Purification, Properties and Immunological Detection of a Bromoperoxidase–Catalase from *Streptomyces venezuelae* and from a Chloramphenicol-nonproducing Mutant

By MARTIN KNOCH, KARL-HEINZ VAN PÉE, LEO C. VINING†
AND FRANZ LINGENS*

*Institut für Mikrobiologie der Universität Hohenheim, Garbenstraße 30,
D-7000 Stuttgart 70, FRG*

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A new bromoperoxidase–catalase was purified from the chloramphenicol-producing actinomycete *Streptomyces venezuelae* ISP 5230. The homogeneous enzyme showed brominating activity, catalase activity and a very low peroxidase activity. The spectral properties and pH dependence of the catalase activity showed similarities to conventional catalases. In contrast to other haem-bromoperoxidases, the bromoperoxidase–catalase was stable when treated with an ethanol/chloroform mixture. Gel filtration gave an estimated M_r of 127 000–136 000. SDS-PAGE showed a single band corresponding in mobility to a species with an M_r of 61 000. The pI was estimated to be 4.5. The bromoperoxidase–catalase was not present in active form in a mutant of *S. venezuelae* ISP 5230, blocked in the chlorination step of chloramphenicol biosynthesis. However, an inactive species of the enzyme was detected in crude extracts of the mutant by using antibodies. From these results it is concluded that this bromoperoxidase participates in the chlorination step during chloramphenicol biosynthesis.

INTRODUCTION

Many fungi, bacteria and other micro-organisms produce a diversity of chlorinated products. However, most of the halogenating enzymes isolated are bromoperoxidases (Neidleman & Geigert, 1986). These usually resemble the bromoperoxidase from *Streptomyces phaeochromogenes* (van Pée & Lingens, 1985) in being haem-containing enzymes, but in the last few years, nonhaem-haloperoxidases have been detected in several organisms, including bacteria (van Pée *et al.*, 1987; Wiesner *et al.*, 1986). Haem-bromoperoxidases catalyse the oxidation of a wide spectrum of organic substrates in the presence of hydrogen peroxide, and also catalyse the oxidation of halide ions. However, only the chloroperoxidase of the mould *Caldariomyces fumago* (Morris & Hager, 1966) and the nonhaem-chloroperoxidases of *Curvularia inaequalis* (Liu *et al.*, 1987) and of *Pseudomonas pyrrocinia* (Wiesner *et al.*, 1986, 1988) catalyse chlorination. Although haloperoxidases are presumed to function in the biosynthesis of naturally occurring halometabolites, only the chloroperoxidases of *C. fumago* and *P. pyrrocinia* have so far been shown to participate *in vivo*.

Streptomyces venezuelae produces the chlorinated antibiotic chloramphenicol. Although much is known about chloramphenicol biosynthesis (Vining & Westlake, 1984), little information about the chlorination step is available. It has been suggested that malonyl-CoA is the substrate that is chlorinated, but the evidence is equivocal and does not exclude the possibility that chlorination follows acylation (Simonsen *et al.*, 1978).

Here we describe the isolation and some properties of a bromoperoxidase–catalase from *S. venezuelae*, and we present evidence that this enzyme exists only as an inactive protein in a mutant blocked in the chlorination step (Doull *et al.*, 1985).

† Present address: Department of Biology, Dalhousie University, Halifax, Nova Scotia B3H 4J1, Canada.

METHODS

Chemicals. Monochlorodimedone, *o*-dianisidine (3,3'-dimethoxybenzidine), nitro blue tetrazolium, phenol red and phosphatase-conjugated anti-rabbit IgG were purchased from Sigma. Hydrogen peroxide (30%, v/v) was from Merck. L-Isoleucine was a gift from Degussa. 1-Methyl-2-(3,5-dibromo-2-methoxyphenyl)pyrrole was a gift from H. Pudleiner and H. Laatsch, University of Göttingen, FRG.

