Free and membrane-bound forms of bacterial cytochrome c_4

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Cytochrome c_4 was isolated from cells of *Pseudomonas aeruginosa*, *Pseudomonas stutzeri* and *Azotobacter vinelandii*. The dihaem nature, M_r of approx. 20000 and ferrohaem spectra in the region of the α - and β -peaks define this family of cytochromes c . The behaviour of the holocytochromes in SDS was atypical, but removal of the haem groups resulted in a normal migration. In all three organisms most of the cytochrome c_4 was tightly bound to the membrane, but some free cytochrome was detected. The membrane-attached cytochrome could be extracted with butanol, and this solubilized form was then indistinguishable in properties from the free form. Denitrifying rather than aerobic growth conditions hardly affected the total cytochrome c_4 in the two pseudomonads, but there was slightly more free form and less membrane-attached form in denitrifying growth. The nature of the attachment of cytochrome c_4 to the membrane is discussed and a model is proposed for the process of solubilization.

INTRODUCTION

Many soluble bacterial cytochromes c have been characterized (Meyer & Kamen, 1982). It is proposed that these are located in the periplasmic space of Gram-negative bacteria (Wood, 1983), and function as the immediate electron donors to terminal oxidation systems, which may be either free or membrane-attached (Pettigrew & Moore, 1987). However, little attention has been paid to c -type cytochromes attached to the membrane. As has been shown in some systems (Ward *et al.*, 1983; Bosma *et al.*, 1987) and as will be apparent in the present paper, there is often a multiplicity of such cytochromes, the functions of which are not known.

Cytochrome c_4 was isolated after butanol extraction from *Azotobacter vinelandii* (Tissières, 1956; Swank & Burris, 1969) and has been reported in some pseudomonads (Ambler & Wynn, 1973; Ambler & Murray, 1973). It is a dihaem cytochrome c of M_r approx. 20000, and the amino acid sequences of the cytochromes c_4 from *A. vinelandii* and *Pseudomonas aeruginosa* are known (Ambler, 1980; Ambler *et al.*, 1984). However, in many respects the family is poorly characterized. Thus diagnostic properties have not been identified, the nature of the membrane attachment has not been studied and the association of the cytochrome with different growth conditions is not known. The present paper identifies the characteristic features of the group by studying cytochromes c_4 from four bacteria, namely *A. vinelandii*, *Ps. aeruginosa*, *Pseudomonas stutzeri* and *Alcaligenes* sp. We also investigate the presence and properties of both free and membrane-attached forms of the protein under different growth conditions as a preliminary to a functional characterization of this widespread component of bacterial respiratory chains.

MATERIALS AND METHODS

Growth of bacteria

The organisms used were *Pseudomonas aeruginosa* (N.C.T.C. 10332), *Pseudomonas stutzeri* (Stanier 224, A.T.C.C. 17591), *Azotobacter vinelandii* (strain o, A.T.C.C. 12837) and *Alcaligenes* sp. (formerly *Pseudomonas denitrificans*) (N.C.I.B. 11015).

The pseudomonads and *Alcaligenes* sp. were grown aerobically in a 10-litre microfermenter (New Brunswick) 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 at 32 °C. For anaerobic growth with nitrate, cultures were initially grown aerobically in the above medium to a cell density approx. 30 % that of the stationary phase, and then NaNO_3 (5 g/l) was added aseptically and aeration was stopped. This protocol was found to be essential for denitrifying growth of *Ps. stutzeri*, which did not survive low-level inoculation into nitrate-containing medium (see also the note in the legend to Fig. 8). *A. vinelandii* was grown with vigorous aeration in a modified Burks medium (Newton *et al.*, 1953). Cells were harvested at early stationary phase.

Purification and quantification of cytochromes

The basic strategy for quantification of free and membrane-attached cytochromes c is outlined in Scheme 1.

Frozen cells (20–40 g) were thawed, suspended in 5 vol. of 10 mM-sodium phosphate buffer, pH 7, containing 1 mM- MgCl_2 and 2 mg of DNAase (type II; Sigma Chemical Co.). The cells were disrupted by passage through a French pressure cell at 83 MPa. In the case of *Ps. aeruginosa*, a second pass through the French press

Abbreviations used: PAC_4 , PSC_4 , AVC_4 and ALC_4 , cytochromes c_4 from *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Azotobacter vinelandii* and *Alcaligenes* sp. respectively.

