Purification and Characterization of a Novel Bacterial Non-heme Chloroperoxidase from *Pseudomonas pyrrocinia*

(Received for publication, February 18, 1988)

Wolfgang Wiesner, Karl-Heinz van Pée, and Franz Lingens

From the Institut für Mikrobiologie der Universität Hohenheim, Garbenstrasse 30, D-7000 Stuttgart 70, Federal Republic of Germany

The first bacterial chloroperoxidase that is capable of catalyzing the chlorination of indole to 7-chlorodimedone was detected in *Pseudomonas pyrrocinia* ATCC 15958, a bacterium that produces the antifungal antibiotic pyrrolnitrin (Wiesner, W., van Pée, K.-H., and Lingens, F. (1986) *FEBS Lett.* 209, 321–324). Here we describe the purification and characterization of this bacterial non-heme chloroperoxidase. The enzyme was purified by DEAE-cellulose chromatography at different pH values, molecular sieve chromatography, and Bio-Gel HTP hydroxylapatite. After the last purification step, chloroperoxidase was homogeneous by polyacrylamide gel electrophoresis and ultracentrifugation. Based on gel filtration and ultracentrifugation results, the molecular weight of the enzyme was 64,000 ± 3,000. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed a single band with the mobility of a 32,000 molecular weight species. Therefore, in solution at neutral pH, this chloroperoxidase is a dimer. The enzyme did not exhibit any absorbance in the visible region of the spectrum. The isoelectric point was 4.1. Chloroperoxidase was specific for I⁻, Br⁻, and Cl⁻ and was not inhibited by azide, but was inhibited by cyanide and F⁻. This procaryotic chloroperoxidase catalyzed the bromination of monochlorodimedone but not its chlorination and has no peroxidase or catalase activity. The pH optimum of the enzyme was between 4.0 and 4.5, and the enzyme was stable between pH 3.5 and 8.5 and showed no loss of activity when incubated at 60 °C for 2 h. Chloroperoxidase also chlorinated 4-(2-amino-3-chlorophenyl) pyrrole to yield aminopyrrolnitrin, the immediate precursor of pyrrolnitrin. This suggests very strongly that chloroperoxidase is involved in the biosynthesis of the antibiotic pyrrolnitrin.

Haloperoxidases are divided into three groups according to their specificity for halide ions: chloroperoxidases, bromoperoxidases, and iodonperoxidases. Chloroperoxidase which was isolated from *Caldariomyces fumago* by Hager and co-workers (1, 2), catalyzes the chlorination, bromination, and iodination of various compounds through electrophilic substitution (3, 4) and addition mechanism (5, 6). Bromoperoxidases, which are specific for Br⁻ and I⁻, have been detected and purified from several marine algae, such as *Rhodomela larix* (7), *Penicillus capitatus* (8), and from bacteria such as *Pseudomonas aureofaciens* (9), *Streptomyces phaeochromogenes* (10), and *Pseudomonas pyrrocinia* (11). The prosthetic group of these haloperoxidases is protoporphyrin IX. However, recently several non-heme haloperoxidases have been isolated and characterized. A non-heme bromoperoxidase was purified from the seaweed *Corallina pilulifera* (12) and a non-heme, vanadium-containing bromoperoxidase was isolated from the marine alga *Aspergillus nodosum* (13). A non-heme, zinc-containing chloroperoxidase was detected in the dematiaceous hyphomycete *Curvularia inaequalis* (14). A non-heme bromoperoxidase was isolated from the 7-chlorotetraycine-producing actinomycete *Streptomyces aureofaciens* (15).

In our search for chlorinating enzymes in bacteria, which normally produce chlorinated metabolites, we found that *Pseudomonas pyrrocinia*, a bacterium that produces the antifungal antibiotic pyrrolnitrin, has a heme-containing bromoperoxidase (11) and a chloroperoxidase (16). Here we describe in detail the purification and characterization of this chlorinating enzyme, which is the first procaryotic chloroperoxidase.

**EXPERIMENTAL PROCEDURES**

**Materials**—DEAE-cellulose DE52 ion exchange resin was purchased from Whatman (Maidstone, Kent, Great Britain). DEAE-Sephadex A-25, Sephadex G-200, molecular weight standards for SDS-polyacrylamide gel electrophoresis and standards for analytical isoelectric focusing were from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). Hydroxylapatite was obtained from Bio-Rad Laboratories (Munich, Germany). Plates for analytical isoelectric focusing were from LKB (Bromma, Sweden). Ultrafiltration membranes (Diaflo, PM-30) were obtained from Amicon Corp. (Danvers, MA). Hydrogen peroxide (30% v/v) was purchased from Merck (Darmstadt, Germany). Monochlorodimedone was prepared from dimedone by chlorination with sodium hypochlorite (2). α-Dianisidine (3,3'-dime-thoxybenzidine) was from Sigma (Taufkirchen, Germany). 4-(2-Amino-3-chlorophenyl)pyrrole was prepared from 7-chlorotryptophan according to van Pée et al. (17). Aminopyrrolnitrin was prepared from pyrrolnitrin by reduction with sodium dithionite (18).

