Cytochrome c_{550} from *Pseudomonas aeruginosa*

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In cells of *Pseudomonas aeruginosa* A.T.C.C. 17933 grown on ethanol the synthesis of a soluble *c*-type cytochrome, together with quinoprotein ethanol dehydrogenase, is induced. The cytochrome, with an α -absorption band at 550 nm, was purified to homogeneity. The molecular mass of the monomeric protein is 15 kDa, the pI is 4.8, and it contains one haem prosthetic group. The midpoint potential of the autoxidizable, but not auto-reducible, cytochrome is 280 mV. Cytochrome c_{550} mediates electron transfer between quinoprotein ethanol dehydrogenase and ferricyanide. In a system composed of membrane

particles with *NN'NN'*-tetramethyl-*p*-phenylenediamine oxidase activity and quinoprotein ethanol dehydrogenase, oxygen consumption is only observed in the presence of cytochrome c_{550} . This indicates the participation of the cytochrome in the electron-transport chain linked to quinoprotein ethanol dehydrogenase in *P. aeruginosa*. The electron transport from ethanol dehydrogenase to oxygen is inhibited by myxothiazol and antimycin, indicating that a cytochrome bc_1 -like complex is involved.

INTRODUCTION

Pseudomonas aeruginosa produces a number of haem-containing proteins. Besides more complex enzymes like cytochrome *c* peroxidase (Singh and Wharton, 1973), cytochrome *cd*, a soluble nitrite reductase (Yamanaka, 1964) and cytochrome *co*, a membrane-bound oxidase (Matsushita et al., 1982a; Yang, 1982), some low-molecular-mass *c*-type cytochromes have been described (Horio, 1958; Wood and Willey, 1980; Matsushita et al., 1982b; Pettigrew and Brown, 1988). When grown on ethanol as sole source of carbon and energy, several members of the genus *Pseudomonas* produce quinoprotein ethanol dehydrogenases (QEDH) (Groen et al., 1984; Rupp and Görisch 1988; Görisch and Rupp, 1989).

The dimeric QEDH of *P. aeruginosa* A.T.C.C. 17933 (Rupp and Görisch, 1988), despite its altered substrate specificity, resembles in its enzymic properties quinoprotein methanol dehydrogenase (QMDH; EC 1.1.99.8) of Gram-negative methylotrophic bacteria. The natural electron acceptor of QMDH is cytochrome c_1 , a *c*-type cytochrome of low pI (Beardmore-Gray et al., 1983). Recently an NAD⁺-dependent MDH was found in Gram-positive methylotrophic *Bacillus* species (Arfman et al., 1989).

From acetic acid bacteria and *Pseudomonas testosteroni* ethanol dehydrogenases are isolated as quinohaemoproteins (Adachi et al., 1978a; Groen et al., 1986). The dimeric QEDH from *P. aeruginosa* A.T.C.C. 17933 contains no haem. However, when the organism is grown on ethanol as sole carbon and energy source, a soluble cytochrome c_{550} is also induced, in addition to QEDH.

EXPERIMENTAL

Chemicals

Phenazine methosulphate (PMS) and 2,6-dichlorophenol-indophenol (DCPIP) were obtained from Fluka (Buchs, Switzerland);

Tris, cytochrome *c* from horse heart (type VI) and cytochrome c_{551} from *P. aeruginosa* were from Sigma (München, Germany). Acrylamide and bisacrylamide were purchased from Serva (Heidelberg, Germany) and DEAE-Sephacel, Sephadex G-50, Chelating-Sepharose, Sephadex G-25 (prepacked PD 10 column), low-molecular-mass (LMM) marker kit, Ampholine PAG-plates and Immobiline dry plates, broad-pI and low-pI kit were from Pharmacia/LKB (Uppsala, Sweden). All other chemicals were obtained from Merck (Darmstadt, Germany) of the highest purity available.

QEDH

Homogeneous QEDH was prepared as described previously (Rupp and Görisch 1988).

