Electron Paramagnetic Resonance Studies on Conformational States and Metal Ion Exchange Properties of Vanadium Bromoperoxidase†

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ABSTRACT: An electron paramagnetic resonance (EPR) study was carried out to examine structural aspects of vanadium-containing bromoperoxidase from the brown seaweed *Ascophyllum nodosum*. At high pH, the reduced form of bromoperoxidase showed an apparently axially symmetric EPR signal (g_0 = 1.969 and $A_0 = 86.8 \times 10^{-4}$ cm^{-1}) with 16 hyperfine lines. When the pH was lowered, a new EPR spectrum was formed (g_0 = 1.970 and $A_0 = 92.6 \times 10^{-4}$ cm^{-1}) in which the values for $g_\parallel$ and $g_\perp$ were hardly affected, whereas both $A_\parallel$ and $A_\perp$ showed a considerable increase. When EPR spectra of the reduced enzyme were recorded in the pH range from 4.2 to 8.4, it appeared that these changes were linked to a functional group with an apparent pK_a of about 5.4. In D_2O this value for the pK_a was 5.3. It is suggested that these effects arise from protonation of histidine or aspartate/glutamate residues near the metal ion. The values for the isotropic hyperfine coupling constant of the reduced enzyme at both high and low pH are also consistent with a ligand field containing nitrogen and/or oxygen donor atoms. When reduced bromoperoxidase was dissolved in D_2O or $H_2{}^{17}O$ instead of $H_2{}^{16}O$, vanadium(IV) hyperfine line widths were markedly affected, demonstrating that water is a ligand of the metal ion. Furthermore, it is shown that hydrogen peroxide and bromide were not able to oxidize reduced bromoperoxidase. Together with previous work these findings suggest that vanadium(IV) is not involved in catalytic turnover and confirm the model in which the vanadium(V) ion of the native enzyme only serves to bind both hydrogen peroxide and bromide. After excess vanadate was added to a homogeneous preparation of purified bromoperoxidase, the extent of vanadium bound to the protein increased from 0.5 to 1.1, with a concomitant enhancement of enzymic activity. Finally, it is demonstrated that both vanadate (VO_4^{3-}) and molybdate (MoO_4^{2-}) compete for the same site on apobromoperoxidase. Molybdate, however was unable to restore enzymic activity, and binding of this metal ion to the V site in the apoenzyme therefore resulted in a loss of halogenating activity.

Haloperoxidases are known to catalyze the peroxidative oxidation of iodide, bromide, and/or chloride ions, which in the presence of a nucleophilic halogen acceptor gives rise to a halogenated compound. A variety of haloperoxidases from different sources have been investigated, such as bacteria (Van Pee & Lingens, 1985), fungi (Morris & Hager, 1966), marine invertebrates (Jannum et al., 1981), marine algae (Hewson & Hager, 1980), and mammals (Bolscher et al., 1984). Many of these haloperoxidases contain heme at the active site (Morrison & Schonbaum, 1976). However, the haloperoxidases identified so far in brown seaweeds and several red seaweeds did not possess a heme prosthetic group, as was verified by Vilter (1983), Itoh et al. (1986), and us (Wever et al., 1985; de Boer et al., 1986a,b; Krenn et al., 1987). Reconstitution experiments showed that the transition metal vanadium is essential for the halogenating activity for most of these algal enzymes (Vilter, 1984; de Boer et al., 1986a,b; Krenn et al., 1987). Moreover, when purified enzyme preparations were analyzed by electron paramagnetic resonance (EPR)[1] and atomic absorption spectrophotometry, it was shown that vanadium was present in the bromoperoxidases from *Ascophyllum nodosum* and *Laminaria saccharina* (de Boer et al., 1986a,b). With the establishment of vanadium-containing bromoperoxidases it was demonstrated for the first time that this transition metal is involved in enzyme-catalyzed redox reactions. The involvement of vanadium in enzymically catalyzed redox reactions has recently been reviewed by Wever et al. (1987).

Native vanadium bromoperoxidase contains the vanadium ion in the 5+ oxidized state. An EPR spectrum of vanadium(IV) is obtained after reduction of the resting enzyme with sodium dithionite (de Boer et al., 1986a,b). This EPR spectrum possesses axial symmetry consisting of two sets of eight hyperfine lines due to the coupling of the unpaired electron with the nuclear spin of vanadium ($I = {}^7/_2$). Since EPR spectral data of reduced bromoperoxidase resemble those of several inorganic oxovanadium(IV) complexes, it was inferred that in the reduced enzyme the vanadium ion is coordinated to an axially terminal oxo ligand. Here, we report on the effects of solvents such as D_2O and $H_2{}^{17}O$ on EPR spectra of reduced bromoperoxidase at both high and low pL. Resulting EPR spectral data are discussed with regard to possible coordinating ligands of the vanadium ion in bromoperoxidase.

Materials and Methods

Bromoperoxidase from the brown seaweed *A. nodosum* was purified as described by Wever et al. (1985) with modifications (de Boer et al., 1986a). Purified enzyme preparations were dissolved in 0.1 M Tris–sulfate/0.1 M sodium sulfate (pH 8.3).

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[1] Abbreviations: EPR, electron paramagnetic resonance; L, unspecified isotope of hydrogen; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; MCD, 2-chlorodimedone.

