R. W., Blackburn, N. J., Knowles, P. F., & Hasnain, S. S. (1987) *J. Am. Chem. Soc. 109*, 7157–7162.

Szentivanyi, H., & Stomberg, R. (1983) *Acta Chem. Scand. A 37*, 709–714.

Tracey, A. S., & Gresser, M. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 609–613.

van der Hoek, M. J., Werner, W., van Zuylen, P., Dobson, B. R., Hasnain, S. S., Worgan, J. S., & Luickx, G. (1986) *Nucl. Instrum. Methods Phys. Res., Sect. A 246*, 380–384.

Vilter, H., & Rehder, D. (1987) *Inorg. Chim. Acta 136*, L7–L10.

Wever, R., Plat, H., & de Boer, E. (1985) *Biochim. Biophys. Acta 830*, 181–186.

Wever, R., Krenn, B. E., de Boer, E., Offenberg, H., & Plat, H. (1988) in Proceedings of the Fourth International Symposium on Oxidases and Related Redox Systems (King, T. E., Mason, H. S., & Morrison, M., Eds.) pp 477–493, Liss Inc., New York.

Wong, J., Lytle, F. W., Messmer, R. P., & Maylotte, D. H. (1984) *Phys. Rev. B30*, 5596–5610.