The cellular location and specificity of bacterial cytochrome *c* peroxidases

Celia F. GOODHEW, Iain B. H. WILSON,* Dominic J. B. HUNTER and Graham W. PETTIGREW†

Department of Preclinical Veterinary Sciences, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, U.K.

The locations of cytochrome *c* peroxidase and catalase activities in the two Gram-negative bacteria *Pseudomonas stutzeri* (N.C.I.B. 9721) and *Paracoccus denitrificans* (N.C.I.B. 8944) were investigated by the production of spheroplasts. In both species the cytochrome *c* peroxidase was predominantly periplasmic: 92 % of total activity in *Ps. stutzeri* and 98 % of non-membrane-bound activity in *Pa. denitrificans* were found in this cellular compartment. In contrast, the catalase was mostly in the cytoplasmic fraction. Purification of the *Pa. denitrificans* cytochrome *c* peroxidase showed it to be the haem *c*-containing polypeptide of M_r 42000 that has already been described by Bosma, Braster, Stouthamer & Van Verseveld [(1987) Eur. J. Biochem. **165**, 665–670] but was not identified by them as a peroxidase. The visible-absorption spectra of the enzyme closely resemble those of cytochrome *c* peroxidase from *Pseudomonas aeruginosa* but the donor specificity is different, with the *Pa. denitrificans* enzyme preferring the basic mitochondrial cytochromes *c* to the acidic cytochromes *c*-551 and reacting well with the *Pa. denitrificans* cytochrome *c*-550.

INTRODUCTION

H_2O_2 is formed as a result of incomplete reduction of O_2 and may be removed by catalase in a dismutation reaction or by peroxidase in a process of reduction to water. In a study of the catalase-negative microaerobe *Campylobacter mucosalis* we showed that H_2O_2 , formed in substantial amounts during respiration, was removed by a cytochrome *c* peroxidase that receives electrons from a small basic monohaem Class I cytochrome *c*-553 (Goodhew *et al.*, 1988). On the basis of Wood's (1983) proposal that *c*-type cytochromes are periplasmic in location, we proposed that the peroxidase must also be periplasmic. Spheroplast formation, which allows release of periplasmic components from Gram-negative bacteria while maintaining cytoplasmic integrity (Ferguson, 1988), would be a direct method for confirming this proposal, but *C. mucosalis* proved to be fragile under conditions of spheroplast formation.

In some other bacteria there is an apparent duplication of mechanisms for H_2O_2 removal in that they have cytochrome *c* peroxidase and are catalase-positive. One possibility is that these enzymes function in the periplasm and cytoplasm respectively. On the basis of a survey of a selection of Gram-negative bacteria (I. Wilson, unpublished work), we chose two organisms, *Pseudomonas stutzeri* strain 9721 and *Paracoccus denitrificans* strain LMD 52.44, that contained both catalase and cytochrome *c* peroxidase activities and also were amenable to spheroplast analysis.

The two best-studied cytochrome *c* peroxidases are from *Pseudomonas aeruginosa* and *Saccharomyces cerevisiae*. The former is a dihaem *c*-type cytochrome of M_r 33690 (302 amino acid residues) (Rönnberg *et al.*, 1989). One haem is high potential and acts as an electron-accepting pole of the molecule, and the other is the peroxidatic centre (Rönnberg & Ellfolk, 1979; Rönnberg *et al.*, 1980; Pettigrew & Moore, 1987). The enzyme is therefore very different in prosthetic structure from that of *S. cerevisiae*, which contains a single non-covalently bound protohaem IX in a polypeptide of M_r 33419 (293 amino acid residues)

(Takio *et al.*, 1980). The *S. cerevisiae* enzyme shows a strong preference for basic cytochrome *c* donors, whereas the *Ps. aeruginosa* enzyme reacts best with the acidic cytochromes *c*-551.

MATERIALS AND METHODS

Growth of cells

Pseudomonas stutzeri 9721 (N.C.I.B. 9721) was grown aerobically in a medium containing trisodium citrate (5 g/l), KH_2PO_4 (1 g/l), $MgSO_4$ (0.5 g/l) and yeast extract (4 g/l) adjusted to pH 7 with NaOH at 30 °C in 1-litre flasks on an orbital shaker. Cells were collected at A_{600} 1.5–1.9 in an Alfa-Laval centrifuge, were washed once with 10 vol. of 10 mM-sodium phosphate buffer, pH 7, and were resuspended at 0.5 g wet wt. of cells/ml in the same buffer.