All other chemicals used were of analytical grade and were obtained from commercially available sources.

**Microorganism and Culture Conditions**—*P. pyrrocinia* ATCC 15958, from which chloroperoxidase was isolated, was grown for 3 days at 30 °C under aeration (0.3 v/v/min) and stirring (150 rpm) in a 100-liter fermentor that was inoculated with 5 x 11 cultures from the late exponential growth phase. The mineral salt medium described by Lübbe et al. (19) was used. Cells were harvested by centrifugation, washed twice with 0.1 M potassium phosphate buffer, pH 7.0, and stored at −20 °C.

**Enzyme Assays and Definition of Units**—Brominating activity was measured by the method used by Heson and Hager (20) with monochlorodimedone (44 μM) as substrate in the presence of H₂O₂ (7.2 mM) and bromide (82 mM) and a suitable amount of enzyme in 0.1 M sodium acetate buffer, pH 5.5. The reaction was started by the addition of enzyme. The decrease in monochlorodimedone absorbance at 290 nm (ε = 1.99 × 10⁶ M⁻¹ cm⁻¹) with time was recorded on a

---

* This work was supported by the Bundesministerium für Forschung und Technologie under the contract BCT 383 and by the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

* The abbreviation used is: SDS, sodium dodecyl sulfate.
Chloroperoxidase from *P. pyrrocinia*

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein mg</th>
<th>Activity BPO*</th>
<th>Activity CPO</th>
<th>Specific activity BPO/CPO</th>
<th>Yield BPO/CPO %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>13,889</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DEAE-cellulose, pH 7.0</td>
<td>144.3</td>
<td>60.6</td>
<td>0.418</td>
<td>100</td>
<td>60/16</td>
</tr>
<tr>
<td>DEAE-Sephadex, pH 8.5</td>
<td>36</td>
<td>36.6/9.5</td>
<td>1.07/0.26</td>
<td>33/24</td>
<td>116</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>10.5</td>
<td>19.9/14.3</td>
<td>2.62/1.59</td>
<td>101/12</td>
<td></td>
</tr>
<tr>
<td>Bio-Gel HTP</td>
<td>1.7</td>
<td>5.8/7.3</td>
<td>3.24/4.23</td>
<td>101/12</td>
<td></td>
</tr>
</tbody>
</table>

* BPO, bromoperoxidase.
* CPO, chloroperoxidase.

Prepared from 250 g of cells (wet weight).

ND, not determined.

---

**FIG. 1.** Polyacrylamide gel electrophoresis of chloroperoxidase from *P. pyrrocinia* under nondenaturing (A) and denaturing conditions (B). Polyacrylamide gels (7.5%) were prepared by the method described by Fehrnstrom and Möberg (23). Each gel contained 15 μg of protein and was stained with Coomassie Blue R-250. SDS-polyacrylamide gels (10%) were prepared by the method described by Laemmli (24). The protein standards used were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

---

**FIG. 2.** Equilibrium ultracentrifugation of purified chloroperoxidase. Relative protein concentration was measured spectrophotometrically at 280 nm. The enzyme (0.3 mg in 500 μl of 5 mM potassium phosphate buffer, pH 7.0) was centrifuged to sedimentation equilibrium at 28,000 rpm. The distance from the axis of rotation (x) is expressed in centimeters.

---

Uvikon 810 spectrophotometer (Kontron). One unit (1 unit) of bromoperoxidase activity was defined as the formation of 1 μmol of monobromomonochlorodimedone/min. Catalase activity was measured at 240 nm, and peroxidase activity was measured with o-dianisidine at 25 °C. The detailed reaction conditions were described in the previous paper (11). Assay of the oxidation of iodide was carried out according to the method of Alexander (21).

4-(2-Amino-3-chlorophenyl)pyrrole was chlorinated in a 100-ml assay containing 4-(2-amino-3-chlorophenyl)pyrrole (50 μM), potassium chloride (8.2 mM), H₂O₂ (7.2 mM), and 100 milliunits of purified chloroperoxidase in 0.1 M sodium acetate buffer, pH 4.0. The reaction was started by the addition of H₂O₂. After incubation for 18 h at 30 °C, the reaction mixture was extracted twice with 40 ml of ethyl acetate.