Bacterial strains. *Streptomyces venezuelae* ISP 5230 (ATCC 10712) wild-type and its chloramphenicol-nonproducing mutant strain Cml-2 were used (Doull *et al.*, 1985).

Growth conditions and preparation of cell extracts. The strains were maintained and propagated routinely on malt-medium. Cells for preparation of extracts were grown in a medium containing glucose, isoleucine and basal salts as described by Doull *et al.* (1985) for chloramphenicol production. Portions (50 ml) of medium were inoculated with 0.1 ml of a well-grown culture and incubated on a rotary shaker at 30 °C. After 48 h, these cultures were each transferred aseptically into 2 l Erlenmeyer flasks containing 500 ml of medium and incubated for a further 96 h. Cultures of the mutant strain were grown for only 72 h because of their much better growth. Cells were harvested by centrifugation, yielding about 6 g (wet wt) l⁻¹ and frozen at -18 °C. For disruption, one part of cells (wet wt) was resuspended in two parts (by wt) of 0.1 M-ammonium acetate buffer (pH 6.5) and sonicated with a Branson sonifier for fifteen 30 s periods. After centrifugation at 22 100 g and 4 °C for 30 min, the supernatant was retained and the cell debris was resuspended in an equal amount of buffer and sonicated as above. The homogenate was centrifuged as above and the supernatants were combined.

Enzyme assays. Brominating activity was measured by the method of Hewson & Hager (1980) with monochlorodimedone (44 mM) as substrate in the presence of H₂O₂ (1.8 mM), bromide (100 mM) and a suitable amount of enzyme in 500 mM-sodium acetate buffer (pH 5.5). The peroxidase assay was done as described by Claiborne & Fridovich (1979) with *o*-dianisidine hydrochloride (48 µM) as substrate in the presence of H₂O₂ (7.2 mM) and a suitable amount of enzyme in 100 mM-sodium acetate buffer (pH 5.5). The oxidation of *o*-dianisidine was followed at 460 nm. Catalase activity was assayed spectrophotometrically at 240 nm (Beers & Sizer, 1952) with H₂O₂ (6.5 mM) as substrate in 100 mM-sodium acetate buffer (pH 5.5). All assays were started by adding the enzyme and absorbance changes were measured against appropriate blanks with a recording spectrophotometer. Specific activities were calculated using the molar extinction coefficient of the substrates or the products, respectively. One unit of activity is defined as the disappearance of 1 µmol substrate or the formation of 1 µmol of product min⁻¹ (mg protein)⁻¹.

Analysis of the bromoperoxidase-catalase by PAGE and analytical isoelectric focusing. For electrophoresis of the native enzyme, 7.5% (w/v) polyacrylamide gels, pH 7.5, according to Maurer (1968) were used. PAGE in the presence of 0.1% SDS was done according to Laemmli (1970). Proteins in the Pharmacia low molecular mass kit were used as standards. For analytical isoelectric focusing, an LKB thin-layer polyacrylamide gel (5%, w/v) was used in the pH range 4.0-6.5. As protein standards, the Pharmacia isoelectric focusing calibration kit (2.5-6.5) was used. The pI was determined by comparison with the migration rates of standard proteins. All electrophoresis was done with an LKB 2117 Multiphore apparatus.

Staining of gels. Gels were stained for protein with Coomassie Blue R250 and for peroxidase activity with *o*-dianisidine to form brown bands (Shannon *et al.*, 1966). To detect brominating activity, phenol red was used as a substrate; this is brominated to bromophenol blue (Loo *et al.*, 1964). The assay mixture contained 0.5 M-sodium acetate buffer (pH 5.5), hydrogen peroxide (7.2 mM), 0.1 M-sodium bromide and 0.002% phenol red. For enzyme-bound haemferric ions, benzidine dye was used as described by Clarke (1964).