Paracoccus denitrificans (A.T.C.C. 19367, N.C.I.B. 8944, L.M.D. 52.44) was grown aerobically in a medium containing sodium succinate (13.5 g/l), KH_2PO_4 (4 g/l), K_2HPO_4 (6 g/l), $MgSO_4$ (0.2 g/l), $CaCl_2$ (0.04 g/l), sodium molybdate (0.15 g/l), $MnSO_4$ (0.001 g/l), NH_4Cl (1.6 g/l). $FeSO_4 \cdot 7H_2O$ (0.005 g/l) was added from a stock solution (1.1 g/l in 5 mM-citric acid). The medium (10 litres) was sparged with air (2 litres/min) in a New Brunswick Microfermenter and cells were harvested, washed and resuspended as described above. Higher aeration gave smaller yields of cytochrome *c* peroxidase.

Formation of spheroplasts

The formation of spheroplasts is described in the legend to Fig. 1.

Isocitrate dehydrogenase assay

Isocitrate dehydrogenase (EC 1.1.1.42) was determined by the method of Bernt & Bergmeyer (1974). NADPH production was monitored at 340 nm with a Philips PU 8740 spectrophotometer.

* Present address: Glycobiology Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

† To whom correspondence should be addressed.

Fig. 1. Cellular location of *c*-type cytochromes in *Ps. stutzeri* and *Pa. denitrificans*

The periplasmic (P), cytoplasmic (C) and membrane (M) fractions from (a) *Ps. stutzeri* 9721 and (b) *Pa. denitrificans* and the spheroplast washes (SW1 and SW2) of the latter were subjected to SDS/PAGE (15% polyacrylamide) according to the procedure of Laemmli (1970). Spheroplasts of *Ps. stutzeri* were formed as described by Hunter *et al.* (1989), based on the method of Wood (1978). Conditions were 100 mg of fresh cells/ml in medium containing 0.5 M-sucrose, 40 mM-Tris/HCl buffer, pH 8, 4 mM-EDTA, 10 mM-MgCl₂ and 5 mg of lysozyme/g of cells. The MgCl₂ was added after 2 min exposure to EDTA at 30 °C and the incubation was continued for 30 min. Spheroplasts were centrifuged at 15000 *g* for 30 min at 4 °C and lysed in 20 mM-Tris/HCl buffer, pH 8, for 15 min at room temperature. The membranes and the cytoplasm were separated by centrifugation at 15000 *g* for 30 min at 4 °C. In the case of *Pa. denitrificans*, the cell suspension was steadily diluted with 1 vol. of water (30 °C) during the initial 2 min exposure to EDTA. Final concentrations after MgCl₂ addition were therefore half those quoted above. In addition, the spheroplast pellet of *Pa. denitrificans* was washed twice in 20 mM-Tris/HCl buffer, pH 8, containing 0.25 M-sucrose at 50 mg of spheroplasts/ml (based on the original wet weight of cells that gave rise to the spheroplasts) to give spheroplast washes SW1 and SW2. The traces are absorbance scans at 690 nm following SDS/PAGE and haem detection by the method of Goodhew *et al.* (1986). Key: CCP, cytochrome *c* peroxidase; *c*₅, cytochrome *c*₅; *c*-551, cytochrome *c*-551; *c*₄, cytochrome *c*₄; *c*-550, cytochrome *c*-550; *c*', cytochrome *c*'. *M_r* values were obtained by comparison with marker proteins BSA (*M_r* 66000), ovalbumin (*M_r* 45000), carbonic anhydrase (*M_r* 29000) and cytochrome *c* (*M_r* 12000). Since the cytochrome *c*₅ (*M_r* 11000) and cytochrome *c*-551 (*M_r* 8000) of *Ps. stutzeri* lie outside the standard range, they are shown in parentheses, but the *M_r* values for these proteins are well established (Pettigrew & Moore, 1987).

