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The role of cytochrome c_4 in bacterial respiration

Cellular location and selective removal from membranes

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The cellular location of cytochrome c_4 in *Pseudomonas stutzeri* and *Azotobacter vinelandii* was investigated by the production of spheroplasts. Soluble cytochrome c_4 was found to be located in the periplasm in both organisms. The remaining cytochrome c_4 was membrane-bound. The orientation of this membrane-bound cytochrome c_4 fraction was investigated by proteolysis of the cytochrome on intact spheroplasts. In *P. stutzeri*, 78% of the membrane-bound cytochrome c_4 could be proteolysed, whilst 82% of the spheroplasts remained intact, suggesting that the membrane-bound cytochrome c_4 is on the periplasmic face of the membrane in this organism. Cytochrome c_4 was not susceptible to proteolysis on *A. vinelandii* spheroplasts, in spite of being digestible in the purified state. Cytochrome c_5 was shown to have a similar cellular distribution to cytochrome c_4. Selective removal of cytochrome c_4 from membranes of *P. stutzeri* was accomplished by the use of sodium iodide and propan-2-ol, with the retention of most of the ascorbate–TMPD (*NNN'N'*-tetramethylbenzene-1,4-diamine) oxidase activity associated with the membrane. Sodium iodide removed most of the cytochrome c_4 from *A. vinelandii* membranes with retention of 62% of the ascorbate–TMPD oxidase activity. Cytochrome c_4 could be returned to the washed membranes, but with no recovery of this enzyme activity. We conclude that cytochrome c_4 is not involved in the ascorbate–TMPD oxidase activity associated with the membranes of these two organisms.

INTRODUCTION

Cytochromes *c* play a central role in bacterial electron transport systems (Pettigrew & Moore, 1987). Many soluble cytochromes *c* have been characterized, and Wood (1983) has proposed that these cytochromes are located in the periplasm of Gram-negative bacteria. This proposal has some experimental support; thus *Paracoccus denitrificans* cytochrome *c*-550 (Scholes *et al.*, 1971), *Thermus thermophilus* HB8 cytochrome *c*-552 (Lorence *et al.*, 1981), *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas capsulata* cytochromes c_2 (Prince *et al.*, 1975), *Paracoccus denitrificans* cytochrome cd_1 (Alefounder & Ferguson, 1980), *Pseudomonas aeruginosa* cytochrome cd_1 (Wood, 1978), *Desulphovibrio vulgaris* (Marburg) cytochrome c_3 (Badziong & Thauer, 1980) and cytochrome *c* peroxidase from pseudomonads (C. F. Goodhew, D. J. B. Hunter, I. B. H. Wilson and G. W. Pettigrew, unpublished work) are all periplasmic cytochromes.

Membrane-bound cytochromes *c* have received less attention and their location and orientation are uncertain in most cases. According to Wood (1983), these cytochromes *c* should also be located in the periplasm, either attached to the periplasmic face of the cell membrane or, if part of an integral membrane protein, as a periplasmic-facing domain. An example of a protein expected to have this orientation is cytochrome c_1. This protrudes into the intermembrane space in mitochondria (Leonard *et al.*, 1981) and its bacterial counterparts are expected to be periplasmic-facing (Pettigrew & Moore, 1987).

Cytochrome c_4 is a dihaem cytochrome of M_r 20000, with a low α/β radio and a low α absorbance in the ferrohaem form (Pettigrew & Brown, 1988). It has been purified from *Azotobacter vinelandii* (Tissieres, 1956; Swank & Burris, 1969), and from *Pseudomonas aeruginosa* and *Pseudomonas stutzei* (Pettigrew & Brown, 1988), and has been reported in other pseudomonads and an *Alcaligenes* species. Pettigrew & Brown (1988) showed that the majority of cytochrome c_4 in *P. aeruginosa*, *P. stutzeri* and *A. vinelandii* was membrane-bound, with a small portion soluble. According to the proposal of Wood (1983), we would expect the soluble portion of cytochrome c_4 to be located in the periplasm and the membrane-bound portion to be on the periplasmic face of the cell membrane. The present paper uses spheroplast formation and proteolysis of intact spheroplasts to address this question.

The function of cytochrome c_4 is unknown. A role in denitrification is unlikely as it is found in the strict aerobe *A. vinelandii*. Jurtshuk *et al.* (1981) have proposed that cytochrome c_4 is the *c*-type component of the *co*-type cytochrome oxidase of *A. vinelandii*. Yang (1986) found that pure cytochrome *o* showed no reactivity with molecular oxygen, whereas a partially pure preparation containing a *c*-type cytochrome, presumed to be cytochrome c_4, showed a reaction with oxygen and possessed TMPD (*NNN'N'*-tetramethylbenzene-1,4-diamine) oxidase activity. However, there was no characterization of this *c*-type component, and the proposal of Jurtshuk *et al.* (1981) remains unsupported by firm evidence. This paper investigates the effect of selective removal of cytochrome c_4 from spheroplast membranes prepared from *A. vinelandii* and *P. stutzeri* on the ascorbate–TMPD oxidase activity of these membranes.

Abbreviations used: TMPD, *NNN'N'*-tetramethylbenzene-1,4-diamine; PAGE, polyacrylamide-gel electrophoresis; ICDH, isocitrate dehydrogenase.