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Apobromoperoxidase preparations were prepared as reported by Vilter (1984). The inactivated enzyme was dissolved in 0.1 M Tris-HCl/0.1 M sodium sulfate (pH 8.3). Enzymic activity was measured as described by Wever et al. (1985), and protein content was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

The pL titrations of bromoperoxidase were done in 0.1 M sodium citrate/0.1 M sodium sulfate (pL 4.2–7.0), dissolved in either H_2O or D_2O (Merck, 99.75% enriched in D), and in 0.1 M sodium acetate/0.1 M sodium sulfate (pH 4.2–5.9), dissolved in H_2O. Citrate as well as acetate buffers are known to display only minor pH changes on freezing (Orii & Morita, 1977). The citrate buffers in D_2O were adjusted to the desired pD by using DCl or NaOD solutions in D_2O. To obtain enzyme solutions at the desired pL, purified bromoperoxidase samples (~300 μM) were centrifuged through a column of Sephadex G-25 equilibrated with either the citrate or acetate buffer, according to the method originally described by Penefsky (1979). The pL was measured in the column effluent with a Philips PW 9408 pH meter equipped with an Ingold Combi glass calomel micro pH electrode. The meter was standardized against H_2O buffers, and pD values were obtained by adding 0.4 to the meter reading. EPR samples were prepared by reduction with sodium dithionite, after which the solutions were frozen in liquid nitrogen.

Dithionite-free reduced bromoperoxidase preparations (20 μM) were also prepared by the method of Penefsky (1979) (Sephadex G-25 in 0.1 M sodium acetate/0.1 M sodium sulfate, pH 5.0) to remove excess of the reducing agent. The column effluent containing vanadium(IV) bromoperoxidase was incubated with hydrogen peroxide (2 mM), or with hydrogen peroxide (2 mM) and potassium bromide (100 mM), or with an equal volume of water; samples for EPR were frozen in liquid nitrogen.

Exchanging $H_2{}^{17}O$ (Amersham, 45% enriched in ^{17}O) for $H_2{}^{16}O$ was done by lyophilizing concentrated bromoperoxidase preparations (227 μM in 0.1 M Tris–sulfate/0.1 M sodium sulfate, pH 8.3). Freeze-dried enzyme preparations were dissolved in $H_2{}^{16}O$ and $H_2{}^{17}O$ and allowed to equilibrate for 6 h at room temperature. Specific activities obtained before lyophilization and after dissolving the freeze-dried samples in $H_2{}^{16}O$ and $H_2{}^{17}O$ were identical. EPR samples were prepared as mentioned above. EPR spectra of reduced bromoperoxidase before lyophilization were identical with those of the freeze-dried enzyme dissolved in $H_2{}^{16}O$, showing equal g values, hyperfine coupling constants, and resonance line widths.

A solution of vanadate ($VO_4{}^{3-}$) was prepared by dissolving V_2O_5 (Aldrich) in diluted NaOH which was subsequently adjusted to pH 7.0 by adding HCl. A molybdate solution was prepared by dissolving Na_2MoO_4 (Merck) in water.

The effect of molybdate on the reconstitution of apobromoperoxidase with vanadate was studied by incubating concentrated samples of the inactivated enzyme (166 μM) with vanadate (130 μM) and molybdate in the concentration range from 0 to 50 mM. After 24 h at room temperature, the enzymic activity was measured and the samples were reduced with 5 mM sodium dithionite. EPR spectra were recorded and intensities of vanadium(IV) bromoperoxidase signals were determined by measuring peak-to-peak signal heights of the first derivative $m_I = -1/2$ hyperfine line,[2] which contains both parallel and perpendicular features. The proportionality constant between intensity of this central $m_I = -1/2$ hyperfine line and vanadium(IV) bromoperoxidase concentration was determined by comparing $m_I = (-5/2)_\parallel$ signal intensities (obtained by double integration) of reconstituted, reduced bromoperoxidase and a standard of $VOSO_4$ dissolved in HCl (Aasa & Vänngård, 1975; Albracht, 1984; Chasteen, 1981).

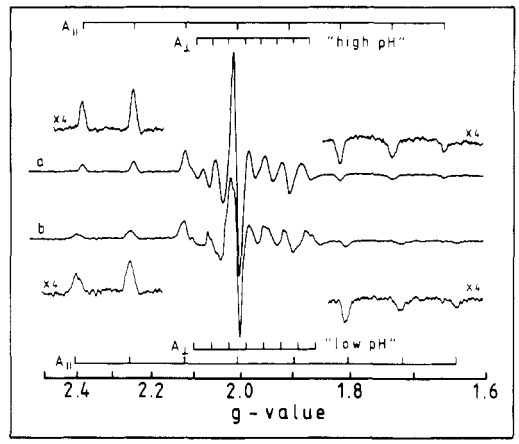

FIGURE 1: X-band EPR spectra of reduced bromoperoxidase in H_2O, at pH 8.4 (trace a; 140 μM enzyme) and at pH 4.2 (trace b; 226 μM enzyme). Both the $A_\parallel$ and $A_\perp$ resonance lines of the high-pH and low-pH features are indicated in the figure. Instrument settings: microwave frequency, 9255 MHz; microwave power, 2 mW; modulation width, 1.0 mT; temperature, 64 K.

