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The vanadium chloroperoxidase from the fungus, *Curvularia inaequalis*

Evidence for the involvement of a histidine residue in the binding of vanadate

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The binding of vanadate to the novel vanadium chloroperoxidase from *C. inaequalis* was investigated. Reconstitution experiments of apo-chloroperoxidase by vanadate at different pH values showed that in the pH 6–7 range an acid/base group is present which affects the binding of the vanadate. It is proposed that this group is a histidine. This hypothesis was tested by specifically modifying this residue using diethylpyrocarbonate. In the apo-enzyme 9 histidines were modified, whereas in the holo-enzyme 6 histidines were modified. Modification with diethylpyrocarbonate had no effect on the chlorinating activity of the holo-enzyme, but when the apo-enzyme was modified the reactivation by vanadate was strongly inhibited. We conclude that histidine in the active site of chloroperoxidase is involved in the binding of vanadate.

Chloroperoxidase; Vanadate; Histidine; Prosthetic group; Diethylpyrocarbonate

1. INTRODUCTION

Chloroperoxidases are members of the group of enzymes called haloperoxidases. These enzymes are able to oxidize halides in the presence of hydrogen peroxide. For a long time it was believed that haloperoxidases were heme-containing enzymes [1–3] but in 1984 it was discovered that the seaweed, *Ascophyllum nodosum*, contained a bromoperoxidase that was vanadium dependent [4]. Shortly after that it was demonstrated that vanadium was the prosthetic group in most bromoperoxidases from seaweed [5–7].

Various biophysical techniques such as ESE, EXAFS and EPR were used to study the interaction of the prosthetic group with the protein in the vanadium bromoperoxidase from *A. nodosum*. ESE experiments showed that in the near vicinity of the vanadium atom in the reduced bromoperoxidase a nitrogen atom is present [8] which is likely to belong to an imidazole group [9]. Measurements of the kinetics of hydrogen peroxide binding to the vanadium bromoperoxidase from *A. nodosum* showed that a group with a pK_a of 5.9 governed this binding [9]. A general property of these vanadium bromoperoxidases is that the vanadium can be removed by dialysis against phosphate-containing buffers. The enzymic activity is fully restored by addition of orthovanadate (VO_4^{3-}). However, a study in which the binding of vanadate to the apo-enzyme over a pH range from 5.9 to 8.5 was studied, failed to demonstrate an ionizable group in this range for the bromoperoxidase from *A. nodosum* [10].

Selective modification of amino acids possibly involved in the binding of vanadate to the enzyme may give information as to the nature of the active site. In this study the binding of vanadate as a function of pH was studied and also selective modification of histidines by diethylpyrocarbonate was used as a tool to assess the nature of the residues involved in the binding of the prosthetic group to the chloroperoxidase of the fungus, *Curvularia inaequalis*.

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Abbreviations: DEP, diethylpyrocarbonate; MES, 2-[*N*-morpholino] ethane sulfonic acid; MOPS, 3-[*N*-morpholino] propane sulfonic acid; ESE, electron spin echo; EXAFS, extended X-ray absorption fine structure; EPR, electron paramagnetic resonance.

2. MATERIALS AND METHODS

Our studies were carried out with the vanadium chloroperoxidase from *Curvularia inaequalis* which was obtained from the Centraal Bureau voor Schimmelcultures (CBS, Baarn, The Netherlands) strain no. 102.42. Growth of the fungus and isolation of the enzyme were performed according to [11] except that after the DEAE-column chromatography a phenyl Sepharose hydrophobic interaction column was also used to which the enzyme was bound and was eluted from with a gradient of 2–0 M NaCl in 50 mM Tris-Cl (pH 8.3). The enzymatic activity of the chloroperoxidase was determined spectrophotometrically on a Varian Cary-17 spectrophotometer by measuring the chlorination of 50 μM monochlorodimedone ($\varepsilon = 20.2$ $mM^{-1} \cdot cm^{-1}$ at 290 nm) [12]. All activity measurements were carried out in 0.1 M sodium acetate (pH 5.5), 5 mM sodium chloride and 1 mM hydrogen peroxide. Amino acid analysis using an HP 1090 Aminoquant was performed by Eurosequence in Groningen, The Netherlands, on freeze-dried samples.

To determine the K_d for vanadate, chloroperoxidase was inactivated by dialysis against 100 mM potassium citrate (pH 3.8), 100 mM potassium phosphate and 2 mM EDTA. After incubation the sample was dialysed against Tris-SO_4 (50 mM, pH 8.3). During this treatment the chloroperoxidase loses vanadate and enzymic activity.