MATERIALS AND METHODS

Growth of bacteria

The organisms used in this study were *Pseudomonas stutzeri* (Stainer 224, A.T.C.C. 17591) and *Azotobacter vinelandii* (strain o, A.T.C.C. 12837). *P. stutzeri* was grown aerobically at 32 °C in a medium containing trisodium citrate (5 g/l), potassium dihydrogen phosphate (1 g/l), magnesium sulphate (0.5 g/l) and yeast extract (Oxoid; 4 g/l), adjusted to pH 7.0, in 11 flasks on an orbital shaker or, for the washing experiments, in a 10 l microfermentor (New Brunswick) with sterile air at 10 l/min. *A. vinelandii* was grown in a modified Burks medium (Newton *et al.*, 1953) in the 10 l microfermentor, with vigorous aeration (15 l of air/min). Cells of each type were harvested at early stationary phase, at A_{600} = 1.2–1.5, by centrifugation at 4000 *g* for 30 min at 4 °C. They were washed once, by resuspension in 5 vol. of 10 mM-Tris/HCl, pH 8, at 20 °C followed by recentrifugation, and were resuspended to 0.5 g of cells/ml in 10 mM-Tris/HCl, pH 8, at 20 °C.

SDS/polyacrylamide-gel electrophoresis and haem staining

SDS/PAGE and detection of haem *c* by peroxidase action on 3,3′,5,5′-tetramethylbenzidine used the method of Goodhew *et al.* (1986). Quantification of the amount of staining of each band was by means of absorbance scans at 690 nm using a Shimadzu CS-930 t.l.c. scanner.

Isocitrate dehydrogenase assay

Isocitrate dehydrogenase (ICDH; EC 1.1.1.42) was assayed by the method of Bernt & Bergmeyer (1974). Production of NADPH was monitored at 340 nm using a Philips PU-8740 spectrophotometer.

Ascorbate–TMPD oxidase assay

Ascorbate–TMPD oxidase activity was measured using a Clark oxygen electrode, thermostatted at 25 °C. The reaction was initiated by the addition to the chamber of a 50 μl sample, containing 10 mM-sodium phosphate, pH 7.0, 1 mM-EDTA, 1 mM-ascorbate, 0.3 mM-TMPD and water to a total volume, after the addition of sample, of 2 ml. The initial rate of activity was recorded.

Production of spheroplasts

Spheroplasts were produced from both organisms by treatment of freshly harvested cells with lysozyme and EDTA as described in the legend to Fig. 1. Spheroplast membranes could then be obtained by lysis of the spheroplasts using a low ionic strength plus EDTA treatment, liberating the cytoplasmic contents of the cells.

Proteolysis of spheroplasts

Spheroplasts were produced from each organism by lysozyme/EDTA treatment and were then proteolysed as described in the legends to Figs. 2 and 3. Proteases were chosen which cleaved cytochrome c_4 into well-defined fragments; thus subtilisin was used with *P. stutzeri* spheroplasts and thermolysin was used with *A. vinelandii* spheroplasts.

Removal of cytochrome c_4 from spheroplast membranes using sodium iodide

Cytochrome c_4 was removed from spheroplast membranes using sodium iodide, as described in the legend to Fig. 4. Return of cytochrome c_4 to the membrane was accomplished by dialysing purified cytochrome c_4, or cytochrome c_4 removed from membranes by iodide treatment, together with cytochrome c_4-depleted membranes, suspended in 10 mM-sodium phosphate, pH 7.0, containing 2 M-sodium iodide (*P. stutzeri*), or in 10 mM-sodium phosphate alone (*A. vinelandii*). Dialysis was performed against two changes each of 1 litre of 10 mM-sodium phosphate, pH 7.0, for 2 h per change. Soluble cytochrome c_4 was then separated from that returned to the membrane by centrifugation (11000 *g*, 20 min, 4 °C) and the membrane pellets were resuspended in 10 mM-sodium phosphate, pH 7.0. Ascorbate–TMPD oxidase activities could then be measured, and the amount of cytochrome c_4 reconstituted could be assessed by means of SDS/PAGE followed by haem staining and absorbance scans at 690 nm.

Removal of cytochrome c_4 from spheroplast membranes using propan-2-ol

P. stutzeri spheroplast membranes (15 mg of protein/ml) were centrifuged from their storage medium (11000 *g*, 30 min, 4 °C) in 2 ml aliquots and were resuspended in 2 ml of the appropriate percentage (v/v) of propan-2-ol in 10 mM-sodium phosphate, pH 7.0. After incubation at 0 °C for 30 min, the samples were centrifuged (11000 *g*, 30 min, 4 °C) to separate the solubilized proteins from the membranes. The latter were each resuspended in 2 ml of 10 mM-sodium phosphate, pH 7.0. Ascorbate–TMPD oxidase activity and the percentage of cytochrome c_4 remaining on the membrane were then measured.

RESULTS

Location of cytochromes c_4 and c_5 in *Azotobacter vinelandii* and *Pseudomonas stutzeri*

Formation of spheroplasts. Successful spheroplast formation in both species is indicated by two features (Table 1). Firstly, little ICDH, an enzyme accepted to be a cytoplasmic marker (Wood, 1978), is released into the periplasmic fraction by lysozyme/EDTA treatment. This indicates that few spheroplasts were lysed by the treatment. Secondly, very little cytochrome *c*-551 is present in the 'membrane' fraction. This cytochrome is known to be soluble (Pettigrew & Brown, 1988) and its absence from the membrane fraction indicates that very few whole cells remain after lysozyme/EDTA treatment. These results show that the lysozyme/EDTA treatment is effective in releasing the periplasmic contents whilst allowing retention of cytoplasmic integrity. A low ionic strength step was then used to lyse the spheroplasts.