EPR spectra were recorded on a Varian E-9 spectrometer, coupled to an HP 2100 computer via a PDP 11-03 microcomputer for data aquisition. The instrument was operating at X-band frequency (ca. 9.2 GHz) with 100-kHz magnetic field modulation. Temperature, magnetic field, and microwave frequency were measured as described by Albracht (1984) and Wever et al. (1974). In the pH-titration experiment carried out in H_2O, EPR $m_I = (-7/2)_\parallel$ line shapes were simulated by using a Fortran program for $S = 1/2$ powder-type systems (Beinert & Albracht, 1982). In this pH-titration experiment signal intensities were calculated from these simulated parallel $m_I = -7/2$ hyperfine lines, as was previously outlined by Chasteen (1981). In the pD-titration experiment carried out in D_2O, signal intensities were calculated from the experimental $m_I = -7/2$ parallel hyperfine lines (Chasteen, 1981). Room temperature EPR experiments were performed in flat quartz cells at a microwave power of 200 mW.

Results

Electron paramagnetic resonance (EPR) spectra of reduced bromoperoxidase were markedly pH dependent. This is shown in Figure 1 for frozen solutions of vanadium(IV) bromoperoxidase in citrate buffer at pH 8.4 and 4.2. As can be seen in this figure, reduced bromoperoxidase at pH 8.4 showed an EPR spectrum only composed of a single set of 16 hyperfine lines ("high-pH" resonances). At pH 4.2, these high-pH resonances were almost completely replaced by a new set of 16 hyperfine lines (designated "low-pH" resonances). Spectral parameters for both the high-pH and low-pH forms of reduced bromoperoxidase are given in Table I. The EPR data for both forms indicate that $g_\parallel$ and $g_\perp$ hardly responded to pH changes whereas $A_\parallel$ and $A_\perp$ both showed a considerable increase upon lowering the pH. The values for the high-pH form were the same as those reported for reduced bromoperoxidase in Tris buffer at pH 8.3 (de Boer et al., 1986a).

The appearance of a new set of 16 hyperfine lines at low pH was also found when acetate was used instead of citrate buffers. In Table I it is shown that similar values for g and the hyperfine coupling constants were found for both pH conformations of reduced bromoperoxidase in either citrate or acetate buffers. This indicates that the appearance of a

[2] m_I labeling assumes a negative nuclear hyperfine interaction.

Table I: EPR Parameters of Reduced Bromoperoxidase

experimental conditions[a]	$g_\parallel$[b]	$g_\perp$	g_0[c]	$A_\parallel$[d]	$A_\perp$	A_0[e]
H_2O, citrate, high pH	1.948	1.979	1.969	160.1	50.2	86.8
H_2O, citrate, low pH	1.950	1.980	1.970	167.5	55.1	92.6
H_2O, acetate, high pH	1.952	1.979	1.967	159.6	51.1	87.3
H_2O, acetate, low pH	1.953	1.981	1.967	165.7	54.5	91.6
D_2O, citrate, high pD	1.951	1.980	1.971	160.5	51.2	87.7
D_2O, citrate, low pD	1.949	1.980	1.970	165.8	55.6	92.4

[a] Data obtained from EPR spectra of reduced bromoperoxidase in 0.1 M sodium citrate/0.1 M sodium sulfate in the pL range 4.2–8.4 or in 0.1 M sodium acetate/0.1 M sodium sulfate in the pH range 4.2–5.9. [b] Reported g values are ±0.002. [c] Calculated from $g_0 = (g_\parallel + 2g_\perp)/3$. [d] A values, listed in units of 10^{-4} cm^{-1}, are ±1.0. [e] Calculated from $A_0 = (A_\parallel + 2A_\perp)/3$.

new set of resonances in going from high to low pH is independent of the buffer system used. Furthermore, the spectral parameters for both forms completely differed from those of complexes of vanadyl acetate ($g_0 = 1.971$, $A_0 = 100.6 \times 10^{-4}$ cm^{-1}) and vanadyl citrate [$g_0 = 1.969$, $A_0 = 95.4 \times 10^{-4}$ cm^{-1}; see also Chasteen et al. (1973)]. Also, room temperature EPR spectra of reduced bromoperoxidase indicated that both pH forms are protein bound. At pH 5.0 (citrate buffer) normal axially symmetric spectra were obtained as expected for slow-tumbling protein molecules, giving rise to near-rigid-limit solution EPR spectra (Chasteen, 1981). Therefore, the high-pH and low-pH resonances did not originate from vanadyl–buffer chelates and obviously arose from the vanadium active site of bromoperoxidase. Moreover, a linear relationship existed between the intensity of EPR first derivative absorption curves and enzymic activity of different sample preparations in the pH range from 4.2 to 8.4 (not shown). The linear relationship between the amount of vanadium(IV) incorporated in the enzyme as judged from EPR spectra of reduced bromoperoxidase and enzymic activity has been demonstrated before (de Boer et al., 1986a,b). Furthermore, normal kinetic behavior of bromoperoxidase sample preparations in this pH range was observed when enzymic activity was measured under standard assay conditions (see Materials and Methods). This demonstrates that pH values as low as 4.2 do not irreversibly denature the enzyme. This conclusion was also drawn from steady-state kinetic work on bromoperoxidase in the pH range from 3.7 to 7.6 (Wever et al., 1985). In addition to this, it was noticed that the effect of pH on EPR spectra of reduced bromoperoxidase was completely reversible. Readjustment of the pH of different sample preparations, from pH 4.2 and 5.0 to pH 8.0, completely restored the original EPR spectra only composed of high-pH features (not shown).