Purification of bromoperoxidase. (1) *DEAE-cellulose DE 52.* After treatment with 1.5% (w/v) streptomycin sulphate and centrifugation, the crude extract was applied to a DEAE cellulose DE 52 column (5.5 × 11 cm) equilibrated with 0.15 M-sodium acetate buffer (pH 5.5). The column was washed and then eluted with a gradient (600 ml) of 0.15-0.7 M-sodium acetate buffer (pH 5.5). Fractions (4.2 ml) were assayed for protein (*A*₂₈₀), peroxidase and catalase activity. Those with at least 25% of the activity of the most active fraction were pooled, and the pH was adjusted to 6.5.

(2) *Phenyl-Sepharose CL-4B.* Ammonium sulphate was added to the enzyme solution to 1.0 M. The solution was then applied to a phenyl-Sepharose CL-4B column (14 × 2.5 cm) equilibrated with 0.5 M-ammonium sulphate in 10 mM-ammonium acetate buffer, pH 6.5. Protein was eluted with a descending gradient of 0.5-0 M-ammonium sulphate (600 ml, pH 6.5). Fractions were assayed for protein (*A*₂₃₀) and catalase activity, and the active fractions were pooled.

(3) *Hydroxyapatite.* Without dialysis, the pooled enzyme solution was applied to a hydroxyapatite column (14 × 2.5 cm) equilibrated with 10 mM-potassium phosphate buffer (pH 6.8), and eluted with a gradient (400 ml; 10-100 mM) of the same buffer. Fractions (2.8 ml) were screened for bromoperoxidase activity as well as for protein (*A*₂₁₅). The active fractions were pooled.

(4) *Sepharose Q Fast Flow.* The enzyme was applied to a Sepharose Q column (1 × 13 cm), equilibrated with 50 mM-ammonium acetate buffer (pH 5.6). The column was eluted with a gradient (400 ml) of 0-400 mM-KCl in the same buffer. Fractions were assayed and pooled as in step (3). The solution was dialysed against 10 mM-

ammonium acetate buffer (pH 6.5) and concentrated using an Amicon concentrator with a PM-30 membrane.

(5) *Phenyl-Sepharose CL-4B*. To remove the last impurities, hydrophobic chromatography with phenyl-Sepharose was repeated with a small column (0.8 × 4.5 cm).

Preparative PAGE. For production of antisera, the nearly homogeneous enzyme solution from different preparations after four steps of purification was subjected to preparative PAGE. The protein solution was concentrated to a volume of approximately 200 µl before being applied to a 4 mm polyacrylamide gel and electrophoresed as described by Maurer (1968). The brownish band was cut out and stored at -18 °C.

Immunological procedure. (a) *Production of antisera*. The frozen gel pieces from the preparative PAGE were cut into four parts containing approximately 0.25 mg bromoperoxidase each, pulverized and suspended in a total volume of 1 ml of phosphate-buffered saline (PBS; 0.13 M-NaCl, 0.007 M-Na₂HPO₄, 0.003 M-NaH₂PO₄). The probe was given to a rabbit as described by Spielman *et al.* (1974). After the third booster injection, the rabbit had to be bled, because it had developed abscesses at the injection sites. The serum was checked for precipitating antibodies using the agar double-diffusion test (Ouchterlony, 1949).

(b) *Electroblotting of proteins*. Proteins were separated by native or SDS-polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose filter (0.45 µm pore size; Schleicher and Schuell) with an LKB Multiphor II NovaBlot. The blotting was done as described in the LKB instruction manual. The discontinuous electrode-solution system for isoelectric focusing gels was used for native gels; a continuous buffer system was used for SDS-PAGE.

