^{51}V NMR Investigation of a Vanadate(V)-dependent Peroxidase From *Ascophyllum nodosum (L.) Le Jol*

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A peroxidase which is able to catalyse brominations and iodinations has recently been isolated from the marine alga *Ascophyllum nodosum* [1]. In contrast to many other peroxidases, an *Ascophyllum nodosum* peroxidase (A.n.I.), as shown by the lack of a Soeret band in the electron absorption spectrum [2, 3], does not belong to the hemoproteids. The most unusual property of A.n.I. is possibly its specific reactivation by vanadata(V) after de-activation with complexing buffers [4, 5], a fact which leads to the suggestion of vanadate having a distinct function at the active site of the peroxidase [4] and thus being the prosthetic group of the enzyme [5]. This assumption has essentially been supported by the ESR detection of V(IV) in reduced peroxidase [5]. Reduction may well, however, lead to changes in the coordination sphere of the vanadium centre. We have therefore undertaken a ^{51}V NMR study of the unreduced form in order to obtain first information on the active site of A.n.I. and its possible interaction with iodide.

Although ^{51}V NMR spectroscopy is a well established method for the investigation of diamagnetic vanadium compounds [6], a fact which is mainly due to its almost exclusively favourable nuclear properties [nuclear spin = 7/2, quadrupole moment = -0.052×10^{-28} m^2, natural abundance 99.76%, relative receptivity = 0.38 (constant B_0) and 5.52 (constant ν_0)], there have been only sporadic reports dealing with ^{51}V NMR studies in systems with a (potential) biological relevance. Among these are recent papers on the interaction between vanadate and alcohols [7], carbonic acids [8], phosphoric acid [9], and uridine and ribonuclease [10]. The present study is the first case where a ^{51}V resonance for an enzyme relying on vanadate is reported**.

The material used in our study was extracted from *Ascophyllum nodosum* and purified by an improved procedure described elsewhere [11]. The specific activity as iodoperoxidase [12] was 930(30), and as bromoperoxidase [3] 200(20) U/mg. This is a higher activity than observed in samples of earlier preparations [2, 3]. The protein contents were determined by UV and by the methods of Lowry and Bradford [22]. A solution containing 0.8 mg A.n.I. was dialysed twice for 24 h against the 25-fold volume of 2 mM tris-buffer (tris(hydroximethyl)aminomethane, Serva), pH 8.5, containing Na_3VO_4 (Merck; concentration 20 μM). For the ^{51}V NMR experiments, the samples were concentrated by use of immersible CX-30^{TM} single-use ultra filtration units (Millipore) to a final concentration of 77(2) mg/ml (0.82 mM, assuming a molar mass for the enzyme of 95 000). The vanadium contents, as determined by atomic absorption spectroscopy, were 74.7 μg V/g enzyme solution (*ca.* 1.46 mM), which amounts to about 2 V atoms per molecule of A.n.I. This is in accord with ESR evidence for bromoperoxidase from *Laminaria saccharina* [13] and also from *Ascophyllum nodosum* (R. Wever, Amsterdam, personal communication).

Results and Discussion

The results of the NMR measurements are summarized in Table I; illustrative spectra are displayed in Figs. 1–3. The complexation capacity of the tris-buffer towards vanadate is small. A 1.6 mM vanadate solution containing a tenfold excess of tris, exhibits one main signal at −555.0 ppm, which can be assigned to $H_xVO_4^{(3-x)-}/HV_2O_7^-$ [14]. A low-field signal of low intensity (*cf.* Fig. 1) is probably produced by vanadate-tris interaction.

Peroxidase (0.82 mM) in tris-buffer solution and with its original vanadium contents (1.46 mM) shows a broad signal (Fig. 2a, **A**) at −1070(30) ppm which shifts to *ca.* −1250(80) ppm as the sample is treated with additional vanadate (Fig. 2a, **B**). Line widths at half-height, $W_{1/2}$, are around 6 kHz. We have also been able to detect, in the original, untreated solution, a sharp signal ($W_{1/2}$ = 105 Hz) at −539.4 ppm, corresponding to free HVO_4^{2-} in apparent slow exchange with peroxidase-bound vanadate. This signal gains intensity only after addition of more than the twofold excess of vanadate (overall vanadium concentration > 3 mM), indicating that added vanadium is also bound unspecifically to the protein. Vanadate-protein binding can also be observed in the vanadate/albumin system (Fig. 2b), for which, along with a broad resonance shifting between −710(80) and −970(40) ppm for albumin-bound vanadate, a

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**A vanadium resonance for enzyme-bound vanadate has also been detected in the vanadate/phosphoglycerate mutase system (M. J. Gresser, Burnaby, B.C., personal communication).