Catalase assay

Catalase (EC 1.11.1.6) was determined by the method of Rorth & Jensen (1967). O₂ evolved from the catalytic reaction of catalase with H₂O₂ was measured by means of the Clark oxygen electrode at 25 °C. Initial concentrations of O₂ and H₂O₂ were 0.25 mM and 2 mM respectively. Decomposition of H₂O₂ to O₂ follows a first-order progress curve and velocities were derived from the initial reaction rate.

Cytochrome *c* peroxidase activity of spheroplast fractions

Cytochrome *c* peroxidase activity was measured by the decline in the absorbance at the α -band of ferrocytochrome *c* on addition of a cell extract and H₂O₂. Ferrocytochromes *c* were prepared by reduction in 1 mM-sodium ascorbate and removal of excess reductant by passage through Sephadex G-25 equilibrated in 10 mM-sodium phosphate buffer, pH 7, containing 1 mM-EDTA. The assay mixture contained approx. 5 μ M-ferrocytochrome *c*, 17 μ M-H₂O₂, 10 mM-sodium phosphate buffer, pH 7, and 1 mM-EDTA. Horse cytochrome *c* was used as the donor in the assay of *Pa. denitrificans* extracts, and *Ps. aeruginosa* cytochrome *c*-551 in the assay of *Ps. stutzeri* extracts.

Purification of cytochrome *c* peroxidases

The cytochrome *c* peroxidase of *S. cerevisiae* was isolated as described by Pettigrew & Seilman (1982); that from *Ps. aeruginosa* was obtained by using the procedure of Foote *et al.* (1983) and had a final purity index (*A*₄₀₈/*A*₂₈₀ ratio) of 4.2. The *Pa. denitrificans* enzyme was purified from the periplasmic extract of 12 g of cells by ion-exchange chromatography on DEAE-cellulose as described in the legend to Fig. 2. This and further steps were carried out at 4 °C. Fractions containing the peroxidase were pooled and applied to a hydroxyapatite column (8 cm $\times$ 2 cm) and the bound material was eluted with a linear gradient (600 ml) of sodium phosphate buffer, pH 7, from 1 mM to 100 mM. Fractions containing the cytochrome *c* peroxidase were diluted with water to a concentration of 5 mM-sodium phosphate, re-adsorbed on a DEAE-cellulose column (1 cm $\times$ 2 cm) and eluted with 10 mM-Tris/HCl buffer, pH 8, containing 500 mM-NaCl. Molecular exclusion chromatography was carried out on this solution on a column (82 cm $\times$ 2.5 cm) of Sephadex G-75-40 equilibrated in 20 mM-Tris/HCl buffer, pH 8, containing 100 mM-NaCl. The final product had a purity index (*A*₄₀₈/*A*₂₈₀ ratio) of 4.5.

Fig. 2. Separation of the *c*-type cytochromes present in the periplasm of *Pa. denitrificans*

A periplasmic extract of *Pa. denitrificans* (12 g wet wt. of cells) prepared as described in the legend to Fig. 1 was passed directly through a DEAE-cellulose column (8 cm $\times$ 2 cm) equilibrated in 10 mM-Tris/HCl buffer, pH 8. The adsorbed proteins were eluted with a linear salt gradient of 600 ml from 10 mM-Tris/HCl buffer, pH 8, to 10 mM-Tris/HCl buffer containing 400 mM-NaCl. Cytochromes were eluted at 190 mM-NaCl (cytochrome *c*-550), 240 mM-NaCl (cytochrome *c'*) and 300 mM-NaCl (cytochrome *c* peroxidase, CCP). (a) The profile shows the absorbance at 410 nm of the eluate fractions from the DEAE-columns. (b) Selected fractions were subjected to SDS/PAGE (15% polyacrylamide) followed by haem staining as indicated in the legend to Fig. 1. M_r values are from Fig. 1. The subunit of cytochrome *cd*₁ (nitrite reductase) was tentatively identified as the haem-staining band at M_r 60000. Only a little of this enzyme is present under the growth conditions used.