Scheme 1. Scheme for the separation of cytochromes c_4 and c -551 from other cytochromes c

Boxes indicate the points at which the amount of a particular cytochrome was determined. Subscripts 'f' and 'm' denote free and membrane-bound species. The lanes indicated in parentheses refer to Fig. 2. The portion of the scheme in broken lines was not carried out with *Ps. aeruginosa* extracts because the amount of cytochrome c_4 was so small.

greatly diminished the viscosity. The broken-cell suspension was centrifuged at 100 000 g for 60 min and the membrane pellet was homogenized in 10 vol. of 5 mM-Tris/HCl buffer, pH 8, and stored frozen. The French-press supernatant was acidified to pH 5 with 1 M-acetic acid and centrifuged at 10 000 g for 30 min. The acidified supernatant was neutralized with 2 M- NH_3 , desalted on Sephadex G-25 into 5 mM-Tris/HCl buffer, pH 8, and adsorbed on a DEAE-cellulose (Whatman DE52) column (2.5 cm $\times$ 3 cm) equilibrated in the same buffer. The adsorbed material was eluted with 0.2 M-sodium phosphate buffer, pH 7, to give concentrate A (proteins not attached to the membrane and soluble at pH 5).

The acidified pellet was suspended in 10 vol. of water and reneutralized with 2 M- NH_3 . This procedure solubilizes any acid-insoluble proteins such as cytochrome cd_1 and cytochrome c peroxidase, and the membrane vesicles remained as a fine suspension. The mixture was desalted on Sephadex G-25 into 5 mM-Tris/HCl buffer, pH 8, and adsorbed on a DEAE-cellulose (Whatman DE52) column (2.5 cm $\times$ 3 cm) equilibrated in the same

buffer. The small membrane vesicles were not adsorbed on this column, and the unadsorbed material was acidified to pH 5 and the precipitate collected by centrifugation as before. These aggregated membranes were added back to the original French-press pellet. The adsorbed material was eluted with 0.2 M-sodium phosphate buffer, pH 7, to give concentrate B (proteins not attached to the membrane and insoluble at pH 5).

The combined membrane suspension in 5 mM-Tris/HCl buffer, pH 8, was homogenized with an equal volume of butan-1-ol (in a top-drive blender), and the emulsion formed was centrifuged at 10 000 g for 30 min. After centrifugation the tubes contained a denatured protein pellet, a red aqueous lower phase, a lipid interface and a butanol upper layer. The butanol still present in the aqueous phase was removed by passage down Sephadex G-25 in 5 mM-Tris/HCl buffer, pH 8, and the cytochromes were adsorbed and eluted from a DEAE-cellulose (Whatman DE52) column as described above to give concentrate C (membrane proteins solubilized by butanol).

Each of the three concentrates was applied separately to a Sephadex G-75 column (2.5 cm $\times$ 85 cm) equilibrated with 20 mM-Tris/HCl buffer, pH 8, containing 100 mM-NaCl and previously calibrated with proteins of known M_r values. Fractions in the region of M_r 20000 contained cytochromes c_4 and c_5 and were combined, diluted with 10 vol. of water, reduced with 1 mM-sodium ascorbate and adsorbed on a DEAE-cellulose (Whatman DE52)

Fig. 1. Molecular-exclusion chromatography of cytochromes c from nitrate-grown *Ps. aeruginosa*

The three concentrates described in the Materials and methods section were applied separately to a Sephadex G-75 column (2.2 cm $\times$ 85 cm) equilibrated in 20 mM-Tris/HCl buffer, pH 8, containing 100 mM-NaCl, and 3.7 ml fractions were collected. Concentrate A contained cytochromes c that were not attached to the membrane and were soluble at pH 5. Concentrate B contained cytochromes c that were not attached to the membrane and were insoluble at pH 5. Concentrate C contains cytochromes c that were solubilized by butanol from the membrane. The $\bullet$ symbols denote fractions containing cytochrome cd_1 (panel b, fraction 29) and cytochrome c peroxidase (CCP) (panel b, fraction 36), which were used for the SDS/polyacrylamide-gel electrophoresis of Fig. 2 (lanes b and c respectively).

column (1.5 cm $\times$ 10 cm) equilibrated in 5 mM-Tris/HCl buffer, pH 8, containing 1 mM-ascorbate. The adsorbed cytochromes were eluted with a linear salt gradient (400 ml) of 0–100 mM-NaCl in the same buffer. This column separated cytochrome c_4 and cytochrome c_5 .

Fractions from the Sephadex G-75 column in the region of M_r 10000 contained cytochrome $c-551$ and no other haem c protein.