Bacterial strain and culture conditions

P. aeruginosa A.T.C.C. 17933 was grown aerobically at 37 °C in 2 litre Erlenmeyer flasks in 500 ml of a mineral medium supplemented with 0.5% (v/v) ethanol (Rupp and Görisch, 1988). As alternative carbon sources we used glucose (0.5%, w/v), or succinate as disodium salt (0.5%, w/v). The complex medium used contained 5 g of yeast extract, 5 g of peptone, 3 g of meat extract and 5 g of NaCl/litre. The batch cultures were shaken at 175 rev./min.

Cells were harvested at the end of the exponential growth phase by centrifugation, washed in 20 mM potassium phosphate buffer (PPB), pH 7.2, and stored at –20 °C.

Enzymic activities of the membrane particles

NN'NN'-Tetramethyl-*p*-phenylenediamine (TMPD) oxidase activity of membranes was determined polarographically in the presence of ascorbate with a Clark-type oxygen electrode (DW 1; Bachofer G.m.b.H., Reutlingen, Germany) as described by Rafael (1986).

Abbreviations used: BTP, Bistris propane; DCPIP, 2,6-dichlorophenol-indophenol; i.e.f., isoelectric focusing; LMM, low molecular mass; PAG, polyacrylamide gel; %T, total acrylamide concentration; %C, bisacrylamide; PPB, potassium phosphate buffer; PMS, phenazine methosulphate; QEDH, quinoprotein ethanol dehydrogenase; (Q)MDH, (quinoprotein) methanol dehydrogenase; TMBZ, 3,3',5,5'-tetramethylbenzidine; TMPD, *NN'NN'*-tetramethyl-*p*-phenylenediamine.

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NADH oxidase and succinate oxidase activities of membrane particles were measured polarographically in 100 mM PPB, pH 7.0, with 1–3 mg of membrane protein in a total volume of 1 ml. The reaction was started by adding substrate to a final concentration of 1 mM. The inhibitors antimycin A and myxothiazol were added in methanolic solution to the reaction chamber with a microsyringe. Methanol is not oxidized by the membrane preparation.

Measurement of QEDH activity

PMS/DCPIP assay

The test was performed as described previously (Rupp and Görisch, 1988).

Ferricyanide reductase assay

$K_3[Fe(CN)_6]$ reductase activity of the system QEDH/cytochrome c_{550} was measured in 10 mM Mops, pH 7.0, with 0.45 μ M QEDH, 2 μ M cytochrome c_{550} and 1 mM $K_3[Fe(CN)_6]$ in a total volume of 1 ml. The reaction was started by adding ethanol to a final concentration of 1 mM. The absorbance decrease was monitored at 420 nm. The absorption coefficient (ϵ_{420}) of $K_3[Fe(CN)_6]$ is 1.0 mM⁻¹·cm⁻¹ (Hommel and Kleber, 1984).

Reconstitution of an ethanol-oxidizing system

A Clark-type oxygen electrode was used to determine oxygen consumption rates of the ethanol-oxidizing system. Membrane vesicles (2.2 mg/ml), QEDH (0.54 μ M) and cytochrome c_{550} (6.35 μ M) were mixed in a total volume of 0.64 ml of 20 mM BTP, pH 7.0. The reaction was started by adding 20 μ l of an ethanol solution to a concentration of 200 μ M.

Spectrophotometry

Spectra were recorded with a Perkin-Elmer lambda 5 spectrophotometer at 25 °C.

Electrophoresis

SDS/PAGE was performed as described by Schägger and von Jagow (1987). Separating gels [16.5% total acrylamide (% T)/bisacrylamide (3% C)] with spacer gel (10% T/3% C) and stacking gel (4% T/3% C) were used. Proteins were stained with Coomassie Brilliant Blue G-250. Haem-containing proteins were detected by peroxidase staining with 3,3',5,5'-tetramethylbenzidine (3,3',5,5'-TMBZ) by the method of Thomas et al. (1976), modified as described by Goodhew et al. (1986).