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TABLE I. ^{51}V NMR Data[a]

Compound/system	{V} Concentration (mM)	Molar ratio {V}/protein	$\delta(^{51}V)$[b] (ppm)	$W_{1/2}$[c] (Hz)	Assignment
{V}–tris(16 mM)–D_2O[d]	1.6		−524.6	180	{V}–tris
			−538.3[e]	56	HVO_4^{2-}
			−564.3	130	T_2 and/or T_4[f]
			−567.3	80	
{V}–A.n.I.(0.82 mM)–tris–H_2O[g]	1.5	1.8	−555.0[h]	105	$H_xVO_4^{(3-x)-}/HV_2O_7^-$
			−1070(30)	5300	{V}–A.n.I.
	2.0	2.5	−1230(60)	5800	{V}–A.n.I.
	2.9	3.6	−1250(80)	[i]	{V}–A.n.I.
	5.0	6.1	−535(30)	3000	HVO_4^{2-}
			−1290(70)	6500	{V}–A.n.I.
{V}–im(0.13 M)–H_2O	16.7		−573(8)	[i]	T_4–T_6[f]
{V}–A.n.I.(0.724 mM)–im–H_2O[j]	1.4	1.8	−1090(60)	[i]	{V}–A.n.I.
{V}–A.n.I.–im–KI(9 mM)–H_2O	1.3	1.8	−1270(45)	4600	{V}–A.n.I.
{V}–albumin(1.2 mM)[k]–tris–H_2O	0.45	0.4	−1000(60)	[i]	{V}–albumin
	4.0	3.3	−570(40)	1300	HVO_4^{2-}
			−710(80)	8200	{V}–albumin
	8.0	6.6	−560(30)	1100	HVO_4^{2-}
			−970(40)	2100	{V}–albumin

Complex[l]	$\delta(^{51}V)$	$W_{1/2}$
$[VO_2(oxalate)_2]^{3-}$[m]	−520	500
$[VO(O_2)(oxalate)_2]^{3-}$	−595	250
$[VO(O_2)_2(oxalate)]^{3-}$[n, o]	−695	380
$[VO(O_2)_2CO_3]^{3-}$[n, p]	−720	280
$[VO(O_2)(CO_3)_2]^{3-}$[q]	−760	320
$[VO(O_2)_2(acetate)]^{3-}$[n]	−705	1250
$[VO(O_2)(acetate)_2]^{3-}$[q]	−750	680

[a]If not indicated otherwise, data were obtained on a Varian DP wideline spectrometer at B_0 = 1.4280 T [$B(VOCl_3)$ = 1.4273 T; ν_0 = 15.97 MHz] and sweep widths of typically 4 mT, maximum rf setting, modulation amplitude 0.05 to 0.4 mT, scan number *ca.* 300, scan time 2 min, time constant 0.1 to 0.3 s. The samples were contained in 14 mm tubes. Abbreviations: {V} = vanadate, used as Na_3VO_4 (Merck); tris = tris(hydroxymethyl)aminomethane buffer 0.2 M (pH = 8.5); im = imidazole/HCl buffer 0.2 M, pH = 6.2; A.n.I. = peroxidase from *Ascophyllum nodosum*. [b]Relative $VOCl_3$ neat. [c]Width at half-height; estimated error *ca.* 10%. [d]Obtained on a Bruker AM 360 spectrometer at 94.67 MHz, sweep width 125 kHz, relaxation delay 0.5 s, pulse width 10.9 μs, scan number 89 000. The samples were contained in 8 mm diameter inserts of 0.42 ml capacity (Wilmad) fitted into rotating 10 mm vials. For the measurements of vanadate-peroxidase, the lock (D_2O) was applied externally. [e]Main signal, taking about 98.5% of the total intensity. [f]T_n refers to *n*-nuclear vanadate species with a tetrahedral coordination of V. For assignments see ref. 14. [g]Final pH = 8.0. [h]Obtained on a Bruker AM 360 (footnote d), 95 000 scans. [i]Not determined due to overmodulation. [j]Final buffer concentration 0.134 M, final pH = 6.6. [k]Bovine serum albumin (Merck), M = 67 000. [l]Data obtained on a Bruker DP 60 instrument at 1.4282 T. Typical measuring parameters deviating from those in footnote a: sweep range 0.5 mT, modulation amplitude 8 μT, *ca.* 3 scans, scan time 5 min. Typical concentrations 0.2 M. Error for δ: ±5 ppm, for $W_{1/2}$ 10%. [m]Prepared according to ref. 20. The oxalate bounds over its two carboxylato groups, forming a chelate 5-ring. [n]Prepared according to ref. 21. [o]In this complex, the oxalate is possibly bound via one of its carboxylato groups only, forming a chelate 4-ring (η^2-mode). [p]Reportedly, the O_2^{2-} and CO_3^{2-} ligands are η^2-bound [21]. *Cf.* Fig. 3 for details. [q]Tentative assignments based on the change in the ^{51}V NMR spectra on addition of excess carbonate/acetate or boiling down part of the peroxide.