This standard procedure was applicable to all the extracts studied, with only minor modifications. The quantities of cytochrome $c-551$ and cytochrome c_4 were determined spectrophotometrically after the Sephadex G-75 column stage and the DE52 DEAE-cellulose chromatography respectively.

The strategy of purification and the determination of quantity is summarized in Scheme 1, and, to illustrate the method, profiles of absorbance at 410 nm for the elution pattern from the Sephadex G-75 column for nitrate-grown *Ps. aeruginosa* are shown in Fig. 1. An assessment of the cytochromes c present at particular stages was made by haem staining after SDS/polyacrylamide-gel electrophoresis (Fig. 2). In this case cytochrome cd_1 (nitrite reductase) and cytochrome c peroxidase were major components of the acid-insoluble proteins of concentrate B (Fig. 1b and lanes b and c respectively of Fig. 2). In this organism very little cytochrome c_4 was present unattached to the membrane (Figs. 1a and 1b and lane d of Fig. 2), and for this reason no DE52 DEAE-cellulose chromatography was carried out. To obtain an estimate of the amount of unattached cytochrome c_4 present, the amount of cytochrome c_4 plus cytochrome c_5 was estimated from the combined fractions in the 20000- M_r region of the Sephadex G-75 column (Fig. 1a) and the proportion of cytochrome c_4 was calculated from the relative haem staining of cytochromes

Fig. 2. SDS/polyacrylamide-gel electrophoresis of the cytochromes c from nitrate-grown *Ps. aeruginosa*

The arrow indicates the top of the gel (15% acrylamide, 0.4% methylenebisacrylamide). Lane a, concentrate A; lane b, fraction 29 from Fig. 1(b); lane c, fraction 36 from Fig. 1(b); lane d, pooled cytochrome $c_4 + c_5$ fractions from Fig. 1(a); lane e, pooled cytochrome $c-551$ fractions from Fig. 1(a); lane f, membranes; lane g, butanol extract; lane h and lane i, from the second and first peaks respectively from the DEAE-cellulose salt-gradient chromatography of the cytochrome $c_4 + c_5$ pooled fractions of Fig. 1(c); lane j, pooled cytochrome $c-551$ fractions from Fig. 1(c); lane k, purified cytochrome c_4 . Bands were detected by haem staining.

c_4 and c_5 after SDS/polyacrylamide-gel electrophoresis (Fig. 2, lane d).

Although the cytochromes c_4 isolated by this procedure were free from other cytochromes, they still contained non-cytochrome proteins. For studies on properties, complete purification of cytochromes c_4 was achieved by using a pH-gradient elution from CM-cellulose (Ambler & Wynn, 1973).

The cytochromes c_4 from *Ps. aeruginosa*, *Ps. stutzeri*, *A. vinelandii* and *Alcaligenes* sp. are below abbreviated to PAC_4 , PSC_4 , AVC_4 and $ALCC_4$ respectively.

Haem staining after SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis followed by detection of haem c by peroxidase action on 3,5,3',5'-tetramethylbenzidine was as described by Goodhew *et al.* (1986). This was used as a routine method for the assessment of cytochrome c components during purifications, only one example of which is shown (Fig. 2). Protein staining after SDS/polyacrylamide-gel electrophoresis was as described previously (Goodhew *et al.*, 1986).

Spectrophotometry and redox potentiometry

Native spectra were obtained in 0.1 M-sodium phosphate buffer, pH 7, with a Cary 219 spectrophotometer. Pyridine ferrohaemochromes were formed in 2 M-pyridine/0.15 M-NaOH by addition of $Na_2S_2O_4$, and their concentration was determined by using an absorption coefficient at 550 nm of $31.18 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Bartsch, 1971). Potentiometric redox titrations were carried out as previously described (Leitch *et al.*, 1985).

Amino acid analysis

Amino acid analysis of the purified cytochromes was carried out on a Locarte analyser after hydrolysis in 6 M-HCl *in vacuo* for 20–70 h at 105 °C and calculated relative to haem content determined by the pyridine ferrohaemochrome method. Cysteine was measured as cysteic acid after removal of the haem group (Ambler & Wynn, 1973), performic acid oxidation (Hirs, 1967) and acid hydrolysis.