(c) *Immunological detection of blotted proteins*. After transfer of the proteins, the nitrocellulose sheet was submerged in a solution of skimmed milk (5%, v/v) in PBS for 2 h to block the remaining binding sites. The bromoperoxidase-catalase antiserum was diluted 1:100 in blocking solution and the blot was incubated overnight at room-temperature on a rocking platform. The sheet was washed with five aliquots of PBS within 30 min. Alkaline-phosphatase-conjugated anti-antibodies (Sigma) were diluted 1:2000 in blocking solution, added to the nitrocellulose blot and incubated for another 2 h. Further washing and developing of the nitrocellulose blot was done as described by Blake *et al.* (1984) with 5-bromo-4-chloroindolyl phosphate as a substrate for the alkaline phosphatase reaction. The reaction was stopped after 10 min.

M_r determination. The *M_r* of purified bromoperoxidase-catalase was estimated by molecular-sieve chromatography with a 95 × 2.5 cm column of Sephadex G-200. The column was standardized with ferritin (*M_r* 440 000), gamma globulin (150 000), bovine serum albumin (68 000) and ovalbumin (44 000). The column was equilibrated with 50 mM-ammonium acetate buffer (pH 6.8).

Protein determination. Protein was determined by the Lowry method using bovine serum albumin as a standard.

Spectroscopy. Absorption spectra were recorded on a Uvicon 810 spectrophotometer (Kontron Analytik). For GC-MS, a Varian 3700 mass spectrometer was used. For X-ray fluorescence spectra, a Perkin Elmer model 400 atomic absorption spectrometer was used.

Treatment with chloroform/ethanol. This was done by the method described by Nadler *et al.* (1986). A solution of purified bromoperoxidase-catalase was mixed with the solvents in the proportions enzyme solution/chloroform/ethanol (10:5:3, by vol.) and vortexed for 10 min at room-temperature. After centrifugation for 5 min to separate the two phases, the upper phase, which contained the enzyme, was assayed for activity.

Bromination of 1-methyl-2-(3,5-dibromo-2-methoxyphenyl)pyrrole by the bromoperoxidase-catalase. To demonstrate the brominating ability of the bromoperoxidase-catalase, we used 1-methyl-2-(3,5-dibromo-2-methoxyphenyl)pyrrole as a substrate. This particular phenylpyrrole derivative was used because it is very sensitive to bromination, but gives no brominated product in the absence of bromoperoxidase. The assay mixture contained 1-methyl-2-(3,5-dibromo-2-methoxyphenyl)pyrrole (0.02 mM), hydrogen peroxide (7.2 mM), potassium bromide (0.5 M) and 100 mU of purified bromoperoxidase in sodium acetate buffer (0.5 M, pH 4.75). After 16 h at room-temperature, the reaction mixture was extracted twice with ethyl acetate. A blank without enzyme was treated in the same way. Reaction products were characterized by GC-MS, with a Varian 3700 mass spectrometer (80 eV). A 25 m glass capillary column SE-30 was used. The temperature of the oven was run from 120–240 °C (10 °C min⁻¹); the temperatures of the injector and the detector were 250 and 300 °C, respectively.

RESULTS

Enzyme purification

No brominating activity could be detected in the crude extract. In the first purification step fractions assayed for catalase activity exhibited two peaks (Fig. 1). Only one of these was associated with peroxidase activity. After further purification of the material in the other peak region, brominating activity (monochlorodimedone assay) was found to accompany the catalase activity. Even in purified material from this peak region no peroxidase activity with *o*-dianisidine was ever detected in the absence of bromide by the spectrophotometric assay. The results of the purification of this component are summarized in Table 1.

Fig. 1. First ion-exchange chromatography on DEAE-cellulose DE 52 showing the separation of the two catalase activities present in crude extracts of *S. venezuelae*. Fractions of 4.2 ml were collected. O, Absorbance at 280 nm; ●, catalase activity; □, peroxidase activity; —, sodium acetate buffer (pH 5.5) concn.

Table 1. *Summary of the purification of bromoperoxidase-catalase from S. venezuelae*

The enzyme was purified from 142 g bacteria (wet wt). —, Could not be determined; BPO, bromoperoxidase.