signal for HVO_4^{2-} arises at higher vanadate concentrations.

The shift values for A.n.I.-bound vanadate are the highest observed to date for vanadium(V), exceeding those for $VOF(NO_3)_2$ (−826 ppm [15]) and VF_5 (−875 ppm [16]). Generally, large shielding values can be expected if the ligand functions in the coordination sphere exhibit large electronegativities χ (low polarizabilities) [16] and, in fact, almost linear δ/χ correlations have been established for VOZ_3 and VOZ_4^- complexes, where Z = Br, Cl, NR_2, OR and F [6, 16]. It is also evident, however, that these correlations are only valid within a series of complexes where all members have the same coordination number and where strains due to bulky ligands or small chelate-ring structures are negligible. For example, the compounds VO_3OH^{2-} [14], $\overline{VO(OH)_2\text{-}OCH_2CH_2O}$ [7] and $VO_2(oxalate)_2^{3-}$ [9, 17, 18] all

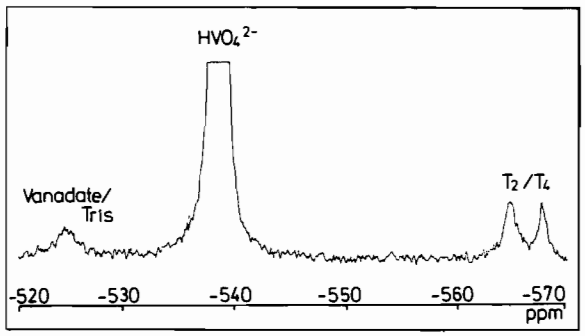

Fig. 1. 94.67 MHz $^{51}V\{^1H\}$ NMR spectrum of 1.6 mM Na_3VO_4 in 16 mM tris-buffer at pH *ca.* 8.5. The signal for HVO_4^{2-} covers about 98.5% of the total intensity. T_2 and T_4 are divanadate and tetravanadate, respectively.

have the same $\delta(^{51}V)$ around −530 ppm. On the other hand, monofunctional ligands bonding in the η^2 mode induce a drastic increase of shielding. Examples are $VO_3(O_2)^{3-}$ (−769 ppm [19]), VOF_3-NO_3^- (−778 [15]) and the carbonato and carboxylato complexes in Table I with three- (O_2^{2-}) and four-membered rings (NO_3^-, CO_3^{2-}, $CH_3CO_2^-$).

A possible explanation for the consistently high shielding of the ^{51}V nucleus in A.n.I.-bound vanadate therefore is the participation of at least two η^2-bound carboxylato groups in a six- or seven-coordinate environment built up exclusively by the highly electronegative oxygen functions.

