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ORIGINAL ARTICLE

Esther Garcia-Rodriguez · Takashi Ohshiro
Toshiaki Aibara · Yoshikazu Izumi
Jennifer Littlechild

Enhancing effect of calcium and vanadium ions on thermal stability of bromoperoxidase from *Corallina pilulifera*

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Abstract Bromoperoxidase from the macro-alga *Corallina pilulifera* is an enzyme that possesses vanadate in the catalytic center, and shows a significant thermostability and stability toward organic solvents. The structural analysis of the recombinant enzyme overexpressed in yeast revealed that it contains one calcium atom per subunit. This has been confirmed by inductively coupled plasma emission spectrometry experiments. The study of the effect of metal ions on the apo-enzyme stability has shown that the calcium ion significantly increased the enzyme stability. In addition, vanadate also increased the thermostability and strontium and magnesium ions had similar effects as calcium. The holo-enzyme shows high stability in a range of organic solvents. The effect of the different ions and solvents on the structure of the enzyme has been studied by circular dichroism experiments. The high stability of the enzyme in the presence of organic solvents is useful for its application as a biocatalyst.

Keywords Bromoperoxidase · Thermostability · Calcium · Vanadate

Introduction

Haloperoxidases catalyze the halogenation of organic substrates in the presence of hydrogen peroxide. These enzymes are thought to function in the biosynthesis of halogenated natural products. They are classified into three groups on the basis of the cofactor requirement. One of these is the vanadium-containing enzymes, and the remaining two classes comprise the heme-containing and metal-free enzymes. Haloperoxidases are named after the most electronegative halide they can use, that is, chloroperoxidase (CPO) utilizes chloride, bromide, and iodide, whereas bromoperoxidase (BPO) utilizes bromide and iodide.

Vanadium-dependent haloperoxidases bind vanadate as a prosthetic group and have been isolated from a number of marine algae, some lichens and fungi. Amongst the enzymes from marine algae, BPO from the red alga *Corallina pilulifera* has been extensively characterized [1–5] and the complementary DNAs for two distinct BPO isomers have been cloned [6]. One of them (*bpo1*) consists of 598 amino acids with a calculated molecular mass of 65,312 Da and has been expressed in *Escherichia coli* and *Saccharomyces cerevisiae* [7]. The BPO from *C. officinalis* is similar to the *C. pilulifera bpo1* with 92.1% sequence identity, and the X-ray structures of both enzymes have been determined [8–10]. Other vanadium-dependent BPOs from algae [11–13] and CPOs from fungi [14–16] have also been studied in detail. Sequence alignment indicates that amino acid sequences are highly conserved in the vanadate-binding site, but similarities in the remaining regions are very low.

Vanadium-dependent haloperoxidases were shown to be thermostable and stable in organic solvents [17]. Since the oxidative reactions by these enzymes have been reported using a variety of compounds as substrates [18–20], they are considered as useful and stable biocatalysts. The analysis of the three-dimensional structure of BPO from *C. pilulifera* suggests that one calcium ion per subunit is bound in a loop at the top of the active-site cleft [10]. The finding of a calcium ion in the enzyme molecule is specific for the *Corallina* BPOs, and it suggests an important role in the enzyme's structure. In this study, we investigated the importance of calcium and other metal ions for protein stability.

E. Garcia-Rodriguez · J. Littlechild (✉)
Henry Wellcome Building for Biocatalysis,
School of Biological and Chemical Sciences,
University of Exeter,
Exeter, EX4 4QD, UK
E-mail: j.a.littlechild@exeter.ac.uk
Tel.: +44-1392-263468
Fax: +44-1392-263434

T. Ohshiro · T. Aibara · Y. Izumi
Department of Biotechnology,
Tottori University,
Tottori 680-8552, Japan

Materials and methods

Preparation of the apo-enzyme and enzyme assay

Enzyme purifications from *C. pilulifera* and the recombinant *S. cerevisiae* were performed according to the previously reported methods [1, 7]. In order to prepare the apo-enzyme, the purified enzyme solution was dialyzed against 100 mM citric acid–potassium dihydrogenphosphate buffer (pH 3.8) with 10 mM ethylenediaminetetraacetic acid, and then it was neutralized by dialysis against 50 mM tris(hydroxymethyl)aminomethane (Tris) sulfate buffer (pH 7.4).

The enzyme activity was determined at 30°C spectrophotometrically by following the bromination of monochlorodimedone (MCD, $\epsilon = 19.9\ mM^{-1}\ cm^{-1}$ at 290 nm) as described previously [21]. The reaction mixture contained 100 mM 2-morpholinoethanesulfonate buffer (pH 6.5), 100 mM KBr, 60 μM MCD, 2 mM H_2O_2, 1 mM Na_3VO_4, and the apo-enzyme.

Effects of metal ions and organic solvents on the enzyme stability

Prior to the heat treatment, the apo-enzyme was preincubated with the relevant metal ion for 2 h at 30°C. The heat treatment was carried out for 20 min at each temperature. The effect of calcium and vanadate ions was examined according to the following procedures. The apo-enzyme was preincubated with 1 mM Na_3VO_4or 1 mM $CaCl_2$ or both of them, and subjected to heat treatment. The apo-enzyme preincubated with only calcium and that without the preincubation were further incubated with 1 mM Na_3VO_4 for 2 h at 30°C and then the enzyme activity was determined (Scheme 1).

