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(Model) studies on vanadate-dependent bromo/iodoperoxidase from *Ascophyllum nodosum*

VO^{2+} is not incorporated into the active site

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Vanadate-dependent peroxidase A.n.I, the main isoenzyme (M_r = 100 kDa) from the seaweed, *Ascophyllum nodosum*, contains 2 V per enzyme molecule (as shown by ICP-MS metal analysis) after complete reconstitution with vanadate (V), possibly distributed in a 1:1 ratio between the surface and active site. VO^{2+} is only weakly associated to the surface of A.n.I. There is no transport channel for VO^{2+}. The EPR spectrum of the reduced holoenzyme is anisotropic (axial) already at room temperature, with EPR parameters similar to those of VO^{2+} complexes of small model peptides such as Ala-His, Gly-Tyr, Gly-Ser, Gly-Glu, Ser-Gly and Phe-Glu. The complex formation between Ala-His and $H_2VO_4^-$ in water has also been investigated (by ^{51}V NMR); the formation constant at pH 7.2 amounts to 266(28) M^{-1}.

Vanadate-dependent peroxidase; Vanadyl-peptide complex; Vanadate-peptide complex; Electron paramagnetic resonance; ^{51}V Nuclear magnetic resonance spectroscopy

1. INTRODUCTION

Vanadate-dependent haloperoxidases are common in many species of marine brown algae [1,2] of which the most intensively investigated is the *Ascophyllum nodosum* isoenzyme, A.n.I [1,3]. This remarkable non-heme enzyme catalyzes the bromination and iodination of organic substrates by peroxide using an inorganic halide as halogen source. In the absence of a suitable substrate, singlet oxygen is evolved from H_2O_2 [4]. A.n.I (M_r = 100 kDa) is especially rich in acidic amino acids. It can be inactivated by dialysis against EDTA/citrate buffer, and reactivated with vanadate (V). Phosphate, arsenate and molybdate are inhibitors for A.n.I. The reduced form of the holoenzyme, containing VO^{2+}, is inactive, but quickly regains activity by re-oxidation when exposed to air.

Reconstitution of the apoenzyme with vanadate (V) is a slow process, taking about 24 h until full activity is regained. This has led to the assumption [5] that there is a shuttle mechanism (Fig. 1) by which vanadate is gradually incorporated into the active site (III in Fig. 1). Hydrogen-bonding (I) and coordination (II) of vanadate to the surface of A.n.I have been detected by means of ^{51}V NMR spectroscopy [5]. Since, under physiological conditions, vanadate ($H_2VO_4^-$) is easily reduced to vanadyl (VO^{2+}) by, e.g. glutathion or ascorbate, the question arises (i) whether VO^{2+} is also bound non-specifically to the protein surface, and (ii) whether there is a mechanism by which VO^{2+} is transported into the active center.

We have approached this problem using EPR spectroscopy. Several simple dipeptides have been employed as model compounds, containing serine, histidine, tyrosine or glutamine as one of the peptide constituents, and hence amino acids which have been discussed as constituents of the active site coordination sphere of vanadium. These model studies have been supplemented by ^{51}V NMR investigations of the system vanadate/Ala-His. Coordination of an N ligand (probably histidine) to the active site vanadium has been deduced from an electron spin echo study [7]. We also present new analytical data regarding the vanadium content of the holoenzyme.

2. MATERIALS AND METHODS

A.n.I was isolated from *Ascophyllum nodosum* and purified as described earlier (see [8] for an overview). The apoenzyme had a residual activity of 4.2%. The following compounds were obtained from commercial sources: dipeptides (Serva), $Na_3[VO_4]$ (Janssen), $VOSO_4.5H_2O$ (Merck). pH values (conditioned with citrate buffer 0.1 M), enzyme, peptide and vanadium concentrations are indicated in the figure captions. The enzyme concentrations were calculated for a molecular weight for A.n.I to be 100 kDa [8], and the protein contents were