In order to test whether there is an interaction of halide ions (Br^-, I^-) and the prosthetic group in A.n.I., we have also investigated the A.n.I.–iodide systems under conditions where the peroxidase activity is close to the optimum (pH 6.2 [12], molar ratio {V}/A.n.I. 7/1). A.n.I. in imidazole/HCl buffer* shows the same signal at −1090(60) ppm as has been observed for the tris-buffer system. Hence, no changes occur on changing the buffer and pH (and by Cl^-, the oxidation of which is not catalysed by the enzyme). In contrast, addition of iodide shifts the signal to higher field by about 250 ppm (Fig. 2a, C). Direct binding of I^- to vanadium(V) should, according to the shift/electronegativity relation mentioned above [16], shift the signal to low field. The interaction of iodide therefore has to be interpreted in terms of an alteration in the coordination sphere of vanadium, possibly by a change from the η^1- to the η^2-mode of a carboxylato group of an acidic constituent of the protein matrix.

*The activity of the enzyme in this buffer drops to *ca.* 90% during a 24 h period.

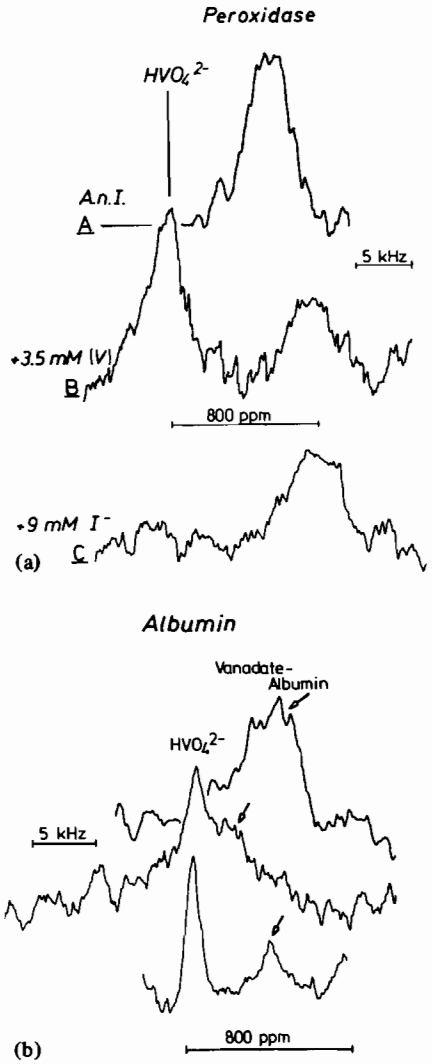

Fig. 2. (a) ^{51}V NMR spectra of peroxidase from *Ascophyllum nodosum*, reactivated with vanadate in tris-buffer (see text), pH = 8.5, showing enzyme-bound vanadate (A, total concentration 1.46 mM). Spectrum B represents the situation after addition of a 3.4-fold excess of vanadate (total V concentration 5.0 mM). C shows the spectrum of A.n.I. in imidazole buffer (pH = 6.6) after addition of KI (overall iodide concentration 9 mM). (b) ^{51}V NMR spectra of 1.2 mM bovine serum albumin in tris-buffer, containing 0.45, 4 and 8 mM (top to bottom) vanadate. All spectra were obtained at 15.97 MHz on a Varian DP 60 wide-line instrument. See Table I for data and further details.

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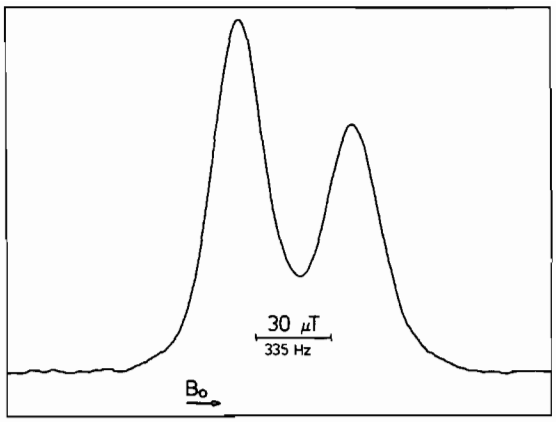

Fig. 3. 15.97 MHz ^{51}V NMR spectrum of a sample of $[VO(O_2)_2CO_3]^{3-}$ prepared according to ref. 21. The signal to low field disappears on addition of excess CO_3^{2-} or on boiling the aqueous solution, and regains intensity to the expense of the high-field signal as H_2O_2 is added. The high-field signal is therefore tentatively assigned to $[VO(O_2)(CO_3)_2]^{3-}$.

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