Production of antisera and Western blotting

Antibodies were raised in rabbits by intramuscular injection of 100 μg of purified cytochrome c_4 in Freund's complete adjuvant, followed by two booster injections of 50 μg each after 1 week and 2 weeks. Antisera were collected after 3–4 weeks by bleeding of the ear vein. Western blotting from SDS/polyacrylamide electrophoretic gels was carried out as described in the Bio-Rad Transblot cell instruction pamphlet, with Tween 20 (0.1%) and Marvel milk powder (2%) as blocking agents, 1/100–1/500 dilutions of antisera and detection by goat anti-(rabbit IgG) antibody coupled to horse-radish peroxidase (Scottish Antibody Production Unit).

RESULTS

Properties of the purified cytochromes c_4

SDS/polyacrylamide-gel electrophoresis. The oxidized holocytochromes c_4 migrated anomalously fast in SDS/polyacrylamide-gel electrophoresis (Fig. 3). This was especially pronounced with PAC_4 , where M_r is approx.

Fig. 3. SDS/polyacrylamide-gel electrophoresis of purified cytochromes c_4

The arrow indicates the top of the gel (15% acrylamide, 0.4% methylenebisacrylamide). Lanes a–f were loaded with 0.35 nmol of purified cytochromes c_4 . 2-Mercaptoethanol, when present, was 1% (v/v). Lane a, $ALCC_4$ + 2-mercaptoethanol; lane b, $ALCC_4$; lane c, AVC_4 ; lane d, PAC_4 ; lane e, PSC_4 ; lane f, apo- PSC_4 ; lane g, PSC_4 + 2-mercaptoethanol; lane h, bovine serum albumin (M_r 66000), yeast cytochrome c peroxidase (M_r 34000), myoglobin (M_r 17000), cytochrome c (M_r 12000) and *Ps. aeruginosa* c-551 (M_r 9000). Bands were detected by protein staining.

17000 from the gel but is 19900 from the amino acid sequence (Ambler, 1980). However, the cytochrome c_4 could be converted into a slower-migrating form by reduction with 2-mercaptoethanol, which probably results in loss of the ferrous iron from the haem groups (Wood, 1981). A slower-migrating form was also obtained by removal of the haem groups. These effects are shown in Fig. 3 for PSC_4 , where the oxidized holocytochrome, 2-mercaptoethanol-reduced cytochrome and apocytochrome have M_r values of 17800, 24500 and 21400 respectively. The cytochrome c_4 -like protein from *Alcaligenes* sp. (Shidara, 1980) showed much less of an effect with 2-mercaptoethanol treatment (Fig. 3, lanes a and b).

The holocytochromes c_4 from *Ps. aeruginosa*, *Ps. stutzeri* and *A. vinelandii* were also anomalous in their multiple banding (Fig. 3). This effect was abolished by removal of the haem group (lane f) or by reduction with 2-mercaptoethanol (lane g). In the case of PAC_4 , the slower of the two bands could be converted into the faster one by heating the sample at 90 °C in SDS (Fig. 4). This conversion was more complete if the cytochrome was initially in the reduced state (notice that this was pre-reduction followed by removal of the excess reducing agent, unlike the 2-mercaptoethanol treatment described above, which leads to iron loss from the haem in the presence of SDS).

Visible absorption spectra. The α/β regions (500–580 nm) of the ferrocytochrome c_4 spectra are shown in

Fig. 4. SDS/polyacrylamide-gel electrophoresis of cytochrome c_4 from *Ps. aeruginosa*

Scans of absorbance at 690 nm in a gel (15% acrylamide, 0.4% methylenebisacrylamide) stained for haem after electrophoresis of samples of *Ps. aeruginosa* cytochrome c_4 were obtained by using a Shimadzu t.l.c. scanner (model CS930). Samples of the oxidized holocytochrome were treated in SDS either at 20 °C or at 90 °C for 4 min. Samples of the reduced holocytochrome, prepared by treatment with 1 mM-ascorbate followed by removal of excess reductant, were similarly treated with SDS either at 20 °C or at 90 °C for 4 min. The top (start) of the gel is to the left of each trace.

Fig. 5. Included also in the Figure are the pyridine ferrohaemochrome spectra at equivalent concentration (broken lines). The features of low α/β -peak ratio and low α absorbance (see also Table 1) are shared by all the cytochromes c_4 studied, and these features distinguish the group from the cytochromes c -551 (e.g. Fig. 5f) and most other cytochromes c .

Progressive reduction revealed no complexity in the α -peaks of AVc_4 and PAC_4 (Leitch *et al.*, 1985), but with PSc_4 the two haem groups are spectroscopically distinct (Fig. 5d). The ferricytochromes c_4 exhibit a near-i.r. band around 700 nm, indicative of methionine-iron co-ordination (Schechter & Saludjian, 1967), and the absorption coefficients (Table 1) suggest that this co-ordination is present for both haem groups.