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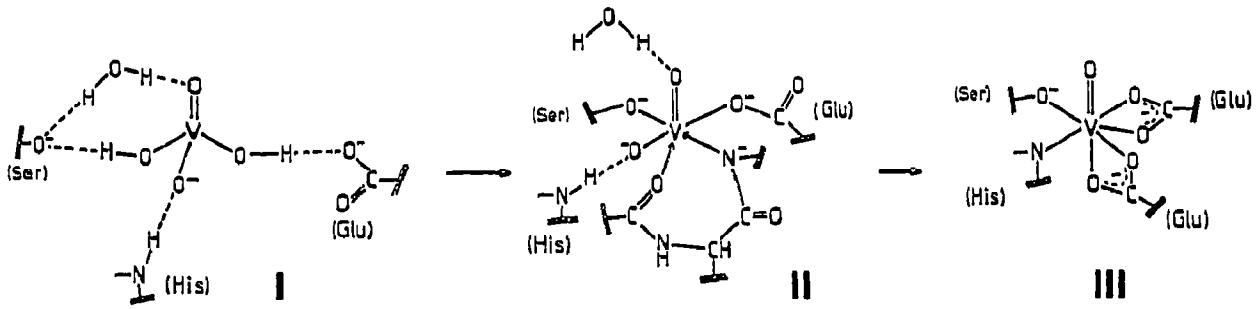

Fig. 1. Schematic representation of a stepwise incorporation of vanadate into the active site of A.n.I. (I) The tetrahedral vanadate ion is linked to the protein surface by hydrogen bonds. (II) Side-chain and/or back-bone functions coordinate to the vanadium center, increasing its coordination number. (III) Tentative formulation for the active site, based on XAS, EPR/spin-echo, and ^{51}V NMR data from the literature (see [6] for a survey).

determined by UV (260 nm) measurements. Reduction was carried out with a ca. 4-fold molar excess of sodium dithionite.

The following assays were employed for the ^{51}V NMR measurements: (i) constant c(V) = 3 mM, c(Ala-His) = 1–17 mM; (ii), constant c(Ala-His) 9 mM c(V) = 1–17 mM. All samples for NMR were prepared in H_2O/D_2O, 2/1, with an ionic strength of 0.2 M (NaCl); pH/D = 7.2 (30 mM HEPES buffer). Room temperature ^{51}V NMR spectra were scanned on a Bruker AM 360 spectrometer at 94.73 MHz. Chemical shifts $\delta(^{51}V)$ are given relative to $VOCl_3$. EPR spectra were recorded on a Bruker ECS 106 spectrometer (equipped with a Bruker microwave bridge ECS 041 MR and an ESP 300 data system) at 9.78 GHz (room temperature) or 9.45 GHz (14 K) at a microwave power of 5.03 mW.

Metal analyses (by ICP-MS) were obtained for (a) 0.51 μM holoenzyme and (b) 2.3 μM apoenzyme in citrate buffer, pH 7.0. Samples (100 μl) were digested with 500 ml of HNO_3 (suprapure) at 60°C and diluted with double-distilled H_2O to a final volume of 10 ml. All glassware was rinsed with 0.1 M HNO_3 and 1 mM EDTA, and then at least ten times with water.

3. RESULTS AND DISCUSSION

Fig. 2 shows the room temperature EPR spectra of the following systems: reduced holoenzyme (A), VO^{2+} in citrate buffer (B), reduced apoenzyme plus VO^{2+} (C and D). The EPR parameter for (A) are listed together with data for VO^{2+}–protein complexes from recent literature, and with data for model compounds in Table 1. Spectra of complexes formed between VO^{2+} and model dipeptides are depicted in Fig. 3. Reduced holo-A.n.I already exhibits an axial spectrum at room temperature, indicating immobilization of the active site VO^{2+} under ambient temperature conditions. The g and A values (obtained at pH 7) are larger in our sample than those previously reported for the 77 K spectra at pH 8.2 [3,9]. They resemble those of the model peptide complexes, which is suggestive of a coordination mode similar to II and III in Fig. 1.