Isoelectric focusing (i.e.f.)

I.e.f. was performed on Ampholine PAG-plates, pH 3.5–7.5, and Immobiline Dry plates pH 4.0–7.0 according to the manufacturer's instructions. For calibration broad-pI and low-pI kits were used.

Molecular-mass determinations

The molecular mass of the native protein was determined on a Sephadex G-75 column (2.6 cm × 75 cm) by the procedure of

Andrews (1964). The following marker proteins were used: horse heart cytochrome (molecular mass 12.4 kDa), α -chymotrypsinogen, (25 kDa) and haemoglobin (64.5 kDa).

The molecular mass of denatured cytochrome c_{550} was determined by SDS/PAGE. The LMM kit and, in addition, cytochrome c from horse heart (12.4 kDa) and cytochrome c_{551} from *P. aeruginosa* (9 kDa) were used as standard.

Protein determination

Protein was measured by the method of Sedmak and Grossberg (1977), with cytochrome c from horse heart as standard.

Detection of cytochromes

Cytochromes in the column eluates were detected by means of their absorption at 410 nm. They were quantified by the method of Rieske (1967) using a $\Delta\epsilon_{550}$ value of ($\Delta\epsilon_{550}$) 19.1 mM⁻¹·cm⁻¹.

Cytochrome concentrations in membranes were determined from the difference spectrum of dithionite-reduced and ammonium persulphate-oxidized samples using the following absorption coefficients and wavelength pairs (Azzi and Gennis, 1986): haem a , 610–630 nm, $\Delta\epsilon$ 24.0 mM⁻¹·cm⁻¹; haem b , 560–575 nm, $\Delta\epsilon$ 23.4 mM⁻¹·cm⁻¹; haem c , 551–542 nm, $\Delta\epsilon$ 18.7 mM⁻¹·cm⁻¹.

Haem determination

Extraction with butan-2-one/HCl was performed as described by Teale (1959). Pyridine ferrohaemochromogene was prepared as described by Paul et al. (1953). The haem content was calculated from the difference spectrum of the reduced-minus-oxidized pyridine haemochromogene complex, using a $\Delta\epsilon_{549}$ value of 19.1 mM⁻¹·cm⁻¹.

Metal analysis

Samples of purified protein (7.2 and 14.4 nmol) were extensively dialysed against twice-distilled water. The iron content was determined with an energy-dispersive X-ray fluorescence spectrometer (System 77; Finnigan International, Sunnyvale, CA, U.S.A.).

Autoreduction

Cytochrome c_{550} was tested for the effect of pH-dependent autoreduction as described by O'Keeffe and Anthony (1980). The following 25 mM buffers were used: ammonium acetate, pH 4.0 and 5.0; PPB, pH 6.0 and 7.0; Tris/HCl, pH 8.0 and 9.0; and glycine/NaOH, pH 10.0 and 11.0.

Autoxidation

A sample of cytochrome c_{550} (0.1 mg/ml) in 66 mM PPB, pH 7.0, was reduced by adding a few crystals of $Na_2S_2O_4$. Excess reductant was removed by desalting on a Sephadex G-25 column. The sample was exposed to air and the absorbance change at 550 nm was monitored.

Potentiometric titrations

Cytochrome c_{550} (1 μ M) was reduced and oxidized under oxygen-free nitrogen in 0.3 M PPB, pH 7.0, with DCPIP (350 μ M) as mediator. Reductive titrations were performed by stepwise addition of a 30 mM $Na_2S_2O_4$ solution. In oxidative titrations

the solution was reduced by adding $\text{Na}_2\text{S}_2\text{O}_4$ in adequate concentration, before the stepwise oxidation with a 20 mM solution of $\text{K}_3[\text{Fe}(\text{CN})_6]$. The midpoint potential was calculated by the method of Dutton (1978).