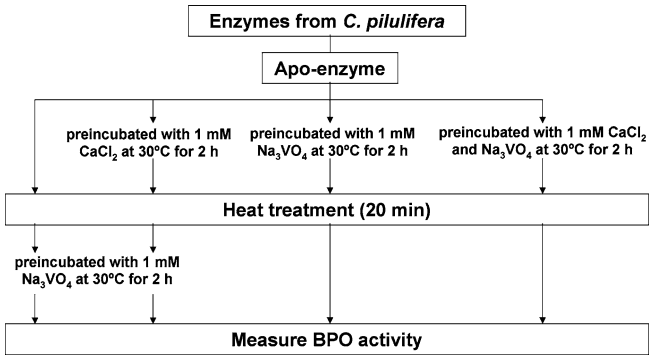
When the effects of other metal ions were examined, the apo-enzyme was preincubated with 1 mM Na_3VO_4 and 1 mM of each metal compound [LiCl, NaCl, KCl, RbCl, $AgNO_3$, $MgCl_2$, $MnCl_2$, $FeSO_4$, $CoCl_2$, $NiCl_2$, $SrCl_2$, $BaCl_2$, $(CH_3COO)_2Zn$, or $AlCl_3$], and then the heat treatment was carried out before the enzyme assay.

In order to compare the thermostability of the enzyme in the presence of different concentrations of Ca^{2+} and Mg^{2+}, experiments were carried out at concentrations between 0.1 and 5.0 mM $CaCl_2$ and $MgCl_2$ at various temperatures up to 85°C. The other experimental conditions were as described already.

In the case of investigating the effects of molybdate and tungstate, the apo-enzyme was preincubated with 1 mM Na_2MoO_4 or Na_2WO_4 for 16 h at 30°C before the heat treatment. The enzyme was further preincubated with 1 mM Na_3VO_4 for 2 h at 30°C, and then the enzyme activity was determined.

When the effect of organic solvents was examined, the apo-enzyme was preincubated with 1 mM Na_3VO_4 or 1 mM Na_3VO_4 and 1 mM $CaCl_2$. Then the organic solvents methanol, ethanol, acetone, dioxane, tetrahydrofuran, dimethylformamide, acetonitrile, and dimethylsulfoxide were added to the enzyme solution to 80% v/v. After the mixture had been kept at 30°C for the given period, the enzyme activity was determined. As the control experiment, after the apo-enzyme had been treated with the organic solvents as described already, the enzyme was preincubated with 1 mM Na_3VO_4 before the enzyme assay.

All of the activity measurements were carried out in triplicate.



Scheme 1 Experimental procedure used to obtain the results represented in Fig. 1

Circular dichroism experiments

Circular dichroism (CD) experiments were carried out using a JASCO J-720 spectrometer for the following metal and ion combinations with the apo-enzyme, apo-enzyme with vanadate, apo-enzyme with molybdate, tungstate, and phosphate. The buffer used for these experiments was 1 mM Tris sulfate buffer (pH 7.4) and the concentration of the apo-enzyme was 0.05 mg/ml for the analysis of far-UV spectra and 0.5 mg/ml for the analysis of near-UV spectra.

Since the CD spectra could not be measured in the presence of excess amounts of vanadate, the apo-enzyme was preincubated with 1 mM sodium orthovanadate, and the enzyme solution was dialyzed against 1 mM Tris sulfate buffer (pH 7.4) to remove the excess vanadate. The other salts, sodium molybdate, sodium tungstate, and sodium dihydrogenphosphate, were added at 1 mM to the protein solution. These salts did not prevent the analyses of CD spectra when they were added at this concentration. In addition, experiments were carried out with the apo-enzyme and 1 mM aluminum chloride in the presence and absence of vanadate.

The effect of the solvents on the structure of the apo-enzyme in the presence of vanadate was determined for the solvents methanol, ethanol, and dimethylsulfoxide at 80% (v/v) concentration. The final protein concentration was 0.05 or 0.5 mg/ml.

Results

Effects of calcium and vanadate ions on the thermostability of the enzyme

First, we analyzed the calcium content by inductively coupled plasma emission spectrometry in the two enzyme preparations: the enzyme purified from *C. pilulifera* and the recombinant enzyme overexpressed in yeast. The metal content for the apo-enzyme was also determined as a control experiment. The metal analysis indicated that both BPO enzymes had almost one mole of calcium per subunit and that the apo-enzyme did not contain any calcium (Table 1). We did not determine the calcium content in the native apo-enzyme owing to the small amount of enzyme available.

We also examined the effect of calcium and vanadate ions on the thermostability of the enzyme. The preincubation with calcium and vanadate ions significantly enhanced the apo-enzyme stability (Fig. 1). The temperatures at which 50% of the initial activity was maintained were 92, 87, 75, and 57°C with the enzyme preincubated with vanadate and calcium, with only calcium, with vanadate alone, and without either metal ion, respectively. The apo-enzyme increased its thermostability by 35°C with the addition of both calcium and vanadate.

