Isolation and Properties of Cytochrome *c* Peroxidase from *Pseudomonas denitrificans*

By Andrew F. W. COULSON and Robin I. C. OLIVER

Department of Molecular Biology, University of Edinburgh, King's Buildings, Mayfield Road, Edinburgh EH9 3JR, Scotland, U.K.

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The isolation of cytochrome *c* peroxidase, cytochrome *c*₄, cytochrome *c*-551 and azurin from *Pseudomonas denitrificans* is described. The peroxidase has a molecular weight of 63 000 and an isoelectric point of 5.6. Its absorption spectrum suggests that it contains two haem *c* groups/molecule. Preliminary steady-state kinetic data are reported with cytochromes *c*-551 and *c*₄ and azurin as the second substrate.

The haem-containing peroxidases are of two types. The best known are those containing protohaem, such as catalase, the plant peroxidases, yeast cytochrome *c* peroxidase etc. (Yonetani, 1976). The mechanisms of these enzymes all involve the intermediacy of relatively stable higher-oxidation states of the haem and, perhaps, of its surrounding protein. Cytochrome *c* peroxidases (cytochrome *c*-H₂O₂ oxidoreductase, EC 1.11.1.5) have been reported to occur in a variety of bacterial species (Lenhoff & Kaplan, 1956; Yamanaka & Okunuki, 1970), and one of these (from *Pseudomonas aeruginosa*) has been purified and characterized by Soininen *et al.* (1973, and earlier papers in the same series). This enzyme contains haem *c*, and these authors did not detect any similarity in mechanism to the haem *b* peroxidases.

We wished to establish whether these haem *c*-containing peroxidases represent a new mechanistic class, but could not isolate the enzyme in high yield from the organism used by Ellfolk, Soininen and co-workers (apparently because of proteolytic degradation of the enzyme during the preparation). For this reason, and also to see how widely distributed the enzyme might be, we have sought an alternative source of the enzyme.

We now report the isolation and characterization of cytochrome *c* peroxidase from *Pseudomonas denitrificans*, and the isolation of three potential substrates of the enzyme (two cytochromes and an azurin) from the same source.

Experimental

Instruments

The spectrophotometers used were a Unicam model SP.1800 instrument fitted with a model AR25 pen recorder, and a Unicam model SP.800 instrument fitted with a home-built scale expander (maxi-

mum full-scale sensitivity 0.1 *A* units; back-off 0–2.0 *A* units) and Perkin–Elmer 2.5 mV pen recorder. The pH-meter was a Corning EEL model 100 instrument that was calibrated each day with pH 4.00 and pH 7.00 buffers.

Buffers

Ammonium acetate buffers were prepared by adjusting the pH of a solution of acetic acid of the stated molarity to the stated pH with 2M-NH₃ solution. Glycine buffers contained the stated molarity of glycine, and were adjusted in pH by addition of strong acid or alkali. Phosphate buffers were made by mixing solutions of Na₂HPO₄ and NaH₂PO₄, each of the stated molarity, to give the stated pH.

Chromatography media

CM-cellulose and DEAE-cellulose of the stated type number were made by Whatman Biochemicals (Maidstone, Kent, U.K.). Sephadex and agarose (Sephacrose) gel were obtained from Pharmacia (Uppsala, Sweden).

Gel electrophoresis

Polyacrylamide-gel electrophoresis, with glycine or β -alanine buffers, was carried out by the procedures described by Brewer & Ashworth (1969), and SDS/polyacrylamide-gel disc electrophoresis by the method of King & Laemmli (1971). Iron-containing proteins were identified on gels by staining with anisidine/H₂O₂.

Ultrafiltration

Millipore apparatus of appropriate volume (142 mm, 90 mm or 25 mm ultrafiltration cells) was used with membrane types PSAC (for cytochromes and azurin) or PSED (for cytochrome *c* peroxidase).

Abbreviation used: SDS, sodium dodecyl sulphate.

Organisms and culture conditions

Pseudomonas denitrificans N.C.I.B. 9496 was obtained as a freeze-dried culture from the Torrey Research Station. *Pseudomonas aeruginosa* N.C.T.C. 10332 was obtained as a freeze-dried culture from the Central Public Health Laboratory. Both strains were maintained on nutrient agar plates. The organisms were grown in batches on the nitrate medium described by Lenhoff & Kaplan (1956). Single colonies were used to inoculate 10 ml of sterile medium, and cultures of successively 100 ml, 500 ml and 9 × 500 ml were prepared from this with 24 h growth at 37°C between inoculations. Three milk churns, each containing 40 litres of medium prepared by mixing 10-fold-concentrated medium with autoclaved tap-water, were inoculated with 3 × 500 ml cultures and incubated at 37°C for 24 h or less, unstirred and without aeration. Large-scale cultures were also grown in a 300-litre fermenter operated in this department by Dr. S. G. Hughes and Mr. T. Bruce. Cells were harvested with a Sharples continuous centrifuge, and were either acetone-dried immediately or stored frozen at -20°C.