Analytical Procedures—For gas chromatography-mass spectrometry a mass spectrometer Varian 3700 (Varian, Bremen, Germany) with a 25-m glass capillary column SE-30 was used. The temperature of the oven was run from 100 to 240 °C (10 °C/min); the temperatures of injector and detector were 250 and 300 °C, respectively.

Protein Determination—Protein concentrations were assayed by the method of Lowry et al. (22) with bovine serum albumin as standard.

Preparation of Crude Extracts—Crude extracts were prepared as described previously (16).

Purification of *P. pyrrocinia* Chloroperoxidase—All steps were performed at 4 °C. The crude extract was dialyzed against 10 mM potassium phosphate buffer, pH 7.0, for 18 h. The solution was passed onto a column (2.7 × 50 cm) of DEAE-cellulose DE52; equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The sample was washed onto the column with 1500 ml of this buffer and a 400-ml gradient (10-500 mM potassium phosphate buffer, pH 7.0) was applied. Fractions (3.2 ml) were assayed for protein (A₂₈₀) and brominating activity. Those fractions with an activity of more than 25% of the most active fraction were pooled and dialyzed against 10 mM potassium phosphate buffer, pH 8.5, for 18 h. The pooled material was applied to a DEAE-Sephadex A-25 column (2.5 × 24 cm), equilibrated with 10 mM potassium phosphate buffer, pH 8.5. The
unadsorbed fractions were collected and concentrated by ultrafiltration with a PM-30 membrane (Amicon) and then applied onto a Sephadex G-200 column (3.0 x 94 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.0. Fractions of 3.2 ml were collected and assayed as before. Fractions with bromination activity higher than 25% of the most active fraction were pooled and dialyzed against 5 mM potassium phosphate buffer, pH 7.0, for 18 h, before being adsorbed onto a column (2.5 x 24 cm) of Bio-Gel HTP hydroxyapatite equilibrated with 5 mM potassium phosphate buffer, pH 7.0. The unadsorbed fractions were collected and concentrated by ultrafiltration with an Amicon concentrator with a PM-30 membrane. Polyacrylamide Gel Electrophoresis—Analytical polyacrylamide gel electrophoresis under nondenaturing conditions at pH 8.9 was performed in horizontal 7.5% polyacrylamide gels with a LKB 2117 Multiphor apparatus (LKB Product AB, Bromma, Sweden) according to the method of Fehrnstrom and Moberg (23). SDS-gel electrophoresis was performed by the method of Laemmli (24) in a 10% gel, pH 8.3, containing 0.1% SDS. Prior to electrophoresis, samples and marker proteins were treated with 5% SDS and 5% mercaptoethanol at 100°C for 5 min. After electrophoresis the gel was stained for protein with Coomassie Brilliant Blue R-250. Peroxidase active bands in the gel were routinely detected by the o-dianisidine hydrogen peroxide staining technique developed by Shannon et al. (25).

Isoelectric Focusing—Analytical isoelectric focusing in thin-layer plates of 5% polyacrylamide, containing a 2.4% solution of ampholines in the pH range of 3.5-9.5, was carried out using a LKB Multiphor System according to the manufacturer’s instruction (26). Isoelectric focusing was carried out at 10°C until the voltage increased from 150 to 1000 V. Prior to staining, the gels were placed in a destaining solution of methanol/acetic acid/distilled water (3:1:6) for 15 min. Staining was performed in 0.2% Coomassie Brilliant Blue R-250. The pI of chloroperoxidase was determined by comparing the protein’s migration rate with those of standard proteins of known pIs (Pharmacia).

Molecular Weight Estimation and Analytical Ultracentrifugation—The molecular weight of chloroperoxidase was estimated by molecular sieve chromatography with a 3 x 94 cm column of Sephadex G-200, standardized with ferritin (Mr = 440,000), immunoglobulin (Mr = 150,000), bovine serum albumin (Mr = 67,000), and cytochrome c (Mr = 13,000). The column was equilibrated with 50 mM potassium phosphate buffer, pH 7.0.