Effect of various metal ions on the thermostability of the enzyme

A variety of metal ions were tested to examine their effect on the thermal stability of the BPO enzyme. They were added to the preincubation mixture at 1 mM together with the recombinant apo-enzyme and 1 mM Na_3VO_4. Ca^{2+} was the most effective, and other divalent cations such as Sr^{2+} and Mg^{2+} were effective to some extent (Fig. 2). The univalent or trivalent metal ions such as Ag^{+} and Al^{3+} lowered the thermostability of the enzyme when compared with the control. In addition, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, and Ba^{2+} had slightly positive effects, whereas Li^{+}, Na^{+}, K^{+}, and Rb^{+} had no effect and Zn^{2+} lowered the thermostability (data not shown).

The results of the experiments to determine the effect of the variation of Ca^{2+} and Mg^{2+} concentrations on the thermostability of the enzyme are shown in Fig. 3. At calcium concentrations of 1 mM or less the enzyme was less stable at 85°C. In the presence of magnesium the enzyme is less stable above 65°C at all concentrations.

Table 1 Determination of calcium in bromoperoxidase by inductively coupled plasma emission spectrometry

	Enzyme		Apo	
	Wild	Recombinant	Wild	Recombinant
Mol Ca/mol subunit	0.976	0.828	ND	0

ND not determined

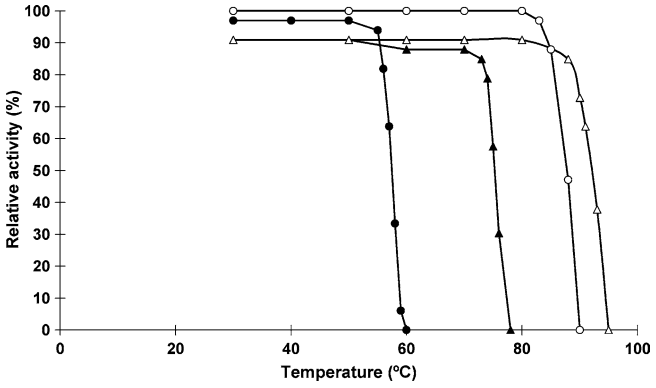


Fig. 1 Effects of calcium and vanadate ions on the thermostability of apo-bromoperoxidase (*BPO*). The apo-enzyme (13.3 μg protein/ml) was preincubated with 1 mM Na_3VO_4 (*filled triangles*), 1 mM $CaCl_2$ (*open circles*), both of them (*open triangles*), or without metal ion (*filled circles*) for 2 h at 30°C, and was subjected to heat treatment for 20 min at the indicated temperature. The enzyme not preincubated with vanadate was further incubated with 1 mM Na_3VO_4 for 2 h at 30°C. The enzyme activity was determined as described in "Materials and methods." In all cases with or without metal ions, the values of the enzyme activity indicated as 100% were the same

Effects of tungstate and molybdate ions on the thermostability of the enzyme

To examine whether or not the positive effect on the thermostability was specific to vanadate, two analogous metal ions, WO_4^{2-} and MoO_4^{2-}, were tested (Fig. 4). Both of these ions did not enhance the stability and the presence of molybdate clearly reduced the enzyme activity

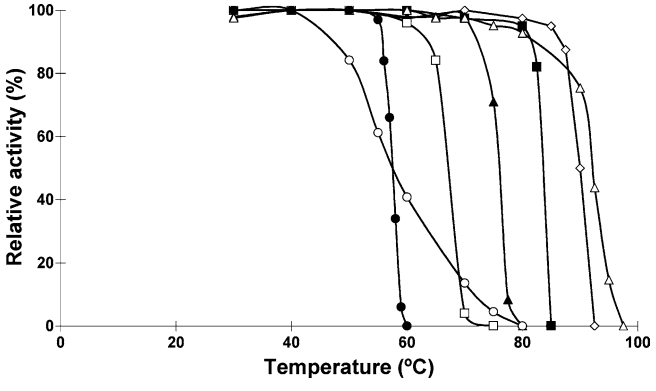
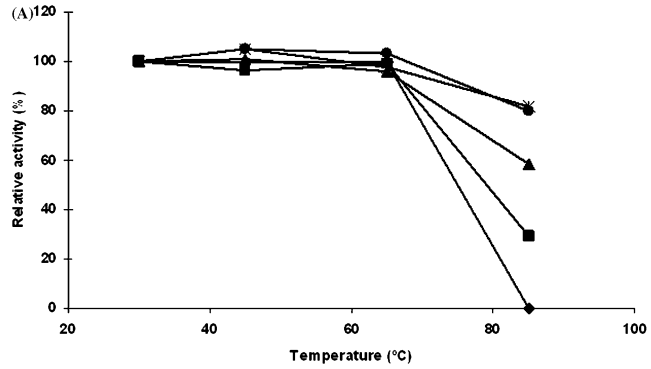


