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^{14}N-coordination to VO^{2+} in reduced vanadium bromoperoxidase, an electron spin echo study

Eize de Boer*, Cornelus P. Keijzers+, Adri A.K. Klaassen+, Eduard J. Reijerse+, David Collison°, C. David Garner° and Ron Wever

Laboratory of Biochemistry, University of Amsterdam, PO Box 20151, 1000 HD Amsterdam, +*Department of Molecular Spectroscopy, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands* and °*Chemistry Department, University of Manchester, Manchester M13 9PL, England*

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Vanadium bromoperoxidase from the brown seaweed *Ascophyllum nodosum* was studied with electron spin echo envelope modulation (ESEEM) spectroscopy. After comparing the Fourier transformed (FT) ESEEM spectra with those of a number of vanadyl model compounds, it could be concluded that nitrogen is present in the equatorial plane of the vanadyl cation of reduced bromoperoxidase (^{14}N frequencies occurred at 3.1, 4.2, 5.3 and 8.1 MHz). Furthermore, the FT-ESEEM spectra of reduced bromoperoxidase exhibited an intense ^{1}H modulation (13.8 MHz), which was completely replaced by a deuterium modulation at ~2 MHz when bromoperoxidase was dissolved in D_2O, instead of H_2O. These latter data confirm earlier EPR experiments on reduced bromoperoxidase [(1988) Biochemistry 27, 1629–1635], showing that the oxo-vanadium (IV) ion is coupled to exchangeable protons.

Vanadium; Bromoperoxidase; ESEEM; *(Ascophyllum nodosum)*

1. INTRODUCTION

Many organisms are able to synthesize halogenated metabolites. These halometabolites are formed upon oxidation of halides by haloperoxidases and hydrogen peroxide, in the presence of a nucleophilic reagent. Halometabolites appear to be involved in chemical defence mechanisms since they display considerable antibacterial and anticellular activities [1]. Most haloperoxidases contain haem at the active site [2]. However, vanadium(V) is essential for the brominating activity of bromoperoxidases purified from several marine algae [3–6] and also from a lichen [7].

Correspondence address: R. Wever, c/o G.E.E. van Noppen, FIL, Publications Secretary, Laboratory of Biochemistry, University of Amsterdam, PO Box 20151, 1000 HD Amsterdam, The Netherlands

* *Present address:* Duphar BV, PO Box 2, 1300 AA Weesp, The Netherlands

Vanadium bromoperoxidase from *Ascophyllum nodosum* has been extensively studied by electron paramagnetic resonance (EPR) spectroscopy [4,5,8]. An EPR spectrum of the enzyme can be obtained after reduction of the vanadium(V) to vanadium(IV) ($3d^1$), using sodium dithionite. The rhombically distorted nature of this X-band (~9 GHz) EPR spectrum [8], which showed coupling of the unpaired electron with ^{51}V ($I = 7/2$), and its associated parameters [4,5,8] are consistent with the presence of a terminal oxo group and are indicative of oxygen and/or nitrogen donor atoms in the coordination sphere [15]. Therefore, it is of interest to verify whether or not nitrogen is present in the immediate vicinity of the vanadyl cation.

In the present communication we report on the studies of nuclear modulations in the electron spin echo (ESE) decay envelope of reduced bromoperoxidase. These nuclear modulations arise when a paramagnetic centre is coupled to surrounding nuclear spins, such as ^{1}H and ^{14}N [9,10]. By Fourier transformation of this electron spin echo envelope modulation (ESEEM) decay pat-

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tern, it is then possible to construct an electron nuclear double resonance (ENDOR)-like spectrum, composed of the nuclear magnetic resonance (NMR) frequencies of the nuclei which are coupled to the unpaired electron [9,10]. These spectra proved to be useful in identifying ligands of paramagnetic metal ions in a variety of metalloproteins [11–14].