In preparation for the ultracentrifugation run, purified chloroperoxidase was dialyzed against 2 liters of 50 mM potassium phosphate buffer, pH 7.0, for 20 h. The dialysis buffer was then used as the reference solution in the ultracentrifuge run. Equilibrium ultracentrifugation was performed in a Beckman model E ultracentrifuge. Centrifugation was carried out at 16.7 °C at a rotor velocity of 28,000 rpm. Relative protein concentrations were measured spectrophotometrically at 280 nm. Calculation was performed according to the method outlined by Yphantis (27). The value of 0.735 cm3/g was used for the partial specific volume. The molecular weight of chloroperoxidase subunits was obtained by SDS-polyacrylamide gel electrophoresis.

Determination of Metal Content—The metal determination was carried out with an energy-dispersive x-ray fluorescence apparatus, system 77 (Finnigan International Inc., San Jose, CA). Prior to determination, the purified chloroperoxidase was dialyzed against 5 mM ammonium acetate buffer, pH 6.8, for 20 h, which was used as a blank. The method used is suitable for the simultaneous detection of

![Graph](https://via.placeholder.com/150)

**Fig. 3. Absorption spectrum of non-heme chloroperoxidase from P. pyrocinia.** The spectrum was recorded in 5 mM potassium phosphate buffer, pH 7.0, and the concentration of the enzyme was 0.45 mg/ml.

### Table II

*Values obtained by extrapolating to time 0, assuming first order decay.*

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Number of residues/subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24-h hydrolysate</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>37.46</td>
</tr>
<tr>
<td>Threonine a</td>
<td>16.17</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>30.82</td>
</tr>
<tr>
<td>Glycine</td>
<td>31.55</td>
</tr>
<tr>
<td>Alanine</td>
<td>29.54</td>
</tr>
<tr>
<td>Valine</td>
<td>20.03</td>
</tr>
<tr>
<td>Methionine</td>
<td>5.48</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>7.81</td>
</tr>
<tr>
<td>Leucine</td>
<td>22.86</td>
</tr>
<tr>
<td>Lysine</td>
<td>15.36</td>
</tr>
<tr>
<td>Histidine</td>
<td>12.58</td>
</tr>
<tr>
<td>Arginine</td>
<td>13.10</td>
</tr>
<tr>
<td>Tryptophan b</td>
<td></td>
</tr>
<tr>
<td>Cysteine c</td>
<td></td>
</tr>
</tbody>
</table>

* a Values obtained by extrapolating to time 0, assuming first order decay.
* b Tryptophan was determined by the method described by Liu (31).
* c Cysteine was determined as cysteic acid according to the method described by Hirs (29).
the following metals: titanium, manganese, chromium, iron, vanadium, nickel, copper, zinc, lead, and selenium.

Absorption Spectra—Absorption spectra were measured in cuvettes of 1 cm path length with a Uvikon 810 spectrophotometer from Kontron (Eching, Germany).

Determination of Partial Amino Acid Sequence—The partial sequence of the N-terminal end was determined on an Applied Biosystem model 470 A gas-phase protein sequencer connected to a 120 A on-line high performance liquid chromatography.

Amino Acid Analysis—The determination of amino acids was carried out with a Biotronic LC 6000 amino acid analyzer. Samples were hydrolyzed in evacuated, sealed tubes in 6 N HCl at 110 °C for 24, 48, 72, and 96 h (28). Half-cysteine was determined as cysteic acid after hydrolysis with p-toluenesulfonic acid (31).

Isoelectric Point—The isoelectric point of the purified enzyme was determined to be 4.1 by analytical isoelectric focusing.

Absorption Spectrum—Fig. 3 shows the ultraviolet-visible absorption spectrum of purified chloroperoxidase. The enzyme did not exhibit any significant absorption bands corresponding to heme or flavin in the visible region of the spectrum. It only showed a protein absorbance at 278 nm; this indicates that this chloroperoxidase is a non-heme enzyme and is thus completely different from previously isolated bacterial haloperoxidases, which are all hemoproteins with the exception of bromoperoxidase from *S. aureofaciens* (15).

Determination of Metal—Since the visible spectrum of chloroperoxidase suggested that the enzyme is a non-heme protein, the content of metal was determined by x-ray fluorescence. Based on a molecular weight of 64,000, the content of zinc and iron was found to be 0.2 and 0.1 atoms/molecule of enzyme, respectively. The metals titanium, manganese, chromium, vanadium, nickel, copper, lead, and selenium were not present in the enzyme preparation.

Partial Amino Acid Sequence—The NH2-terminal amino acid sequence of the purified enzyme was NH2-X-Tyr-Val-Thr-Thr-Lys-Asp-Asn-Val-Glu-Ile-Phe-Tyr-Lys-Asp-Trp-Gly-Thr-His-Asp-Ala-Gln-Pro-Ile-. The first amino acid (X) could not be identified properly.