Acetone drying

Fresh or freshly thawed cell paste was blended in 700 ml batches (in an explosion-proof Waring Blendor) with acetone chilled to -20°C, with 2.5 litres of acetone to 100 ml of cell paste. The cell powder was filtered off under water-pump vacuum and dried in a desiccator continuously evacuated with a water pump until the smell of acetone was not detectable.

Extraction of cell powder

The acetone-dried powder, in 70 g batches, was blended in a Waring Blendor with 700 ml of 0.1 M-ammonium acetate buffer, pH 7, at 45°C. The mixture was incubated for 10 min at 45°C, blended again, cooled to about 35°C, mixed with 1 mg of bovine pancreatic ribonuclease (Miles-Seravac, Maidenhead, Berks., U.K.), cooled in ice and centrifuged at 12000g for 1 h.

Assay procedures

Protein concentrations were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Substrates

The reducing substrate used for assay of the peroxidase was *Ps. aeruginosa* cytochrome *c*-551, isolated (together with azurin) by a method similar

to that developed by Ambler (1973). The pH of the centrifuged extract of cells in ammonium acetate buffer was adjusted to 3.9 by the cautious addition of 50% (v/v) acetic acid with continuous stirring and cooling in ice. The precipitate was removed by centrifuging at 23000g for 30 min. The supernatant was diluted 10-fold with distilled water, and cytochromes and azurins were removed from it by allowing it to pass rapidly, under gravity, through a short wide column (e.g. 2 cm × 5 cm for the extract from 70 g of cell powder) of CM-23 CM-cellulose, equilibrated with 0.05 M-ammonium acetate buffer, pH 3.9. The mixed coloured proteins were eluted from the column with 0.05 M-ammonium acetate buffer, pH 7. The pH of the eluate was adjusted to pH 3.9 with acetic acid, and then diluted with distilled water until its conductivity was no more than that of 0.05 M-ammonium acetate buffer, pH 3.9. This material was passed through a CM-52 CM-cellulose column 10–15 cm long and of such a diameter that the cytochrome bound in a layer 1–2 cm deep at the top of the column. The pH of the eluting buffer (0.05 M-ammonium acetate) was raised in small steps until the cytochrome *c*-551 and azurin separated and moved down the column at a suitable rate.

Cytochrome *c*-551

Fractions containing cytochrome *c*-551 were collected and pooled. The cytochrome was concentrated by adjusting the pH and conductivity of the pooled material to match those of 0.05 M-ammonium acetate buffer, pH 3.9, binding the cytochrome to the smallest possible column of CM-52 CM-cellulose equilibrated with the same buffer, and eluting with 0.05 M-ammonium acetate buffer, pH 7. The purity of the cytochrome was assessed by measuring the ratio of the absorbance at 551 nm in a dithionite-reduced sample to that at 280 nm in the untreated sample. CM-cellulose chromatography was repeated, if necessary, until this ratio was greater than 1. The material was shown to be homogeneous on SDS/polyacrylamide disc gels at pH 8.9 and on β -alanine gels at pH 4.

Azurin

The pooled azurin-containing fractions were concentrated by ultrafiltration and $(\text{NH}_4)_2\text{SO}_4$ was added to 75% saturation. Any precipitate at this stage was removed by centrifugation, and the solution was saturated with $(\text{NH}_4)_2\text{SO}_4$. Under these conditions pure azurin is completely but slowly precipitated.

Reduced samples of the cytochrome or azurin were prepared by treating small volumes of the concentrated material with a few crystals of $\text{Na}_2\text{S}_2\text{O}_4$, and removing excess reducing agent by passage

through a small Sephadex G-25 column (1 cm × 10 cm), eluted with 0.1 M-phosphate buffer, pH 6.5.

Peroxide solutions were made by dilution from 30% (v/v) H₂O₂ (Superoxol; from British Drug Houses, Poole, Dorset, U.K.). The concentration of the stock solution was measured by titration against KMnO₄.

Assay procedure

The concentration of the stock solution of reduced *Ps. aeruginosa* cytochrome *c*-551 was measured by diluting a suitable volume (e.g. 50 μl) with 1 ml of 0.1 M-phosphate buffer, pH 6.5, and measuring the absorbance at the 551 nm peak before and after complete reduction with Na₂S₂O₄ and complete oxidation with K₃Fe(CN)₆. These absorbances were used to calculate the percentage of the protein, in the stock solution, that was in the reduced form. The total concentration of cytochrome was calculated from the absorbance of the fully reduced solution and from the change in absorbance on complete oxidation, by using absorption coefficients of 26.9 and 7.9 mm⁻¹·cm⁻¹ for the reduced and oxidized forms respectively. The absorption coefficients were measured by titration of reduced cytochrome against H₂O₂ in the presence of yeast cytochrome *c* peroxidase.