This effect of pH on EPR spectra of reduced bromoperoxidase was also observed when H_2O was replaced by D_2O; frozen solution spectra in citrate buffer at pD 6.9 and 4.2 are shown in Figure 2. Also when D_2O was employed, EPR spectra of reduced bromoperoxidase at high pD (pD 6.9) were composed of 16 hyperfine lines ("high-pD" resonances). These high-pD resonances lost intensity when the pD was lowered, and at pD 4.2 the spectrum almost exclusively consisted of a new set of 16 hyperfine lines (designated "low-pD" resonances). Spectral parameters for both forms are given in Table I. As was also observed for reduced bromoperoxidase in H_2O, both $g_\parallel$ and $g_\perp$ were not affected by pD changes, whereas $A_\parallel$ and $A_\perp$ increased significantly upon lowering the pD. Within experimental error, indentical values for g and the hyperfine coupling constants were found for reduced bromoperoxidase at high and low pL in either H_2O or D_2O. Since the hyperfine splitting parameters $A_\parallel$ and $A_\perp$ are sensitive measures of the coordination environment of the vanadium(IV) ion, the increase in A_0 by about 4.9×10^{-4} cm^{-1} in solutions containing H_2O or D_2O indicates that the metal site geometry changed in going from high to low pL (Chasteen, 1981; Boucher et al., 1969).

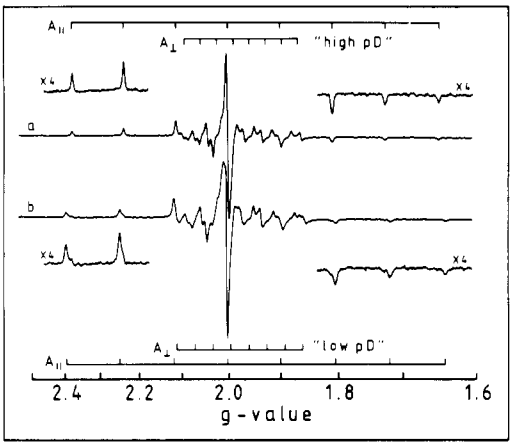

FIGURE 2: X-band EPR spectra of reduced bromoperoxidase in D_2O, at pD 6.9 (trace a; 226 μM enzyme) and at pD 4.2 (trace b; 310 μM enyzme). Both the $A_\parallel$ and $A_\perp$ resonance lines of the high-pD and low-pD features are indicated in the figure. Instrument settings: microwave frequency, 9255 MHz; microwave power, 2 mW; modulation width, 0.5 mT; temperature, 68 K.

In addition to this effect of pD on EPR spectra of reduced bromoperoxidase, both forms in D_2O showed a marked reduction in vanadium(IV) hyperfine line widths when compared to spectra of the reduced enzyme in H_2O at both high and low pH (see Figures 1 and 2). A major source contributing to the line width of frozen solutions of vanadyl species is unresolved superhyperfine coupling arising from coordinating nuclear spins, such as H ($I = {}^1/_2$), D ($I = 1$), and ^{14}N ($I = 1$) (White & Chasteen, 1979; Albanese & Chasteen, 1978). Thus, the observed decrease in line width in sample preparations containing D_2O can be explained in terms of interacting exchangeable hydrogens near the metal ion. Since $g_N{}^D$ equals $0.1538 g_N{}^H$, coupling constants for deuterium are much smaller than those for hydrogen, which would explain the reduction in vanadium(IV) hyperfine line widths upon exchanging deuterons for protons. Other effects contributing to vanadyl hyperfine line widths in H_2O and D_2O were considered by Albanese and Chasteen (1978).

By the use of D_2O, resolution of the spectra in Figure 2 is highly improved. Due to this enhancement of resolution the presence of a small amount of in-plane anisotropy becomes visible. The effect of this rhombicity on EPR parameters is relatively small, and within resolution of the perpendicular hyperfine lines $g_x - g_y$ is of the order of 0.009 and $A_x - A_y$ is about 0.5×10^{-4} cm^{-1}.

When $H_2{}^{17}O$ was employed instead of $H_2{}^{16}O$, a marked increase in vanadium hyperfine line widths was observed. For instance, the parallel $m_I = -{}^5/_2$ hyperfine line of reduced bromoperoxidase increased in line width from 1.87 mT in $H_2{}^{16}O$ to 2.25 mT when $H_2{}^{17}O$ was used (Figure 3). In Figure 3 the effect of D_2O is also shown by a decrease in hyperfine line width to 0.98 mT. This effect of $H_2{}^{17}O$ on vanadium(IV) hyperfine line widths arose from the coupling of the unpaired electron with the nuclear spin of ^{17}O ($I = {}^5/_2$). This indicates that at least part of the exchangeable hydrogens contributing to EPR hyperfine line widths of vanadium(IV) bromoperoxidase originate from the presence of L_2O near the paramagnetic species.

The appearance of a second set resonance signals upon lowering the pL was most pronounced in the low-field region

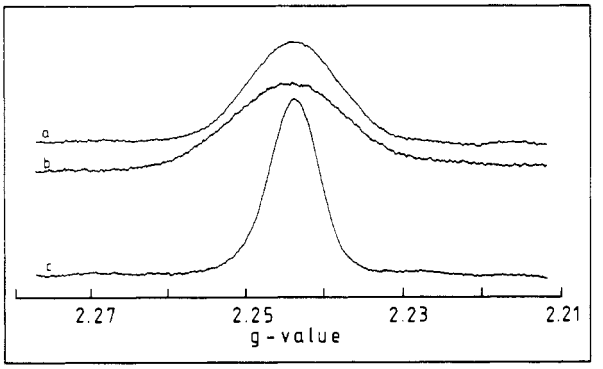

FIGURE 3: Effect of deuterium and ^{17}O-labeled water analogues on $m_I = -5/2$ parallel resonance line widths of reduced bromoperoxidase. Bromoperoxidase (in 0.1 M Tris–sulfate/0.1 M sodium sulfate, pH 8.3) was dissolved in $H_2{}^{16}O$ (trace a), $H_2{}^{17}O$ (trace b), and D_2O (trace c) at 155 μM. Equal intensities were obtained for all three EPR spectra. EPR spectra of bromoperoxidase dissolved in $H_2{}^{17}O$ (45% enriched in oxygen-17) were normalized to 100% ^{17}O contribution. Average traces of 15 scans are shown, normalized to a microwave frequency of 9255 MHz. Line widths are 1.87 ($H_2{}^{16}O$), 2.25 ($H_2{}^{17}O$), and 0.98 mT (D_2O). Instrument settings: microwave power, 2 mW; modulation width, 0.5 mT; temperature, 60 K.