Location of the soluble forms of cytochromes c_4 and c_5. In both organisms, cytochrome c_4 is predominantly membrane-bound. However, 15–25% is soluble, in agreement with the results of Pettigrew & Brown (1988) (Table 1). The soluble cytochrome c_4 appears almost entirely in the periplasmic fraction of each organism (Fig. 1). These periplasmic fractions show no sign of other membrane cytochromes *c*, indicating that the presence of cytochrome c_4 is not the result of contamination of the

Table 1. Location of *c*-type cytochromes in *P. stuzeri* and *A. vinelandii*

The figures in parentheses are the amounts of cytochromes *c* after purification as described in Pettigrew & Brown (1988). P, periplasm; C, cytoplasm; M, membranes. Relative areas were obtained by weighing excised peaks from the 690 nm absorbance scans of Fig. 1. The amount is given in nmol of protein/100 g of cells. This was calculated assuming linearity of haem staining (see Goodhew *et al.*, 1986) and using a known amount of cytochrome standard. Note that cytochrome c_4 is dihaem and therefore gives twice the staining intensity per mol as a monohaem cytochrome such as cytochrome *c*-551. The percentage is calculated from the proportion found in a particular fraction relative to the total. M/P is the ratio of membrane-bound to periplasmic cytochrome. For the associated figures in parentheses, M/P is the ratio of membrane-bound to soluble cytochrome *c* after purification according to Pettigrew & Brown (1988).

	P			C			M			
	Relative area	Amount	%	Relative area	Amount	%	Relative area	Amount	%	M/P
P. stutzeri:										
Cytochrome c_4	1.4	97 (35)	14	0.3	21	3	8.0	556 (205)	83	5.7 (5.9)
Cytochrome c_5	4.2	583	35	1.8	250	15	6.0	833	50	1.4
Cytochrome *c*-551	20.7	2874 (1610)	92	1.6	222	7	0.2	28 (35)	1	0.01 (0.02)
ICDH			5			77			18	
A. vinelandii:										
Cytochrome c_4	5.1	123 (79)	21	0.8	19	3	18.1	433 (315)	75	3.5 (4.0)
Cytochrome *c*-551	18.7	900 (246)	91	0.8	38	4	1.1	53 (14)	5	0.06 (0.06)
ICDH			0.1			97			3	

periplasm by membranes. Very small amounts of cytochrome c_4 (3%) are apparently present in the cytoplasmic fractions (Table 1), but these are accompanied by traces of other membrane cytochromes (see e.g. Fig. 1*a*). Thus they are probably the result of low level contamination by membranes.

Cytochrome c_5 is predominantly membrane-bound in *P. stutzeri*. However, a sizeable fraction appears in the periplasm (Table 1). In *A. vinelandii*, quantification of the amount of cytochrome c_5 in the periplasm was not possible, as there is complexity in the haem-staining pattern in the cytochrome c_5 region of this fraction (Fig. 1*b*). Purification studies have indicated that both peaks in this region contain cytochromes which are spectrally identical to cytochrome c_5. However, as the exact identity of these peaks was not certain, the amount of cytochrome c_5 could not be obtained. In addition to the periplasmic and membrane-bound cytochrome c_5, some appears in the cytoplasmic fractions of both organisms in amounts which cannot be explained either by periplasmic cytochrome c_5 trapped adventitiously in the spheroplast pellet or by membrane contamination. This is discussed below.

A further observation in *A. vinelandii* is that protohaem is detected in the periplasmic fraction in addition to that in the membrane fraction (Fig. 1*b*). This cannot be due to contamination of the periplasmic fraction by membrane *b*-type cytochromes since, as noted previously, there is no sign of contamination of the periplasm by membrane *c*-type cytochromes.

Orientation of membrane-bound forms of cytochrome c_4 and c_5. Proteolysis of intact spheroplasts was used to investigate the membrane orientation of cytochromes c_4 and c_5. Digestion intensities are expressed as the ratio of protein/enzyme (w/w). It should be noted that 'protein' refers to the total amount of protein assumed to be accessible to the protease. In the case of a purified cytochrome this is simply the amount of cytochrome present. However, in the case of spheroplasts, accessible protein was assumed to comprise that in the periplasm plus the amount of added lysozyme plus that in the membranes. This is an overestimation of the actual amount of accessible protein as an unknown proportion of the membrane protein will be cytoplasmic-facing, deeply buried in the membrane, or otherwise inaccessible to the protease.

P. stutzeri. Subtilisin was chosen for proteolysis of *P. stutzeri* cytochrome c_4 as it produces fragments of approximately equal size after digestion at an intensity of 20:1 protein/enzyme (w/w) (Fig. 2). In addition, it possesses the advantage of being easily inhibitable by phenylmethanesulphonyl fluoride. Of total membrane-bound cytochrome c_4, 78% is proteolysed by subtilisin under these conditions, a similar yield to proteolysis of the pure cytochrome under similar conditions. This degree of removal of cytochrome c_4 was confirmed by Western blotting of subtilisin-treated membranes, using an anti-(*P. stutzeri* cytochrome c_4) antiserum, which showed that only 19% of the cytochrome c_4 remained on the membranes after subtilisin treatment (results not shown). Approximately two-thirds of the 78% loss in haem-staining activity can be accounted for by the appearance of the band labelled c_4(s), which corresponds to the subtilisin fragments of pure cytochrome c_4 (Fig. 2). These fragments remain attached to the membrane after proteolysis.

