Purification and Molecular and Catalytic Properties of Bromoperoxidase from *Streptomyces phaeochromogenes*

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A bromoperoxidase has been isolated and purified from the chloramphenicol-producing actinomycete *Streptomyces phaeochromogenes*. The purified enzyme was homogeneous as determined by polyacrylamide gel electrophoresis. The prosthetic group of the bromoperoxidase was ferriprotoporphyrin IX. Based on gel filtration results the molecular weight of the enzyme was $147\,000 \pm 3000$. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis showed a single band having the mobility of a 72500 molecular weight species. Therefore, in solution at neutral pH, the bromoperoxidase behaved as a dimer. The isoelectric point was 4.0. The spectral properties of the native and reduced enzyme are reported. The homogeneous enzyme also had peroxidase and catalase activity.

INTRODUCTION

Bromoperoxidases, enzymes which can use bromide ions in the presence of hydrogen peroxide and a halogen acceptor for the catalytic formation of carbon–halogen bonds, have been isolated from several marine algae (Ahern *et al.*, 1980; Baden & Corbett, 1980; Manthey & Hager, 1981) and from the bacterium *Pseudomonas aureofaciens* (van Pée & Lingens, 1985). The bacterial enzyme showed many differences from the algal enzymes. Surprisingly, although chlorinated metabolites are produced by marine algae (Faulkner, 1977) and bacteria (Neidleman, 1975), only brominating but not chlorinating enzymes have so far been isolated from these sources. Therefore we decided to use the chloramphenicol-producing actinomycete *Streptomyces phaeochromogenes* to obtain more information about bacterial halogenation.

METHODS

Reagents. Hydrogen peroxide (30%, v/v) was purchased from Merck and *t*-butyl hydroperoxide (80%, v/v) was from Fluka. Monochlorodimedone was prepared from dimedone by chlorination with sodium hypochlorite (Hager *et al.*, 1966). *o*-Dianisidine (3,3'-dimethoxybenzidine) was from Sigma and L-isoleucine was a gift from Degussa, Konstanz, FRG.

Organism and culture conditions. *Streptomyces phaeochromogenes* NRRL B-3559 was from Northern Regional Research Laboratories, Peoria, Ill., USA. The mineral salt medium described by Chatterjee *et al.* (1983), with glucose as the carbon source and L-isoleucine as the nitrogen source, was used. The chloramphenicol-producing strain was grown in 2 litre Erlenmeyer flasks, containing 1 litre of medium, at 30 °C for 96 h on a rotary shaker. Cells were harvested by centrifugation, yielding about 12 g (wet wt) l⁻¹.

Enzyme assays. Halogenating activity and peroxidase activity were measured essentially as described for the bromoperoxidase from *Pseudomonas aureofaciens* (van Pée & Lingens, 1985).

Purification of *S. phaeochromogenes* bromoperoxidase. The crude extract was prepared by suspending one part of cells (wet wt) in two parts of 50 mM-sodium acetate buffer, pH 5.5, and disrupting with a Branson sonifier J-17 A for six 30 s periods. The cell debris was removed by centrifugation for 30 min at 22100 g and 4 °C. Ammonium sulphate was added to the crude extract to 40% saturation, and after stirring for 20 min the precipitate was removed by centrifugation and discarded. Ammonium sulphate was added to the supernatant to 70% saturation, and stirred for 20 min. The precipitate was removed by centrifugation, dissolved in 50 mM-sodium acetate buffer, pH 5.5, and dialysed against 5 litres 50 mM-sodium acetate buffer, pH 5.5, for 15 h. After centrifugation the

dialysed enzyme was adsorbed to a DEAE-cellulose DE52 column (5×10 cm) equilibrated with 50 mM-sodium acetate buffer pH 5.5. The sample was washed onto the column with 800 ml of this buffer and a 500 ml gradient of 0–0.6 M-KCl in 50 mM-sodium acetate buffer, pH 5.5, was applied. Then a further 250 ml 0.6 M-KCl in 50 mM-sodium acetate buffer, pH 5.5, were passed through the column. Fractions (3.4 ml) were assayed for protein (A_{280}) and haloperoxidase activity. Those fractions (115–138), having an activity of more than 30% of the maximal activity, were pooled.