Amino acid compositions. The amino acid compositions calculated on the basis of haem content by the pyridine ferrohaemochrome method are shown in Table 2. Only a value of 2 mol of haem/mol gave estimates of M_r in agreement with the results of SDS/polyacrylamide-gel electrophoresis.

By using the Cornish-Bowden (1983) approach to the

Fig. 5. Visible absorption spectra of the cytochromes c_4 in the region of the α - and β -bands

The native spectra (continuous lines) are for cytochromes c (3.2 μ M) reduced with a pinch of $Na_2S_2O_4$ in 0.1 M-sodium phosphate buffer, pH 7. The spectra of pyridine ferrohaemochromes (broken lines) are for cytochromes c at the same concentration reduced with a pinch of $Na_2S_2O_4$ in 2 M-pyridine/0.15 M-NaOH. The peak absorbance of the pyridine ferrohaemochrome spectra is 0.1 (550 nm). (a) butanol-solubilized AVc_4 ; (b) free AVc_4 ; (c) butanol-solubilized PAC_4 ; (d) butanol-solubilized PSc_4 ; (e) butanol-solubilized $ALCc_4$; (f) free *Ps. stutzeri* cytochrome c -551.

estimation of sequence similarity, PAC_4 , PSc_4 and AVc_4 pairings fall below the value of 40% divergence, thus indicating sequence homology (Fig. 6). The c_4 -like cytochrome from *Alcaligenes* sp. has a more ambivalent pattern of analysis, although it does show relatedness to AVc_4 (32% predicted sequence divergence).

Immunological cross-reactivity. Western-blot analysis of the purified cytochromes c_4 with antisera raised against AVc_4 and PSc_4 is shown in Fig. 7. Weak cross-reactivity was seen among PAC_4 , PSc_4 and AVc_4 (lanes b, c and d) but not against the cytochrome c_4 -like protein from *Alcaligenes* sp. (lanes a). This is in agreement with the tenuous sequence similarities predicted in Fig. 6.

Table 1. Spectral and potentiometric properties of the cytochromes c_4

Values in parentheses for the spectral data are millimolar absorption coefficients. Values in parentheses for the potentiometric data are the contributions of each component to the absorbance change at the α -peak. Abbreviation: N.D. not determined.

	AV c_4		PA c_4	PS c_4		ALC c_4
	Membrane-bound	Free	Membrane-bound	Membrane-bound	Free	Membrane-bound
α -Peak	550.5 (48.8)	550.5 (48.6)	550.5 (44.2)	550 (44.4)	550.5 (44.2)	551 (45.6)
β -Peak	522 (35.6)	522.5 (35.4)	522 (32.4)	521.5 (36.8)	521.5 (35.9)	522 (39.3)
α/β -Peak ratio	1.37	1.37	1.36	1.21	1.23	1.16
α -Peak/270 nm ratio	1.31	1.29	1.31	1.18	N.D.	1.25
Near-i.r. band	704 (1.7)	N.D.	702 (1.72)	701 (1.7)	N.D.	N.D.
E_m (mV)	317 (56 %)	315 (60 %)	322 (50 %)	300 (41 %)	305 (44 %)	275 (40 %)
	263 (44 %)	240 (40 %)	268 (50 %)	190 (59 %)	215 (56 %)	230 (60 %)

Table 2. Amino acid compositions of the cytochromes c_4

The experimental values were obtained as the nmol of amino acids present in a 20 h hydrolysate and calculated relative to the haem content determined by the pyridine ferrohaemochrome method. The sequences for AV c_4 and PA c_4 are from Ambler *et al.* (1984) and Ambler (1980) respectively. The values for cysteine were obtained after oxidation to cysteic acid in the apoprotein. They were calculated relative to phenylalanine. Tryptophan was estimated from the remainder absorbance at 280 nm after subtracting the known contributions of tyrosine residues and the two haem groups. An absorption coefficient of 27.7 mM⁻¹·cm⁻¹ (two haem groups) for the latter was derived from spectroscopy of well-characterized cytochromes c . Abbreviation: N.D., not determined.