Normally, the $3d^1$ ground state of vanadyl (oxovanadium(IV)) compounds is orbitally non-degenerate with no electronic excited states nearby [15,16]. Due to the fact that this d_{xy} ground state is essentially non-bonding, nuclear hyperfine coupling arising from the ligands is usually small [17,18]. Obviously, ESEEM requires the presence of a hyperfine interaction. In order to observe modulations it is necessary, however, that the sum of the anisotropic nuclear interactions (hyperfine and/or nuclear quadrupole) is of the same order of magnitude as the sum of the isotropic nuclear interactions (hyperfine and Zeeman). Therefore, this is an ideal technique for the identification of ^{14}N in a coordination position with a low electron density (unpaired electron in non-bonding orbital or ^{14}N in a higher coordination sphere) [19,20]. Previously, the technique has been used to study ^1H interactions in VO^{2+} complexes with water and methanol [21], and was applied to determine hyperfine interactions in vanadyl acetylacetonate with nitrogen-containing donor bases [22].

The results of this investigation indicate that the vanadyl cation in reduced bromoperoxidase is indeed coupled to nitrogenous ligands and, moreover, the amplitude Fourier transforms of the nuclear modulation patterns clearly show the presence of weak ^1H superhyperfine coupling, arising from exchangeable protons near the paramagnetic centre.

2. MATERIALS AND METHODS

Bromoperoxidase was purified from the brown seaweed *A. nodosum* as described by Wever et al. [23], with modifications [4]. Purified enzyme preparations were dissolved in 0.1 M potassium citrate/0.1 M potassium sulphate (pH 7.0). Enzymic activity was measured as described before [23], and protein content was determined by the method of Lowry et al. [24]: with bovine serum albumin as a standard. The preparation used in the ESEEM experiments was 2.3 mM in bromoperoxidase or 2.3 mM in vanadium [8], with a specific activity of 112 μmol of 2-chlorodimedone brominated per min per mg of protein.

$[VO(meox)_2]$, oxobis(2-methylquinolin-8-olato) vanadium(IV), was prepared by the method of Shiro and Fernando [25]. A single crystal X-ray diffraction study [25] showed that the molecule possessed crystallographically required two-fold site symmetry. [VO(salen)], oxo-*N,N'*-bis(salicylidene)ethylenediamine vanadium(IV), and [VO(salophen)], oxo-*N,N'*-bis(salicylidene)-*o*-phenyldiamine vanadium(IV), were prepared by adding ethanolic solutions of the appropriate Schiff base to an equimolar aqueous solution of vanadyl sulphate. The resultant precipitates were separated by filtration, washed with ether, then pentane and dried in air. Schiff bases were prepared by the condensation of stoichiometric quantities of salicylaldehyde and the appropriate diamine in absolute ethanol. Satisfactory elemental analyses (for C, H, N and V) were obtained for all complexes by the staff of the Microanalytical Laboratory of the Chemistry Department at the University of Manchester. Vanadyl sulphate was obtained from BDH Ltd (England) and all organic reagents from Aldrich Chemical Co. Ltd (England) and were used without further purification.

The resonator used was a stripline resonator, which was completely filled with the sample solution. Bromoperoxidase was reduced with sodium dithionite in the cavity, and subsequently the filled cavity was quickly frozen in liquid nitrogen. The vanadyl compounds were dissolved in dichloromethane/toluene (1:2).

The ESEEM spectra were measured using the stimulated echo sequence ($90°-\tau-90°-T-90°-\tau$–echo) [9–11], where τ was fixed (300, 380, 400 or 480 ns) and T was scanned from 0 to 5 or 10 μs, with increments of 10 ns. The repetition time was 8 ms. All measurements were performed at 10 K. Details concerning the spectrometer and handling of data have been described previously [26].

