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High-resolution XANES studies on vanadium-containing haloperoxidase: pH-dependence and substrate binding

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High-resolution X-ray absorption vanadium K-edge spectra were recorded for samples of vanadium-containing bromoperoxidase from the brown alga, *Ascophyllum nodosum*, at pH 9, 7, 5 and 4, as well as for enzyme samples containing the substrates, hydrogen peroxide and bromide. The well-resolved features of the XANES spectra are discussed. The pH-dependence of the structure of the active site has been studied revealing no significant change of the absorption features. We were able to detect an interaction of H_2O_2 with the vanadium site of the bromoperoxidase using XAS spectroscopy, whereas addition of bromide causes no energy shift of the XANES spectrum. The possible role of vanadium during the enzymatic reaction is discussed on the basis of our results.

Vanadium; Haloperoxidase; XAS; XANES; *Ascophyllum nodosum*

1. INTRODUCTION

Marine and terrestrial organisms, such as algae and lichens, produce a variety of halogenated organic compounds [1] which exhibit antibiotic activity to presumably counteract bacterial attacks. Haloperoxidases, mostly heme-type but also vanadate-dependent metalloproteins, catalyze the formation of a hypohalite intermediate starting from halide ions and hydrogen peroxide. The hypohalite intermediate itself or HOX acts as the actual halogenating agent (Eqn. 1).

$$H_2O_2 + X^- \xrightarrow{\text{haloperoxidase}} OX^- + H_2O$$
$$OX^- + R\text{–}H \longrightarrow R\text{–}X + OH^- \qquad (1)$$

The enzyme, as isolated from the brown alga *Ascophyllum nodosum*, has a molecular weight of 100 kDa [2]. As shown by various spectroscopic studies (EXAFS, ^{51}V NMR, EPR), vanadium-dependent bromoperoxidase (V-BrPO) contains vanadium(V) surrounded mainly by O-ligands as donor groups in its active state [3]. Dithionite-reduced V(IV)-BrPO is irreversibly inactive. To date, the detailed structure and role of the active site of V-BrPO is not known. Binding of hydrogen peroxide as one of the substrates (H_2O_2 or Br^-) at the vanadium center has been postulated [4] but could not be detected by earlier X-ray absorption measurements [5,6]. The optimum proton concentration for the enzyme is between pH 5 and 6.5. Below pH 5, V-BrPO is slowly inactivated, presumably upon loss of vanadate. No correlation to structural changes upon pH variation has been performed.

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2. EXPERIMENTAL

V-BrPO was prepared according to Vilter [2]. The enzyme samples contained 2 mM vanadium (except enzyme samples at pH 7.2 which contained 1.2 mM vanadium). The enzyme was dissolved in 50 mM Tris-HCl buffer (pH 9 and 7.2) and 40 mM sodium citrate buffer (pH 5 and 4). Each sample contained 20% glycerol. Addition of 30 mM H_2O_2 (pH 7.2 and 5) or 30 mM KBr (pH 7.2) was used to study substrate influences on the X-ray absorption spectra. All samples were transferred to an XAS cell, made of a 25.4 × 3.6 × 1.5 mm piece of Lucite with a 22 × 3.3 × 1 mm window backed with 0.038 mm Kapton (polyimide) film to hold the sample and were frozen in liquid nitrogen immediately afterwards. Enzyme activity (800–1,000 U · mg^{-1}) was measured before and after the XANES experiments; no effect on the enzyme activity (< 50 U · mg^{-1}) was observed.

High resolution vanadium K-edge absorption spectra of the enzyme samples were recorded in fluorescence mode (13 element Ge detector; Canberra Inc.) on beamline 7-3 during a dedicated run of SPEAR (3 GeV, 50-95 mA) at the Stanford Synchroton Radiation Laboratory (SSRL). The synchroton X-ray beam from SPEAR was monochromatized with a Si[220] double crystal (50% detuned at 6,250 eV) and a 1 mm entrance slit, which yielded a reproducibility of 0.2 eV at 5 keV. The samples were held at 10 K during the measurements, using an Oxford Instruments CF1208 continuous-flow, liquid He cryostat.