Amino acid	Amino acid composition (mol/2 mol of haem)					
	AV c_4		PA c_4		PS c_4	ALC c_4
	Experimental	Sequence	Experimental	Sequence	Experimental	Experimental
Asp	22.9	21	18.4	18	19.3	19.4
Thr	7.9	9	9.7	10	5.5	5.2
Ser	7.8	10	9.6	10	8.6	6.2
Glu	18.1	18	14.8	15	19.8	20.8
Pro	10.6	11	7.1	7	9.2	13.0
Gly	22.2	25	21.3	23	27.8	17.8
Ala	28.7	29	26.9	28	23.0	29.8
Val	5.1	5	5.6	6	7.8	7.6
Met	4.4	6	4	6	4.8	4.8
Ile	5.7	6	7.5	9	4.8	7.4
Leu	12.2	14	13.6	14	14.6	13.4
Tyr	5.8	6	3.7	4	5.2	4.8
Phe	3.7	4	4.9	5	4.0	3.2
His	4.2	4	6.8	6	4.0	3.0
Lys	9.8	11	10.3	11	10.8	8.0
Arg	6.7	7	4.9	5	6.2	5.8
Cys	3.9	4	3.7	4	3.9	N.D.
Trp	0	0	0	0	(0.6)	N.D.
M_r ...		19652		19900	19900	19300

Cytochrome c_4 contents in different cells and under different growth conditions

Cytochromes c_4 from a variety of sources were quantified after a purification scheme designed to minimize the number of steps and therefore optimize the yield. In both pseudomonads under both aerobic and denitrifying conditions, and in *A. vinelandii*, cytochrome

c_4 was predominantly membrane-bound, in contrast with the almost entirely unattached cytochrome c -551 (Fig. 8). The change from aerobic to denitrifying conditions in both pseudomonads was associated with a lower content of membrane-bound cytochrome c_4 and, in the case of *Ps. stutzeri*, a corresponding increase in the unattached cytochrome c_4 was noted. The relationship between the

Fig. 6. Prediction of the degree of sequence identity between the cytochromes c_4 .

The amino acid compositions of the cytochromes c_4 were compared by using the statistical treatment of Cornish-Bowden (1983). Values indicate predicted percentage sequence divergence in paired comparisons. Values below 40% indicate a high probability of sequence relationship (Cornish-Bowden, 1983). The value of 21 in parentheses for the AVc_4 - PAC_4 comparison is the actual percentage sequence divergence calculated from the known amino acid sequences (Ambler, 1980; Ambler *et al.*, 1984).

Fig. 7. Western-blot analysis of cytochromes c_4 .

Oxidized holocytochromes c_4 (25 pmol) after SDS/polyacrylamide-gel electrophoresis (15% acrylamide, 0.4% methylenebisacrylamide) were transferred to nitrocellulose and probed by using antisera raised either against PSc_4 (at 1/500 dilution) or against AVc_4 (at 1/100 dilution). For the former case, the lanes contained: lane a, $ALCc_4$; lane b, AVc_4 ; lane c, PAC_4 ; lane d, butanol-solubilized PSc_4 ; lane e, free PSc_4 . For the anti- AVc_4 experiment the lanes contained: lane a, $ALCc_4$; lane b, butanol-solubilized AVc_4 ; lane c, free AVc_4 ; lane d, PAC_4 ; lane e, PSc_4 . Staining was by a goat anti-(rabbit IgG) antibody coupled to peroxidase in the presence of chloronaphthol and H_2O_2 .

Fig. 8. Contents of cytochromes c_4 and $c-551$ under different growth conditions and in different bacteria

The amounts of cytochrome $c-551$ and cytochrome c_4 were determined spectrophotometrically in the final pooled fractions indicated in Scheme 1, and expressed relative to 100 g wet wt. of cells. *Ps. aeruginosa* (PA) and *Ps. stutzeri* (PS) were grown under aerobic (O) and denitrifying (N) conditions. In the latter mode, because of the high inoculum (30%) of aerobic cells, some proteins associated with aerobic growth may persist in the final stationary denitrifying culture. However, one such protein, a cytochrome c of M_r 32000, was found to be present in the denitrifying culture at only 13% the amount found in aerobic cells (Goodhew *et al.*, 1986), indicating that the problem is a small one. *A. vinelandii* can be grown only aerobically. Amounts of free cytochromes are indicated by bars above the horizontal plane; membrane-attached cytochromes are shown by bars below this plane. The broken line for cytochrome $c-551$ in nitrate-grown *Ps. aeruginosa* indicates that the amount of this cytochrome apparently attached to the membrane can be accounted for by free cytochrome in adventitious liquid.

membrane-bound and the unattached cytochromes c_4 is explored further in the following section.