A 2.5 ml volume of 0.1 M-phosphate buffer, pH 6.5, was placed in a 1 cm-path-length cuvette, and enough of the stock cytochrome solution (e.g. 50 μl) to give a final concentration of reduced cytochrome of 10 μM was added and thoroughly mixed. Then 10 μl of 10 mM-H₂O₂ was added (experiment showed that at this concentration the reaction rate is not sensitive to H₂O₂ concentration), and the rate of oxidation was observed at 551 nm to allow correction for the catalysed rate, and to check that this basal oxidation rate (which is very low for pure and fresh samples of cytochrome) was not excessive. The reaction was initiated by addition of 10 μl of a suitable dilution of the enzyme solution, and the initial rate of oxidation was measured. The enzymic activity was conveniently expressed in units of absorbance change/min. All assays were carried out at room temperature. It was found that the activity measured in this way was proportional to the enzyme concentration. The possibility of interference by cytochrome oxidase was checked by carrying out the assay with omission of H₂O₂, and no such activity was observed at this pH.

Steady-state kinetics

Observations of the steady-state rate of oxidation of reduced cytochrome and azurin by H₂O₂ in the presence of cytochrome *c* peroxidase were made by variations of the procedure used for the enzyme assay. All reactions were carried out at room temperature in 0.1 M-phosphate buffer, pH 6.5.

Results and Discussion

Release of cytochrome c peroxidase from cells of Ps. denitrificans

No attempt was made to measure the activity of the enzyme in intact cells. Cytochrome *c* peroxidase, cytochrome and azurin were all released by buffer extraction of acetone-dried powder, by using the same procedure as that described for the extraction of cytochromes and azurin from *Ps. aeruginosa*. Extraction of the enzyme by disruption of cells in a French press [in 0.1 M-phosphate buffer, pH 6.5, at 0°C under 90 MPa (6 tons) pressure] or by ultrasonic disintegration (30 ml of cell paste diluted to 50 ml with 0.1 M-ammonium acetate buffer, pH 6.5, subjected to 10 min treatment with a Dawe type 7530A sonicator in 30 s bursts with cooling in ice) gave in each case about 25% higher yield of enzyme. However, preparation of acetone-dried powders was used in all subsequent experiments, since it is convenient for large-scale use and gives a good yield of cytochromes and azurin.

Characterization of cytochrome c peroxidase in crude extracts

When the aqueous extract (cooled in ice) was acidified with acetic acid, precipitation of protein began at about pH 5. Enzyme activity could not be detected in the supernatant after centrifugation of cell extracts whose pH had been adjusted in this way to 3.9. More than 90% of the enzyme activity was recovered by redissolving the precipitate in neutral buffer. The enzyme was partly characterized in a concentrated solution made by redissolving the precipitate in the minimum volume of pH 7 buffer.

Isoelectric point. Isoelectric focusing was carried out in an LKB apparatus of total volume 110 ml, with Ampholine of pH range 3–10, in accordance with the manufacturer's directions (LKB Instruments, Croydon, Surrey, U.K.). When the current had ceased to fall and the coloured protein bands were sharp, there were several broad irregular bands of colourless precipitated protein near the bottom (acid end) of the column and two faint transparent red bands nearer the middle. The column was drained into 60 equal fractions of volume about 2 ml each and the pH and enzymic activity were measured for each fraction. The u.v.-absorbance maxima for the coloured components were at 410 nm, and the absorbance at this wavelength was measured for each fraction. The results are shown in Fig. 1, which demonstrates that the enzymic activity and the two haem-containing proteins coincide at positions corresponding to isoelectric points 5.1 and 5.3. Apparently the less-abundant component is more active, at least after the electrofocusing procedure.

Fig. 1. Isoelectric focusing of crude *Ps. denitrificans* cytochrome *c* peroxidase pH (○), enzyme activity (●) and A_{410} (▲) of fractions from an isoelectric-focusing experiment carried out as described in the text.

Molecular weight. It was important to establish that the enzyme was soluble before attempts were made to isolate it. Accordingly a sample of the acid precipitate, redissolved as in step 1 of the isolation procedure described below, was chromatographed on a column (0.8 cm × 35 cm) of Sephadex G-200 eluted with 0.1 M-phosphate buffer, pH 7. The enzyme activity was measured in successive 0.25 ml fractions, and reached its peak at fraction 50. Comparison of the elution behaviour with that of Blue Dextran and mammalian cytochrome *c* allowed the molecular weight to be estimated as 100 000 (Andrews, 1970).