3. RESULTS AND DISCUSSION

The EPR spectrum of the frozen solution of reduced bromoperoxidase consists of two groups of hyperfine lines [4,5,8]. A group of eight low-intensity lines, corresponding to the parallel ($g_\parallel$) orientation, which coincides with the V = O direction, and eight high-intensity lines, mainly associated with the perpendicular ($g_\perp$) orientation (fig.1). The modulation decay envelopes were studied at two values of the external magnetic field, H_o, corresponding to the $m_I = (-3/2)_\perp$ hyperfine line and to the central $m_I = (-1/2)_{\parallel,\perp}$ hyperfine line, indicated as a and b, respectively, in fig.1. In principle, the major contribution to the $m_I = (-3/2)_\perp$ signal is due to those molecules, which have the V = O bond perpendicular to H_o, whereas the central $m_I = (-1/2)_{\parallel,\perp}$ hyperfine line covers both parallel and perpendicular orientations [15,21]. Due to a low signal-to-noise ratio it was not possible to obtain ESEEM spectra from the $g_\parallel$ regions of the EPR spectrum.

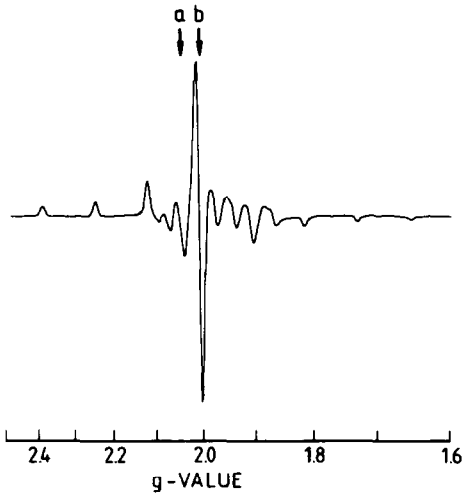

Fig.1. X-band (~9 GHz) frozen solution EPR spectrum of reduced vanadium bromoperoxidase. Magnetic field settings corresponding to the $m_I = (-3/2)_\perp$ (a) hyperfine line, as well as to the central $m_I = (-1/2)_{\parallel,\perp}$ (b) hyperfine line are marked on the figure. The sign of the vanadium hyperfine tensor components is assumed to be negative. The spectrum was obtained with a Varian E-9 EPR spectrometer. Instrument settings: temperature, 60 K; microwave power, 2 mW; modulation width, 1 mT.

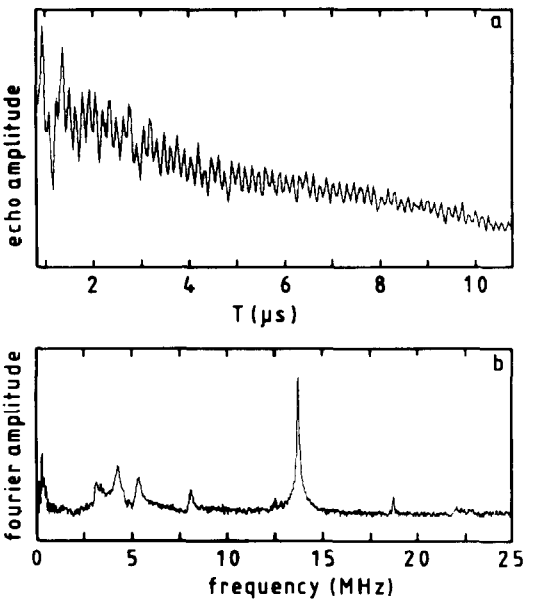

Fig.2. Time-domain (a) and Fourier transform of the echo envelope (b), obtained by the stimulated three-pulse ESEEM method, for reduced vanadium bromoperoxidase. Data were obtained by pulsing on the central EPR $m_I = (-1/2)_{\parallel,\perp}$ hyperfine line (marked b in fig.1). The time-domain data were obtained by averaging 1500 pulses for each time point. Other conditions were: H_o, 0.324 T; microwave frequency, 9043 MHz; τ, 380 ns; pulse width, 20 ns.