Reaction with CO

A sample of cytochrome c_{550} (6.9 μM) was reduced by adding a small amount of ascorbate. CO was bubbled through the sample cuvette for 60 s and nitrogen through the reference cuvette. Spectra were recorded every 120 s. After 8 and 22 min the exposure to CO was repeated and the spectra recorded after 14 and 45 min. CO-binding haem components in membrane particles were quantified by measuring the reduced-plus-CO-minus-reduced spectrum of a sample with 1.6 mg of protein/ml after 45 min. An absorption coefficient ($\Delta\epsilon$) of $170 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 407–428 nm was used (Wood, 1984).

Purification of cytochrome c_{550} and isolation of membrane particles

Wet cells of *P. aeruginosa* (30 g) suspended in 30 ml of 50 mM Tris/HCl, pH 7.9, were disrupted by sonication with a Branson J 17 A ultrasonic disintegrator for 15 min. The solution was centrifuged at 6000 g for 20 min to remove intact cells. Membrane particles were collected by ultracentrifugation for 1 h at 60000 g . The resulting pellet was resuspended in 50 mM PPB, pH 7.0, with 10 mM MgCl_2 , and centrifuged again. The washed membrane pellet was suspended in the same buffer to a final protein concentration of 34 mg/ml. The membrane fraction was stored at -20°C . The supernatant containing soluble proteins was used for further purification of the cytochrome.

The protein concentration was adjusted to 20 mg/ml by dilution before adding solid $(\text{NH}_4)_2\text{SO}_4$ to give 40% saturation. The precipitate was collected at 39000 g and 5°C in a refrigerated centrifuge for 30 min. The pellet was discarded and the supernatant was brought to 75% saturation with $(\text{NH}_4)_2\text{SO}_4$. After repeating the centrifugation step the pellet was resuspended in 5 mM Tris/HCl, pH 8.0, and dialysed overnight against the same buffer. The dialysis residue was applied on to a DEAE-Sephacel column (2.6 cm $\times$ 15 cm) equilibrated with 5 mM Tris/HCl, pH 8.0. Cytochrome was bound as a red layer at the top of the column. Elution was performed with a linear gradient from 0 to 0.7 M NaCl in the same buffer. Fractions containing cytochrome were pooled, concentrated in an Amicon ultra-filtration chamber with a YM 10 membrane, and loaded on to a Sephadex G-50 column (2.6 cm $\times$ 100 cm) equilibrated with 20 mM Tris/HCl/100 mM NaCl, pH 8.0. After elution the cytochrome fractions were applied on to a Cu^{2+} -loaded Chelating-Sepharose column (1 cm $\times$ 6 cm) equilibrated with 66 mM PPB/0.1 M NaCl, pH 7.0. Bound cytochrome was eluted with a linear gradient of 0–2 M NH_4Cl . After desalting with a Sephadex G-25 column, final purification was achieved by repeating the anion-exchange step on a Mono Q HR 5/5 f.p.l.c. column using the same buffer and elution conditions as with DEAE-Sephacel chromatography.

RESULTS

Growth conditions

Cytochrome c_{550} was found in *P. aeruginosa* when the organism was grown on ethanol. In addition, a cytochrome c with an α -band at 554 nm was present. This minor component amounted to less than 5% of the soluble cytochrome c content. Cells grown

on glucose, succinate or nutrient broth and subjected to the same purification procedure contained no cytochrome c_{550} , but did contain other soluble cytochromes such as c_{551} , c_4 or cytochrome peroxidase. These cytochromes were easily distinguished from cytochrome c_{550} by their molecular mass, spectral properties and their behaviour during ion-exchange chromatography.

Purification procedure

By using the purification procedure described in the Experimental section, the yield of homogeneous cytochrome c_{550} was 4.2 mg from 30 g of wet cells (Table 1).

Spectrum

The absorption spectrum of the purified cytochrome is shown in Figure 1. The spectrum with an α -band at 550 nm is typical for c -type cytochromes. A shoulder at 695 nm in the oxidized form suggests the presence of methionine as sixth ligand of the haem iron.