Isolation of cytochrome c peroxidase: typical complete procedure

Step 1: extraction of cells. Fresh or freshly thawed cell paste of *Ps. denitrificans* was acetone-dried as described for *Ps. aeruginosa*, with 25 ml of acetone/ml of cell paste. The acetone-dried powder was blended in a Waring Blendor of 700 ml capacity at 45°C with 0.1 M-ammonium acetate buffer, pH 7, with 10 ml of buffer/g of powder. The mixture was incubated for 10 min at 45°C, blended again, cooled to about 35°C, mixed with 1 mg of bovine pancreatic ribonuclease, cooled in ice and centrifuged at 12 000g for 1 h. The pH of the supernatant was adjusted to 3.9 by the cautious addition of 50% (v/v) acetic acid with continuous stirring and cooling in ice. The precipitate was removed by centrifuging at 23 000g for 30 min. The supernatant was reserved for the extraction of

cytochromes and azurin. The precipitate was redissolved by stirring it overnight at 4°C in a small volume of 0.1 M-phosphate buffer, pH 7 (the pH being re-adjusted to 7 by addition of base as necessary).

Step 2: chromatography on Sephadex G-75. The crude mixture was chromatographed on a column (7 cm × 80 cm) of Sephadex G-75 eluted with 0.1 M-phosphate buffer, pH 6.5. Fractions of volume 10 ml were collected and assayed, and those containing the enzyme were pooled and dialysed against distilled water in the cold. The dialysis was continued until the conductivity was no greater than that of 10 mM-glycine buffer, pH 8.

Step 3: column chromatography on DE-11 DEAE-cellulose. The dialysis residue was passed under gravity through a column (2.5 cm × 15 cm) of DE-11 DEAE-cellulose equilibrated with 0.01 M-glycine buffer, pH 8. The eluate was assayed; all the enzyme was bound to the column. The enzyme was eluted with the same buffer made 0.1 M in $(\text{NH}_4)_2\text{SO}_4$; 10 ml fractions were collected and assayed, and those containing the enzyme were pooled and dialysed against distilled water at 0°C.

Step 4: chromatography on DE-52 DEAE-cellulose. The dialysed material was passed through a column (2.5 cm × 12 cm) of DE-52 DEAE-cellulose equilibrated with 0.01 M-glycine buffer, pH 8.0. The enzyme bound as a narrow brown band at the top of the column. It was eluted with a linear gradient of $(\text{NH}_4)_2\text{SO}_4$ (0–0.1 M in the same buffer). The total volume of the eluate was 500 ml. Fractions of volume 7 ml were collected. Fig. 2 shows the results of

Fig. 2. *Chromatography of Ps. denitrificans cytochrome c peroxidase on DE-52 DEAE-cellulose*
Enzyme from step 3 of the purification procedure (see the text) was chromatographed on a DE-52 DEAE-cellulose column (2.5 cm $\times$ 12 cm) equilibrated with 0.01 M-glycine buffer, pH 8.0. Elution was with a linear gradient (0–0.1 M; total volume 500 ml) of $(\text{NH}_4)_2\text{SO}_4$ in the same buffer. A_{280} ($\circ$), A_{410} ($\bullet$) and enzyme activity ($\blacktriangle$) were measured. The diagonal line indicates the measured conductivity of the effluent from the column.

measurements of absorbance and enzyme activity on the fractions. Enzyme-containing fractions were pooled and concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation [the enzyme is precipitated in 50–75% saturated $(\text{NH}_4)_2\text{SO}_4$, and, as judged by gel electrophoresis, the principal remaining impurity (see below) is not fully precipitated under these conditions], by ultrafiltration or by binding the protein to the minimum volume of DE-52 DEAE-cellulose and eluting it with 1 M-phosphate buffer, pH 7.

Step 5: chromatography on Sephadex G-100. Samples of the concentrated enzyme solution of 1–2 ml volume were chromatographed on a pair of Sephadex G-100 columns of diameter 1.5 cm and total length 190 cm. The columns were eluted with 0.1 M-phosphate buffer, pH 6.5. A typical elution curve is shown as Fig. 3. Evidence described below shows that the fractions with the highest activity are essentially homogeneous.

Table 1 summarizes the course of a purification carried out by this procedure.

Experiments related to isolation

DEAE-cellulose chromatography. Isoelectric focusing had shown that the enzyme has an acidic isoelectric point, but a large number of experiments were required before conditions were found in which the enzyme could be chromatographed on anion-exchangers. The enzyme from the redissolved and

dialysed acid precipitate was not adsorbed on columns of DEAE-cellulose at pH 7 or 8 or QAEA (quaternary aminoethylamino)-Sephadex at pH 9 or 10 equilibrated with 10 mM-phosphate solution. Equilibration of these resins with 1 mM buffers was found to be impracticable.