Fig. 2 Effects of various metal ions on the thermostability of apo-BPO. The apo-enzyme (0.98 μg protein/ml) was preincubated with 1 mM Na_3VO_4 (*filled triangles*), 1 mM Na_3VO_4 + 1 mM $AgNO_3$ (*open squares*), 1 mM Na_3VO_4 + 1 mM $CaCl_2$ (*open triangles*), 1 mM Na_3VO_4 + 1 mM $MgCl_2$ (*filled squares*), 1 mM Na_3VO_4 + 1 mM $SrCl_2$ (*diamonds*), 1 mM Na_3VO_4 + 1 mM $AlCl_3$ (*open circles*), or without metal ion (*filled circles*) for 2 h at 30°C, and was subjected to heat treatment for 20 min at the indicated temperature. The enzyme activity was determined as described in "Materials and methods." In all cases with or without metal ions, the values of the enzyme activity indicated as 100% were the same



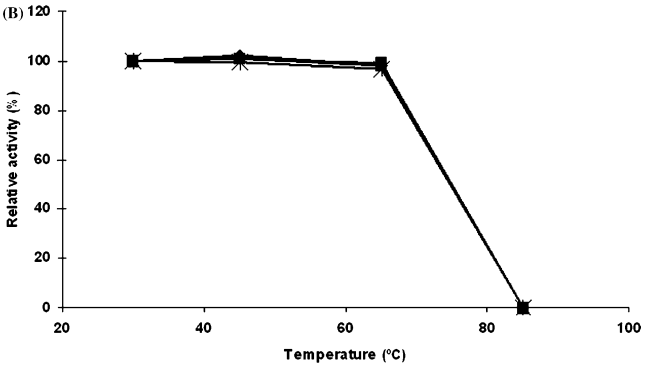


Fig. 3 a Effect of the concentration of calcium ions on the thermostability of apo-BPO. The apo-enzyme (13.3 μg protein/ml) was preincubated with 0.1 mM $CaCl_2$ (*diamonds*), 0.5 mM $CaCl_2$ (*squares*), 1.0 mM $CaCl_2$ (*triangles*), 2.5 mM $CaCl_2$ (*circles*), and 5.0 mM $CaCl_2$ (*stars*) for 2 h at 30°C, and was subjected to heat treatment for 20 min at the indicated temperature. **b** Effect of the concentration of magnesium ions on the thermostability of apo-BPO. The apo-enzyme (13.3 μg protein/ml) was preincubated with 0.1 mM $MgCl_2$ (*diamonds*), 0.5 mM $MgCl_2$ (*squares*), 1.0 mM $MgCl_2$ (*triangles*), 2.5 mM $MgCl_2$ (*circles*), and 5.0 mM $MgCl_2$ (*stars*) for 2 h at 30°C, and was subjected to heat treatment for 20 min at the indicated temperature

at every temperature tested, suggesting an inhibitory effect.

Effect of polar organic solvents on the stability of the enzyme

We also examined the effects of calcium and vanadate ions on the stability of BPO toward polar organic solvents. After the apo-enzyme with Na_3VO_4 and the apo-enzyme with Na_3VO_4 and $CaCl_2$ had been preincubated in 80% v/v organic solvents miscible with water at 30°C for the indicated periods, the enzyme activity was measured in each case. The apo-enzyme preincubated with only vanadate and with both vanadate and calcium was stable to ethanol, tetrahydrofuran, acetonitrile, and acetone, whereas dimethylsulfoxide, dimethylformamide, and dioxane inactivated both forms of the enzyme. It is not surprising that dioxane inactivates the enzyme since its polarity is very high and as such it will distort the essential water layer around the enzyme. CD experiments using the apo-enzyme with vanadate and 80% dimethylsulfoxide

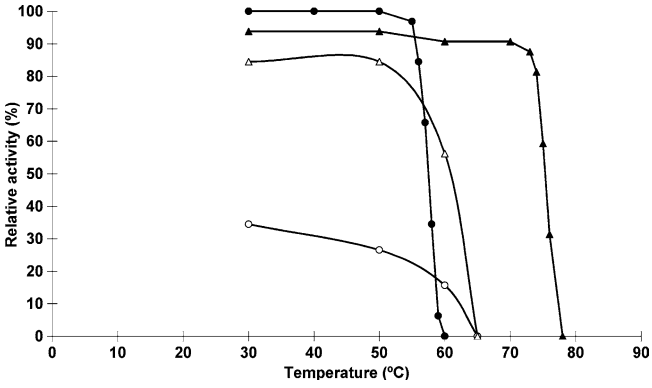
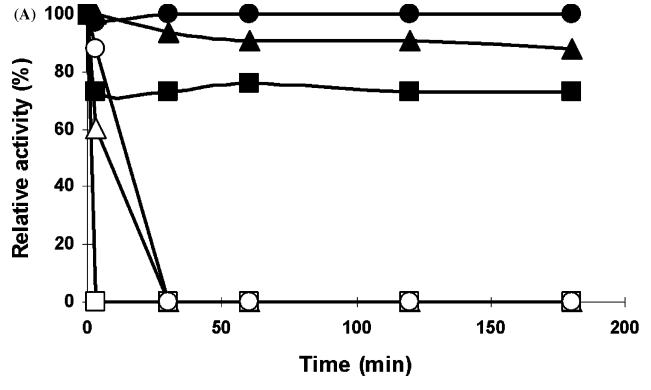
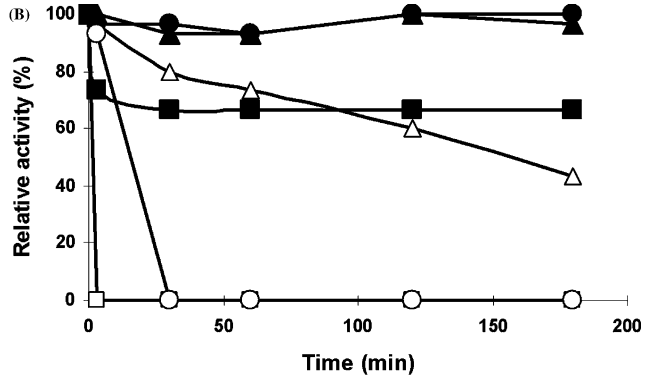