Amino Acid Composition—The results of the amino acid analysis are presented in Table I. The amino acid composition of the enzyme was characterized by the predominance of the acidic amino acids over the basic residues and a high histidine content. The enzyme contains 1 cystein residue/subunit. With Ellman’s reagent, no free thiols could be detected; thus we conclude that the subunits are connected via a disulfide bridge. The molecular weight for chloroperoxidase calculated from the amino acid composition data in Table II was 33,910, which is consistent with that (32,000) obtained by SDS-polyacrylamide gel electrophoresis.

Enzymatic Reactions and Kinetic Properties—The enzymatic reactions with F-, Cl-, and Br- were examined by the monochlorodimedone assay and that with I- by the formation of I2. The enzyme oxidized I- and catalyzed the bromination of monochlorodimedone, but did not catalyze the chlorination or fluorination of this substrate. The addition of F- to the monochlorodimedone assay at pH 5.5 resulted in partial inhibition, but Cl- had no effect on the bromination of this substrate. The purified chloroperoxidase had a specific activity of 4.2 units/mg for the bromination of monochlorodimedone and had no peroxidase or catalase activity.

### RESULTS

Purification of Chloroperoxidase—The results of the purification procedure are summarized in Table I. As this chloroperoxidase only catalyzes the bromination of monochlorodimedone but not its chlorination, the fractions were assayed for brominating activity throughout the purification. No brominating activity was detected in crude extracts. However, when the eluate of the DE52 column was assayed, brominating activity was found. The pooled fractions also had peroxidase and catalase activity, because the chloroperoxidase was eluted together with the bromoperoxidase (11). These two haloperoxidases could be separated on a DEAE-Sephadex A-25 ion-exchange column with potassium phosphate buffer, pH 8.5.

The purified chloroperoxidase gave a single band (RF = 0.3) on polyacrylamide gel electrophoresis (Fig. 1A). When stained for peroxidase activity with o-dianisidine, no activity could be detected.

Molecular Weight and Subunit Structure—Chloroperoxidase was homogeneous in centrifugal fields, generated at 28,000 rpm. The Yphantis plot is shown in Fig. 2. The molecular weight was estimated to be 64,000 from the slope and using 0.735 cm3/g as the partial specific volume (v). The molecular weight obtained from gel chromatography on Sephadex G-200 was 62,000. The subunit weight of chloroperoxidase was estimated to be 32,000 from SDS-polyacrylamide gel electrophoresis (Fig. 1B). Chloroperoxidase was thus revealed as a dimer, whose subunit weight is 32,000 and whose aggregate weight is 64,000 ± 3,000.

Isoelectric Point—The isoelectric point of the purified enzyme was determined to be 4.1 by analytical isoelectric focusing.

Absorption Spectrum—Fig. 3 shows the ultraviolet-visible absorption spectrum of purified chloroperoxidase. The enzyme did not exhibit any significant absorption bands corresponding to heme or flavin in the visible region of the spectrum. It only showed a protein absorbance at 278 nm; this indicates that this chloroperoxidase is a non-heme enzyme and is thus completely different from previously isolated bacterial haloperoxidases, which are all hemoproteins with the exception of bromoperoxidase from *S. aureofaciens* (15).

Determination of Metal—Since the visible spectrum of chloroperoxidase suggested that the enzyme is a non-heme protein, the content of metal was determined by x-ray fluorescence. Based on a molecular weight of 64,000, the content of zinc and iron was found to be 0.2 and 0.1 atoms/molecule of enzyme, respectively. The metals titanium, manganese, chromium, vanadium, nickel, copper, lead, and selenium were not present in the enzyme preparation.

Partial Amino Acid Sequence—The NH2-terminal amino acid sequence of the purified enzyme was NH2-X-Tyr-Val-Thr-Thr-Lys-Asp-Asn-Val-Glu-Ile-Phe-Tyr-Lys-Asp-Trp-Gly-Thr-His-Asp-Ala-Gln-Pro-Ile-. The first amino acid (X) could not be identified properly.

Amino Acid Composition—The results of the amino acid analysis are presented in Table II. The amino acid composition of the enzyme was characterized by the predominance of the acidic amino acids over the basic residues and a high histidine content. The enzyme contains 1 cystein residue/subunit. With Ellman’s reagent, no free thiols could be detected; thus we conclude that the subunits are connected via a disulfide bridge. The molecular weight for chloroperoxidase calculated from the amino acid composition data in Table II was 33,910, which is consistent with that (32,000) obtained by SDS-polyacrylamide gel electrophoresis.