RESULTS

Cellular locations of cytochrome *c* peroxidase and catalase activities

Spheroplasts of *Ps. stutzeri* 9721 were produced as described in the legend to Fig. 1. Only 4% of the cytoplasmic marker enzyme isocitrate dehydrogenase was found in the periplasmic fraction, indicating that the spheroplasts were largely intact (Table 1a). Release of the periplasmic cytochromes (Fig. 1a) shows that this lack of release of isocitrate dehydrogenase is not simply due to the cells remaining intact after lysozyme and EDTA treatment. Similarly, successful spheroplast formation was achieved for the

224 strain of *Ps. stutzeri* (Hunter *et al.*, 1989), but this strain lacks the cytochrome *c* peroxidase. In *Ps. stutzeri* 9721, 92% of the cytochrome *c* peroxidase activity was found in the periplasmic fraction (Table 1a). The catalase shows a less clear-cut distribution but is predominantly cytoplasmic (67%).

In the case of *Pa. denitrificans*, intact spheroplasts were again obtained (5% isocitrate dehydrogenase in the periplasmic fraction) (Table 1b) and these were stable to further washing (7% total isocitrate dehydrogenase release with two iso-osmotic washes) (Table 1b). The distribution of catalase in this experiment follows closely that of isocitrate dehydrogenase, indicating that the catalase is purely cytoplasmic in location. The cytochrome *c*

Table 1. Cellular location of cytochrome *c* peroxidase and catalase

(a) *Ps. stutzeri* 9721

	Distribution (% of summed activities)			Total activity (μ mol of substrate converted/min per g of cells)
	Periplasmic	Cytoplasmic	Membranes	
Isocitrate dehydrogenase	4	94	2	50.5
Catalase	15	67	19	279.2
Cytochrome <i>c</i> peroxidase	92	5	3	2.5

(b) *Pa. denitrificans*

	Distribution (% of summed activities)					Total activity (μ mol of substrate converted/min per g of cells)
	Spheroplast washes			Cytoplasmic	Membranes	
	Periplasmic	SW1	SW2			
Isocitrate dehydrogenase	5	5	2	85	3	45.3
Catalase	6	5	2	79	7	1640
Cytochrome <i>c</i> peroxidase	98	0.5	0.3	1	N.D.*	481.2

* Not determined. The cytochrome *c* peroxidase activity of *Pa. denitrificans* membranes could not be assayed because of high cytochrome *c* oxidase activity.

Fig. 3. SDS/PAGE of purified *Pa. denitrificans* and *Ps. aeruginosa* cytochrome *c* peroxidase and the effect of haem removal

SDS/PAGE according to the method of Laemmli (1970) was performed in 15% polyacrylamide gels. The gel was stained with Coomassie Brilliant Blue R (Goodhew *et al.*, 1986). Lane A, *Ps. aeruginosa* holoenzyme (0.1 nmol); lane B, *Pa. denitrificans* holoenzyme (0.1 nmol); lane C, *Pa. denitrificans* apoenzyme (0.1 nmol); lane D, *S. cerevisiae* holoenzyme (0.1 nmol); lane E, M_r standards BSA (M_r 66000), ovalbumin (M_r 45000), carbonic anhydrase (M_r 29000), myoglobin (M_r 17000) and horse heart cytochrome *c* (M_r 12000); lane F, *Pa. denitrificans* holoenzyme (0.5 nmol); lane G, *Pa. denitrificans* apoenzyme (0.5 nmol). Haem was removed to form the apoenzyme of *Pa. denitrificans* by treatment with HgCl_2 (Ambler & Wynn, 1973).

peroxidase, on the other hand, is periplasmic (98% of non-membrane-bound activity), as was found for *Ps. stutzeri*. It is not easy to assay the membrane fraction for cytochrome *c* peroxidase activity owing to the presence of the very active cytochrome *c* oxidase. On the basis of the gel scans of Fig. 1(b), it is probable that a portion of the cytochrome *c* peroxidase remains attached to the membrane.