After incubation, the spheroplasts treated with protease were 82% intact (Table 2). This indicates that the

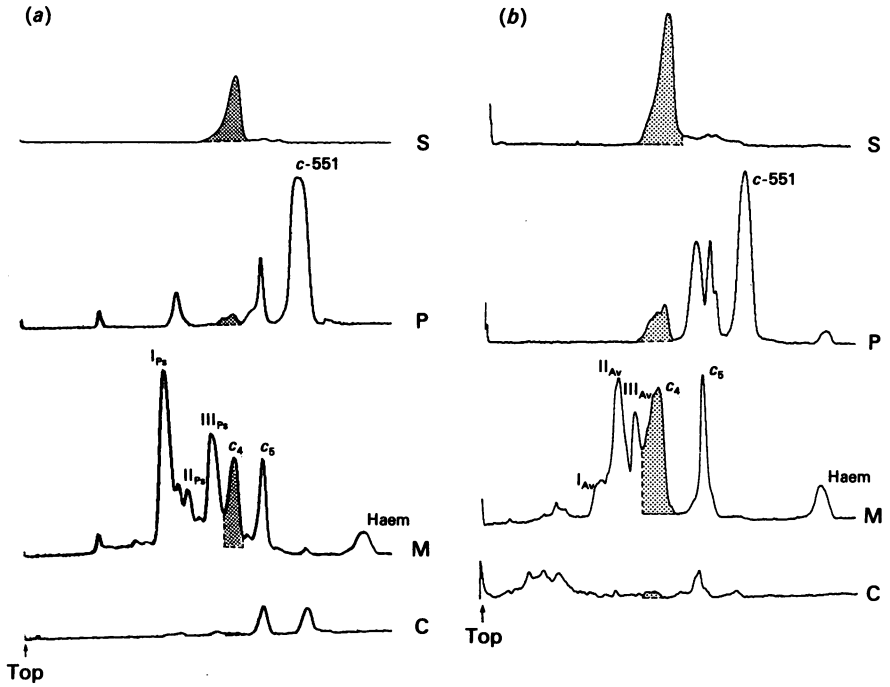

Fig. 1. Location of *c*-type cytochromes in *Azotobacter vinelandii* and *Pseudomonas stutzeri*

The periplasmic (P), cytoplasmic (C) and membrane (M) fractions from (*a*) *P. stutzeri* and (*b*) *A. vinelandii* were obtained as follows and subjected to SDS/PAGE. Washed cells (5 g; 10 ml), resuspended to 0.5 g cells/ml in 10 mM-Tris/HCl, pH 8, at 20 °C, were added to a sucrose/Tris/lysozyme/EDTA solution of volume 35 ml. The reaction mixture was incubated for 2 min at 30 °C, then $MgCl_2$ (5 ml; 100 mM) was added to give final concentrations of 0.5 M-sucrose, 40 mM-Tris/HCl, pH 8, at 20 °C, 5 mg of lysozyme (Sigma) per g of cells and 10 mM-$MgCl_2$, in a total volume of 50 ml. The final concentrations of EDTA during the production of spheroplasts were (*a*) 4 mM and (*b*) 5 mM. The mixture was then incubated for a further 30 min at 30 °C. Centrifugation (11000 *g*, 20 min, 4 °C) separated the periplasmic fraction (P) from the spheroplasts. Lysis of the spheroplasts was then accomplished by resuspending the spheroplast pellet in 50 ml of 10 mM-Tris/HCl, pH 8/2 mM-EDTA at 20 °C. After 15 min at 20 °C, $MgCl_2$ was added to a final concentration of 4 mM. The lysed spheroplasts were then centrifuged (19000 *g*, 30 min, 4 °C) to separate the cytoplasmic fraction (C) from the membranes (M). The latter were then resuspended in 50 ml of 10 mM-Tris/HCl, pH 7.3, at 20 °C. In the case of *A. vinelandii*, an additional low-speed centrifugation (1500 *g*, 5 min, 4 °C) was required after spheroplast lysis, to remove a bulky white jelly which otherwise contaminated the membranes. A second centrifugation (19000 *g*, 30 min, 4 °C) separated the glassy red membranes (M) from the clear cytoplasmic fraction (C). Samples were then subjected to SDS/PAGE on a gradient gel (10–25% polyacrylamide). The traces are absorbance scans at 690 nm following haem detection by the method of Goodhew *et al.* (1986). Three major membrane *c*-type cytochromes may be common to both species and are labelled: (*a*) I_{Ps} (38000), II_{Ps} (32000), and III_{Ps} (25000) and (*b*) I_{Av} (37000), II_{Av} (31000), and III_{Av} (25000). M_r values were obtained by comparison with marker proteins: ovalbumin, 45000; yeast cytochrome *c* peroxidase, 34000; myoglobin, 17000; horse heart cytochrome *c*, 12000 and *Pseudomonas aeruginosa* cytochrome *c*-551, 9000. These gave a linear plot of log M_r against mobility in spite of the gradient nature of the gel. Cytochromes c_4, c_5 and *c*-551 in both organisms were identified by comparison with purified samples of these proteins. Cytochrome c_5 is monomeric in SDS in agreement with the results of Carter *et al.*, (1985). Scans labelled S are lanes loaded with purified cytochrome c_4 from each organism. Only small amounts of cytochrome cd_1 were detected in the periplasm of *P. stutzeri* under these growth conditions (small peak of highest M_r).

subtilisin had access to the cytoplasmic face of the membranes in only 18% of the spheroplasts.