Enzymatic Reactions and Kinetic Properties—The enzymatic reactions with F-, Cl-, and Br- were examined by the monochlorodimedone assay and that with I- by the formation of I2. The enzyme oxidized I- and catalyzed the bromination of monochlorodimedone, but did not catalyze the chlorination or fluorination of this substrate. The addition of F- to the monochlorodimedone assay at pH 5.5 resulted in partial inhibition, but Cl- had no effect on the bromination of this substrate. The purified chloroperoxidase had a specific activity of 4.2 units/mg for the bromination of monochlorodimedone and had no peroxidase or catalase activity.
Chloroperoxidase from P. pyrrocinia

Fig. 5. A, the pH dependence of the bromination reaction as a function of H$_2$O$_2$ concentration. The reaction was started by the addition of H$_2$O$_2$ to the sample which contained 0.1 M sodium acetate buffer, pH 3.0-5.5, or 0.1 M ammonium acetate buffer, pH 5.5-7.0, 44 μM monochlorodimedone and chloroperoxidase. The NaBr concentration was 82 mM. [H$_2$O$_2$] (mM): X—•—•—•—•, 5; ○—○—○, 7.2; △—△—△—△, 10; ▲—▲, 25; □—□—□—□, 50. B, the pH dependence of the bromination reaction as a function of the bromide concentration. The H$_2$O$_2$ concentration was 7.2 mM. [NaBr] (mM): X—•—•—•—•, 10; ○—○—○, 25; ■—■—■—■, 82; △—△—△—△, 100; ▲—▲—▲—▲, 200; □—□—□—□, 500.

Fig. 6. Effect of temperature on the bromoperoxidase activity. Chloroperoxidase was incubated in 10 mM ammonium acetate buffer, pH 7.0, at 60 (○—○—○), 65 (●—●—●), or 70 °C (□—□—□). At variable time intervals, samples were taken and assayed for bromoperoxidase activity. Assay conditions were described under "Experimental Procedures."

Fig. 7. Chlorination of 4-(2-amino-3-chlorophenyl)pyrrole by chloroperoxidase (CPO).

The apparent $K_m$ values for bromide ions and hydrogen peroxide were calculated from Lineweaver-Burk plots. The $K_m$ value for bromide was 3.0 × 10$^{-2}$ M (Fig. 4A). The apparent $K_m$ value for hydrogen peroxide was 4.7 × 10$^{-3}$ M. An inhibition by hydrogen peroxide was observed at concentrations higher than 50 mM (Fig. 4B).

The rate of the bromination reaction of monochlorodimedone done by chloroperoxidase showed optima between pH 4.0 and 4.5, the positions of which were determined using different concentrations of Br$^-$ and H$_2$O$_2$. Fig. 5 illustrates that the enzyme had distinct activity optima, which, at a fixed concentration of Br$^-$ (Fig. 5A), shifted to higher pH values, when the concentration of H$_2$O$_2$ was increased. When the concentration of Br$^-$ was increased and that of H$_2$O$_2$ was kept at a fixed concentration, the optima also shifted to higher pH values (Fig. 5B). It can be seen from Fig. 5A that concentrations of H$_2$O$_2$ up to 50 mM did not inhibit the enzyme. Fig. 5B shows that high concentrations of Br$^-$ (above 100 mM) at pH 3.5 inhibited the enzyme completely.

Effect of Various Compounds—The effect of various compounds on the bromination reaction was examined by adding each compound to the reaction mixture. The metal ions, MnSO$_4$, ZnSO$_4$, NiSO$_4$, CuSO$_4$, FeSO$_4$, and NH$_4$VO$_3$, tested at a concentration of 10 mM, showed no influence on the enzyme activity. The enzyme was inhibited to 50% by potassium cyanide (10 μM) and fluoride (0.18 mM). However, the enzyme regained its full activity when it was preincubated in the presence of cyanide and H$_2$O$_2$ for some time. The length of time necessary for preincubation to regain full activity depended on the cyanide concentration and the amount of enzyme used. Sodium azide, EDTA (5 and 10 mM final concentration) and Cl$^-$ did not affect the enzyme activity.