The resulting 81 ml protein solution were dialysed against 5 litres 50 mM-Tris/HCl, pH 8.9, and passed onto a DEAE-cellulose DE52 column (2.7×5 cm) equilibrated with 50 mM-Tris/HCl pH 8.9. The sample was washed onto the column with 610 ml of this buffer and was then eluted with a linear gradient of 500 ml 0–0.5 M-KCl in 50 mM-Tris/HCl, pH 8.9. Those fractions (57–69) having an activity of more than 20% of the maximal activity were pooled and dialysed against 5 litres 5 mM-potassium phosphate buffer, pH 7.0, for 15 h. The dialysed protein solution was adsorbed to a Bio-Gel HTP hydroxylapatite column (1.5×8 cm) equilibrated with 5 mM-potassium phosphate buffer, pH 7.0. The sample was washed onto the column with 120 ml of this buffer and eluted with 200 ml of a linear gradient (5–50 mM) of potassium phosphate buffer pH 7.0. Those fractions (47–70) having more than 25% of the maximal haloperoxidase activity were pooled and concentrated to 2.3 ml using an Amicon concentrator with a PM-30 membrane. After dialysing against 1 litre sample buffer the enzyme was further purified by preparative polyacrylamide gel electrophoresis on 7.5% (w/v) gels under non-dissociating conditions at pH 7.5 (Maurer, 1968).

The brownish band was cut out, immersed in 5 vols 10 mM-potassium phosphate buffer, pH 7.0, homogenized and stirred for 2 h at 4 °C. After centrifugation the extraction was repeated and the acrylamide fragments were removed by centrifugation. The supernatants were concentrated to 2 ml using an Amicon concentrator with a PM-30 membrane and dialysed against 5 litres 10 mM-potassium phosphate buffer, pH 7.0, for 15 h.

Analysis of the purified bromoperoxidase by PAGE. Analytical PAGE under non-denaturing conditions at pH 8.9 was done in a horizontal 7.5% (w/v) polyacrylamide gel (Fehrström & Moberg, 1977) with an LKB 2117 Multiphor apparatus. SDS-PAGE was done by the method of Laemmli (1970).

Analytical isoelectric focusing in thin-layer plates of 5% (w/v) polyacrylamide containing a 2.4% (w/v) solution of ampholines in the pH range 3.5–9.5 was done using an LKB Multiphor system according to the manufacturer's instructions (Winter *et al.*, 1977). Gels were stained for protein with Coomassie Blue R250 and for peroxidase activity with *o*-dianisidine. The pI of bromoperoxidase and the molecular weight of the subunits were determined by comparing the migration of the protein with those of standard proteins.

Protein determination. Protein concentrations were measured by the method of Lowry using bovine serum albumin as a standard.

Molecular weight determination. The molecular weight of bromoperoxidase was estimated by molecular sieve chromatography with a 94×3 cm column of Sephadex G-200, standardized with ferritin (mol. wt 440000), gamma globulin (150000), bovine serum albumin (68000), and cytochrome *c* (13000). The column was equilibrated with 50 mM-potassium phosphate buffer, pH 7.0.

Spectroscopy. Absorption spectra were recorded on a Uvicon 810 spectrophotometer (Kontron). Reduced pyridine haemochromogen was prepared by the method described by Falk (1964).

RESULTS

Enzyme purification

Previous work has shown that bacterial bromoperoxidases can not be detected in crude extracts or after ammonium sulphate fractionation (van Pée & Lingens, 1984, 1985). They only can be detected after removal of interfering enzymes. In the case of bromoperoxidase from *Streptomyces phaeochromogenes* this was achieved by the use of a DEAE-cellulose DE52 ion-exchange column with sodium acetate buffer, pH 5.5. The results of the purification procedure are summarized in Table 1. The purified enzyme gave a single band on PAGE under non-denaturing (R_F 0.42) and denaturing conditions (Fig. 1), when the gels were loaded with 20 µg protein. The comparison with standard proteins yielded a molecular weight of 72500 for the subunits (Fig. 1*b*). In isoelectric focusing experiments the enzyme exhibited a single band when 20 µg protein was applied to the gel. The isoelectric point was estimated to be 4.0.