In order to find out whether VO^{2+} is bound to the protein surface, we have treated the apoenzyme in an N_2 atmosphere with an equimolar amount of VO^{2+}. The EPR spectrum (not shown in Fig. 2) is identical to that of VO^{2+} in citrate buffer (B in Fig. 2) indicating that, if any, only weak and non-specific binding and/or rapid exchange (beyond the μs domain) between bound and free vanadyl takes place. VO^{2+} apparently is less effectively bound to the protein surface than $H_2VO_4^-$, for which a binding constant of 16(7) M^{-1} has been reported [5]. An EPR spectrum representing a superposition of the spectra of reduced holoenzyme (A) and VO^{2+}/citrate (B) is observed if the apoenzyme is treated with dithionite before addition of VO^{2+} (C and D in Fig. 2). The EPR features are due to residual active site vanadium in the apoenzyme and/or insufficiently strict anaerobic conditions during assay preparation. Neither (C) nor (D) changed during 24 h at room temperature, or after incubation at 50°C (to enhance transport rates) for 1 h, i.e. no external VO^{2+} was transported into the active site. The reason may be the inappropriate effective size ($[VO(H_2O)_5^{2+}$) of the vanadyl cation.

An intriguing question in this context concerns the number of vanadium atoms that can be taken up by

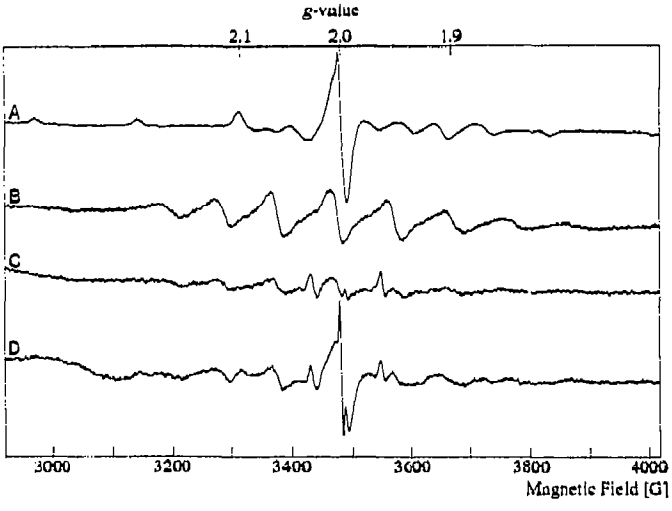

Fig. 2. Room temperature EPR spectra of (A) reduced holoenzyme (c(protein) = 0.24 mM), (B) VO^{2+} (4 mM) in citrate buffer (0.2 M, pH 7), (C) pre-reduced apoenzyme (0.47 mM) + VO^{2+} (0.5 mM) after 20 h of incubation, and (D) apoenzyme (0.47 mM) + VO^{2+} (1 mM) + $S_2O_4^{2-}$ (5 mM) 1 h after assay preparation. All enzyme samples were prepared in 0.1 M sodium citrate buffer, pH 7.0. The sharp central peak in D corresponds to the thionite radical.