Effect of Temperature and Stability—The brominating activity of chloroperoxidase in the monochlorodimedone assay was measured at various temperatures from 15 to 75 °C. Maximum activity was measured at approximately 60 °C. When the enzyme was incubated in 10 mM ammonium acetate buffer, pH 6.8, at various temperatures for variable time intervals (5 min–2 h), chloroperoxidase proved to be thermostable at 60°C for 2 h. When the enzyme was incubated at 70 °C for 10 min, only 50% of its activity remained (Fig. 6). The enzyme could also be stored in 0.1 M sodium acetate buffer, pH 6.8, at -70°C for at least 1 month.
buffer, pH 3.5–5.5, 0.1 M ammonium acetate buffer, pH 6.0–7.0, 0.1 M potassium phosphate buffer, pH 7.0–8.5, at 4 °C or in 0.1 M ammonium acetate buffer, pH 6.8, with 5 mM sodium azide at room temperature for over 2 months without any loss of activity.

Chlorination of 4-(2-Amino-3-chlorophenyl)pyrrole—The chlorination of 4-(2-amino-3-chlorophenyl)pyrrole is illustrated in Figs. 7 and 8. The elemental compositions of the compounds obtained were established by gas chromatography-mass spectrometry. The molecular ion of the major product (Fig. 8B) appeared as a triplet at m/e 226/228/230 (intensity ratio, 9:6:1). This is characteristic for a dichloro-substituted compound. These spectral data and the retention time were identical with those from an authentic sample of aminopyrrolnitrin, prepared from pyrrolnitrin by reduction with sodium dithionite (18). The molecular ion of the second product (Fig. 8C) appeared as a triplet at m/e 270/272/274 (intensity ratio, 3:4:1). This is characteristic for a monobromomonochloro-substituted compound.

**DISCUSSION**

*P. pyrocinia* produces two halogenating enzymes (16). One of these was identified as a heme containing bromoperoxidase (11). The second haloperoxidase was not inhibited by NaN₃ and was able to catalyze the chlorination of indole to 7-chloroindole (16). As this enzyme did not catalyze the chlorination of monochlorodimedone, it was isolated as a bromoperoxidase. The two brominating activities could be separated using DEAE-Sephadex A-25. The chloroperoxidase has now
been successfully purified to homogeneity by ion-exchange chromatography, molecular sieve chromatography, and adsorption chromatography.

The molecular weight of the purified enzyme was approximately 64,000 with a subunit weight of 32,000. Thus, chloroperoxidase is a dimer as are the previously isolated bacterial bromoperoxidases, which, however, are much larger, with subunit weights of 72,500–77,000 (9–11). The subunits of this chloroperoxidase are probably connected via a disulfide bridge.

However, unlike these prokaryotic bromoperoxidases, chloroperoxidase has no absorption in the Soret region of the spectrum and is not inhibited by sodium azide. Thus, chloroperoxidase is a non-heme enzyme, whereas the bacterial bromoperoxidases are heme enzymes with the exception of the bromoperoxidase from *S. aureofaciens* (15).

The non-heme algal bromoperoxidase from *A. nodosum* contains 0.4 atoms of vanadium/molecule of enzyme (13). The non-heme bromoperoxidase from the alga *C. pilulifera* contains 2.3 atoms of iron plus 1.6 atoms of magnesium (12), and the non-heme fungal chloroperoxidase from *C. inaequalis* contains 0.7 atoms of iron plus 2.2 atoms of zinc/molecule of enzyme (14). The chloroperoxidase from *P. pyrrocinia* described here, however, was deficient in metal ions. It contained only 0.2 atoms of zinc and 0.1 atoms of iron/molecule of protein. Vanadium could not be detected in the enzyme. The bromoperoxidase from *A. nodosum* could be inactivated by EDTA at low pH and reactivated by the addition of vanadium.

Such an inactivation could not be achieved with chloroperoxidase, and the addition of several metal ions had no effect on the enzyme activity. Similar results were obtained for the bromoperoxidase from *S. aureofaciens*, which is the only other bacterial non-heme haloperoxidase isolated until now (15). The lack of heme and metal raises the question how the enzyme catalyzes the halogenation reactions. However, as we could not inactivate the enzyme reversibly, this question cannot be answered clearly. It could be that the enzyme has lost most of its iron or zinc during purification, but then we would expect to get higher activity after addition of these metals.

The predominance of glutamic acid and aspartic acid over basic residues in the amino acid composition of the enzyme was similar to that found in other haloperoxidases (1, 8, 12), however, this predominance is not as great as in the bromoperoxidase from *C. pilulifera* so that it does have a higher isoelectric point (pH 4.1) than does the *C. pilulifera* enzyme (12).