Three conditions were finally found to be necessary for effective chromatography. The first is the Sephadex G-75 gel-filtration step, which probably removed competing anions of moderate molecular weight; the second is the use of glycine buffers (presumably zwitterionic glycine is a very poor competitor for anion-binding sites on the resin) and the third is the binding to and release from DEAE-cellulose in a batch process. At this step the enzyme binds in a very broad band, presumably because tightly bound competing proteins occupy most of the binding sites. The eluted enzyme binds as a narrow band to the DE-52 DEAE-cellulose column, and can then be successfully chromatographed.

CM-cellulose chromatography. Initially, conditions were not found in which the enzyme would bind to CM-cellulose. After it was found that the enzyme after step 4 of the preparation contained a single intractable impurity, the device of using zwitterionic buffers was attempted with CM-52 CM-cellulose, with 10 mM-glycine buffer, pH 6.0. Fig. 4 shows the course of a chromatographic experiment under these conditions. The enzyme is clearly separating from its principal impurity, even on a short column, but a

Fig. 3. Chromatography of *Ps. denitrificans* cytochrome *c* peroxidase on Sephadex G-100

Enzyme from step 4 of the purification procedure (see the text) was chromatographed on a pair of Sephadex G-100 columns (1.5 cm $\times$ 190 cm total length). Elution was with 0.1 M-phosphate buffer, pH 6.5. A_{280} (○), A_{410} (●) and enzyme activity (△) were measured.

large fraction of the enzyme remained irreversibly bound to the column (and was visible as a brown band).

'Hydrophobic' chromatography. Hydrophobic adsorbents (Shaltiel & Er-El, 1973) were prepared by reaction of aliphatic amines, $H_2N-[CH_2]_n-NH_2$, with CNBr-activated agarose gel, and equilibrated with 0.05 M-phosphate buffer, pH 6.5. Samples of the partially purified enzyme (after the Sephadex G-75 step of the standard preparation) were dialysed against distilled water, and poured through short columns (1 cm $\times$ 1 cm) of the substituted Sepharose. The activity of the effluent was measured; after the column was adsorbing no more enzyme, small volumes of successively more concentrated salt solutions were passed through the column, and the activity of the effluent was measured. No binding of the enzyme was observed for adsorbents with $n < 4$; for $n \geq 4$ the enzyme was bound, but could not be eluted with saturated NaCl or $(NH_4)_2SO_4$.

Affinity chromatography. A 2.5 g (wet wt.) portion of Sepharose 4B was activated with 0.25 g of CNBr at pH 10.5–11.0 under the conditions described by Shaltiel & Er-El (1973). The activated Sepharose was allowed to react for 20 h at 4°C with 0.85 μ mol of *Ps. aeruginosa* cytochrome *c*-551 in 0.1 M- $NaHCO_3$, pH 8.0. The modified Sepharose was then washed with 0.1 M-glycine buffer, pH 8.0, until the effluent was colourless, and equilibrated with 10 mM-phosphate buffer, pH 6.5. Dilute solutions of cytochrome peroxidase (the less-pure fractions from the final Sephadex G-100 chromatography step were used) were passed through columns of the modified Sepharose and the effluent was assayed. Small quantities of the enzyme appeared to be bound, but the activity was not recovered by washing the column

Table 1. Purification of cytochrome *c* peroxidase from *Ps. denitrificans*

Units of enzyme activity are μ mol of cytochrome *c*-551 oxidized/min under standard conditions described in the text. The relative specific activity is derived from the ratio of enzyme activity to A_{280} . R_z is the ratio of the Soret absorbance to A_{280} .

Step	Volume (ml)	Activity (units/litre)	Total activity (units)	A_{280}	A_{410}	Relative specific activity	R_z	Yield (%)
Cell extract	1500	4000	6000	40	—	1.0	—	100
Redissolved acid precipitate	120	47 000	5600	200	—	2.3	—	93
After Sephadex G-75 chromatography	200	25 000	5000	27	—	9.2	—	83
After DE-11 DEAE-cellulose chromatography	500	3000	1500	0.5	0.26	60	0.52	25
After DE-52 DEAE-cellulose chromatography	100	17 000	1700	0.82	1.15	210	1.4	28
After Sephadex G-100 chromatography	30*	14 000*	400*	0.30*	1.02*	450	3.4	25

* Refers to the material of highest purity; practically no protein is lost in this step.

Fig. 4. Chromatography of *Ps. denitrificans* cytochrome *c* peroxidase on CM-52 CM-cellulose

Enzyme from step 4 of the purification procedure (see the text) was chromatographed on a CM-52 CM-cellulose column (1 cm × 3 cm) equilibrated with 10 mM-glycine buffer, pH 6.0. Elution was with a linear concentration gradient of NaCl (0.1–0.25 M), total volume 80 ml. Fractions of volume 3 ml were collected, and A_{280} (●), A_{410} (○) and enzyme activity (▲) were measured. The diagonal line shows the measured conductivity of the effluent from the column.

with 1 M-NaCl, with saturated $(\text{NH}_4)_2\text{SO}_4$ or with 0.1 % sodium dodecyl sulphate.