Fig. 4 Effects of tungstate and molybdate ions on the thermostability of apo-BPO. The apo-enzyme (13.3 μg protein/ml) was preincubated with 1 mM Na_3VO_4 (*filled triangles*), 1 mM Na_2MoO_4 (*open circles*), 1 mM Na_2WO_4 (*open triangles*), or without metal ion (*filled circles*) for 2 h at 30°C, and was subjected to heat treatment for 20 min at the indicated temperature. The enzyme except for that preincubated with vanadate was further incubated with 1 mM Na_3VO_4 for 2 h at 30°C. The enzyme activity was determined as described in "Materials and methods." In all cases with or without metal ions, the values of the enzyme activity indicated as 100% were the same

Fig. 5 Effect of polar organic solvents on BPO stability. The apo-enzyme (3.9 μg protein/ml) was preincubated with 1 mM Na_3VO_4 (**a**) or 1 mM Na_3VO_4 + 1 mM $CaCl_2$ (**b**) for 2 h at 30°C, and the resultant holo-enzyme was subjected to ethanol (*filled triangles*), methanol (*open triangles*), acetone (*filled squares*), dioxane (*open circles*), or dimethylsulfoxide (*open squares*) at 30°C for the indicated periods. Each organic solvent was added to 80% to the enzyme solution. As the control experiment, the holo-enzyme was incubated at 30°C without any organic solvent for the indicated periods (*filled circles*). The enzyme activity was determined as described in "Materials and methods."





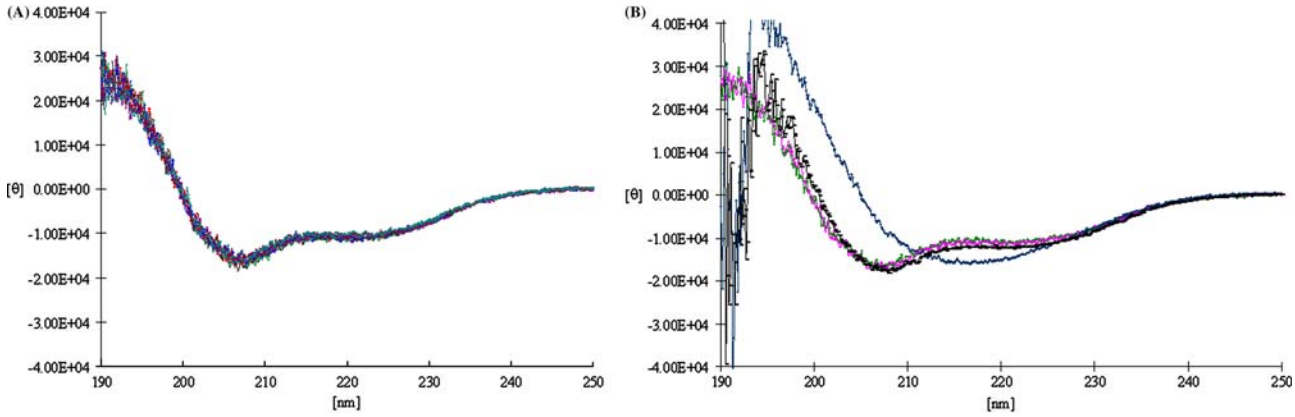


Fig. 6 **a** Circular dichroism (*CD*) spectra of the apo-enzyme (*red*) in the presence of vanadate (*green*), calcium (*blue*), vanadate and calcium (*black*), phosphate (*purple*), molybdate (*brown*), and tungstate (*cyan*). **b** CD spectra of the apo-enzyme (*green*) in the presence of vanadate alone (*pink*), vanadate and methanol (*blue*), and vanadate and ethanol (*black*). The conditions were as described in "Materials and methods." In both cases the apo-enzyme alone was used as a control

showed that the structure of the enzyme was disrupted (data not shown). It is noteworthy that the enzyme preincubated with both vanadate and calcium showed higher stability against methanol than that with only vanadate. The results for ethanol, methanol, dimethylsulfoxide, acetone, and dioxane are shown in Fig. 5. The apo-enzyme activity was completely lost after exposure to the organic solvents for 15 min with the exception of acetone. In this case the enzyme retained 70% activity after exposure for 3 h (data not shown).