Spectral properties

The visible absorption spectra of the native and reduced bromoperoxidase are given in Fig. 2. The native bromoperoxidase spectrum had maxima at 404, 496 and 634 nm with millimolar extinction coefficients (ϵ_{mM}) of 132.4, 18.3 and 11.0, respectively. The dithionite-reduced bromoperoxidase had a Soret band at 438 nm with a ϵ_{mM} of 85.2. The visible absorption

Fig. 1. SDS-PAGE of bromoperoxidase from *Streptomyces phaeochromogenes* under non-denaturing (a) and denaturing (b) conditions. Each gel contained 20 μ g protein and was stained with Coomassie Blue R250. The standard proteins in the left-hand lane of (b) were (molecular weights in parentheses): phosphorylase B (92500), bovine serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000), soybean trypsin inhibitor (21500) and lysozyme (14400).

Table 1. Summary of the purification of bromoperoxidase from *Streptomyces phaeochromogenes*

The enzyme was purified from 275 g bacteria; —, could not be determined.

Purification step	Total vol. (ml)	Total protein (mg)	Total activity (units)	Specific activity [units (mg protein) ⁻¹]	Percentage yield	Purification (fold)
Crude supernatant	1155	1155	—	—	—	—
40–70% (NH ₄) ₂ SO ₄ precipitation	100	650	—	—	—	—
DEAE-cellulose DE52 ion-exchange chromatography:						
pH 5.5	81	113	6.8	0.06	100	1.0
pH 8.9	47	32.9	3.6	0.11	52.9	1.8
Bio-Gel HTP hydroxyl-apatite	2.3	7.8	2.6	0.34	38.2	5.7
Preparative PAGE	2.0	0.8	1.1	1.4	16.2	23.3

spectrum of the ferrous enzyme had a peak at 557 nm with a shoulder at 592 nm. The ϵ_{mM} values were 11.5 and 6.1, respectively. When reduced in alkaline pyridine, bromoperoxidase yielded a haemochromogen whose spectrum was identical with that of protohaem IX (Fig. 3). On the basis of the absorbance at 557 nm (ϵ_{mM} 34.4; Falk, 1964), haem content of 0.91 molecules per dimeric molecule of bromoperoxidase was calculated. The purified enzyme had an A_{404}/A_{274} ratio of 0.4 which is consistent with the low haem content.

Reactions catalysed by bromoperoxidase

Bromoperoxidase in the presence of hydrogen peroxide and bromide catalysed the bromination of monochlorodimedone, but not the chlorination or fluorination of this organic substrate. When hydrogen peroxide was substituted by *t*-butyl hydroperoxide, no brominating

Fig. 2

Fig. 2. Absorption spectra of native and reduced bromoperoxidase. The spectra of native (—) and reduced (---) bromoperoxidase ($1.3 \mu\text{M}$) were recorded in 25 mM-potassium phosphate buffer, pH 7.0.

Fig. 3

Fig. 3. Pyridine haemochromogen spectrum. Bromoperoxidase was present at $1.2 \mu\text{M}$ final concentration. The method of haemochromogen preparation is described in Methods.

activity could be detected. The enzyme activity, however, was not inhibited by *t*-butyl hydroperoxide. Bromoperoxidase in the absence and presence of bromide was able to catalyse the oxidation of *o*-dianisidine, pyrogallol and *o*-phenyldiamine. Catalase activity could also be measured under the same conditions as peroxidase and haloperoxidase activity. With *t*-butyl hydroperoxide instead of hydrogen peroxide neither peroxidase nor catalase activity could be detected.

pH optima of the different catalytic activities of bromoperoxidase

The pH optima of the three catalytic activities of this bromoperoxidase were slightly different (Fig. 4). The optima for the brominating and peroxidase activities were at pH 5.0, whereas the optimum for the catalase activity was at pH 7.0.