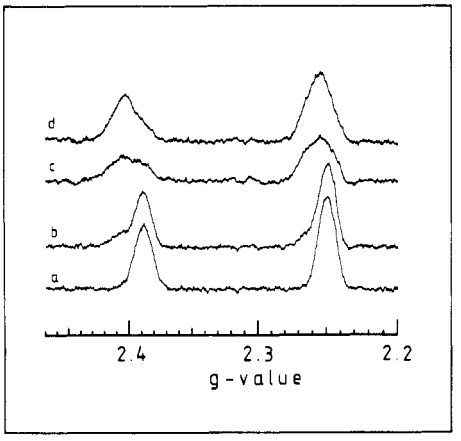

FIGURE 4: Effect of pH on $m_I = -5/2$ and $-7/2$ parallel hyperfine lines of reduced bromoperoxidase. Bromoperoxidase (140–226 μM in H_2O) was dissolved in 0.1 M sodium citrate/0.1 M sodium sulfate. pH values: a, pH 8.4; b, pH 5.9; c, pH 5.0; d, pH 4.2. EPR spectra were recorded at identical values for temperature (60 K), microwave power (2 mW), modulation width (1.0 mT), and receiver gain. Average traces of 15 scans are shown, normalized to a microwave frequency of 9255 MHz.

of the EPR spectra. In particular, the parallel $m_I = -5/2$ and $-7/2$ hyperfine lines were nearly completely resolved for both forms. This is shown in detail in Figure 4 for reduced bromoperoxidase in H_2O in the pH region from 4.2 to 8.4. This figure clearly shows that the low-pH form dominated the traces below pH 5, whereas above this pH the high-pH form was most abundant. Hyperfine line widths for both conformations were obtained by comparing simulated line shapes with experimental curves. The best agreement with experimental spectra was obtained by using a Gaussian line shape function with full widths of 1.9 and 2.5 mT at half-height of the $m_I = (-7/2)_\parallel$ first derivative absorption curve for the high-pH and low-pH forms, respectively. Also, when D_2O was employed, both conformations had equal intensities around pD 5 (Figure 5). Due to the decrease in line width of these parallel $m_I = -5/2$ and $-7/2$ hyperfine lines, resolution was highly improved. Full widths of 0.9 and 1.0 mT at half-height were obtained for the high-pD and low-pD $m_I = (-7/2)_\parallel$ curves, respectively.

In the light of the effects of hydrogen on vanadium(IV) hyperfine lines, the observed line width broadening upon

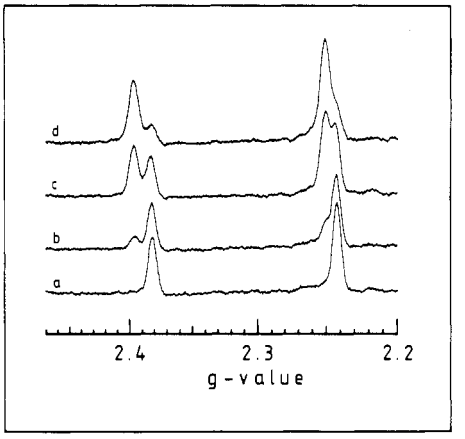

FIGURE 5: Effect of pD on $m_I = -5/2$ and $-7/2$ parallel hyperfine lines of reduced bromoperoxidase. Bromoperoxidase (226–310 μM in D_2O) was dissolved in 0.1 M sodium citrate/0.1 M sodium sulfate. pD values: a, pD 6.9; b, pD 6.0; c, pD 5.4; d, pD 4.2. EPR spectra were recorded at identical values for temperature (67 K), microwave power (2 mW), modulation width (0.63 mT), and receiver gain. Average traces of 15 scans are shown, normalized to a microwave frequency of 9255 MHz.

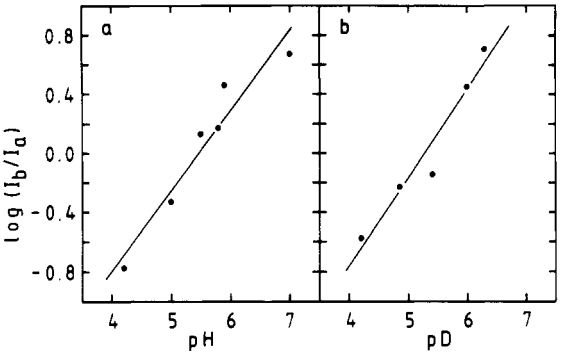

FIGURE 6: Henderson–Hasselbalch plots of the relative intensities of the high-pL and low-pL resonances. Intensities were obtained from different sample preparations in H_2O (part a) and D_2O (part b) and were calculated as mentioned under Materials and Methods. For experimental conditions see Figure 4 and 5. I_b, intensity of the high-pL features; I_a, intensity of the low-pL features.

lowering the pH can be ascribed to protonation of a ligand near the paramagnetic center. In D_2O only minor effects on vanadium hyperfine line widths could be detected in going from high to low pD. Since deuterium hardly contributes to vanadyl hyperfine line widths (Albanese & Chasteen, 1978), this small effect is also in accordance with an ionizing group near the paramagnetic species.