Presence of membrane-bound and free forms of cytochrome c_4

In the cases of *A. vinelandii* and *Ps. stutzeri*, cytochrome c_4 was purified from both the supernatant and pellet of the French-press broken-cell suspensions, indicating the presence of both free and membrane-bound forms. The free cytochrome c_4 in the supernatant was not due to small membrane vesicles, because these could be removed by precipitation at pH 5 and their bound cytochromes c were not adsorbed on DEAE-cellulose. However, because of these vesicles, the presence of free cytochrome c_4 cannot be directly demonstrated in French-press supernatants by gel electrophoresis. Free cytochrome c_4 can be directly demonstrated in supernatants prepared by more gentle methods of cell disruption. For example, cytochromes c_4 and $c-551$ were released by treatment of *A. vinelandii* with lysozyme and EDTA (results not shown). The remaining washed membranes still contained the major part of the cytochrome c_4 but almost no cytochrome $c-551$. There was no trace of the distinctive

membrane-bound cytochromes c in the supernatants, indicating that these were free of small vesicles. Further washing of the membranes released no more cytochrome, indicating that the free and membrane-bound cytochromes c_4 are not in equilibrium.

In contrast, washing aerobically grown *Ps. aeruginosa* cells by the same method released only trace cytochrome c_4 , although most of the cytochrome c -551 and cytochrome c peroxidase were released (results not shown). This is consistent with the results of the purification studies, which found only very small amounts of free cytochrome c_4 in this organism (Fig. 8).

Comparison of membrane-bound and free forms of cytochrome c_4

The properties of free cytochrome c_4 and cytochrome c_4 released by butanol treatment of membranes were compared for the cases of *Ps. stutzeri* and *A. vinelandii*. Insufficient free cytochrome c_4 could be purified from *Ps. aeruginosa* to allow a comparison.

The free and membrane-bound forms were indistinguishable in their α/β spectra (Figs. 5a and 5b for AVc_4). Potentiometric redox titrations of cytochromes c_4 reveal the presence of two haem components with different midpoint potentials and different relative contributions to the spectrum (Leitch *et al.*, 1985). Such analyses gave very similar patterns of redox behaviour for the free and butanol-solubilized forms of both PSc_4 and AVc_4 (Table 1).

Free and butanol-solubilized forms of PSc_4 and AVc_4 were indistinguishable by SDS/polyacrylamide-gel electrophoresis with haem or protein staining (results not shown). The two forms also gave similar patterns and intensities of reaction in Western-blot analysis with antisera raised against the butanol-solubilized forms (Fig. 7).

Thus, by the criteria that we have used, the butanol-solubilized and free forms of cytochrome c_4 are indistinguishable in *A. vinelandii* and *Ps. stutzeri*.

DISCUSSION

SDS/polyacrylamide-gel electrophoresis

Cytochromes c_4 behaved anomalously on SDS/polyacrylamide-gel electrophoresis both in their multiple banding patterns and in their relative migration rate. We propose that, even in SDS, the holoprotein retains some compact structure, leading to a faster migration rate in the gel. This compact structure is disrupted by removal of the iron atoms or of the haem groups, allowing complete unfolding of the protein and a lower migration rate on SDS/polyacrylamide-gel electrophoresis. The multiple banding pattern was also abolished by iron or haem removal, and was influenced by heating. This must therefore be a consequence of partially denatured states of the protein rather than heterogeneity in the preparation.

Analysis of cytochrome c_4 contents by purification

Definitive studies on the effect of growth conditions on the contents of particular bacterial cytochromes c are rare. Two basic approaches are possible, the first being to examine the whole system without fractionation and the second being to purify the individual components. The first approach includes methods such as gel electro-

phoresis, redox potentiometry or deconvolution of complex spectra that can suffer from the problem of secure identification. Other proteins may co-migrate on gels or have similar spectra or redox potentials.

The approach that we have used here is that of purification with the strategy of a minimum number of steps to separate cytochrome c_4 from other cytochromes so as to minimize losses. An important feature of our study is the distinction between membrane-bound and unattached forms of cytochromes. We chose a method (French press) that assured complete cell breakage. Although the membrane vesicles produced could in principle contain trapped but unattached cytochromes, we have, in practice, not found this to be a problem with the organisms studied because essentially no cytochrome c -551 was found in membrane pellets. Thus all free cytochromes were released into the French-press supernatant.

With such a harsh method of breakage, the supernatant is contaminated with small membrane vesicles. However, this does not lead to an overestimate of unattached cytochrome because that associated with vesicles does not behave in the same way during purification as the protein in the free state. The vesicles do represent losses to the membrane, and this was counteracted by acidification of supernatants and return of the sediments to the membranes.