The three-pulse ESE decay envelope as a function of T for reduced bromoperoxidase is shown in fig.2a. This ESEEM spectrum was obtained by pulsing on the central $m_I = (-1/2)_{\parallel,\perp}$ vanadium hyperfine line. The signal shows an essentially monotonic decay of the echo envelope, modulated by a complicated function. The main contribution to this ESEEM is a high frequency component, as became clear after Fourier transformation (FT) (fig.2b). This high-frequency component was visible at 13.8 MHz and was completely replaced by a peak at 2 MHz for a sample of reduced bromoperoxidase dissolved in D_2O (D_2O data not shown). The 13.8 and 2 MHz components are ascribed to the interaction of the unpaired electron with proton and deuteron nuclear spins, respectively (at an external field setting of $H_o = 0.324$ T the free-proton and free-deuteron nuclear frequencies are 13.8 and 2.12 MHz, respectively). Thus, the 13.8 MHz peak is solely attributed to exchangeable protons near to the paramagnetic centre. The 13.8 MHz peak was extremely narrow, indicating that the proton hyperfine coupling is small, which suggests that these protons are only weakly bound to the vanadium(IV) centre. EPR measurements for reduced bromoperoxidase dissolved in D_2O and $H_2{}^{17}O$ have shown that, at least part of, these exchangeable protons are derived from coordinated water [8].

The FT analysis of the ESEEM spectra also revealed the presence of some low-frequency contributions. Fig.2b shows that four transitions were visible at 3.1, 4.2, 5.3 and 8.1 MHz. These frequencies can only be ascribed to the presence of ^{14}N, since the abundance of ^{17}O is too small for detection. Additional measurements of FT-ESEEM spectra for reduced bromoperoxidase were made at settings of $\tau = 300$ and 400 ns. This yielded identical spectra, with variations in relative peak heights as expected from the theory of ESEEM spectroscopy [9]. FT-ESEEM spectral data are given in table 1. This table shows that similar frequencies were obtained for the Fourier transforms at both positions of H_o.

Modulations attributed to the presence of ^{14}N nuclei were also observed in ESEEM spectra of

Table 1

^{14}N frequencies for reduced vanadium bromoperoxidase and some vanadyl complexes

Compound	H_o (T)	f (MHz)	m_I	Orientation	τ (ns)	Observed frequencies (MHz)
Reduced	0.324	9043	−1/2	$g_\parallel + g_\perp$	380	3.1, 4.2, 5.3, 8.1
Bromoperoxidase	0.217	9043	−3/2	$g_\perp$	300	3.0, 4.7, 5.3, 8.6
[VO(meox)$_2$][a]	0.331	9238	−1/2	$g_\parallel + g_\perp$	380	3.2, 4.8, 5.8, 8.4
	0.324	9238	−3/2	$g_\perp$	380	3.2, 4.8, 5.8, 8.4
[VO(salen)]	0.331	9238	−1/2	$g_\parallel + g_\perp$	380	5.0, 5.7, 9.0
[VO(salophen)]	0.331	9238	−1/2	$g_\parallel + g_\perp$	380	5.0, 5.7, 9.0

[a] [VO(meox)$_2$], oxobis(2-methylquinolin-8-olate) vanadium(IV); [VO(salen)], oxo-*N,N'*-bis(salicylidene)ethylenediamine vanadium(IV); [VO(salophen)], oxo-*N,N'*-bis(salicylidene)-*o*-phenylenediamine vanadium(IV)

several VO^{2+} model compounds. Fig.3a shows the ESEEM at H_o = 0.331 T of [VO(meox)$_2$], a trigonal bipyramidal vanadyl compound with two oxygen and two (chemically equivalent) equatorial nitrogen donor atoms [25]. This decay pattern shows several low-frequency components attributable to ^{14}N (fig.3b). The frequencies corresponding to the four major transitions are given in table 1. Table 1 also shows data obtained at H_o = 0.324 T for [VO(meox)$_2$], and includes the ^{14}N frequencies of some other vanadyl compounds. It can be seen that [VO(salen)] and [VO(salophen)] both yielded FT-ESEEM spectra, comparable to those of [VO(meox)$_2$] and reduced bromoperoxidase. The differences in the observed frequencies between the vanadyl compounds assigned to interaction with ^{14}N nuclei may reflect the difference in nitrogen donor atom type; salen and salophen bind to metal ions via imino nitrogen atoms whilst meox contains a quinoline nitrogen. From these FT-ESEEM spectra it is not possible to calculate the number of nitrogen atoms involved in vanadium coordination.