	Volume (ml)	Protein (mg)	Specific activity [U (mg protein) ⁻¹]		Total activity (U)		Yield (%)	Purification (-fold)
			Catalase	BPO	Catalase	BPO		
Crude extract + 1.5% (w/v) streptomycin sulphate	595	2703	—	—	—	—	—	—
DEAE-cellulose DE 52 (pH 5.5)	140	213	3.32	0.002	708	0.48	100	1
Phenyl-Sepharose	107	17.7	20.7	0.049	366	0.87	184	22
Hydroxyapatite	86.0	3.1	85.4	0.255	255	0.78	162	113
Sepharose Q (pH 5.5)	34.8	0.8	279	0.599	235	0.50	104	280
Phenyl-Sepharose	0.7	0.2	963	1.77	212	0.39	81	789

Homogeneity and enzymic activities of bromoperoxidase-catalase

The purified bromoperoxidase-catalase showed a single band on analytical PAGE (Fig. 2, lane A). When stained for brominating activity with phenol red, a blue band developed very quickly (Fig. 2, lane C). Staining for peroxidase activity with *o*-dianisidine (Fig. 2, lane B) and for enzyme-bound ferric iron with benzidine (Fig. 2, lane D) had to be continued for 30 min before faint brownish and blue-brown bands, respectively, became visible. These coincided with the protein band at $R_F = 0.25$. In the spectrophotometric assay peroxidase activity [0.05 U (mg protein)⁻¹] could be measured only in the presence of bromide ions.

The brominating activity of the new enzyme was confirmed by brominating 1-methyl-2-(3,5-dibromo-2-methoxyphenyl)pyrrole. The elemental composition of the compounds obtained was established by GC-MS. The gas chromatogram showed four product peaks, the first of which [retention time (R_t) = 9.0 min] was also observed in assays without enzyme; it did not correspond to a brominated product. The next two peaks (R_t = 10.6 min and R_t = 11.6 min) showed a molecular ion at m/e 503 (35.8%) and isotope peaks at m/e 505 (26%), m/e 501 (27%), m/e 507 (7%) and m/e 499 (6.5%) (Fig. 3A). The intensity ratio (1:4:6:4:1) is characteristic for

Fig. 2. PAGE of native bromoperoxidase-catalase. Top, cathode; bottom, anode. Lane A, Protein staining with Coomassie Blue; B, staining with *o*-dianisidine for peroxidase activity; C, activity staining with phenol red for brominating activity; D, staining with benzidine for enzyme-bound ferric ions.

a tetrabromo-substituted compound and indicates dibromination of the substrate by the bromoperoxidase-catalase. Peak four ($R_t = 13.3$ min) had a molecular ion at m/e 583 and isotope peaks with an intensity ratio of 1:5:10:10:5:1 (Fig. 3B), which is characteristic for a pentabromo-substituted molecule and indicates tribromination of the substrate by the bromoperoxidase-catalase.

Spectral properties of the bromoperoxidase-catalase

The absorption spectrum of the native enzyme is shown in Fig. 4. It had maxima at 403 nm and 616 nm with shoulders at 490 nm and 530 nm. Although addition of $\text{Na}_2\text{S}_2\text{O}_4$ to the enzyme solution did not shift the Soret peak, it caused loss of the enzyme activity.

Molecular properties

When the purified enzyme was subjected to SDS-PAGE, only one protein band was detected. The M_r of the subunits of the *S. venezuelae* bromoperoxidase-catalase was 61 000, as determined by SDS-PAGE. The M_r estimated from gel chromatography on Sephadex G-200 and Superose 12 was 127 000 and 136 000, respectively. Bromoperoxidase-catalase is thus revealed to be a dimer consisting of two subunits of identical size.

Fig. 3. Mass spectra of the reaction products of bromoperoxidase-catalase. Product A, GC peak 2 + 3; product B, GC peak 4.

Fig. 4. Absorption spectrum of native bromoperoxidase-catalase. The spectrum was recorded in 10 mM-ammonium acetate buffer (pH 6.8).