Molecular mass

The molecular mass of the native cytochrome was determined by gel filtration and that of the denatured form by SDS/PAGE. With both methods a molecular mass of 15 kDa was found, therefore the native form is a monomer.

Table 1. Purification of cytochrome c_{550}

	Protein (mg)	Cytochrome c (nmol)	Specific concentration (nmol of haem/mg of protein)	Yield (%)	Purification (fold)
Crude extract*	2830.0	2972	1.05	100	1.0
$(\text{NH}_4)_2\text{SO}_4$ precipitation	1230.0	1460	1.18	49	1.1
DEAE-Sephacel	270.0	1112	4.10	37	3.9
Sephadex G-50	18.3	751	41.00	25	39
Chelating-Sepharose	7.2	452	62.70	15	59.7
Mono Q	4.2	291	69.20	10	65.9

* Starting with 30 g of wet cells.

Figure 1 Absorption spectrum of cytochrome c_{550}

The concentration of cytochrome was 0.1 mg/ml in 50 mM PPB, pH 7.0. —, reduced form; ----, oxidized form. The trace between 600 and 800 nm relates to the short right-hand scale.

pI

In the Ampholine system two red bands with pI values of 4.7 and 4.8 were found. However, a single red band with a pI of 4.8 was observed in the Immobiline system. This band corresponded to a single protein band after Coomassie Brilliant Blue staining.

Haem content

With the pyridine haemochromogene method, a haem content of 0.92 mol/mol of cytochrome was determined; 0.78 mol of iron/mol of protein were calculated by using a molecular mass of 15 kDa. The haem was not extracted with butan-2-one/HCl and the difference spectrum of the reduced minus oxidized pyridine haemochromogene complex showed absorption maxima at 549, 520 and 413 nm, which are typical for *c*-type cytochromes.

Reactivity with CO

Cytochrome c_{550} reacted slowly with CO. Assuming a similar absorbance of the CO complex and the reduced form of the cytochrome (Wood, 1984), the amount of CO binding was about 18% after 14 min and 24% after 45 min. The reduced-plus-CO-minus-reduced difference spectrum of cytochrome c_{550} showed troughs at 551, 522 and 423 nm.

Midpoint potential

Cytochrome c_{550} showed a midpoint potential of 280 mV. No autoreduction occurred at alkaline pH values. It was slowly oxidized by air and was isolated in the oxidized form. Although reducing agents were omitted during the purification procedure, the cytochrome was totally reduced during the first purification steps. A change from the reduced to the oxidized state occurred during metal-chelate affinity chromatography.

Cytochrome c_{550} -mediated ferricyanide reduction

QEDH showed the effect of endogeneous reduction in the artificial-dye test with PMS/DCPIP at pH 9 as described for QMDH (Anthony and Zatman, 1964). Thereby the enzyme was not inactivated. Endogeneous reduction was also observed with cytochrome c_{550} as electron acceptor at pH 7. When the reaction ceased, ethanol was added as substrate. No ethanol-dependent reduction of excess cytochrome c_{550} could be observed. When ferricyanide was added to this system, $[\text{Fe}(\text{CN})_6]^{3-}$ was reduced (Table 2). However, QEDH from *P. aeruginosa* did not reduce ferricyanide directly at pH 7. Ammonia, an activator of QEDH in the artificial-dye test with PMS/DCPIP at pH 9, did not stimulate the electron transfer to $[\text{Fe}(\text{CN})_6]^{3-}$ via cytochrome c_{550} at pH 7.

Characterization of membranes

The reduced-plus-CO-minus-reduced spectrum of membrane particles showed troughs at 428 and 552 nm, with a shoulder at 558 nm, which indicated the presence of an *o*-type oxidase, composed of haem b and c; 0.37 nmol of haem b and 1.02 nmol of haem c were found per mg of membrane protein. The amount of CO-binding haem was about 28% of the total haem b and c content, that is, 0.39 nmol/mg of protein. Haem a, which is reported to be also present in *P. aeruginosa* (Calcott et al., 1975) was found only in trace amounts, with 0.03 nmol/mg of mem-

Table 2. QEDH activities in different assay systems

Specific activities are expressed per mg of QEDH.