Cellular locations of the *c*-type cytochromes

Periplasmic, cytoplasmic and membrane fractions (and, in the case of *Pa. denitrificans*, also spheroplast washes) were subjected to SDS/PAGE and stained for tetramethylbenzidine peroxidase activity (Thomas *et al.*, 1976; Goodhew *et al.*, 1986). This method detects haem groups that are covalently bound to protein and therefore remain attached in denaturing conditions. Some residual retention or transfer of non-covalently bound haem can occur with the *b*-type cytochromes, but in the periplasmic fractions, which are the main concern of the present paper and in which no non-covalent haem is present (Fig. 1a, P, and Fig. 1b, P), the method is quite specific for *c*-type cytochromes.

The periplasmic *c*-type cytochromes of *Ps. stutzeri* 9721 are separated by SDS/PAGE in Fig. 1(a) and are a cytochrome *c*-551 (M_r 8000), cytochrome *c*_s (M_r 11000) and a cytochrome *c* peroxidase (M_r 36000). These identifications were based on M_r values and on absorption spectra after separation by ion-exchange chromatography on DEAE-cellulose and molecular-exclusion chromatography on Sephadex G-75 (results not shown). Almost all the cytochrome *c*-551 (96% on the basis of relative intensity of haem staining) appears in the periplasm. Most of the cytochrome *c*_s (65%) is periplasmic, but there is some present in the cytoplasmic fraction. A similar result was observed with the 224-strain of *Ps. stutzeri* (Hunter *et al.*, 1989) and was explained by the release of membrane-bound cytochrome *c*_s during spheroplast lysis. Almost all the cytochrome *c* per-

Fig. 4. Visible-absorption spectra of cytochrome *c* peroxidase from *Pa. denitrificans*

The spectra are of the final product of the purification of *Pa. denitrificans* cytochrome *c* peroxidase. The oxidised form (Ox.) was the untreated sample in 0.1 M-sodium phosphate buffer, pH 7. The reduced form (Red.) was obtained by addition of a few grains of $\text{Na}_2\text{S}_2\text{O}_4$. The ordinate axis is the absorption coefficient on a per haem basis, the haem concentration being determined by the pyridine haemochrome method (Falk, 1964).

oxidase is periplasmic. The largest membrane cytochrome *c* (M_r 33000) is distinct in electrophoretic mobility and only 3% cytochrome *c* peroxidase activity was found in the membranes.

The separation of the *c*-type cytochromes of *Pa. denitrificans* by SDS/PAGE (Fig. 1b) shows the presence of three cytochromes in the periplasm, which were identified after purification as described below. The cytochrome *c*' (M_r 12000) and the cytochrome *c*-550 (M_r 15000) are solely periplasmic. In contrast, the cytochrome *c* peroxidase (M_r 42000) seems to be partly membrane-attached. Washing the spheroplasts releases further small amounts of the peroxidase, but about 18% remains associated with the membrane as judged by the relative intensity of haem staining (Fig. 1b). Also a proportion (about 15%) appears in the cytoplasmic fraction.

Purification of cytochrome *c* peroxidase of *Pa. denitrificans*

Ion-exchange chromatography on DEAE-cellulose fully resolved cytochrome *c*-550, cytochrome *c*' and cytochrome *c* peroxidase of *Pa. denitrificans* (Fig. 2a). The first of these matched in spectra, M_r on SDS/PAGE and midpoint redox potential the cytochrome *c*-550 first isolated by Scholes *et al.* (1971), sequenced by Timkovich *et al.* (1976) and Ambler *et al.* (1981) and further investigated by Bosma *et al.* (1987). The cytochrome *c*' has not previously been found in *Pa. denitrificans* [although the gels shown by Bosma *et al.* (1987) have a band near M_r 12000 that

Fig. 5. Oxidation of cytochromes *c* by cytochrome *c* peroxidases

Cytochrome *c* peroxidase activity was measured in 10 mM-sodium phosphate buffer, pH 7, containing 1 mM-EDTA at 22 °C by the decrease in absorbance at the α -peak of a ferrocycytochrome *c* donor (5 μ M) on addition of H_2O_2 (18 μ M; H) and a peroxidase preparation (0.03 μ M; P). The cytochrome *c* peroxidase (CCP) preparations were from (a) *S. cerevisiae*, (b) *Pa. denitrificans* and (c) *Ps. aeruginosa*. —, *S. cerevisiae* iso-1-cytochrome *c*; — — —, *Pa. denitrificans* cytochrome *c*-550; ·····, *Ps. aeruginosa* cytochrome *c*-551.