To determine the K_d, the inactivated enzyme was incubated with a range of vanadate concentrations at different pH values. pH values and enzyme concentrations used were: pH 8.75 (100 mM Tris-Cl), enzyme concentration 44.8 nM; pH 8.0 (100 mM Tris-Cl), enzyme concentration 40.5 nM; pH 7.0 (100 mM MOPS), enzyme concentration 129.9 nM; pH 6 (100 mM MES), enzyme concentration 129.9 nM and pH 5.3 (100 mM MES), enzyme concentration 190 nM. After 40 h of incubation the chlorinating activities were measured in the standard assay containing monochlorodimedone. From the enzyme concentration and the fact that maximal activity was reached at a stoichiometry of 1 vanadium per enzyme molecule [11] the amount of vanadate taken up could be calculated. K_d values for vanadate binding were derived from Scatchard plots.

Diethylpyrocarbonate stock solutions were made by dissolving 6.9 M diethylpyrocarbonate in absolute ethanol. Modifications of apo- and holo-chloroperoxidase with DEP were performed in a 1.5 ml quartz cuvet by adding 30 μl of a 510 mM DEP solution in absolute ethanol to 1 ml of the apo- or holo-enzyme preparations. The reaction was followed spectrophotometrically on a Hewlett Packard 8452A diode array spectrofotometer in the wavelength range of 235 to 285 nm. Using a molar extinction coefficient of 3,200 $M^{-1} \cdot cm^{-1}$ at 246 nm [13] the amount of modified histidines could be calculated. Apo-enzyme was prepared as described earlier in this section except that the enzyme was not dialysed against 50 mM Tris-SO_4 but against 50 mM MOPS (pH 7.2). Holo-enzyme was also dialysed against this buffer prior to reaction with diethylpyrocarbonate.

To check the modification of the chloroperoxidase by DEP, 100 mM hydroxylamine, which was dissolved in 0.1 M phosphate buffer (pH 6.8), was added. The protein content of the samples was determined by the method of Bradford [14] using a protein reagent from Bio-Rad with bovine serum albumin as a standard. All chemicals used were of analytical grade. Water was filtered and de-ionised by leading it through an Elgastad B12H (Elga group) and a Milli-Q (Millipore) water-purification system.

3. RESULTS

In Fig. 1 the logarithm of the dissociation constant of the binding of vanadate to the apo-enzyme is plotted against the pH. As can be seen the dissociation constant remains at a level of about 140 nM at pH values 8.75–7 but is rapidly increased at pH values below 7, suggesting that an amino acid residue with a pK_a between 6 and 7 is involved in the binding of the vanadate. Since it is possible that this residue is a histidine, selective chemical modification of histidine was carried out using diethylpyrocarbonate (DEP) to see if modification of this amino acid leads to changes in the vanadate binding to the apo-protein.

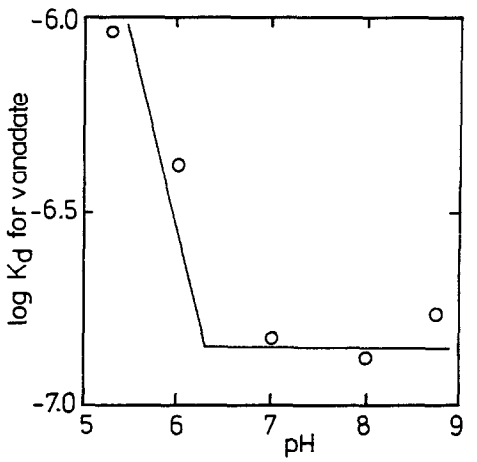

Fig. 1. Log K_d of vanadate as a function of pH. Results were obtained from Scatchard plots, as described in sections 2.

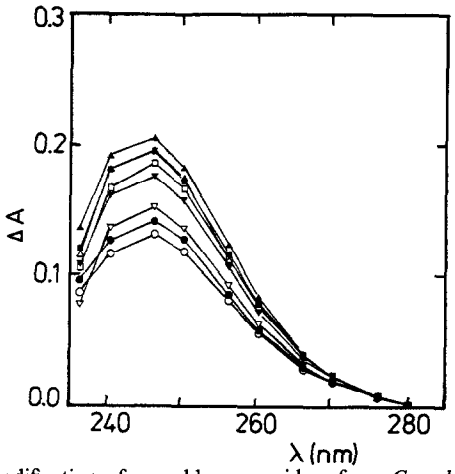

Fig. 2. Modification of apo-chloroperoxidase from *Curvularia inaequalis*: spectral changes induced by DEP. Enzyme concentration 7.6 μM. Spectra were measured after 2 (○—○), 10 (●—●), 25 (▽—▽), 50 (▼—▼), 75 (□—□), 100 (■—■), 125 (△—△) and 150 (▲—▲) s after the addition of DEP. Reaction conditions are as described in section 2.