The activity measurements described previously that were carried out in triplicate were found to have an error of less than 3%.

CD experiments

In all cases for molybdate, tungstate, phosphate, and vanadate, the CD spectra showed no detectable difference to that of the apo-enzyme alone. The spectra are characteristic of the high α-helix content (40%) of the

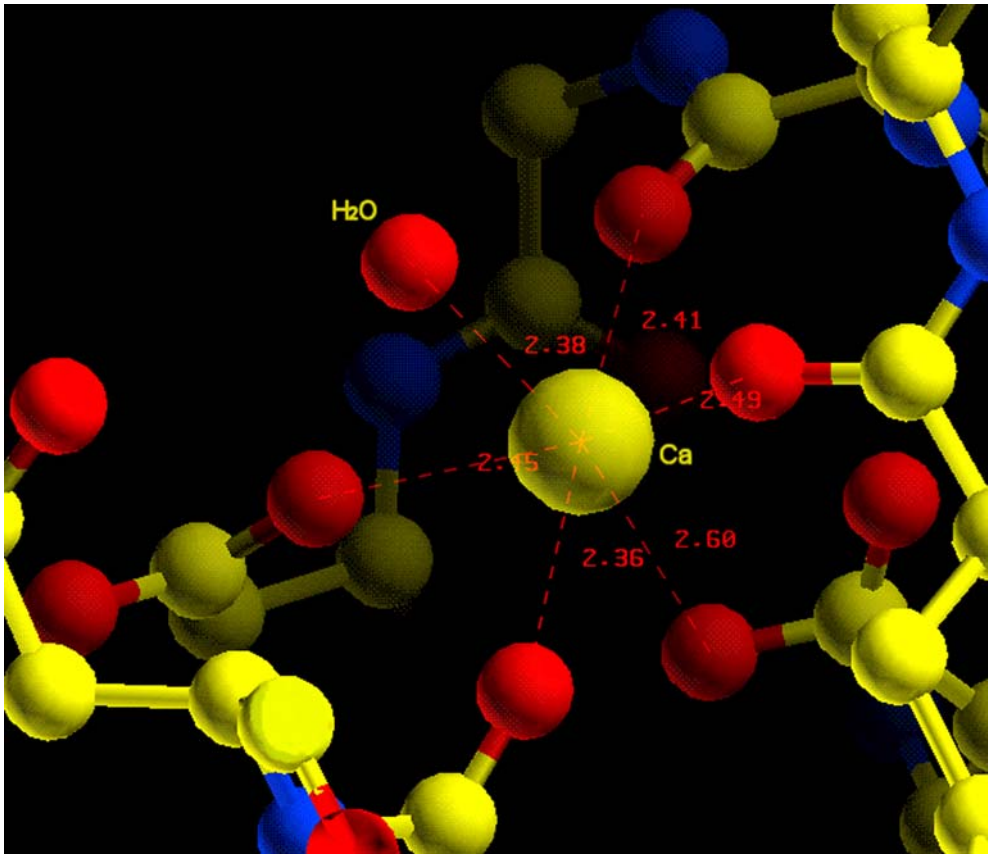


Fig. 7 The calcium ion bound to the loop of *Corallina pilulifera* BPO. Interaction distances between the metal and the protein chain and water are shown in *red*. The image was prepared using the program O [26]

protein showing a prominent negative ellipticity at 208 nm (Fig. 6a).

The addition of 1 mM aluminum chloride to the apo-enzyme showed a small effect on the CD spectrum indicative of some minor structural changes. This was more pronounced in the absence of vanadate (data not shown). The effect of Al^{3+} in reducing the thermostability of the enzyme could be due to its tendency to act as a Lewis acid. This ion has been well characterized in solution and has a high affinity for hydration, attracting oxygen donors and polarizing water. With Al^{3+} free in solution while the temperature is rising, the acidity of the environment around the protein will be affected. This could also explain the similar effect observed with Zn^{2+} on the enzyme's thermostability since it is also a good Lewis acid.

The activity and structure of the apo-enzyme in the presence of vanadate was drastically altered in the presence of 80% methanol and dimethylsulfoxide. This is shown in Fig. 6b, where the CD spectra of the enzyme in ethanol and methanol are compared.

Discussion

The three-dimensional X-ray structural analysis of BPO from recombinant *C. pilulifera* demonstrated that the enzyme had one calcium atom per subunit [10]. Vanadium-dependent BPOs have been shown to be enzymes that are highly stable to temperature and organic solvents [17]. The structural analysis showed that there is a calcium ion in the loop between the amino acid residues at positions 359 and 366, and that it is coordinated by the main chain oxygen atoms of Phe359, Gln361, and Gln368 and the carboxyl groups of Asp363 and Asp366 and a molecule of water [10] (Fig. 7).