Although gentler methods such as lysozyme/EDTA washing overcome this problem of small vesicles, we have found it difficult to obtain reproducible quantitative release of soluble cytochromes. Thus in such experiments the membrane pellet is contaminated by soluble but trapped cytochromes c .

Occurrence of cytochrome c_4 in different bacteria

From the data presented here, cytochromes c_4 are predominantly membrane-attached proteins with M_r approx. 20000, two high-potential haem groups and a ferohaem α -peak near 550 nm of low absorptivity. According to these criteria, the 'cytochrome c -551' isolated by Shidara (1980) from the *Alcaligenes* species originally called '*Pseudomonas denitrificans*' can be described as a cytochrome c_4 . This is in spite of a lack of convincing similarity in amino acid composition (Fig. 6) and an absence of immunological cross-reaction (Fig. 7). Cytochrome c_4 is also present in the strain of *Ps. stutzeri* studied by Kodama & Shidara (1969) and called 'cytochrome c -552(II)'. *Pseudomonas perfectomarinus* contains a cytochrome c -552 with some of the characteristics of cytochrome c_4 but which is different in spectra and in the very low midpoint potential (-180 mV) of one of the two haem groups (Liu *et al.*, 1981). Clarification of the relationship of this cytochrome to known groups awaits sequence determination, but statistical analysis does predict a low-level sequence divergence (37%) from AVc_4 . *Ps. perfectomarinus* also contains a 'cytochrome c -551' with low α/β -peak ratio (Liu *et al.*, 1983) that may be a more typical cytochrome c_4 . In general, very few studies of bacterial membrane cytochromes c have been performed, and cytochrome c_4 may be a more widespread protein than is apparent from its known presence in the pseudomonads, *Alcaligenes* and *A. vinelandii*. For example, Berry & Trumpower (1985) found a membrane-bound c -type cytochrome of M_r 22000 in *Paracoccus denitrificans* that requires further characterization.

Nature of the attachment of cytochrome c_4 to the membrane

Most of the cytochrome c_4 in the organisms that we have studied was firmly attached to the membrane and cannot be removed by washing with aqueous salt solutions or chelating agents. Disruption of the lipoprotein membrane by butanol does release cytochrome c_4 . This could be by breakage of cytochrome-lipid interactions or of cytochrome-protein interactions. We favour the latter, for the following reasons. Firstly, cytochrome c_4 is not a particularly hydrophobic protein [AV c_4 and PA c_4 have polarity indices (Capaldi & Vanderkooi, 1972) of 42 and 41% respectively, rather higher than those of most proteins that are integral within the bilayer]. Secondly, significant portions of AV c_4 and PS c_4 were free, which would not be expected for a protein that partitions into the lipid phase. There was no equilibrium partition because no cytochrome c_4 was removed from membranes by further washing.

Thus we propose that cytochrome c_4 is held to the membrane by hydrophobic interactions with a limited number of specific protein sites. Butanol denatures these sites and releases monomeric cytochrome c_4 indistinguishable in properties from cytochrome c_4 that had not been bound to the membrane. Thus the hydrophobic surfaces by which cytochrome c_4 interacts with its membrane protein site may come together in the solubilized two-domain molecule.

Role of cytochrome c_4 in bacterial respiration

In both *Ps. aeruginosa* and *Ps. stutzeri* slightly more cytochrome c_4 was present on the membrane in aerobic compared with nitrate-grown cells (Fig. 8). This, and the fact that cytochrome c_4 is found in the strict aerobe *A. vinelandii*, may indicate that it is involved in aerobic respiration. We propose that a relatively constant amount of cytochrome c_4 is synthesized regardless of growth conditions but that there are more specific sites available for binding to the membrane in the aerobic cells than in the nitrate-grown cells. Once the sites are saturated, cytochrome c_4 appears as a free component. These sites may be the cytochrome o of the cytochrome co -type oxidase, since Jurtshuk *et al.* (1981) proposed that cytochrome c_4 was the c -type component of this enzyme. However, we note that this is a proposal based on the very indiscriminating criterion of membrane spectra, and requires more secure identification of the c -type cytochrome.

We thank L. Sawyer, D. Hunter and C. Goodhew for helpful comments. The work was supported by Science and

Engineering Research Council Grant GRD45161 and a research studentship for K. R. B. Some of the results with the *Alcaligenes* sp. were obtained by L. Henderson.

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Received 7 October 1987/16 November 1987; accepted 4 February 1988