Analysis of the data was performed on a MicroVax computer using a XAS software package [7]. Vanadium spectra were calibrated internally against the first inflection point of the vanadium foil edge (5,464.0 eV). The reported spectra are background-subtracted,

weighted sums of the data for all scans, and are normalized to give an edge jump of 1.0.

3. RESULTS AND DISCUSSION

High-resolution X-ray absorption vanadium K-edge spectra were recorded for samples of vanadium-containing bromoperoxidase (V-BrPO) from the brown alga, *Ascophyllum nodosum*, at pH 9, 7, 5, and 4, as well as for enzyme samples containing the substrates, hydrogen peroxide and bromide (at pH 7 and 5). The results of those measurements are summarized in Table I.

The most striking feature of vanadium edges [8] is the presence of an intense 'pre-edge' transition at ~ 5,469 eV due to the terminal oxo group as a ligand (V = O) (Fig. 1). This ligand causes a distorted octahedral symmetry around the vanadium center. Therefore, the usually dipole-forbidden 1s → 3d transition becomes dipole-allowed due to a combination of stronger 3d–4p mixing, and overlap of the metal 3d orbitals with the 2p orbitals of the oxygen ligand gaining substantial intensity [9]. This transition is followed sometimes by a weak shoulder on a rising absorption curve that culminates in a strong peak in the vicinity of 5,484–5,485 eV. This strong absorption peak has been assigned as the dipole-allowed 1s → 4p transition [8], the lower energy shoulder as the 1s → 4p shakedown transition [10]. At energies above the 1s → 4p transition, absorption features may arise from a transition to higher np states and multiple scattering. The recorded spectra display a substantially higher resolution than earlier XANES data [5,6], with features such as the shakedown transition not seen before. The high-resolution X-ray absorption spectra allow a detailed comparison with vanadium model compounds and discussion of the coordination type of native and oxidized V-BrPO, as shown elsewhere [11].

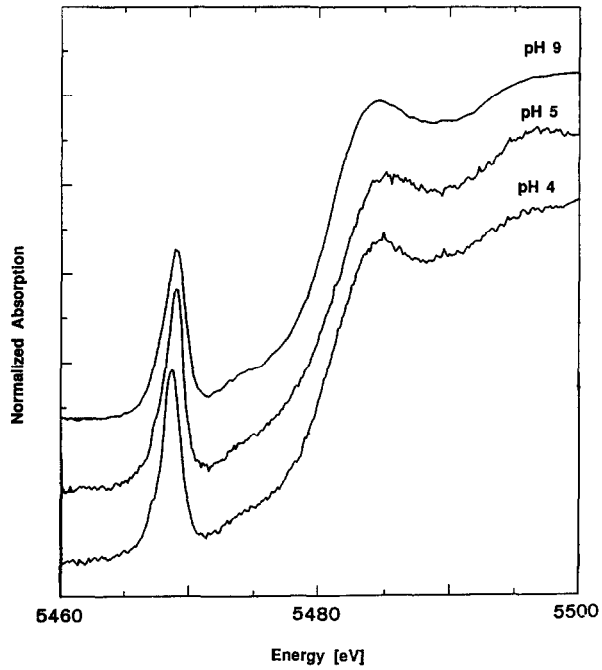


Fig. 1. Dependence of the vanadium K-edge X-ray absorption spectra of vanadium-containing bromoperoxidase on pH.

The dependence of the structure of the active site on pH has been studied. Comparison of the XANES spectra at three different pH's reveals no significant change of the pre-edge and main edge peaks from pH 9 to pH 4 (Fig. 1). However, the 1s → 4p shakedown transition decreases significantly in intensity going from pH 9 to pH 5, indicating minor geometrical changes. In vitro, the enzyme is slowly deactivated at pH 4 [12]. Rapid freezing and measurements at 10 K seem to slow down

Table I

XANES data of vanadium-containing bromoperoxidase (with and without the substrates, H_2O_2 and KBr) at different pH's