System	Units	Specific activity	
		pH 7	pH 9
QEDH (PMS/DCPIP assay)	nmol of DCPIP $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$	50	5930
	nmol of O $_2$ $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$	25	2965
QEDH (cytochrome c_{550} /ferricyanide assay)	nmol of Fe $^{3+}$ $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$	2380	n.d.†
	nmol of O $_2$ $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$	595	n.d.
Reconstituted system* (QEDH, cytochrome c_{550} and membrane particles)	nmol of O $_2$ $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$	58	0

* With one of the reaction components omitted, either QEDH, cytochrome c_{550} or membrane particles, no oxygen was consumed.

† n.d., not determined.

Table 3. Oxidase activities of membrane particles

Specific activities are expressed per mg of membrane protein.

Substrate	Oxygen consumption (nmol of O $_2$ $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$)	Residual activity (%) in the presence of inhibitors (40 nmol/mg)	
		Antimycin	Myxothiazol
Ethanol*	1.7	10	64
NADH	16.3	79	65
Succinate	2.9	6	7
Ferrocycytochrome c_{550}	2.5	n.d.†	n.d.
TMPD/ascorbate	360.0	100	100

* In the presence of QEDH and cytochrome c_{550} as described in the Experimental section, with QEDH or cytochrome c_{550} omitted, no oxygen was consumed.

† n.d., not determined.

brane protein. TMPD oxidase activity of the membrane was not stimulated nor inhibited by cytochrome c_{550} when included in the assay in concentrations from 10.5 to 83.9 nmol of cytochrome c_{550} /mg of membrane protein. Ferrocycytochrome c_{550} however, was oxidized by the membrane particles (Table 3). With succinate or NADH as substrate, membrane particles consumed oxygen. Both oxidase activities were sensitive to the respiratory-chain inhibitors antimycin and myxothiazol, which act in mitochondria at the level of the ubiquinol:cytochrome *c* oxidoreductase complex (Table 3).

Ethanol-dependent respiratory activity in the presence of membrane particles

Ethanol-dependent oxygen consumption was observed in a system containing homogeneous QEDH, homogeneous cytochrome c_{550} and membrane particles. No oxygen consumption was observed if one of the components (QEDH, cytochrome c_{550} or membranes) was omitted from the reaction mixture. The system was active at pH 7, but not at pH 9. Ammonia, an activator of QEDH which is necessary in the artificial-dye test, was not needed for, nor did it stimulate, this ethanol-oxidizing activity at pH 7. Table 2 compares the activities of QEDH in the

different assay systems. The ethanol oxidase system was also sensitive to antimycin and myxothiazol (Table 3).

DISCUSSION

P. aeruginosa and *P. putida* are able to grow on ethanol as sole source of carbon and energy. Under these conditions a dye-linked QEDH is induced (Rupp and Görisch, 1988; Görisch and Rupp, 1989). In addition to the quinoprotein oxidoreductase we find a special low-molecular-mass cytochrome in *P. aeruginosa*. This cytochrome c_{550} is not produced on other carbon sources. Several low-molecular-mass *c*-type cytochromes have been described in *P. aeruginosa* so far (Table 4). They are expressed under a variety of growth conditions, but the physiological function of most of them remains obscure. Cytochrome c_{550} , which is induced together with QEDH, is readily distinguished by its molecular mass from the other long-known soluble cytochrome c_{551} (Horio, 1958).

With its low pI value of 4.8, cytochrome c_{550} resembles the soluble cytochrome c_L , which transfers electrons from quinoprotein methanol dehydrogenases via cytochrome c_H to the terminal oxidase in methylotrophic bacteria (Beardmore-Gray et al., 1983). Recently cytochrome c_L from *Methylobacterium extorquens* AM1 has been sequenced (Nunn and Anthony, 1988). The sequence shows no relation to one of the cytochrome classes I–V established by Ambler (Moore and Pettigrew, 1990). In acetic acid bacteria the tightly membrane-bound EDH complex is composed of a dehydrogenase subunit, a cytochrome *c* subunit, and at least two other protein components (Adachi et al. 1978a,b). Ferricyanide acts *in vitro* as electron acceptor, but the physiological mediator between the enzyme complex and the terminal oxidase is ubiquinone (Matsushita et al., 1992).