In Fig. 2 the spectral effect of the modification of apo-chloroperoxidase by DEP is depicted. As can be seen, upon addition of DEP, a peak with a wavelength maximum at 246 nm is rapidly formed. Tyrosine, which may also be modified by DEP, apparently does not react since modification of tyrosine would lead to a strong absorption decrease at 278 nm ($\varepsilon_{278\,nm}$ = 13,101 $M^{-1} \cdot cm^{-1}$) and no such decrease in the optical spectrum is observed. Similar spectral changes as found in Fig. 2, although smaller in amplitude, were obtained when DEP was allowed to react with holo-enzyme.

To be certain that only modification of histidines and not of lysine residues were causing the increase in absorption at 246 nm, the modified chloroperoxidase was treated with hydroxylamine. The covalent modification of lysines, in contrast to that of the reversible N-carbethoxylation of histidines, is not affected by hydroxylamine and no decrease in absorption will be induced by addition of hydroxylamine. Fig. 3 shows that upon addition of hydroxylamine to DEP-modified chloroperoxidase, the peak at 246 nm decreases readily to a value of zero as expected, showing that the absorption at this wavelength was solely due to modified histidines. When the holo-enzyme was allowed to react with DEP the activity is not affected (results not shown) and this demonstrates that modification does not affect the chlorinating activity.

When the increase in absorption at 246 nm was determined after reaction with DEP, using an extinction coefficient of 3,200 $M^{-1} \cdot cm^{-1}$ [13], it was possible to calculate that in the apo-enzyme 9 histidines were modified whereas in the holo-enzyme it was 6 residues. This dif-

ference suggests that 3 histidines residues are shielded by vanadate and that vanadate at the active site prevents further reaction with DEP. The number of histidines modified in the apo-enzyme fits nicely with the number of histidines which are present in the enzyme according to the amino acid composition. Amino acid analysis on two different preparations of the histidine content shows that 9 residues are present per mol of enzyme (molecular mass of 67 kDa).

When both apo-chloroperoxidase and DEP-treated apo-chloroperoxidase were reactivated by vanadate, the DEP-treated sample reached only 20% of the maximal activity (13.1 U/mg) of the reactivated apo-chloroperoxidase (Fig. 4). When DEP-modified holo-enzyme (13 U/mg) is inactivated its specific activity goes down to 0.2 U/mg. Upon subsequent reactivation with excess vanadate the original activity of the holo-enzyme is restored (12.9 U/mg). However, when apo-enzyme (0.2 U/mg) obtained from modified holo-enzyme, is again treated with DEP the sample now reaches only 20% of the maximal activity (2.8 U/mg). This observation shows that in the holo-enzyme vanadate does indeed shield histidines against modification by DEP. Reactivation by vanadate of DEP-modified apo-enzyme after treatment with hydroxylamine showed an increase in activity for this sample that was equal to the increase in activity observed for normal apo-enzyme (not shown). This again shows that modification of histidines involved in the binding of the vanadate is reversible .

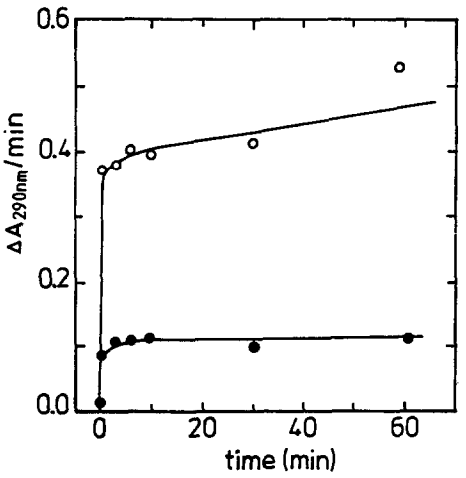

Fig. 4. Reconstitution of the chlorinating activity of apo-enzyme (○—○) and DEP-treated apo-enzyme (●—●) by vanadate. To enzyme samples (4.5 μM) sodium orthovanadate solution (final concentration 625 μM) was added and 15 μl aliquots were taken to measure chlorinating acitivities in the standard acitivity assay.