was not further investigated]. It can be identified on the basis of its characteristic high-spin absorption spectra (R. Gilmour & G. W. Pettigrew, unpublished work). The cytochrome *c* peroxidase was further purified by chromatography on hydroxyapatite and Sephadex G-75 as described in the Materials and methods section. The product had an A_{408}/A_{280} ratio of 4.5 and gave a single band on staining with Coomassie Blue (Fig. 3). By using the haem-staining method after SDS/PAGE it was shown that the haem staining coincides with the protein band, and no free haem was observed (results not shown). This confirms that the haem is covalently bound to the protein. The visible-absorption spectra of the reduced and oxidized forms are shown in Fig. 4.

Cytochrome *c* peroxidase activity

The cytochrome *c* peroxidases of *Ps. aeruginosa*, *Pa. denitrificans* and *S. cerevisiae* were tested for activity with *Ps. aeruginosa* cytochrome *c*-551, *Pa. denitrificans* cytochrome *c*-550 and *S. cerevisiae* iso-1-cytochrome *c* as electron donors (Fig. 5). We have presented the data in a qualitative form because the kinetics of the *Pa. denitrificans* enzyme were complex and have not been fully investigated. The enzyme appeared to lose activity rapidly at high dilutions in the assay mix.

The *Pa. denitrificans* enzyme resembled that from *S. cerevisiae* in showing a marked preference for the basic yeast cytochrome *c* over the acidic *Ps. aeruginosa* cytochrome *c*-551. The *Ps. aeruginosa* enzyme showed the opposite preference. *Pa. denitrificans* cytochrome *c*-550 was rapidly oxidized by both the *Pa. denitrificans* peroxidase and that from *S. cerevisiae*.

DISCUSSION

Cellular location

An important integrating proposal in cytochrome *c* research has been that of Wood (1983), in which *c*-type cytochromes are proposed to be periplasmic in location in Gram-negative bacteria. A considerable body of evidence deriving from two sources now

supports this idea (Ferguson, 1988; Pettigrew & Moore, 1987). The first is the demonstration that signal-peptide sequences are encoded in cytochrome *c* genes, implying that the protein products are secreted from the cell; the second is spheroplast studies, in which the periplasmic contents are released while maintaining cytoplasmic integrity. Cytochrome *c* peroxidase would certainly be expected to be periplasmic on this basis, since not only is it a *c*-type cytochrome itself but it reacts with a cytochrome *c* donor. However, direct evidence has been lacking.

We show here that the cytochrome *c* peroxidases from *Ps. stutzeri* 9721 and *Pa. denitrificans* 8944 are periplasmic enzymes. Thus, with the location of catalase in the cytoplasm (Table 1), the catalase-positive cell has the means of H_2O_2 removal from both the periplasmic and cytoplasmic cellular compartments, so diminishing the risk of peroxide-induced damage.

At an early stage in this investigation we examined the location of the better-known cytochrome *c* peroxidase from *Ps. aeruginosa*. We obtained erratic results, but in several experiments the peroxidase seemed to be present in the cytoplasm. Such a location was also suggested by Wood (1978) on the basis of preliminary spheroplast studies. We think that this illustrates a potential problem with the spheroplast method that can lead to wrong conclusions. *Ps. aeruginosa* seems to be weakened by the lysozyme and EDTA treatment but not sufficiently to release all its periplasmic and peripheral-membrane components. Only at the lysis stage are these components released and are therefore identified as cytoplasmic in location. Such effects may explain why Ellfolk and co-workers originally considered the enzyme to be membrane-associated and used acetone-dried powders to allow efficient extraction (Ellfolk & Soininen, 1970). Whatever the reason for this delayed release in a spheroplast experiment, *Ps. stutzeri* poses less of a problem in this respect. The results of Fig. 1(a) demonstrate that there is no haem *c*-containing protein in the cytoplasm with the same electrophoretic mobility as the peroxidase. The small haem-staining bands present in the cytoplasmic fraction are consistent with a low level of membrane contamination.