Enzyme samples	Pre-edge peak[a]	Peak height[b]	$v_{1/2}$[c]	Intensity[d]	Relative intensity[e]	Main edge[f]	1s → 4p transition[g]	1s → 4p shakedown[h]
VBrPO (pH 9)	5,469.0	0.60	1.8	1.07	0.64	5,480.2	5,484.4	~ 5,474
VBrPO (pH 7)	5,468.9	0.63	1.7	1.07	0.65	5,480.5	5,484.7	~ 5,474
VBrPO (pH 5)	5,468.9	0.59	1.8	1.05	0.63	5,480.2	5,484.7	–
VBrPO (pH 4)	5,469.1	0.57	1.8	1.03	0.60	5,480.7	5,485.1	–
VBrPO (pH 7)/H_2O_2	5,468.8	0.63	1.6	0.95	0.70	5,481.4	5,485.7	–
VBrPO (pH 5)H_2O_2	5,468.8	0.65	1.5	0.98	0.72	5,481.0	5,485.3	–
VBrPO (pH 7)/Br^-	5,468.8	0.62	1.7	1.05	0.65	5,480.4	5,484.5	–

[a] Energy [eV] of the pre-edge peak (± 0.2 eV).
[b] Normalized peak height (± 0.02) of the pre-edge peak.
[c] Width [eV] of the pre-edge at half-maximum height (± 0.2 eV).
[d] Product of the normalized peak height and width at half height ($I \cdot v_{1/2}$).
[e] Relative intensity = peak height of the pre-edge: peak height of the 1s → 4p transition.
[f] Energy of the first inflection point [eV] of the main edge (± 0.2 eV).
[g] Energy [eV] of the 1s → 4p transition (± 0.2 eV).
[h] Energy [eV] of the 1s → 4p shakedown transition (± 0.2 eV).

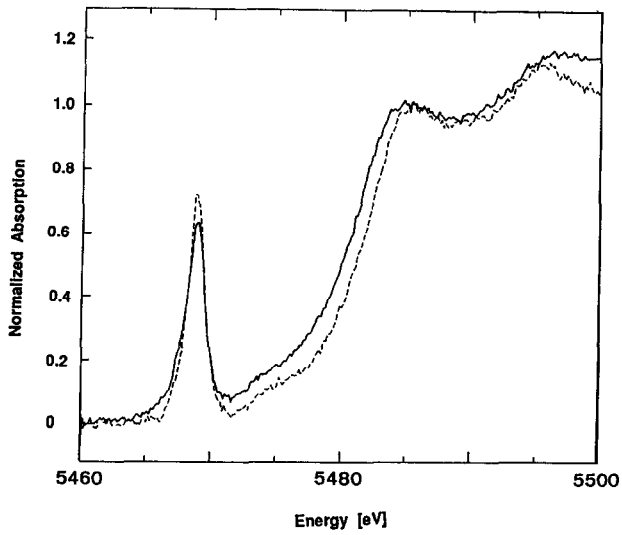


Fig. 2. Vanadium K-edge X-ray absorption spectra of vanadium-containing bromoperoxidase (pH 5, 2 mM vanadium) with (dotted line) and without (solid line) hydrogen peroxide (30 mM).

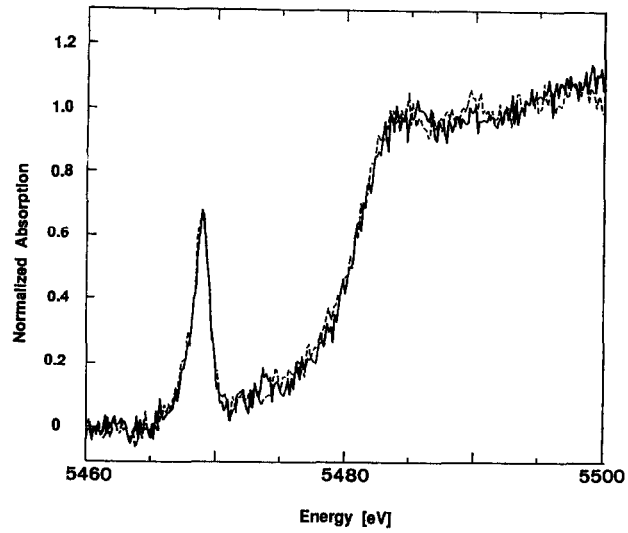


Fig. 3. Vanadium K-edge X-ray absorption spectra of vanadium-containing bromoperoxidase (pH 7.2, 1.2 mM vanadium) with (dotted line) and without (solid line) potassium bromide (30 mM).