Sephadex chromatography. Only one component in the enzyme was observed on polyacrylamide disc gels at pH 8 after DE-52 DEAE-cellulose chromatography. However, chromatography on a column (100 cm × 1.5 cm) of Sephadex G-150 showed that the peak of enzyme activity did not coincide with the peak of absorbance at 280 nm. The pair of columns finally used in the purification procedure was selected on the basis of recycling chromatography experiments on a column of dimensions 32 cm × 1.5 cm. The molecular weights of cytochrome *c* peroxidase and of its impurity were estimated by calibrating the analytical Sephadex G-150 column and the preparative Sephadex G-100 column with mixtures of Blue Dextran, bovine serum albumin, *Staphylococcus aureus* penicillinase, myoglobin, *Ps. aeruginosa* cytochrome *c*-551 and ϵ -dinitrophenyllysine. By using the method described by Andrews (1970) to analyse the results, the molecular weights of the enzyme and of its principal impurity were estimated as $10^{4.85}$ and $10^{4.95}$ and as $10^{4.75}$ and $10^{4.85}$ for the Sephadex G-150 and G-100 columns respectively. Taking the mean of these values gives an estimate of the molecular weights of 63 000 for the enzyme and 79 000 for its impurity.

Isolation of cytochrome and azurin

The procedure used for the isolation of these components was based on that used for the purification of cytochrome *c*-551 and azurin from *Ps.*

aeruginosa. However, these proteins are significantly less abundant in *Ps. denitrificans* (at least under our growth conditions) and there are clearly more components in the mixture of cytochromes. Many of these components are present at low concentration, and we have purified only those that can be isolated in large enough quantities to study their interaction with the cytochrome *c* peroxidase.

Batch adsorption on CM-cellulose

The starting material was the supernatant from the acid precipitation step of the preparation of cytochrome *c* peroxidase. The cytochromes and azurin were adsorbed from the mixture by diluting it and passing it through a column (e.g. 6.5 cm × 6 cm for the material from 100 g of acetone-dried powder) of CM-23 CM-cellulose equilibrated with 0.05 M-ammonium acetate buffer, pH 4.3. The column was eluted with a similar buffer of pH 5.5, and the coloured effluent collected.

Chromatography on CM-cellulose

The pooled mixed proteins from several batches of cells were chromatographed on CM-52 CM-cellulose. The pH and conductivity of the mixture were adjusted to those of 0.05 M-ammonium acetate buffer, pH 3.9, and it was passed through a column (e.g. 3 cm × 14 cm) of CM-52 CM-cellulose equilibrated with the same buffer. The coloured proteins bound as a narrow dark band at the top of the column. The pH of the eluting buffer was raised in

steps, and the successive fractions of coloured materials were collected and pooled. The major components were two kinds of cytochrome, with α -bands in the reduced form at 554 and 551 nm, eluted by buffers of pH 4.5 and 5.5 respectively, and one of azurin eluted more slowly at pH 4.5.

Azurin

It was sometimes found by polyacrylamide-gel electrophoresis that the azurin fraction, having been concentrated by adsorption on and elution from a small column of CM-52 CM-cellulose, was already essentially homogeneous. Otherwise the concentrated material was transferred to 0.01 M-glycine buffer, pH 8.0, by means of a column of Sephadex G-25 and passed through a column (e.g. 2.5 cm $\times$ 9 cm) of DE-52 DEAE-cellulose equilibrated with the same buffer. The azurin was eluted with a concentration gradient of NaCl (0–0.1 M) in the same buffer. The protein was concentrated on a small column of CM-52 CM-cellulose and chromatographed on a column (2.6 cm $\times$ 92 cm) of Sephadex G-75. The absorbance of the effluent was measured at 280 and 625 nm, and the azurin-containing fractions were pooled and the pH was adjusted to 4.6. The solution was made 80% saturated with $(\text{NH}_4)_2\text{SO}_4$, centrifuged and finally saturated with $(\text{NH}_4)_2\text{SO}_4$. The azurin was precipitated slowly and was collected by centrifugation.

Cytochrome c-551

The pooled material was concentrated by ultrafiltration, adjusted in pH and conductivity and bound to a column (2.5 cm $\times$ 15 cm) of CM-52 CM-cellulose equilibrated with 0.05 M-ammonium acetate, pH 4.3. The column was eluted with buffers of slowly increasing pH. A minor component was mobile at pH 4.65, and travelled with the solvent front at pH 4.85. The major component was eluted as a symmetrical single peak at this pH. The spectra of the two materials were very similar, and both were fully oxidized on the column. The pooled fractions containing the major component were saturated with $(\text{NH}_4)_2\text{SO}_4$, and the precipitated protein was re-dissolved in the minimum volume of ammonium acetate buffer, pH 5.1, and chromatographed on a column (2.6 cm $\times$ 90 cm) of Sephadex G-75 equilibrated with the same buffer. The protein was shown to be homogeneous by disc gel electrophoresis in the β -alanine system.