When the logarithms of the relative intensities of the two conformations in either H_2O or D_2O were plotted as a function of pL, straight lines were obtained (Figure 6). These plots show that the relative intensities of the "high-pL" and "low-pL" resonances obeyed the Henderson–Hasselbalch equation [pL = pK_a + log ([base]/[acid])]. This also suggests that the appearance of a second set of resonances at low pL was coupled to an ionizing group at the metal site. From the Henderson–Hasselbalch titration curves apparent pK_a values of 5.4 ± 0.4 and 5.3 ± 0.4 could be calculated for sample preparations containing H_2O and D_2O, respectively.

In earlier work it was suggested that the vanadium(V) ion of native bromoperoxidase serves as a binding site for the substrates hydrogen peroxide and bromide and does not change valence state during catalytic turnover (de Boer et al., 1986b). The conclusion that the oxidation state of the vanadium ion remains unchanged during the peroxidative oxidation of bromide ions is supported by the observation that hydrogen

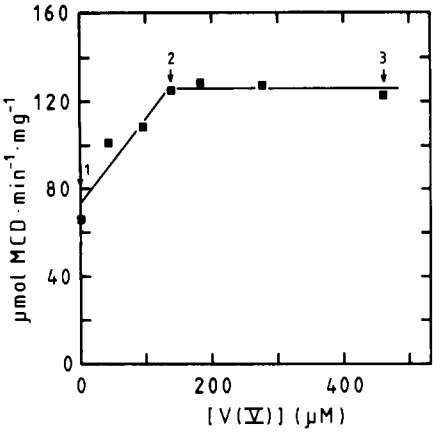

FIGURE 7: Effect of additional vanadium(V) on the specific activity of bromoperoxidase. Purified bromoperoxidase (183 μM) was incubated with various amounts of vanadate; after incubation for 24 h at room temperature the specific activity was measured. The amount of vanadium(V) incorporated in the enzyme was measured by double integration of $m_I = (-^7/_2)_\parallel$ hyperfine lines for three different sample preparations of the reduced enzyme (denoted in the figure with arrows). The extent of vanadium binding was (1) 5.8, (2) 11.6, or (3) 12.4 nmol of V/mg of protein.

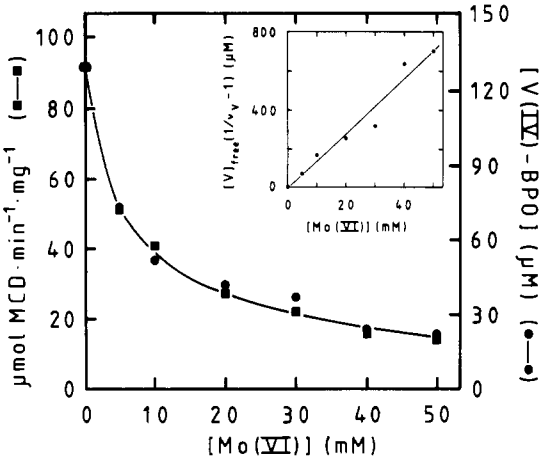

FIGURE 8: Effect of molybdate on the reconstitution of apobromoperoxidase with vanadate. Experimental conditions were as mentioned under Materials and Methods. Specific activity was measured by the bromination of 2-chlorodimedone (μmol of MCD min^{-1} mg^{-1}). The amount of vanadium incorporated in the enzyme is denoted by [V(IV)-BPO]. Insert, EPR spectral intensities of vanadium (IV) bromoperoxidase plotted as $[V]_{free}(1/\tilde{\nu}_V - 1)$, as a function of the amount of molybdate added to the apoenzyme.

peroxide is unable to rapidly oxidize reduced vanadium(IV) bromoperoxidase. In this experiment dithionite-reduced bromoperoxidase was applied to a column of Sephadex G-25 and centrifuged to remove the excess reducing agent. Thereafter, hydrogen peroxide was added to part of the column effluent. EPR spectroscopy showed that the signal intensities of the dithionite-free reduced bromoperoxidase sample preparations were identical, both in the absence and in the presence of hydrogen peroxide (not shown). Also a combination of hydrogen peroxide and bromide did not affect EPR signal amplitudes of vanadium(IV) bromoperoxidase. Previous studies have demonstrated that hydrogen peroxide and bromide were also unable to reduce native bromoperoxidase (de Boer et al., 1986a). The inability of the two substrates to induce redox changes confirms the model in which vanadium(V) only serves to bind hydrogen peroxide and bromide in the peroxidation reaction.