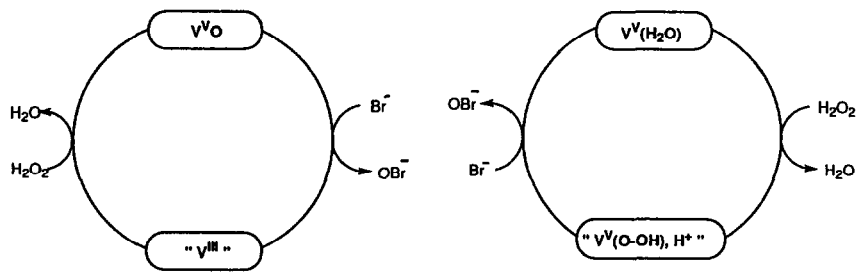
this process since full activity has been established after X-ray irradiation.

The first clue for an interaction of hydrogen peroxide with the metalloprotein came from spectrophotometrical studies [4]. However, no such interaction could be substantiated using X-ray absorption spectroscopy [5,6]. As proposed by Arber et al., low substrate concentrations (10 mM H_2O_2) might have been responsible for this observation [5]. We were able to detect such an interaction of H_2O_2 with the vanadium site of the BrPO at higher substrate concentrations (30 mM H_2O_2) using high-resolution XANES spectroscopy. At pH 7, as well as at pH 5 (Fig. 2), the main edge (inflection point) of the $1s \rightarrow 4p$ transition is shifted by +0.9 and +0.8 eV, respectively (Table I). Considering a reproducibility of 0.2 eV this energy shift is significant.

We therefore propose a direct interaction of the substrate, hydrogen peroxide, with the vanadium(V) center of V-BrPO, leading to small changes in the coordination sphere of the absorber atom. The energy position of the pre-edge does not change upon substrate addition. Slight changes of the relative intensities of the pre-edge to the main peak heights are observable. As shown earlier, the intensity of the pre-edge feature (product of normalized peak height and width at half height, $\nu_{1/2}$) can be related to the coordination number of the vanadium(V) center [11]. An octahedral coordination site for the vanadium center of V-BrPO is proposed using our XANES data. The addition of H_2O_2 to V-BrPO does not result in any substantial change in the coordination number. Therefore, substitution of a water molecule by a hydroperoxo ligand seems reasonable, as proposed by others [13]. The substrate binding is fully reversible, as shown by enzyme purification and subsequent re-measurement of the same sample.

Whereas addition of hydrogen peroxide causes an energy shift of the XANES spectrum of V-BrPO, addition of the second substrate, bromide (30 mM), does not give rise to any change of the edge data of V-BrPO (pH 7) (Fig. 3).

The detailed role of the vanadium center in the BrPO's is not known. Two reasonable mechanisms for the catalytic halide oxidation have been proposed (Scheme 1) [3a]. Vanadium could function as an electron transfer catalyst of the oxidation of bromide by peroxide (Scheme 1A) or a Lewis acid catalyst (Scheme



Scheme 1.

1B). In the first case, bromide coordinates directly at the oxo-vanadium(V) center. Electron transfer occurs, forming hypobromite and a reduced vanadium(III) site. In a second step, two electrons get transfered from the peroxide to reconstitute the active site. In the Lewis acid scheme (B) vanadium(V) coordinates hydrogen peroxide. The hydroperoxo ligand could then oxidize bromide. Vanadium would not formally change its oxidation state and bromide would not need to coordinate at the metal center. Such nucleophilic addition reactions of halides on peroxides with subsequent halide oxidation are known [14].

Our results clearly favor the latter mechanism (Scheme 1B), in which vanadium does not cycle between different oxidation states, but rather acts as a Lewis acid activating the coordinated peroxo or hydroperoxo ligand for electrophilic attack of the halide. This would be one of the very rare examples where a metalloenzyme catalyzes a redox reaction without change of the oxidation state of the involved transition metal (see [15] and references therein).

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