4. DISCUSSION

In this article results are obtained which show that histidine is involved in the binding of vanadate to the chloroperoxidase from the fungus, *Curvularia inaequalis*. The results show that the logarithm of the dissociation constant for vanadate is not linearly dependent on pH as was found for the related vanadium bromoperoxidase from the seaweed, *Ascophyllum nodosum* [10]. The dissociation constant for vanadate in the bromo-peroxidase increases from a value of 55 nM at pH 8.5 to a value of 10 μM at pH 5.9, whereas the dissociation constant for vanadate in the chloroperoxidase stays constant at a value of about 140 nM between pH values 8.75–7.0 but at lower pH an increase to about 1 μM at pH 5.3 is observed. This shows that the the dissociation constants for the binding of vanadate to apo-enzyme in both enzymes are of the same order of magnitude. However, the pH dependence for the chloroperoxidase suggests strongly that an amino acid with a pK_a of about 6 is involved in the binding of vanadate, implying also that there is a difference in environment of the vanadate in both enzymes. It is not likely that ionization of vanadate itself is responsible since the pK_a values for vanadic acid (3.5, 7.8 and 12.5) are such that they do not correlate with the observed pH dependency. An obvious candidate for an amino acid is a histidine residue. To test that histidine is indeed involved a modification study was carried out.

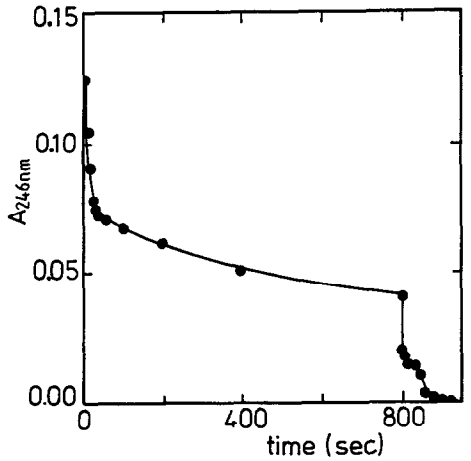

Fig. 3. Time–course of the reaction of hydroxylamine with DEP-modified apo-chloroperoxidase. Hydroxylamine (final concentration 10 mM) was added at 0 and 800 s to DEP modified apo-chloroperoxidase (2.9 μM).

Literature data [13,15–17] show that the method of choice for specific modification of histidine is a reaction with diethylpyrocarbonate leading to a N-carboxyethylation of histidine residues. In this method care should be taken not to modify lysine and tyrosine together with the histidines. Control experiments based on those described by Hager and Blanke [13] ruled out possible reactions with tyrosine and lysine (Figs. 2 and 3). These authors also show [13] that, using the molar extinction coefficient of the N-carboxyethylated histidine, the number of histidines modified in the reaction can be estimated. In our experiments, 9 histidines were modified by the reaction with DEP in the apo-enzyme and in the holo-enzyme 6 histidine residues.

The number of 9 histidines accessible to DEP modification in the apo-enzyme fits nicely the total amount of histidines present in this enzyme based on the amino acid composition. In the apo-enzyme a further 3 histidines are modified compared to the holo-enzyme, leading to the conclusion that these 3 histidines are not accessible in the holo-enzyme and thus that vanadate is shielding these histidines. This conclusion was further supported by the observation that, when modified holo-enzyme was dialysed to remove the vanadate, thus turning it into an apo-enzyme which was subsequently modified with DEP, the sample, upon reconstitution with vanadate, only reached 20% of the maximal chlorinating activity found for the native enzyme. Upon reaction of DEP with holo-enzyme, modification of histidine residues occurs as evidenced by the changes in the optical absorption spectrum. However, this modification does not affect the chlorinating activity of the enzyme. At least 1 of the additional 3 histidines which are modified in the apo-enzyme and which are shielded by vanadate in the holo-enzyme appears to play a role in the binding of vanadate.

When DEP modified apo-enzyme was incubated with vanadate 20% of the maximal chlorinating activity was still observed, suggesting that modification of the histidines with diethylpyrocarbonate is not complete. DEP modified apo-enzyme that was treated with hydroxlamine, which reverses the modifications, showed the same reactivation of the activity by vanadate as the normal apo-protein. This again shows that the histidine modification does indeed account for the lack of reactivation in the modified chloroperoxidase. It is thus clear, from the results shown, that histidine is involved in the binding of the vanadate in the chloroperoxidase from the fungus, *Curvularia inaequalis*.

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