Chloroperoxidase showed an apparent *Km* value of $3 \times 10^{-2}$ M for bromide ions. This value was similar to that of bromoperoxidase from *C. pilulifera*, which is $1.1 \times 10^{-2}$ M (12), bromoperoxidase from *S. aureofaciens* ($5.9 \times 10^{-2}$ M) (15), and of chloroperoxidase from *C. fumago* for chloride ions (2.8 $\times 10^{-2}$ M) (2). However, the *Km* value for H$_2$O$_2$ (4.7 $\times 10^{-2}$ M) was much higher than that of the *C. pilulifera* enzyme (9.2 $\times 10^{-2}$ M) (12) and of chloroperoxidase from *C. fumago* (7.9 $\times 10^{-2}$ M) (2), however, similar to that of *S. aureofaciens* bromoperoxidase (3.1 $\times 10^{-2}$ M) (15). Whereas the algal heme-containing bromoperoxidases were inhibited by hydrogen peroxide at low concentrations, i.e. above 1 mM for the *Penicillium phoeniix* enzyme and in excess of 2 mM for the *P. capitatus* bromoperoxidase (32), the algal non-heme-type bromoperoxidase of *C. pilulifera* was resistant to concentrations of H$_2$O$_2$ up to 8 mM, whereas the chloroperoxidase from *P. pyrrocinia* was not inhibited by H$_2$O$_2$ at concentrations below 50 mM.

In addition, chloroperoxidase was thermostable at 60 °C for 2 h and could be stored at room temperature for over 2 months, in the presence of 5 mM sodium azide, without any loss of activity.

The two fungal chloroperoxidases from *C. fumago* and *C. inaequalis* both catalyze the chlorination of monochloroaromatics, whereas the chloroperoxidase from *P. pyrrocinia* did not catalyze the chlorination, but only the bromination of this substrate. However, the *P. pyrrocinia* chloroperoxidase catalyzes the chlorination of indole to 7-chloroindole (16), whereas the *C. fumago* enzyme is able to catalyze this reaction but produces oxindole when incubated with KCl, H$_2$O$_2$, and indole (33). Thus, the *P. pyrrocinia* enzyme seems to have a much higher substrate specificity than the other chloroperoxidases with respect to the chlorination reaction, but not with respect to bromination. *P. pyrrocinia* chloroperoxidase also catalyzes the chlorination of 4-(2-amino-3-chlorophenyl)pyrrole to aminopyrrolnitrin. The detected monobromo-4-(2-amino-3-chlorophenyl)pyrrole is probably due to very small contaminations of bromide, present in the potassium chloride, and a very high affinity of chloroperoxidase to bromide ions. The isolation of enzymatically formed aminopyrrolnitrin from 4-(2-amino-3-chlorophenyl)pyrrole and 7-chloroindole from indole suggests very strongly that the isolated chloroperoxidase is involved in the biosynthesis of the antibiotic pyrrolnitrin and the 7-chloroindole derivatives, isolated from *P. pyrrocinia* (19).

This was the first time that a chlorinating enzyme could be isolated from procaryotic sources and could be demonstrated as for a bacterial haloperoxidase that is involved in the biosynthesis of an antibiotic. Until now, this was only possible for the eucaryotic chloroperoxidase from *C. fumago*, which is involved in the biosynthesis of caldariomycin (34).

Acknowledgments—We are grateful to H. Sobek for help in determining the molecular weight of chloroperoxidase by sedimentation equilibrium and Dr. K. D. Jany, Institut für Biochemie, Universität Stuttgart, for help with the amino acid analysis. We wish to thank Dr. H. Schreiber, Institut für Physik, Universität Hohenheim, for determining the molecular weight of chloroperoxidase by sedimentation and Dr. K.-H. van Pie, Institut für Biochemie, Universität Stuttgart, for amino acid analysis. We are grateful to H. Sobek for help in determining the molecular weight of chloroperoxidase by sedimentation equilibrium and Dr. K. D. Jany, Institut für Biochemie, Universität Stuttgart, for help with the amino acid analysis. We wish to thank Dr. H. Schreiber, Institut für Physik, Universität Hohenheim, for determining the molecular weight of chloroperoxidase by sedimentation and Dr. K.-H. van Pie, Institut für Biochemie, Universität Stuttgart, for amino acid analysis. We are grateful to H. Sobek for help in determining the molecular weight of chloroperoxidase by sedimentation equilibrium and Dr. K. D. Jany, Institut für Biochemie, Universität Stuttgart, for help with the amino acid analysis. We wish to thank Dr. H. Schreiber, Institut für Physik, Universität Hohenheim, for determining the molecular weight of chloroperoxidase by sedimentation and Dr. K.-H. van Pie, Institut für Biochemie, Universität Stuttgart, for amino acid analysis.
Chloroperoxidase from *P. pyrrocinia*