Both membrane-bound cytochrome c_5 and its soluble form disappear completely on proteolysis (Fig. 2). The disappearance of cytochrome c_5 from the periplasmic and membrane fractions is accompanied by the appearance of a band in the periplasm with a size just greater than that of the cytochrome c_4 fragments [c_4(s)] (Fig. 2, lane c). It is likely that this fragment derives from both soluble and membrane-bound forms of cytochrome c_5. In addition to its effect on cytochromes c_4 and c_5, proteolysis of spheroplasts with subtilisin partially removes the weakly staining band II_{Ps} from the membranes (Fig. 2, lane f). Bands I_{Ps} and III_{Ps} are not affected by the proteolysis (Table 2).

A. vinelandii. Subtilisin does not digest purified native cytochrome c_4 from *A. vinelandii*. Of other proteases tested (trypsin, chymotrypsin, thermolysin and staphylococcal V8 protease), thermolysin alone efficiently digested purified cytochrome c_4 (results not shown) to produce two fragments with an M_r of approx. 11000 and a smaller fragment whose yield increases as the intensity of digestion increases (Fig. 3, lanes e and f). At 50:1 protein/enzyme (w/w), proteolysis was essentially complete, as judged by the disappearance of the cytochrome c_4 band.

Despite this, however, thermolysin, at an intensity of 16:1 protein/enzyme (w/w), produces no loss of haem staining by the membrane-bound cytochrome c_4, indicating that no proteolysis of the cytochrome is taking place

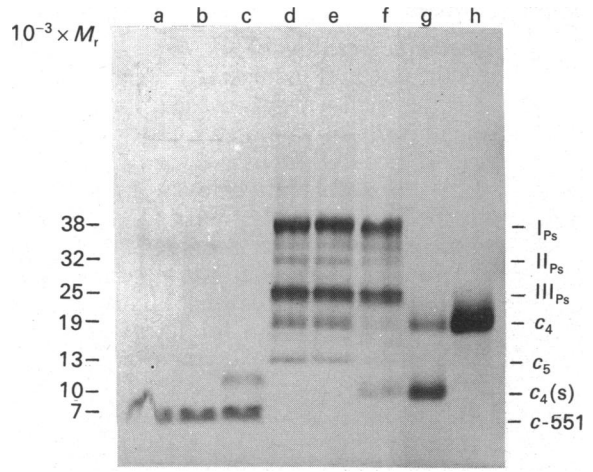

Fig. 2. Proteolysis of cytochrome c_4 on spheroplasts of *P. stutzeri*

P. stutzeri spheroplast suspension (5 ml), prepared as described in the legend to Fig. 1, was exposed to subtilisin at a digestion intensity of 20:1 (w/w) protein/enzyme for 30 min at 30 °C. Phenylmethanesulphonyl fluoride (PMSF) in propan-2-ol was then added to give a 25-fold molar excess over enzyme and a final concentration of 1% propan-2-ol. A control experiment contained no subtilisin. Periplasmic, cytoplasmic and membrane fractions were prepared from the spheroplast suspension as described in the legend to Fig. 1, and samples were subjected to SDS/PAGE. EDTA (3 mM) was used in the production of the spheroplasts. Lanes a and d contain the periplasmic and membrane fractions of untreated spheroplasts. Lanes c and f contain periplasmic and membrane fractions after subtilisin treatment. Lanes b and e contain periplasmic and membrane fractions from the control experiment. Lanes g and h each contain 1 nmol samples of purified cytochrome c_4, untreated (lane h) and treated with subtilisin (lane g) by incubation for 15 min at 30 °C at a digestion intensity of 20:1 (w/w) protein/enzyme. PMSF in propan-2-ol was then added to give a 30-fold molar excess over enzyme. The M_r values of the major membrane bands were calculated as described in the legend to Fig. 1, using the following standards: bovine serum albumin, 66000; yeast cytochrome *c* peroxidase, 34000; myoglobin, 17000; horse heart cytochrome *c*, 12000 and *P. aeruginosa* cytochrome *c*-551, 9000. c_4(s) indicates the subtilisin fragments of cytochrome c_4.

Table 2. The proteolysis of cytochrome c_4 in spheroplasts of *P. stutzeri*

Relative peak areas were obtained by weighing excised peaks from the absorbance scans of Fig. 2. Peak areas of channel f (+subtilisin) were expressed as a percentage of corresponding peaks in channel e (−subtilisin) The percentage value for c_4(s) was calculated relative to the parent c_4 peak of channel e (−subtilisin). Spheroplast intactness was calculated by expressing the combined cytoplasmic and membrane-bound ICDH activities as a percentage of the total activity. The values obtained were: −subtilisin, 72%, +subtilisin, 82%.

	Peak area		
	−Subtilisin	+Subtilisin	%
c_4	4.5	1.0	22
c_4(s)	—	2.2	49
I_{Ps}	13.8	13.1	95
III_{Ps}	10.8	11.1	103