Cytochrome c_4

The pooled fractions from the CM-cellulose column were dialysed against 0.1 M-glycine buffer, pH 8.0, and passed through a column (0.5 cm $\times$ 15 cm)

of DE-52 DEAE-cellulose equilibrated with the same buffer. The coloured protein was bound at the top of the column, and was eluted with a linear concentration gradient of NaCl (0–0.1 M). The coloured fractions were pooled, concentrated by ultrafiltration (PSAC membrane) and chromatographed on a column (2.6 cm $\times$ 90 cm) of Sephadex G-75 equilibrated with ammonium acetate buffer, pH 5.1. The fractions forming the major coloured peak were pooled and concentrated, and the protein was shown to be homogeneous by disc gel electrophoresis in the β -alanine system. A second coloured component, of lower molecular weight, was not abundant enough to be isolated.

Characterization of proteins

Molecular weight. The experiments described above allow the molecular weight of cytochrome c peroxidase to be estimated as 63 000. The molecular weights of the other electron-transport proteins were established in a similar manner by using a column of Sephadex G-75 calibrated with *Bacillus licheniformis* penicillinase, myoglobin, horse heart cytochrome c and *Ps. aeruginosa* cytochrome c -551. The molecular weight of the cytochrome c -551 from *Ps. denitrificans* was estimated as 9000, that of the azurin as 12 000 and that of cytochrome c_4 as 20 000. The minor component of the latter cytochrome had a molecular weight of 8000.

Isoelectric point of isolated cytochrome c peroxidase. A sample of the purified enzyme was electrofocused under the same conditions as those described for the crude extract. The material contained one component of isoelectric point 5.6.

Spectra. The isolated proteins were all in their oxidized (Fe^{3+} or Cu^{2+}) forms. The u.v.- and visible-absorption spectra were observed for purified samples (whose protein concentrations had been measured by the method of Lowry *et al.*, 1951), and the visible-absorption spectra measured again after complete reduction with $\text{Na}_2\text{S}_2\text{O}_4$. Fig. 5 shows the spectra of cytochrome c peroxidase and Fig. 6 those of cytochrome c_4 . The spectra of cytochrome c -551 and azurin were essentially the same as those of the corresponding proteins from *Ps. aeruginosa*. Cytochrome c -551 had a spectral purity ratio $[(A_{\text{red}}^{551} - A_{\text{red}}^{570})/A_{\text{ox}}^{280}]$ of 1.16, and the molar absorption coefficient at 551 nm was 28 600 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ (based on protein concentration measured by the method of Lowry *et al.*, 1951). For the azurin the A_{625}/A_{280} ratio was 0.31.

Steady-state kinetics. The steady-state kinetics of the enzyme were studied by measuring the rate of oxidation of *Ps. aeruginosa* cytochrome c -551 or azurin by H_2O_2 in the presence of cytochrome c peroxidase. The reaction rates were measured by observing the changes in absorbance at 551 nm for

Fig. 5. Spectra of *Ps. denitrificans* cytochrome *c* peroxidase before (B) and after (A) reduction with excess $\text{Na}_2\text{S}_2\text{O}_4$. The protein concentration was 0.44 mg/ml in 0.1 M-phosphate buffer, pH 6.8. The path length was 1 cm.

Fig. 6. Spectra of cytochrome c_4 before (B) and after (A) reduction with excess $\text{Na}_2\text{S}_2\text{O}_4$. The protein concentration was 0.26 mg/ml in 0.1 M-phosphate buffer, pH 6.8. The path length was 1 cm.

the cytochrome and at 625 nm for azurin. Substrate concentrations were chosen in the ranges 1–40 μM - H_2O_2 and 5–40 μM reducing substrate.

Substrate inhibition by cytochrome *c*-551 was observed at concentrations of this substrate greater

than 50 μM . Otherwise the initial rates appeared to conform to the expected equation for a two-substrate reaction:

$$e/v = 1/V + K_a/aV + K_b/bV + K_{ab}/abV$$

where v is the observed reaction rate, e , a and b are the concentrations of enzyme, H_2O_2 and reduced cytochrome c -551 respectively and V , K_a , K_b and K_{ab} are constants. Substrate inhibition prevented the measurement of $1/V$. The apparent K_m for H_2O_2 was measured at a series of values of cytochrome concentration, and from these data the values of K_a and K_b were estimated as 100 and 200 μM respectively. Similar values were obtained when *Ps. aeruginosa* azurin was used as substrate.

At constant (10 μM) concentrations of substrate, it was found that the rates of enzymic oxidation of *Ps. denitrificans* cytochrome c -551 and azurin by H_2O_2 were the same as those of the *Ps. aeruginosa* proteins, whereas the rate of oxidation of cytochrome c_4 was about 30% less.