The coordination of a divalent metal ion has only been observed in BPO from *Corallina* species. A sequence and structure comparison between the BPO from *Ascophyllum nodosum* and that from *C. pilulifera* was carried out in order to study the differences that could lead to the presence of Ca^{2+} in the latter enzyme. Both alignments demonstrated that the calcium ion was bound in a loop that is shorter in the *A. nodosum* enzyme than in *Corallina* BPO. The amino acid sequence in the region of interest differs in length by three amino acids (Fig. 8).

The thermal vibrations in this area are high, and a longer loop region would generally become more unstable (Fig. 9). The interactions between the calcium ion and the protein molecule in the BPO from *C. pilulifera* would be expected to stabilize this loop, and lead to the stabilization of the whole structure. In fact, it can be observed that the corresponding loop in *C. pilulifera*

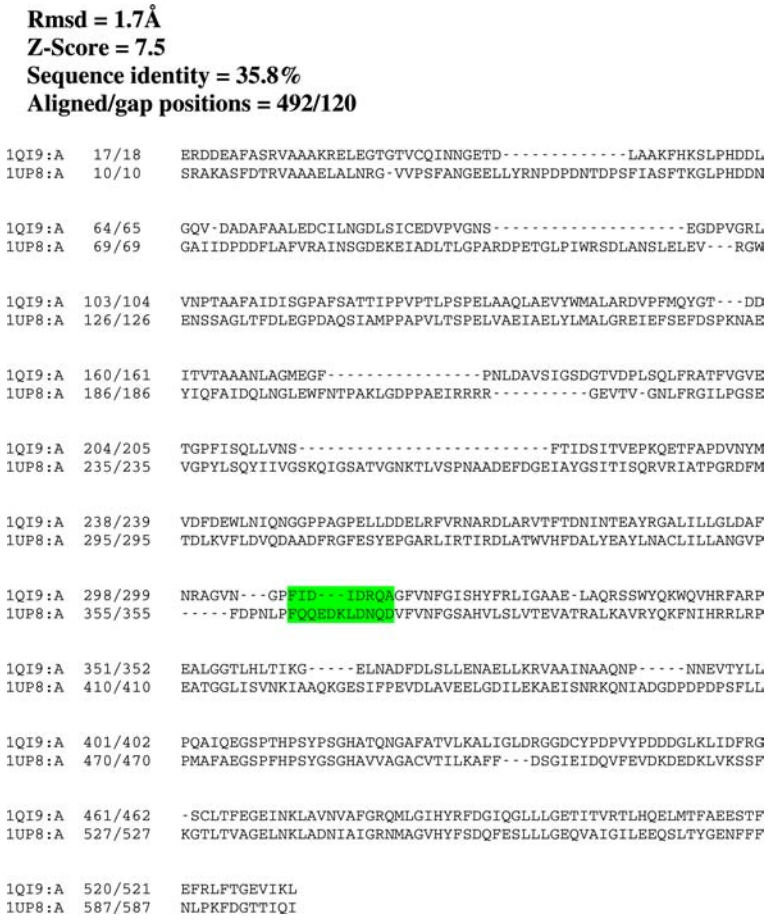


Fig. 8 Structure-based sequence alignment of *C. pilulifera* (1UP8:A) and *Ascophyllum nodosum* (1QI9:A) BPO. The calcium-binding loop and the corresponding region of the *A. nodosum* enzyme are highlighted in *green*. The alignment was carried out using the program CE [27]. Position numbers according to sequence (starting from 1) and according to the Protein Data Bank (*PDB*) are given as sequence/PDB numbering. The alignment parameters are shown at the *top*