The situation is less clear-cut for *Pa. denitrificans*. A sizeable

band of similar mobility to the peroxidase is present in the cytoplasm and also in the membrane fraction. However, there is very little peroxidase activity associated with the cytoplasmic fraction (although this may be difficult to measure if the cytoplasmic catalase is very active), and we have been unable to measure the enzyme activity in the membrane fraction. Thus it is uncertain whether the cytoplasmic and membrane material in question is peroxidase or some other *c*-type cytochrome that happens to possess the same electrophoretic mobility. The question as to whether the peroxidase is a peripheral-membrane protein or exists free in the periplasm may be an important one from the point of view of its interaction with the electron-transfer system, as discussed below.

Properties of the bacterial cytochrome *c* peroxidases

The identification of a cytochrome *c* peroxidase in *Pa. denitrificans* is novel, but the enzyme as a *c*-type cytochrome has been detected by other workers (Husain & Davidson, 1986; Bosma *et al.*, 1987). Bosma *et al.* (1987) observed a *c*-type cytochrome of M_r 45 000 in extracts of *Pa. denitrificans* grown under conditions of limiting O_2 . Separation of this species from other *c*-type cytochromes allowed characterization of its spectra, which closely resemble those obtained in the present paper. The spectra of the *Pa. denitrificans* enzyme (Fig. 4) show the characteristic features of the *Ps. aeruginosa* cytochrome *c* peroxidase (Ellfolk *et al.*, 1983), namely Soret bands that do not cross at the Soret (oxidized) maximum, an α -peak at 555–557 nm with a shoulder at the short-wavelength side, and the presence of a weak high-spin band near 630 nm. These features are also present in the spectra of the *Ps. stutzeri* enzyme partially purified in the present paper (results not shown) and the *Ps. stutzeri* enzyme found in A.T.C.C. 11607 studied by Villalain *et al.* (1984). Other cytochromes *c* that may be members of this spectroscopic group but that have not yet been tested for peroxidase activity are the cytochrome *c*-557(551) from *Alcaligenes faecalis* (Iwasaki & Matsubara, 1971) and the cytochrome *c*-549,554 of *Nitrobacter agilis* (Chaudhry *et al.*, 1981).

The M_r value (39 000) derived for the *Ps. aeruginosa* enzyme from its relative mobility on SDS/PAGE is considerably higher than the formula weight of the amino acid sequence (M_r 33 690; Rönnberg *et al.*, 1989). This effect was noted previously (Soininen *et al.*, 1973; Singh & Wharton 1973), and Rönnberg *et al.* (1989) suggested that the two covalently bound haem groups perturb the hydrodynamic shape of the protein in SDS so that it is anomalously hindered by the gel. However, we have found that removal of the haem groups with $HgCl_2$ (Ambler & Wynn, 1973) slightly decreases the electrophoretic mobility of the protein in SDS (results not shown). This effect is also seen for the *Pa. denitrificans* enzyme (Fig. 3). Thus the anomalous electrophoretic mobility of *Ps. aeruginosa* cytochrome *c* peroxidase in SDS cannot be accounted for by the presence of the haem groups and must arise from some atypical feature of the polypeptide chain itself.

The reactivity of both enzymes of course requires a much more complete investigation with respect to cytochrome *c* concentration, ionic strength and pH. However, under the limited conditions at present employed, the *Pa. denitrificans* enzyme reacts best with the cytochrome *c*-550 from the same source, strongly suggesting that this is the physiological donor. In this connection it is noteworthy that Berry & Trumpower (1985) have shown that a quinol oxidase, which contains the bc_1 complex,

cytochrome aa_3 and a cytochrome *c*-552, can be isolated from *Pa. denitrificans* membranes. This complex may allow the oxidation of ubiquinol by O_2 without the involvement of the soluble cytochrome *c*-550. Thus the principal function of cytochrome *c*-550 in *Pa. denitrificans*, in contrast with the role of its counterpart (cytochrome *c*) in mitochondria, may be to mediate between the membrane electron-transport chain and soluble terminal oxidizing enzymes such as cytochrome *c* peroxidase.

I. B. H. W. thanks the Carnegie Trust for a vacation scholarship, and D. J. B. H. thanks the Medical Faculty, University of Edinburgh, for a Ph.D. studentship. This research was partly supported by a Veterinary Faculty grant.