General Discussion

Electron-transport components

We have described here the isolation of four proteins from *Ps. denitrificans* that are all presumably concerned with electron transport. A variety of such proteins has been detected in bacteria and, as noted above, a number of cytochromes present at low concentration were habitually discarded in these preparations. It is possible to identify the components isolated here as members of (sequence-based) classes proposed by Ambler (1973). The cytochrome c -551 is presumably the protein whose amino acid sequence is given by Ambler & Wynn (1973). This sequence is similar to but not identical with those of the corresponding proteins from six other pseudomonads (including *Ps. aeruginosa*) and *Azotobacter vinelandii*.

Two other acidic cytochromes c are commonly found in pseudomonads. Cytochrome c_4 is a di-haem protein of molecular weight 20000, and cytochrome c_5 a low-molecular-weight (8000) mono-haem protein. On the basis of the molecular weight, spectra and acidity it is reasonable to call the protein referred to here as cytochrome c_4 by that name. The minor component separated from it by gel filtration is probably cytochrome c_5 .

The amino acid sequence of *Ps. denitrificans* azurin has been reported (Ambler, 1973), and the relationship of its sequence to those of other azurins and plastocyanin discussed (Barker *et al.*, 1976).

Cytochrome c peroxidase

Cytochrome c peroxidase may be compared with the enzymes catalysing the same reaction from yeast and from other bacteria.

The yeast enzyme (Yonetani, 1976) is a basic protein of mol.wt. 53000 that contains one haem b

group/molecule of protein. The information available about its mechanism may be summarized by saying that H_2O_2 reacts with the ferric enzyme to give a stable oxidized intermediate with characteristic spectra. This intermediate reacts successively with two molecules of reduced cytochrome c , gaining one electron from each. The *Ps. denitrificans* enzyme is an acidic protein of mol.wt. 63000 containing haem c . If the millimolar absorption coefficient of haem c at the Soret band in the oxidized form is about 100, the protein probably contains two haem groups/molecule. No change in the spectrum of the enzyme was observed on addition of stoichiometric quantities of H_2O_2 . Steady-state kinetics provide no evidence for the simultaneous binding of two molecules of reducing substrate. It seems therefore likely that there are relatively stable enzyme intermediates, but none of the experiments described in the present paper gives any information about their nature.

Two other cytochrome c peroxidases have been purified from bacterial sources and have been described in detail. The enzyme from *Thiobacillus thiooxidans* (Tano *et al.*, 1977) is a protohaem-containing enzyme of mol.wt. 32000 that has been compared with the yeast enzyme. The *Ps. aeruginosa* enzyme was isolated by Ellfolk and co-workers (Soininen *et al.*, 1973). The properties of this enzyme are generally similar to that described in the present paper. The molecular weight is 50000 and the protein contains two haem c groups/molecule. The positions of the absorption maxima in the spectra of the oxidized and reduced enzymes are the same as those reported in the present paper. The activity of the *Ps. aeruginosa* enzyme was the same towards azurin as to cytochrome c -551, but the activity towards cytochrome c_4 was not examined. There are apparent differences in detail in the kinetic properties of two enzymes. That from *Ps. aeruginosa* shows behaviour under steady-state conditions that depends on the order of addition of reagents, and has a substantially lower K_m for H_2O_2 than that reported here. No substrate inhibition was reported.

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References

- Ambler, R. P. (1973) *Syst. Zool.* **22**, 554–565
- Ambler, R. P. & Wynn, M. (1973) *Biochem. J.* **131**, 485–498
- Andrews, D. (1970) *Methods Biochem. Anal.* **18**, 1–53
- Barker, W. C., Schwartz, R. M. & Dayhoff, M. O. (1976) in *Atlas of Protein Sequence and Structure* (Dayhoff, M. O., ed.), vol. 5, suppl. 2, pp. 51–65, National Biomedical Research Foundation, Washington
- Brewer, J. M. & Ashworth, R. B. (1969) *J. Chem. Educ.* **46**, 41–45

- King, J. & Laemmli, U. K. (1971) *J. Mol. Biol.* **62**, 465–477
- Lenhoff, H. M. & Kaplan, N. O. (1956) *J. Biol. Chem.* **220**, 967–970
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 165–175
- Shaltiel, S. & Er-El, Z. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 778–781
- Soininen, R., Ellfolk, N. & Kalkkinen, N. (1973) *Acta Chem. Scand.* **27**, 1106–1107
- Tano, T., Sakai, K., Sugio, T. & Imai, K. (1977) *Agric. Biol. Chem.* **41**, 323–330
- Yamanaka, T. & Okunuki, K. (1970) *Biochim. Biophys. Acta* **220**, 354–356
- Yonetani, T. (1976) *Enzymes 3rd Ed.* **13**, 345–360