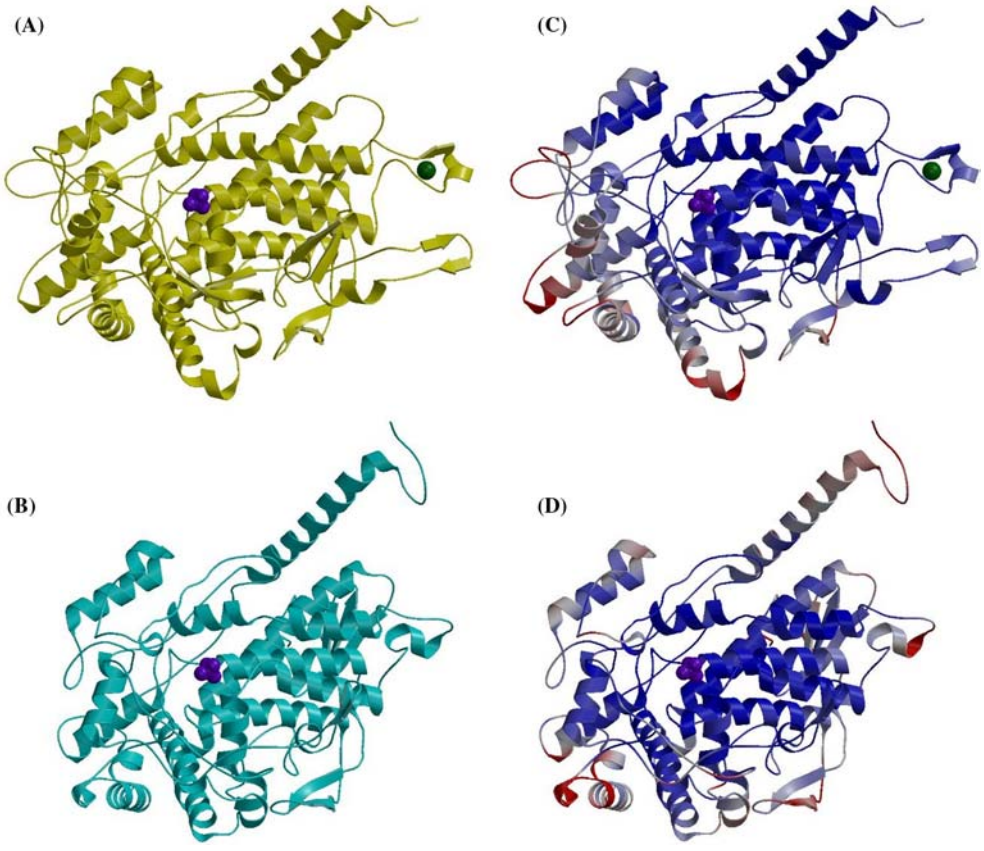


Fig. 9 Structural comparison between the BPO subunit from *C. pilulifera* (**a**, in *yellow*) and from *A. nodosum* (**b**, in *cyan*). Metal ions are represented in CPK mode, vanadate is *purple*, and calcium is *green*. **c**, **d** Both structures are colored by B-factor in a scale from *blue* (low B-factor) to *red* (high B-factor). The image was prepared using the program Bobscript [28] and rendered with Raster3D [29]

shows relatively lower B-factors than that of *A. nodosum*. Other regions with high B-factors are solvent-exposed regions. It should be noted that differences between both enzymes, other than the calcium-binding region, occur in those regions involved in the formation of the dodecameric arrangement in the *C. pilulifera* enzyme, in contrast with the more solvent-exposed dimeric arrangement for the *A. nodosum* BPO.

In this study, we assumed that the calcium present in this enzyme would contribute to the enzyme stability. We confirmed that the addition of calcium ion to the apo-enzyme enhanced the stability, and that the apo-enzyme without metal ions was not so stable.

Besides Ca^{2+}, other divalent cations, such as Sr^{2+} and Mg^{2+}, also increased the thermostability. It was considered that some metals belonging to the alkaline-earth group which enhanced the thermostability would be incorporated into the normal binding site for calcium in the enzyme. The affinity for binding at this site is not only dependent on the charge of the ion, but also on a combination of its size and coordination sphere. A similar phenomenon was observed in the case of a subtilisin-type protease, aqualysin I, from *Thermus aquaticus* YT-1 [22]. Although aqualysin I had calcium-binding sites involved in the thermostability, metal ions such as La^{3+}, Sr^{2+}, and Nd^{3+} were also found to stabilize the enzyme against heat treatment. It was speculated that the calcium-binding site of aqualysin I exhibited the structural flexibility to bind several metal cations of different size and valence.

It was shown that not only calcium but also vanadate enhanced the thermostability of BPO from *C. pilulifera*. We also demonstrated that similar ions such as molybdate and tungstate do not have the same effect on the enzyme stability.

From the reported three-dimensional structure for this enzyme [10] and related enzymes from other species [23, 24], it is known that vanadate coordinates with a histidine residue in the active site. It has also been demonstrated that phosphate acts as an analog for vanadate within the active site owing to its structural similarity. It occupies a similar position in the active site of the enzyme but is only held by hydrogen-bonding interactions [9]. The structure of CPO with tungstate in the active site has been determined [25] and it shows no coordination with the active-site histidine. The results from these experiments can therefore be explained by the thermal movement of the vanadate-binding side chains in the apo-enzyme. The presence of tungstate will compete with vanadate and prevent it from binding the active-site histidine. It will form hydrogen-bonding interactions with the surrounding amino acid side chains, reducing thermal vibrations. The activity is then lowered owing to competition for the active site, but the thermal stability is slightly greater than that of the apo-enzyme. Molybdate is observed to inhibit the activity of

the enzyme, while maintaining the same stability to temperature as tungstate. This inhibition could be caused by a stronger interaction of the molybdate with the active site, competing with vanadate.

The holo-enzyme exhibits high stability in most of the commonly used organic solvents. Particularly notable is the maintenance of 90% activity in ethanol. The presence of calcium is again shown to be of importance in the stability of the enzyme structure, increasing the activity and lifetime of the enzyme in methanol. Results from CD experiments have proven that this solvent drastically affects the structure of the holo-enzyme.

The previous structural study indicated the presence of the calcium ion in the BPO molecule of *C. pilulifera* produced by the recombinant yeast [10]. The experiments carried out in this study clearly demonstrate that the calcium ion contributes to the enzyme stability. The role of vanadate was shown to be important for thermostability as well as activity of the enzyme. The experiments carried out show the high stability in organic solvents will be valuable in the use of this BPO enzyme in biocatalysis.

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