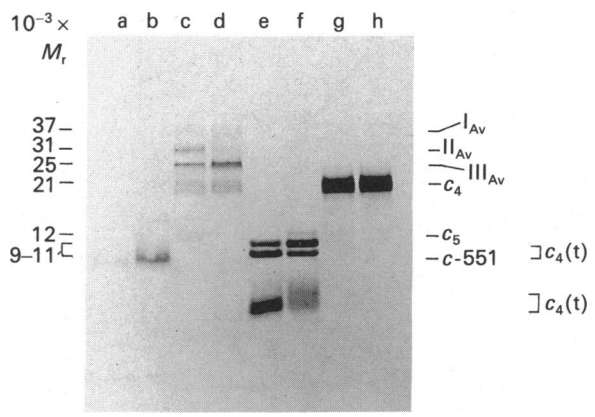

Fig. 3. Proteolysis of cytochrome c_4 on spheroplasts of *A. vinelandii*

A. vinelandii spheroplast suspension (5 ml), prepared using 5 mM-EDTA, was exposed to thermolysin at a digestion intensity of 16:1 (w/w) protein/enzyme for 30 min at 30 °C. Periplasmic, cytoplasmic and membrane fractions were prepared from the spheroplast suspension as described in the legend to Fig. 1, with the difference that no $MgCl_2$ was added after the EDTA lysis stage. This ensured inhibition of the thermolysin after the experiment by EDTA chelation of its active site metal ion. EDTA could not be added immediately after the thermolysin treatment, as premature lysis of the spheroplasts would have occurred. Lanes b and d contain periplasmic and membrane fractions treated with thermolysin as described above. Lanes a and c are corresponding fractions treated as for the proteolysis experiment, but with no addition of thermolysin. Lanes e–h contain 0.5 nmol samples of purified cytochrome c_4 untreated (lane h), treated with a 60-fold molar excess of EDTA over enzyme prior to thermolysin at an intensity of 10:1 (w/w) protein/enzyme (lane g), or treated with thermolysin at digestion intensities of 50:1 (lane f) or 10:1 (lane e) (w/w) protein/enzyme. After 30 min incubation at 30 °C, EDTA was added to those samples not already treated to stop the reaction. The M_r values of the major bands were calculated as described in the legend to Fig. 2. c_4(t) indicates the position of the thermolysin fragments of cytochrome c_4.

on the spheroplasts (Fig. 3, lanes c and d). In contrast, cytochrome c_5 is completely degraded (lane d), and probably contributes to a soluble band (M_r approx. 10000) which appears in the periplasm (lane b). Band II_{Av} is also extensively degraded and may also contribute to this soluble fragment.

The thermolysin-treated spheroplasts released only 6% of their cytoplasmic ICDH, indicating that 94% remained intact during the digestion.

Selective removal of cytochrome c_4 from membranes and effect on ascorbate–TMPD oxidase activity

Selective removal of cytochrome c_4 and cytochrome c_5 from the membranes of the two organisms was accomplished by the use of the chaotropic ion I^-. Other treatments commonly used to wash off extrinsic membrane proteins, such as high ionic strength (10 mM-sodium phosphate, pH 7.0/2.5 M-NaCl), low ionic strength (10 mM-Tris/HCl, pH 7.3) and high pH (10 mM-sodium carbonate, pH 9.5) (Berry & Trumpower, 1985) failed to remove cytochrome c_4 (results not shown). In addition, 30% (v/v) propan-2-ol removed cytochromes

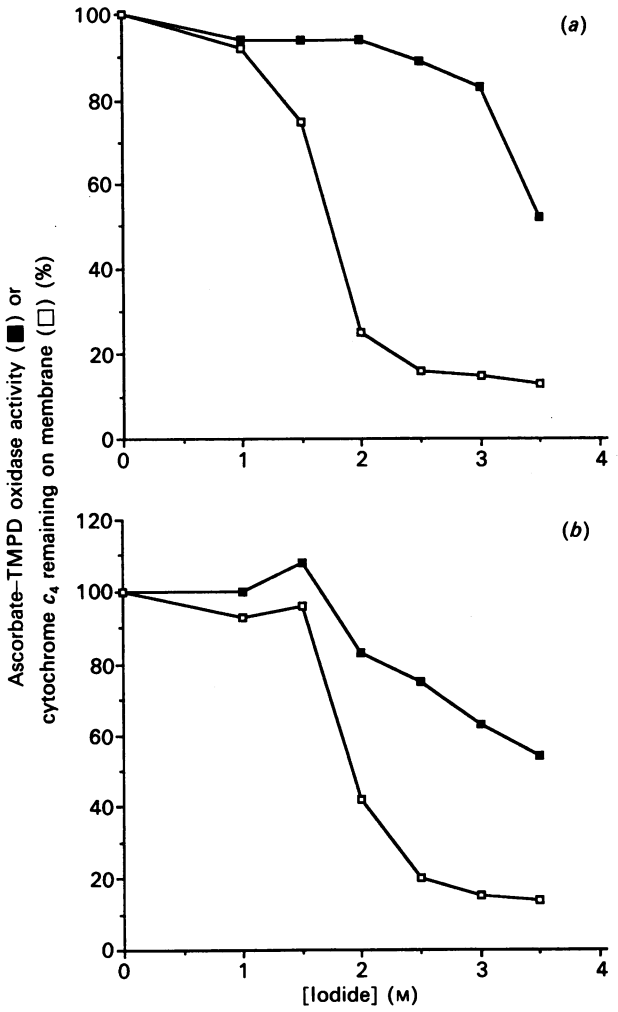

Fig. 4. Removal of cytochrome c_4 from spheroplast membranes of (*a*) *P. stutzeri* and (*b*) *A. vinelandii* by sodium iodide and the effect on ascorbate–TMPD oxidase activity