Bromoperoxidase from *A. nodosum* as purified by us had a low content of vanadium(V) (0.4 mol of V/mol of protein; de Boer et al., 1986a). Since the enzyme preparation was aggregated, it was not possible to incorporate more vanadium in the enzyme [cf. de Boer et al. (1986b)]. When purified bromoperoxidase was incubated with vanadate under conditions of high ionic strength (i.e., by adding 0.1 M sodium sulfate to the Tris buffer), specific activity increased from 66 to 126 μmol of 2-chlorodimedone (MCD) brominated min^{-1} (mg of protein)$^{-1}$ (Figure 7). Samples were taken in this experiment (marked with an arrow in Figure 7) to determine the extent of vanadium binding by EPR. Double integration of $m_I = (-^7/_2)_\parallel$ hyperfine lines of these preparations showed that the amount of vanadium bound to the enzyme increased from 5.8 to 12.0 nmol of V/mg of protein. Assuming a molecular weight of 90000 for the protein molecule (de Boer et al., 1986a), this value of 12.0 nmol of V/mg corresponds to 1.1 mol of V/mol of bromoperoxidase. This indicates that after adding excess vanadate to purified bromoperoxidase, the metal ion was present at the active site in a 1:1 ratio per enzyme molecule.

It has previously been shown that vanadium can be removed from native bromoperoxidase by treatment with EDTA at pH 3.8. Halogenating activity of the apoenzyme reappeared upon adding vanadate while other metal ions, such as Mo(VI), Fe(II), Cu(II), Nb(V), Zn(II), and Ni(II), were not effective (Vilter, 1984; de Boer et al., 1986a,b). Instead, when added to the apoenzyme, molybdate acted as an inhibitor in the reconstitution with vanadate (Figure 8). In this experiment several sample preparations of apobromoperoxidase were incubated with equal amounts of vanadate (VO_4^{3-}) and molybdate (MoO_4^{2-}), which was present in the concentration range from 0 to 50 mM. After prolonged incubation (24 h at room temperature) enzymic activity was measured and the samples were reduced with sodium dithionite, after which EPR spectra were recorded. From the spectra, concentrations of enzyme-bound vanadium(IV) were determined. In Figure 8 it can be seen that both activity and the amount of enzyme-bound vanadium(IV) decreased to the same extent with increasing concentrations of molybdate. The reduction in enzymic activity when reconstitution was carried out in the presence of molybdate suggests that both Mo(VI) and V(V) compete for the same binding site on apobromoperoxidase.

When two ligands (A and B) compete for the same set of (identical) binding sites (n) on, e.g., a macromolecule, each with a different association constant (K_A and K_B), the following equation holds for the molar fraction of ligand A bound to the macromolecule ($\tilde{\nu}_A$) (Steinhardt & Reynolds, 1969):

$$\tilde{\nu}_A = \frac{nK_A[A]_{free}}{1 + K_A[A]_{free} + K_B[B]_{free}}$$

For molybdenum and vanadium competing for one site per protein molecule ($n = 1$) this equation becomes

$$[V]_{free}(1/\tilde{\nu}_V - 1) = 1/K_V - (K_{Mo}/K_V)[Mo]_{free}$$

where $[V]_{free}$ equals the amount of vanadium(V) added to apobromoperoxidase minus the concentration of enzyme-bound vanadium(IV). $\tilde{\nu}_V$ is given by $[V(IV)]_{bound}/[bromoperoxidase]$, and $[Mo]_{free} \sim [Mo]_{added}$, since the binding of molybdenum hardly affects the amount of free molybdenum in solution. When $[V]_{free}(1/\tilde{\nu}_V - 1)$ was plotted as a function of molybdate concentration, a linear curve was obtained (insert, Figure 8). This demonstrates that Mo and V indeed compete for the same binding site on the apoenzyme.[3] The line cuts the ordinate

at the origin, indicating that K_V must be large (>10^6 M^{-1}; because of the spread in the intercept an accurate value for K_V cannot be obtained by this method). Tight binding of vanadium(V) to bromoperoxidase was also suggested by Vilter (1984). Assuming an upper limit for K_V of 10^6 M^{-1}, the association constant for Mo binding is on the order of 14 000 M^{-1}.

Discussion

Since the isotropic g and hyperfine coupling constant A_0 of vanadium(IV) species provide sensitive measures of the metal-binding site, we employed EPR spectroscopy in studying the vanadium-containing bromoperoxidase from *A. nodosum*. In the reduced state this enzyme showed an EPR spectrum typical of a vanadyl cation (VO^{2+}) coordinated to the protein moiety. At pH 8.3 this axially symmetric EPR spectrum exhibited a total of 16 hyperfine lines, indicating the presence of one homogeneous metal coordination environment. Replacing D_2O for H_2O resulted in a significant narrowing of vanadium hyperfine line widths. This indicates that exchangeable hydrogens are present near the vanadium(IV) ion of reduced bromoperoxidase. Since a marked increase in vanadium hyperfine line widths was observed when $H_2{}^{17}O$ was employed instead of $H_2{}^{16}O$, water obviously forms part of the coordination environment of the vanadium(IV) ion. Lowering the pL in solutions of reduced bromoperoxidase in either H_2O or D_2O highly affected EPR spectra, resulting in overlapping resonances with equal intensities at about pL 5.4. This superposition of resonances can be explained by the occurrence of two magnetically nonequivalent metal-binding sites, designated the high-pL and low-pL resonances. In H_2O the appearance of a second set of 16 hyperfine lines at low pH was accompanied by line width broadening. Moreover, the relative intensities of both the high-pL and low-pL forms showed linear Henderson–Hasselbalch curves. From this it is inferred that the pL-dependent changes in EPR spectra of reduced bromoperoxidase are associated with the ionization of a functional group with an apparent pK_a of about 5.4. It may very well be that protonation of this group is responsible for the observed inhibition of enzymic activity at lower pH values (Wever et al., 1985).