QEDH from *P. aeruginosa* shows a sharp activity optimum at pH 9 and depends on ammonia as activator in the artificial-dye-linked assay with PMS/DCPIP (Rupp and Görisch, 1988). However, in a system with homogeneous QEDH, homogeneous cytochrome c_{550} and a membrane preparation of *P. aeruginosa*, which contains a TMPD-dependent oxidase activity, electron transport from ethanol to O_2 is observed at the more physiological conditions of pH 7. Ammonia is not needed as activator, and the system is inactive at pH 9. Omission of cytochrome c_{550} interrupts the electron transport. Membrane particles prepared from *P. aeruginosa* grown on ethanol contain an *o*-type cytochrome oxidase. This finding is in agreement with results reported by Matsushita et al. 1982a, who purified a cytochrome *co* from *P. aeruginosa*.

The *o*-type cytochrome oxidase does oxidize ferrocyclochrome c_{550} , but its activity with TMPD as substrate is not stimulated by the soluble cytochrome. However, QEDH uses ferricyanide as electron acceptor only in the presence of cytochrome c_{550} . Together with the observation that no other soluble cytochromes have been found in *P. aeruginosa* grown on ethanol, the findings above indicate that cytochrome c_{550} accepts electrons from QEDH and passes the reduction equivalents on to ubiquinone, or some other membrane component, which is then oxidized by way of other membrane cytochromes and the *o*-type oxidase.

The membrane particles show oxidase activity with NADH or succinate as substrate, and both reactions are inhibited by antimycin and myxothiazol. The ethanol oxidase activity of the reconstituted system with QEDH, cytochrome c_{550} and membrane particles is also inhibited by antimycin and myxothiazol (Table 3), which indicates the operation of a cytochrome bc_1 -like complex (von Jagow and Link 1986). From the total haem b and c content of the membrane, about 28 % react with CO.

The activity of the reconstituted ethanol-oxidizing system is relatively low when compared with QEDH activity in the cytochrome c_{550} /ferricyanide assay at pH 7. It is also low when compared with the PMS/DCPIP assay at pH 9; however, it is twice the QEDH activity in the PMS/DCPIP test at pH 7 (Table 2). When comparing the oxidase activities of the membranes in Table 3, the ethanol-oxidizing activity is low compared with the NADH oxidase activity, but it amounts to 58 % of the succinate oxidizing activity under these conditions.

Cytochrome c_{550} is a new type of soluble cytochrome *c* in *P. aeruginosa*, which is induced by ethanol and functions as electron acceptor of QEDH. With these properties and its low pI it resembles cytochrome c_L in Gram-negative methylotrophic bacteria. In contrast with cytochrome c_L , however (Beardmore-Gray et al., 1983), cytochrome c_{550} does not use horse heart cytochrome *c* as electron acceptor. To confirm a close relationship between the two proteins, sequence data of cytochrome c_{550} are needed.

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Table 4. LMM *c*-type cytochromes in *P. aeruginosa*

Cytochrome	Molecular mass (kDa)	Midpoint redox potential (mV)	Remarks	Reference
c_{551}	9	286	Soluble	Horio et al. (1960)
c_4	19.9	322	Dihaem, membrane-bound	Pettigrew and Brown (1988)
c_{551}	16.5	310	Membrane-bound	Matsushita et al. (1982b)
c_{555}	11.5	340	Membrane-bound	Matsushita et al. (1982b)
$c_{550.5}$	32	300	Membrane-bound	Wood and Willey (1980)
c_{550}	15	280	Soluble	The present paper

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