DISCUSSION

When crude extracts from *Streptomyces phaeochromogenes* were incubated in the presence of monochlorodimedone, hydrogen peroxide and bromide, no enzyme reaction could be detected. However, when the supernatant solution was incubated in the presence of *o*-dianisidine and hydrogen peroxide, oxidation of the substrate was evident. The same phenomenon was observed with extracts from the bacterium *Pseudomonas aureofaciens* (van Pée & Lingens, 1985) and from the marine alga *Penicillium lamourouxii* (Baden & Corbett, 1980). In the latter case, however, bromoperoxidase activity could already be detected after precipitation and concentration by ammonium sulphate fractionation. With extracts from *S. phaeochromogenes* ammonium sulphate fractionation and ion-exchange chromatography had to be done before brominating activity was detectable. In the case of bromoperoxidase from *Ps. aureofaciens* even a second ion-exchange chromatography step was necessary before brominating activity could be measured. This demonstrates one of the problems with the isolation of bacterial haloperoxidases, which is probably due to the fact that crude extracts contain a very efficient catalase. This catalase

Fig. 4. Brominating, peroxidase and catalase activities of bromoperoxidase as a function of pH. The brominating activity was measured by the monochlorodimedone assay (○). Peroxidase activity was determined by the *O*-dianisidine assay (●). Catalase activity was measured by following the decomposition of hydrogen peroxide at 240 nm (□). Buffering was achieved with 0.1 M-potassium phosphate buffer. 100% activity corresponded to the maximal activity.

Table 2. Comparison of bromoperoxidase from *Streptomyces phaeochromogenes* with bromoperoxidase from *Pseudomonas aureofaciens*, with an algal bromoperoxidase from *Penicillus capitatus*, and with chloroperoxidase from *Caldariomyces fumago*

	<i>Streptomyces phaeochromogenes</i>	<i>Pseudomonas aureofaciens</i>	<i>Penicillus capitatus</i> *	<i>Caldariomyces fumago</i>
$10^{-3} \times$ Mol. wt	144-150	155-158	97	42
Subunits	2	2	2	1
Spectrum peak wave-length (nm)	404 496	405 500 540†	413 495 545†	403 515 542
	634	631	635	650
Haem-type	IX	IX	IX	IX
Chlorination	-	-	-	+
Bromination	+	+	+	+
Oxidation of <i>o</i> -dianisidine	+	+	+	+
Catalase activity	+	+	+	+
pH optimum (bromination)	5.0	5.5	4.0†	2.75

* Data from Manthey & Hager (1981).

† Data from Baden & Corbett (1980).

‡ Shoulder of peak.

competes with bromoperoxidase for hydrogen peroxide. Therefore bromoperoxidase activity can only be detected after removal of the catalase. The molecular weight of the bromoperoxidase from *S. phaeochromogenes* is very similar to that of the other bacterial bromoperoxidases already described (Table 2), but quite different from those of the eukaryotic bromoperoxidases from marine algae (Baden & Corbett, 1980; Manthey & Hager, 1981) and that of the chloroperoxidase from the fungus *Caldariomyces fumago* (Morris & Hager, 1966). Both bacterial bromoperoxidases form a dimer, consisting of two identical subunits, a characteristic they share with only one of the described algal bromoperoxidases. The spectral properties of the bacterial bromoperoxidases parallel those of the algal bromoperoxidases and chloroperoxidase. The prosthetic group of all these enzymes is protoporphyrin IX. Like the eukaryotic haloperoxidases the two bacterial bromoperoxidases also have catalase and peroxidase activity, but their brominating activity is rather low and the two other activities are very high. Like the algal

bromoperoxidases, those from *S. phaeochromogenes* and *Ps. aureofaciens* exhibit no chlorinating activity. This is very surprising, especially as *S. phaeochromogenes* produces chloramphenicol and *Ps. aureofaciens* produces the antifungal antibiotic pyrrolnitrin, both chlorinated metabolites. Geigert *et al.* (1984) postulated a bromonium ion-induced chlorination for the synthesis of chlorinated metabolites in marine algae. Whether this also holds for the formation of chlorinated metabolites from bacteria is not yet known, but will be the subject of further investigations.

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Dedicated to Professor Wolfram Zillig on the occasion of his 60th birthday.

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