The isotropic EPR spectral parameters g_0 and A_0 are largely determined by the vanadium(IV) ligand field strength. For instance, weak-field $VO(H_2O)_5{}^{2+}$ gives rise to $g_0 = 1.964$ and $A_0 = 109.1 \times 10^{-4}$ cm^{-1}, whereas the strong-field complex $VO(S_2CCN)_2{}^{2-}$ yields $g_0 = 1.992$ and $A_0 = 67.3 \times 10^{-4}$ cm^{-1} (Boucher et al., 1969; Chasteen, 1981). From these data it is clear that the spread in g_0 is relatively small, whereas A_0 decreases considerably with increasing field strength. On going from low to high pH, the A_0 of reduced bromoperoxidase decreased by about 4.9×10^{-4} cm^{-1}, and according to the correlation between average ligand field strength and isotropic hyperfine splitting this suggests an increase in field strength of the ligand environment. This is also in accordance with ionization of a functional group near the vanadium ion. When the vanadyl cation was used as an EPR spin probe to investigate metal-binding sites of metalloproteins, EPR line shifts as a function of pH were also employed to study the protonation behavior of first coordination sphere vanadium(IV) ligands in serotransferrin and carbonic anhydrase (Chasteen et al., 1977; Fitzgerald & Chasteen, 1974).

Within a protein backbone apparent pK_a values for histidyl imidazole nitrogens and β- or γ-carboxylic acid side groups of glutamate and aspartate residues are known to vary between 5–7 and 4–5, respectively (Brill, 1977). The apparent pK_a of 5.4 indicates that histidine or aspartate/glutamate residues are likely candidates for coordinating ligands. The possible coordination of vanadium(V) in bromoperoxidase by carboxylic acid side chains was also derived from ^{51}V NMR spectra of the native enzyme (Vilter & Rehder, 1987). Alternatively, it is conceivable that we deal with ionization of a coordinated water molecule (Albanese & Chasteen, 1978). The values for the isotropic hyperfine coupling constant for both the high-pL (87.0×10^{-4} cm^{-1}) and low-pL (92.1×10^{-4} cm^{-1}) conformations are also in accordance with a ligand field of moderate strength, involving oxygen and/or nitrogen donor atoms (Boucher et al., 1969; Chasteen, 1981).

Bromoperoxidase is able to incorporate the metal ion molybdenum(VI) instead of vanadium(V). The molybdenum enzyme, however, does not display any haloperoxidase activity. Although molybdenum is incorporated in bromoperoxidase, it does not have a marked affinity for the enzyme, compared to vanadium. This was evident from the high amount of molybdenum needed to compete with vanadium for the active site of bromoperoxidase. Similarly, it was demonstrated that phosphate (which is a structural analogue of vanadate) forms an inactive complex with apobromoperoxidase (de Boer et al., 1986a).

Earlier it was reported that vanadium was incorporated in the nitrogenases from *Azotobacter vinelandii* and *Azotobacter chroococcum* (Benemann et al., 1972). For N_2 fixation these bacteria require a molybdenum–iron cofactor which is a constituent of the nitrogenase system. It was demonstrated that, under conditions of shortage of molybdenum, vanadium was taken up by the nitrogenase enzyme complexes from these *Azotobacter* species. Although vanadium was present in the nitrogenase system, it did not appear to fulfil a role as a catalyst. Moreover, it was proposed that the effect of vanadium incorporation could be accounted for by structural aspects, such as stabilizing the enzyme and affecting some of its catalytic properties (Burns et al., 1971; Benemann et al., 1972).

Recently, evidence has been presented that both *A. vinelandii* and *A. chroococcum* possess two nitrogen fixation systems: the conventional molybdenum nitrogenase and an alternative nitrogen-reducing enzyme complex. The alternative nitrogenase complexes from both species were shown to contain a vanadium–iron cofactor, analogous to the MoFe cofactor in the conventional enzymes. These VFe nitrogenases completely differed from the conventional nitrogenases with regard to substrate specificity and molecular weights (Hales et al., 1986; Robson et al., 1986; Arber et al., 1987). In contrast to vanadium bromoperoxidase the alternative nitrogenase from *A. vinelandii* did not exhibit an EPR signal derived from the vanadium ion (Hales et al., 1986). Since structural aspects of the vanadium(IV) ion in reduced bromoperoxidase can

[3] After reduction with sodium dithionite the sample preparations turned green, obviously due to the formation of paramagnetic $[MoOCl_5]^{2-}$ (Cotton & Wilkinson, 1972). This complex exhibited an intense rhombic signal around $g = 1.92$, and for this reason it was not possible to obtain an EPR signal derived from any molybdenum(V)–enzyme complex. This strong EPR signal of the molybdenum complex did not disturb vanadium(IV) bromoperoxidase signal intensities which were obtained from the central $m_I = -1/2$ hyperfine line, situated around $g = 2.0$. Signal intensities of vanadium(IV) bromoperoxidase were not affected by the presence of molybdenum, indicating that after addition of ~5 mM sodium dithionite to the preparations containing 0–50 mM molybdate, vanadium(V) bromoperoxidase was reduced prior to molybdenum(VI). At neutral pH free VO^{2+} (i.e., not bound to the enzyme) will precipitate due to the formation of $VO(OH)_2$. Vanadyl hydroxide is not observable by EPR (Chasteen, 1981).

readily be studied by EPR spectroscopy, this technique provides a convenient tool to obtain information on the metal-binding site of this vanadium-containing enzyme.

Registry No. V, 7440-62-2; VO_4^{3-}, 14333-18-7; MoO_4^{2-}, 14259-85-9; bromoperoxidase, 69279-19-2.

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