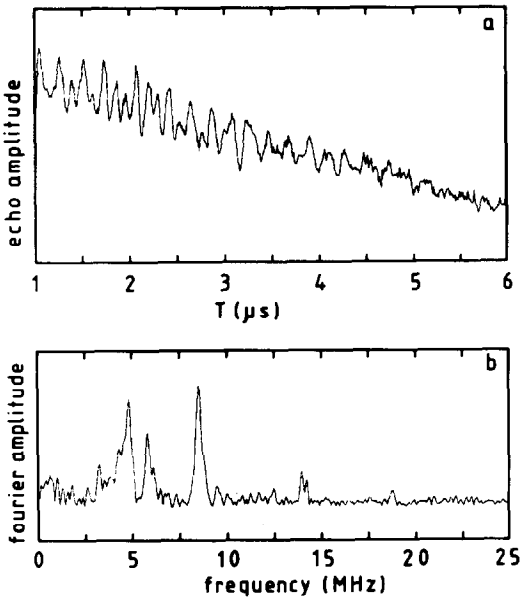

Fig.3. Time-domain (a) and Fourier transform of the echo envelope (b), obtained by the stimulated three-pulse ESEEM method, for [VO(meox)$_2$]. Data were obtained by pulsing on the central EPR $m_I = (-1/2)_{\parallel,\perp}$ hyperfine line. The time domain data were obtained by averaging 100 pulses for each time point. Other conditions were: H_o, 0.331 T; microwave frequency, 9238 MHz; τ, 480 ns; pulse width, 40 ns.

In ENDOR studies on Cu(II), Ag(II), and oxovanadium(IV)-containing complexes of tetraphenylporphyrin (TPP), nitrogen hyperfine interactions were found of 47.0, 67.7 and 6.8 MHz, respectively [27–29]. The large differences between the contact interactions for CuTPP and AgTPP, on the one hand, and VOTPP on the other, were ascribed to the difference in electron distribution of these metal ions (d^9 for Cu(II) and Ag(II), d^1 for VO(II)). In Cu- and AgTPP the unpaired electron resides primarily in the metal $d_{x^2-y^2}$ orbital, which overlaps with the sp^2 hybrid orbitals of the pyrrole nitrogens, explaining the large contact interactions. In VOTPP the unpaired electron is in a molecular orbital in which the metal d_{xy} contribution dominates [17,18]. Marginal overlap of this orbital with ligand orbitals accounts for the small

contact interaction of 6.8 MHz [27]. Due to the large contact interactions of equatorial nitrogen in Cu(II) complexes, modulations of these nuclei are normally not observed in ESEEM spectra [19], but are usually well resolved in EPR. As a consequence, directly coordinated nitrogen of imidazole histidine to Cu(II) in amine oxidase [30] and galactose oxidase [12] did not contribute to the ESEEM decay patterns of these enzymes. Since the contact interactions of equatorial nitrogen ligands in vanadyl complexes are relatively small, they are not resolved in EPR but readily contribute to ESEEM spectra. From this we conclude that in reduced bromoperoxidase nitrogen is also equatorially coordinated to the vanadyl cation. This demonstrates that ESEEM spectroscopy is a powerful tool to study structural aspects of vanadium bromoperoxidase.

The use of ESEEM spectroscopy on reduced vanadium bromoperoxidase and a series of low-molecular mass vanadyl complexes with oxygen and nitrogen donor ligands has further defined the metal environment of this enzyme. These results have confirmed the occurrence of exchangeable protons reported in a previous EPR study [8], but now suggest that only weak binding is involved. The presence of equatorial nitrogen donor atoms in a near-axial electronic environment defined by a terminal oxo group is strongly suggested by comparison with the model compounds and the arguments developed from studies reported for metal-porphyrin complexes.

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