REFERENCES

- Ambler, R. P. & Wynn, M. (1973) *Biochem. J.* **131**, 485–498
- Ambler, R. P., Meyer, T. E., Kamen, M. D., Schichman, S. A. & Sawyer, L. (1981) *J. Mol. Biol.* **147**, 351–356
- Bernt, E. & Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 624–627. Academic Press, New York
- Berry, E. A. & Trumpower, B. L. (1985) *J. Biol. Chem.* **260**, 2458–2467
- Bosma, G., Braster, M., Stouthamer, A. H. & Van Verseveld, H. W. (1987) *Eur. J. Biochem.* **165**, 665–670
- Chaudhry, G. R., Suzuki, I., Duckworth, N. W. & Lees, H. (1981) *Biochim. Biophys. Acta* **637**, 18–27
- Ellfolk, N. & Soininen, R. (1970) *Acta Chem. Scand.* **24**, 2126–2135
- Ellfolk, N., Rönnberg, M., Aasa, R., Andréasson, L. E. & Vänngård, T. (1983) *Biochim. Biophys. Acta* **743**, 23–30
- Falk, J. E. (1964) *Porphyrins and Metalloporphyrins*, p. 240. Elsevier, Amsterdam
- Ferguson, S. J. (1988) in *Bacterial Energy Transduction* (Anthony, C. ed.), pp. 151–182. Academic Press, London
- Foot, N., Thompson, A. C., Barber, D. & Greenwood, C. (1983) *Biochem. J.* **209**, 701–707
- Goodhew, C. F., Brown, K. R. & Pettigrew, G. W. (1986) *Biochim. Biophys. Acta* **852**, 288–294
- Goodhew, C. F., Elkurdi, A. B. & Pettigrew, G. W. (1988) *Biochim. Biophys. Acta* **933**, 114–123
- Hunter, D. J. B., Brown, K. R. & Pettigrew, G. W. (1989) *Biochem. J.* **262**, 233–240
- Husain, M. & Davidson, V. L. (1986) *J. Biol. Chem.* **261**, 8577–8580
- Iwasaki, H. & Matsubara, T. (1971) *J. Biochem. (Tokyo)* **69**, 847–857
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Pettigrew, G. W. & Moore, G. E. (1987) *Cytochromes c: Biological Aspects* (Springer Series in Molecular Biology), pp. 155–160. Springer-Verlag, Heidelberg
- Pettigrew, G. W. & Seilman, S. (1982) *Biochem. J.* **201**, 9–18
- Rönnberg, M. & Ellfolk, N. (1979) *Biochim. Biophys. Acta* **581**, 325–333
- Rönnberg, M., Osterlund, K. & Ellfolk, N. (1980) *Biochim. Biophys. Acta* **626**, 23–30
- Rönnberg, M., Kalkinen, N. & Ellfolk, N. (1989) *FEBS Lett.* **250**, 175–178
- Rorth, M. & Jensen, P. K. (1967) *Biochim. Biophys. Acta* **139**, 171–173
- Scholes, P. B., McLain, G. & Smith, L. (1971) *Biochemistry* **10**, 2072–2076
- Singh, J. & Wharton, D. C. (1973) *Biochim. Biophys. Acta* **292**, 391–401
- Soininen, R., Ellfolk, N. & Kalkinen, N. (1973) *Acta Chem. Scand.* **27**, 1106–1107
- Takio, K., Titani, K., Ericsson, L. H. & Yonetani, T. (1980) *Arch. Biochem. Biophys.* **203**, 615–629
- Thomas, P. E., Ryan, D. & Levin, W. (1976) *Anal. Biochem.* **75**, 168–176
- Timkovich, R., Dickerson, R. E. & Margoliash, E. (1976) *J. Biol. Chem.* **251**, 351–356
- Villalain, J., Moura, I., Liu, M. C., Payne, W. J., LeGall, J., Xavier, A. V. & Moura, J. J. G. (1984) *Eur. J. Biochem.* **141**, 305–312
- Wood, P. M. (1978) *FEBS Lett.* **92**, 214–218
- Wood, P. M. (1983) *FEBS Lett.* **164**, 223–226