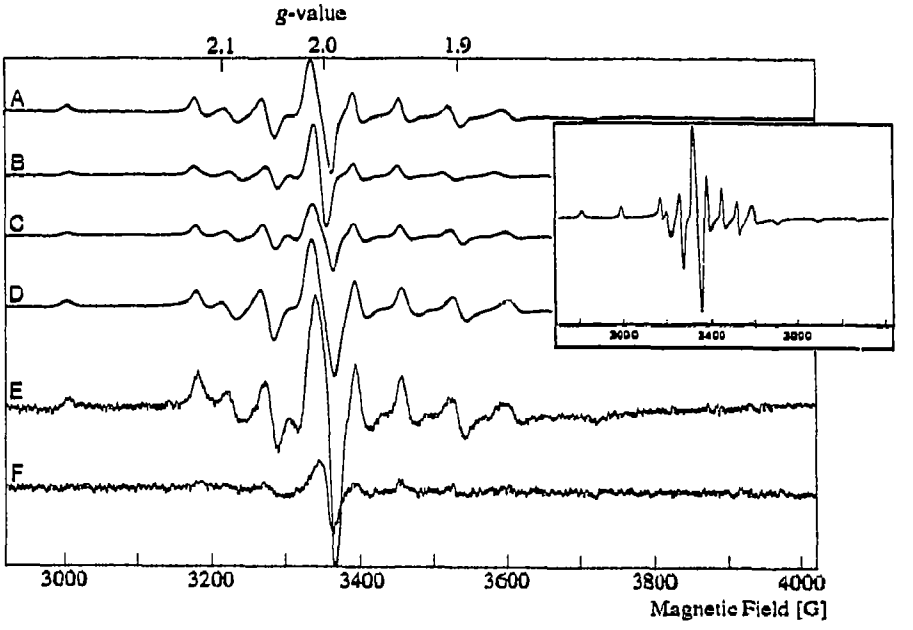

Fig. 3. EPR spectra (14 K) of aqueous vanadate (4 mM)/dipeptide (20 mM) in citrate buffer (0.1 M) after reduction with dithionite. (A) Gly-Ser (pH 7.5). (B) Gly-His (pH 7.5). (C) Phe-Glu (pH 7.5). (D) Gly-Tyr (pH 7.0). (E) Ser-Gly (pH 7.5). (F) Gly-Tyr (pH 8.2). The inset shows D at 150 K, and in a broader range with all of the EPR transitions visible.

vanadate-dependent peroxidases. For the sea-weed *Laminaria saccharina*, 2 V per enzyme molecule have been reported [9]; the reports on vanadium contents of A.n.I are contradictory, ranging from 0.4 [3], through 1 [16], to 2 [17]. Isolation of the enzyme usually is accompanied by a partial loss of vanadium. A renewed analysis of fully reconstituted holo-A.n.I by ICP-MS (inductively coupled plasma mass spectrometry; see Table II for selected data of the metal analysis) revealed 2 V per enzyme molecule (M_r = 100 kDa [8,18]. Since there is no apparent subunit structure these may correspond to 1 surface and 1 active site vanadium.

The model peptides we have used in this study all coordinate to VO^{2+} (Fig. 3). The spectra are isotropic at ambient temperatures, and axially anisotropic at 12 K. The EPR parameters (Table I) suggest a mixed *N/O* coordination sphere [19] and, in the case of Gly-Tyr, a noteworthy pH dependence of both the complex stability and the coordination mode. A decrease in complex stability with increasing pH, and a concomittant change in spectral parameters has also been observed in the system vanadate/Gly-Tyr by ^{51}V NMR [5]. At pH 7.5, only Ala-His shows g and A values significantly deviating from those of the other dipeptides. We have shown earlier that vanadate(V) ($H_2VO_4^-$) forms fairly stable 1:1 complexes with dipeptides such as Gly-Tyr, Phe-Glu

Table I

EPR data for VO^{2+} complexes[a]

Substrate	Ref.	$g_{//}$	$g_\perp$	g_o[c]	$A_{//}$	$A_\perp$	A_o[c]
A.n.I (pH 7.0)[b]	this work	1.95	1.99	1.98	173	60	98
A.n.I (pH 8.2)	[3,9]	1.948	1.978		160.1	50.2	
L.s.I[d]	[10]	1.948	1.979				
Collagen	[11]	1.934	1.965		168.4	61.8	
Xylose isomerase	[12]	1.933	1.980		176.9	68.3	
Transferrin	[13]	1.937	1.968		167.7	57.9	
$[VO(H_2O)_5]^{2+}$		1.934	1.979		182.4	72.0	
$[VO\{N_5\}]^{2+}$[e]	[14]	1.970	2.001		151.1	51.1	
Amavadin	[15]	1.928	1.991	1.970	169.8	49.8	86.5
Gly-Tyr[f] (pH 7.0)	this work	1.930	1.984		180.3	54.4	
Gly-Tyr[g] (pH 7.0)	this work	1.923	1.984		185.0	54.5	
Gly-Tyr[g] (pH 8.2)	this work	1.917	1.981		185.5	53.3	
Gly-Ser[g] (pH 7.5)	this work	1.924	1.983		182.4	52.7	
Ser-Gly[g] (pH 7.5)	this work	1.924	1.983		183.5	52.9	
Phe-Glu[g] (pH 7.5)	this work	1.927	1.984		182.4	52.8	
Gly-His[g] (pH 7.5)	this work	1.933	1.984		176.6	51.9	
Gly-His[b]	this work			1.974			97.5

[a] At 77 K, if not indicated otherwise ([b,f,g]).
[b] At room temp.
[c] Calculated from the $g_{//}/g_\perp$ and $A_{//}/A_\perp$ values, respectively.
[d] Vanadate-dependent peroxidase from *Laminaria saccharina*.
[e] $\{N_5\}$ is a potentially hexadentate nitrogen ligand, coordinating through 4 secondary plus 1 primary amine nitrogens.
[f] At 150 K.
[g] At 14 K.