Spheroplast membranes were produced as described in the legend to Fig. 1 and were adjusted to 15 mg of protein/ml (*P. stutzeri*) or 3 mg of protein/ml (*A. vinelandii*). The iodide treatment method used was common to both organisms. Aliquots (2 ml) of membranes were centrifuged from their storage medium (11000 *g*, 20 min, 4 °C) and were then resuspended in 2 ml of the appropriate concentration of sodium iodide in 10 mM-sodium phosphate, pH 7.0. After incubation for 30 min at 0 °C, 10 mM-sodium phosphate, pH 7.0, was added to give an iodide concentration of 1 M. This allowed the membranes to pellet on centrifugation (11000 *g*, 20 min, 4 °C). The supernatants produced were dialysed against two changes of 1.5 l of 10 mM-sodium phosphate, pH 7.0, for 2 h per change. Supernatants were lyophilized and the freeze-dried material and the membrane pellets were taken up in their original incubation volume (10 mM-sodium phosphate, pH 7.0). ■, Ascorbate–TMPD oxidase activity of the membranes relative to control membranes (10 mM-phosphate, pH 7.0; 100% activity). □, Percentage of cytochrome c_4 remaining on the membrane after each treatment. The amounts of cytochrome c_4 remaining on the membrane were obtained by weighing excised peaks from 690 nm absorbance scans performed after SDS/PAGE and haem detection by the method of Goodhew *et al.* (1986). Variations in staining intensity over the gels were allowed for by normalizing each cytochrome c_4 peak relative to band III_{Ps} or III_{Av}.

c_4 and c_5 from the membranes of *P. stutzeri* but not from those of *A. vinelandii* (results not shown).

Fig. 4 shows the effect of increasing concentrations of iodide ion on the removal of cytochrome c_4 from spheroplast membranes of *P. stutzeri* and *A. vinelandii* and on the ascorbate–TMPD oxidase activities of the membranes. In both organisms, removal of cytochrome c_4 is apparently incomplete, with around 15% remaining on the membrane even when 3.5 M-iodide is used. However, the true amount of cytochrome c_4 remaining is probably less than this, for two reasons. Firstly, there are several minor bands which stain for haem and which migrate close to the position of cytochrome c_4. It is difficult to distinguish the staining by residual cytochrome c_4 from staining of these. Secondly, cytochrome c_4 and band III are not completely resolved. Band III therefore makes some contribution to the cytochrome c_4 staining which is difficult to allow for accurately. For these reasons, the amount of cytochrome c_4 shown as remaining on the membrane after 3.5 M iodide treatment should be regarded as an upper estimate.

Experiments similar to the iodide experiments were performed using propan-2-ol as the washing agent, with *P. stutzeri* spheroplast membranes. Again, removal of cytochrome c_4 was apparently incomplete, probably for the reasons stated previously. However, with 30% propan-2-ol, only 28% of the cytochrome c_4 remained on the membrane, whilst 90% of the ascorbate–TMPD oxidase activity was retained (results not shown).

Both removed and pure cytochrome c_4 could be returned to depleted membranes. In *P. stutzeri*, 65–78% of the original amount could be returned to membranes washed with 2 M-iodide with no effect on the oxidase activity. In *A. vinelandii*, 92% could be returned to membranes washed with 3 M-iodide. No recovery of activity was observed (results not shown).

DISCUSSION

Location and orientation of cytochromes c_4 and c_5

Wood (1983) has proposed that all *c*-type cytochromes are located either in the periplasm or on the periplasmic side of the cell membrane in Gram-negative organisms. This paper provides further evidence for the proposal and also confirms and extends the conclusions of Pettigrew & Brown (1988) who showed that cytochrome c_4 exists in two forms, one soluble and one membrane-bound, in *P. stutzeri* and *A. vinelandii*.

Spheroplasts were successfully produced from both organisms under investigation, allowing the cellular location of the soluble fractions of cytochromes c_4 and c_5 to be determined. Almost all of the soluble cytochromes c_4 and c_5 were found to be located in the periplasm, with a small proportion in the cytoplasm. These apparently cytoplasmic cytochromes are actually the result of contamination of the cytoplasmic fraction by membranes in the case of cytochrome c_4 from both organisms. However, the amount of cytochrome c_5 in the 'cytoplasm' of both organisms is too great to be explained by such contamination, or by periplasmic cytochrome c_5 being trapped in adventitious liquid in the spheroplast pellet, prior to lysis. We propose that this 'cytoplasmic' cytochrome c_5 is actually membrane cytochrome c_5 which has been washed off the spheroplast membranes by the treatment used to lyse the spheroplasts (low ionic strength plus EDTA).

A further proposal of Wood (1983) is that *b*-type cytochromes should not appear in the periplasm of Gram-negative organisms, as their non-covalently-bound haem would be easily lost to the medium. However, protohaem was detected in the periplasmic fraction of *A. vinelandii*. This may suggest that a soluble *b*-type cytochrome exists in the periplasm, but a number of other explanations for the presence of protohaem should be considered. Firstly, the protohaem is not due to the presence in the periplasmic fraction of membrane-bound *b*-type cytochromes attached to contaminating membranes, as no corresponding membrane-bound *c*-type cytochromes are detected. It is possible, however, that certain membrane cytochromes *b* are removed from the spheroplast membranes by the lysozyme/EDTA treatment used to produce the spheroplasts. Another possibility is that protohaem alone is removed by lysozyme/EDTA. Secondly, the protohaem may be the prosthetic group of a soluble, periplasmic peroxidase or of a haemoglobin-like protein.

Investigation of the membrane orientation of a protein requires the use of a modifying agent which is membrane-impermeant. In this respect, proteases are very useful since their size precludes their passing across the membrane. The data presented here demonstrate their use in such an investigation.

Purified cytochromes c_4 from *P. stutzeri* and *A. vinelandii* are cleavable using subtilisin and thermolysin respectively. Both proteases cleave the cytochromes c_4 into two approximately equal halves. In addition, a lower-M_r minor fragment is produced in the case of *A. vinelandii* cytochrome c_4, which increases in amount as the digestion intensity increases. This is presumably the result of further degradation of the fragments. The major fragments produced correspond to the two domains of the dihaem cytochrome, each containing a single haem. The proteases therefore probably act in the interdomain region of the cytochrome (Brown, 1988).