Fig. 5. Native PAGE. Lane A, Crude extract of the non-chlorinating mutant of *S. venezuelae* stained for protein; lane B, Western blot of lane A; lane C, purified bromoperoxidase-catalase.

Fig. 6. Brominating and catalase activities of bromoperoxidase-catalase as a function of pH. Brominating activity was measured using the monochlorodimedone assay. The buffers (0.2 M concentration) were sodium acetate (○, pH 4.0–5.5) and ammonium acetate (●, pH 5.0–6.5). Catalase activity was measured by following the decomposition of hydrogen peroxide at 240 nm. The buffers (0.2 M concentration) were sodium acetate (□, pH 4.0–5.5), potassium phosphate (■, pH 5.5–9.0) and glycine/sodium hydroxide (△, pH 8.5–12).

In isoelectric focusing experiments, the enzyme exhibited a single band with an estimated pI of 4.5, when a wide-range isoelectric focusing gel (pH 3.5–9.5) was used. When a narrow-range isoelectric focusing gel (pH 4.0–6.5) was used, the purified protein showed multiple bands in the range of 4.55–4.35.

Immunological specificity

Antiserum prepared against the purified bromoperoxidase-catalase was examined by the Ouchterlony double-diffusion assay with different bromoperoxidase preparations. A single precipitation band was formed against the homologous antigen when dilutions up to 1:32 of the antiserum were used. On the Western blot after native PAGE, only extracts which contained the bromoperoxidase-catalase revealed a positive reaction with diluted antiserum (Fig. 5c).

When crude extract of the mutant strain was blotted, an immunological reaction occurred with a protein that had exactly the same R_F value as the bromoperoxidase-catalase (Fig. 5b). The same result was obtained by blotting native as well as SDS gels (result not shown).

Effect of pH on the enzyme activities of bromoperoxidase-catalase

The pH optimum for the bromination of monochlorodimedone showed a distinct maximum at pH 4.75, whereas for the catalase activity a broad maximum in the range of pH 6–9 (Fig. 6) was obtained.

Stability after treatment with chloroform/ethanol

After treatment with a mixture of chloroform/ethanol, the enzyme still showed 65% of its initial brominating activity. In addition, it retained its catalase activity. Another bromoperoxidase isolated from *S. venezuelae* was totally denatured by this treatment.

Compounds inhibiting the brominating activity of bromoperoxidase-catalase in the presence of substrates

Potassium cyanide and sodium azide in concentrations of 0.05 mM completely inhibited bromoperoxidase-catalase. Hydroxylamine in the same concentration caused 50% inhibition of the brominating activity; 3-amino-1,2,4-triazole in concentrations of 5 and 40 mM produced inhibitions of 45 and 90%, respectively.

DISCUSSION

From soluble extracts of *Streptomyces venezuelae* a bromoperoxidase-catalase was purified to homogeneity. The enzyme has brominating activity (1.8 U mg⁻¹), catalase activity (963 U mg⁻¹) and insignificant peroxidase activity that is observed only in the presence of

bromide ions (0.05 U mg^{-1}). We termed it a bromoperoxidase-catalase to distinguish it from other bacterial haloperoxidases, such as that isolated from *P. pyrrocinia* (Wiesner *et al.*, 1985) and also present in extracts of *S. venezuelae*, which have high peroxidase activity. Brominating activity could be detected only after the first purification steps, as already observed with other bacterial bromoperoxidases (van Pée & Lingens, 1985). Obviously, there are unknown inhibitory substances present in the crude extract. Previously, catalase was thought to be interfering with the monochlorodimedone assay (van Pée & Lingens, 1985); however, even when catalase is inhibited or removed by heat treatment, inhibition of brominating activity can be observed in cell-free extracts of bacteria (van Pée *et al.*, 1987). The differences in yield for brominating and catalase activity are due to the fact that the pooled fractions after ion exchange chromatography contain an additional catalase, which is removed in later steps.