Table II

Selected analytical ICP-MS data for A.N.I[a]

	V	Mo	I	Br	Zn	Fe	Mg,Cr,Ni	Al
Holoenzyme	1.98(8)	0.20	0.50	<0.01	0.43	0.50	<0.01	0.67
Apoenzyme[b]	0.08(4)	0.11	0.25	<0.01	0.02	1.27	<0.01	0.15

[a] Metal contents are given in atoms per enzyme molecule. If not stated otherwise, the mean deviation amount to ± 13%.
[b] Obtained by dialysis against citrate/EDTA, pH 3.8.

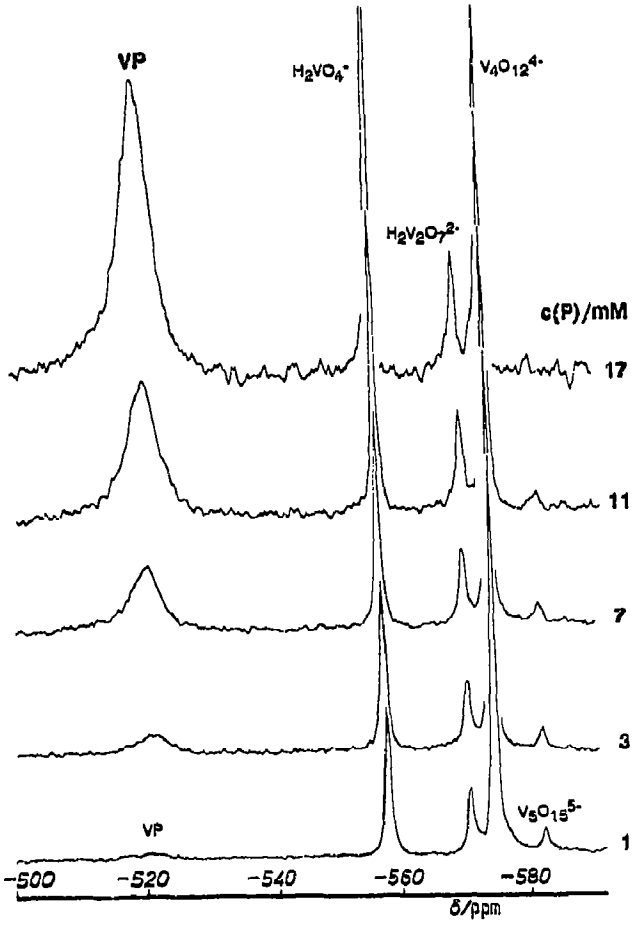

Fig. 4. 94.7 MHz ^{51}V NMR spectra of aqueous solutions of vanadate (3 mM) and Ala-His (c(P) = 1–17 mM; indicated at the right hand margin) at pH 7.2 and an ionic strength of 0.2 M. The uncoordinated oligovanadates are indicated. VP is a vanadate–peptide complex, possibly of the stoichiometry 1:1, with coordination number 5 or 6.

[5] and Gly-Ser [20], with the terminal NH_2 and the peptide linkage participating in coordination, and $\delta(^{51}V)$ between −498 and −510 ppm (relative to $VOCl_3$) typical for this coordination mode.

Ala-His, as shown by the results presented here, again behaves differently. Fig. 4 is a collection of ^{51}V NMR spectra of the system vanadate/Ala-His at a constant overall vanadate concentration c(V) = 3 mM, and varying peptide concentrations c(P) = 1–17 mM. Along with uncomplexed tetrahedral vanadate ions, V_n, that can exist under these conditions, a broad signal corresponding to a vanadate–peptide (VP) complex arises at low field. The $\delta(^{51}V)$ value of −519 ppm suggests participation in coordination with the imidazol-N. A quantitative evaluation leads to a formation constant for a 1:1 complex, at pH 7.2, of 266(28) M^{-1}. This is an order of magnitude more than for complexes formed between vanadate and dipeptides without a side chain function [21,22], but compares with those obtained for Phe-Glu and Gly-Tyr [5,21].

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