Using subtilisin, we have been able to show that 78% of the membrane-bound cytochrome c_4 in *P. stutzeri* is digested, whilst only 18% of the spheroplasts released their cytoplasmic ICDH. The simplest interpretation of these results is that membrane-bound cytochrome c_4 faces the periplasm. Nevertheless, we cannot exclude the possibility that a portion of the cytochrome c_4 faces the cytoplasm. An alternative possibility is that cytochrome c_4 faces the cytoplasm but possesses a transmembrane portion which is attacked by the subtilisin. However, there is no indication of a hydrophobic, membrane-spanning sequence in the amino acid sequence of *A. vinelandii* cytochrome c_4 (Ambler *et al.*, 1984) to which the cytochrome c_4 from *P. stutzeri* is thought to be closely related. Also, a significant proportion of cytochrome c_4 is soluble, which would not be expected for an integral membrane protein. Therefore, the 78% digestion of cytochrome c_4 in 82% intact spheroplasts leads us to conclude that a large proportion, and probably all, membrane-bound cytochrome c_4 is periplasm-facing in *P. stutzeri*.

Using thermolysin, it was not possible to proteolyse cytochrome c_4 on *A. vinelandii* spheroplasts. Thus the cytochrome c_4 must either face the cytoplasm in *A. vinelandii* or be in a position, or of a conformation, not susceptible to cleavage by thermolysin. By comparison with *P. stutzeri*, the latter is more likely.

In both organisms, cytochrome c_5 is digested on the spheroplast membranes, producing a smaller soluble fragment. Membrane-bound cytochrome c_5 is therefore periplasm-facing. In addition, the membrane cytochrome band II is also digested, suggesting that the haem-containing moiety faces the periplasm in this case also. The other membrane cytochromes *c* (bands I and III) are not digested, and must either face the cytoplasm or be otherwise inaccessible to the proteases used.

Involvement of cytochrome c_4 in ascorbate–TMPD oxidase activity

Jurtshuk *et al.* (1981) have proposed that cytochrome c_4 forms the *c*-type component of the *co*-type cytochrome oxidase of *A. vinelandii*. However, this was based on the very undiscriminating criterion of membrane spectra; no positive identification of the *c*-type cytochrome with purified cytochrome c_4 was made. In the present study, the effect on ascorbate–TMPD oxidase activity of selective removal of cytochrome c_4 from membranes of *P. stutzeri* and *A. vinelandii* has been investigated.

In the case of *P. stutzeri*, treatment of membranes with 2–3 M-iodide or 30% (v/v) propan-2-ol results in the removal of at least 75–85% of the cytochrome c_4 with almost no loss of ascorbate–TMPD oxidase activity. Purified cytochrome c_4, or the iodide extract, could be reconstituted with the washed membranes with no recovery of activity.

In *A. vinelandii*, removal of cytochrome c_4 by iodide is associated with loss of ascorbate–TMPD oxidase activity. However, with 3 M-iodide, approx. 63% of the activity is still present, with only 15% of the cytochrome c_4 remaining. Return of purified cytochrome c_4, or of the iodide extract, to the washed membranes results in no recovery of activity.

These results indicate that cytochrome c_4 is probably not essential for ascorbate–TMPD oxidase activity. However, this cannot be stated with complete certainty for two reasons. Firstly, there remains a small residual amount of cytochrome c_4 on the washed membranes. Secondly, the lack of a positive control in the reconstitution experiments means that changes to the cytochrome c_4 during extraction or purification, or changes to the oxidase during washing with iodide, cannot be ruled out.

Cytochrome c_5 is removed by iodide in parallel to the removal of cytochrome c_4 in both organisms. This may indicate some similarity in the mechanism of attachment of the two cytochromes to the membrane and suggests that cytochrome c_5 is also not involved in ascorbate–TMPD oxidase activity.

Chaotropic agents, of which iodide is an example, have been frequently used in the selective removal of membrane proteins (Hatefi & Hanstein, 1974). For example, succinate dehydrogenase from *Rhodospirillum rubrum* has been isolated by the use of 0.85 M-perchlorate (Hatefi & Davies, 1972). Iodide and perchlorate have similar chaotropic potencies (Hatefi & Hanstein, 1974) and the requirement here for higher iodide concentrations indicates that cytochrome c_4 is more tightly bound to the membrane than succinate dehydrogenase.

Conclusions

We conclude that cytochromes c_4 and c_5 are predominantly membrane-bound proteins which face the

periplasmic space. In the case of *P. stutzeri*, almost all the cytochrome c_4 can be removed with no effect on ascorbate–TMPD oxidase activity. In the case of *A. vinelandii*, removal of most of the cytochrome c_4 is accompanied by a loss of 50% of ascorbate–TMPD oxidase activity, but return of the pure cytochrome to the membrane does not allow recovery of activity. Thus we propose that cytochrome c_4 has no involvement in ascorbate–TMPD oxidase activity, in contrast to the proposal of Jurtshuk *et al.* (1981). If the cytochrome *o* in these organisms is a *co*-type oxidase, the *c*-type component is not cytochrome c_4. This component may, however, be one of the other *c*-type membrane bands. The relatively high redox potentials of cytochrome c_4 (in *A. vinelandii*, 317 and 263 mV; in *P. stutzeri*, 300 and 190 mV; Leitch *et al.*, 1985) do, however, suggest that it is positioned close to the terminal oxidase.

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