To be absolutely sure that the isolated enzyme catalysed bromination of organic compounds, brominating activity of the new enzyme was demonstrated by enzymic bromination of 1-methyl-2-(3,5-dibromo-2-methoxyphenyl)pyrrole. This result is important because oxidation of monochlorodimedone by horseradish peroxidase was reported by Griffin & Ashley (1984). It is also known that haloperoxidases can use hydrogen peroxide to oxidize organic substrates, demonstrated with phenol red, which upon oxidation showed a change of absorbance at 610 nm (Glenn & Gold, 1985). Since bromination of phenol red results in a change of absorbance at 592 nm (de Boer *et al.*, 1987), it is difficult to differentiate between the two reactions, and the assay using phenol red is not very reliable for activity staining.

The enzyme described here is proposed to be a haem-protein since its optical spectrum shows a Soret band. The 405/280 nm ratio of 0.6, the faint staining with benzidine and low content of ferric iron [1.55 atoms of iron per molecule of (dimeric) enzyme] is consistent with a low haem content, also shown for other bacterial bromoperoxidases (van Pée & Lingens, 1985). As with other haem-enzymes, the bromoperoxidase-catalase was inhibited by sodium azide and potassium cyanide. The low specific activity for bromination and the broad pH optimum (7–8.5) for bromination of monochlorodimedone were similar to those of other haem-containing bromoperoxidases. The enzyme consists of two subunits of identical size but has a distinctly lower M_r than other bacterial haem-bromoperoxidases. However, the striking difference between this and other comparable enzymes is its very low peroxidase activity, only detectable by activity staining after a long incubation period or in the presence of bromide ions.

Contrary to other bacterial haem-bromoperoxidases, the new bromoperoxidase-catalase resembles in some aspects conventional catalases such as that from *Micrococcus luteus* and the bacterial catalases described by Nadler *et al.* (1986). As is typical for these enzymes, catalase activity is not lowered by sodium dithionite and is independent of pH in the range 5–10. That the enzyme has hydrophobic properties is revealed by its stability in chloroform/ethanol and strong binding to phenyl-Sepharose. Its brominating activity was inhibited by 3-amino-1,2,4-triazole. The (very low) peroxidase activity might be due to the 'bifunctional' peroxidase-catalase nature of catalase described by Schoenbaum & Chance (1976). According to Sichak & Dounce (1986), peroxidase activity could be due to the haematin group as was shown for denatured catalase monomers.

Active bromoperoxidase-catalase was absent in extracts from a mutant strain of *S. venezuelae* blocked in the chlorination step. Immunological experiments revealed no cross-reaction with other bacterial haem-bromoperoxidases characterized up to now. However, blotting experiments with extracts of the mutant strain showed the presence of an inactive bromoperoxidase-catalase. Chloroperoxidase from *Pseudomonas pyrrocinia* catalyses the bromination of monochlorodimedone but not the chlorination of this substrate. However, monodechloroaminopyrrolnitrin [4-(2-amino-3-chlorophenyl)pyrrole], an intermediate of pyrrolnitrin biosynthesis, is chlorinated to aminopyrrolnitrin by this chloroperoxidase (Wiesner *et al.*, 1988). This demonstrates the existence of halogenating enzymes with a high substrate specificity concerning chlorination, but not, however, with respect to bromination. Such enzymes can be shown to have bromoperoxidase activity using the monochlorodimedone assay, whereas their chlorinating activity can only be demonstrated by using a 'natural' substrate. The results described above suggest that the bromoperoxidase-catalase isolated from *S. venezuelae*

and detected in inactive form in the mutant blocked in the chlorination step of chloramphenicol biosynthesis could actually be the chlorinating enzyme that participates in the chlorination step of chloramphenicol biosynthesis. To prove this, however, more information about the nature of the substrate for the chlorination step in chloramphenicol biosynthesis is necessary.

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