Purification and partial characterization of the membrane-bound haem-containing proteins from *Acinetobacter calcoaceticus* LMD 79.41

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Cytoplasmic membranes of *Acinetobacter calcoaceticus* cells, cultured under acetate-limiting conditions, contain cytochrome b_{554} and a cytochrome o -containing oxidase. Both have been purified to homogeneity and characterized. Cytochrome b_{554} is a monomeric protein (molecular mass 70 kDa) with a midpoint potential of +100 mV (in the membrane it has most probably a value of +50 mV). The cytochrome o -containing oxidase seems to be an $\alpha\beta\gamma\delta$ protein since the molecular mass of the native protein was estimated to be 150 kDa and the molecular masses of the subunits, determined by SDS-polyacrylamide-gel electrophoresis, are 55, 41, 33 and 17 kDa. Redox spectroscopy of the purified complex shows the presence of a cytochrome $b_{555/563}$ having a midpoint potential of approximately +160 mV (both in purified form and in the membrane). CO difference spectroscopy shows the presence of a second b -type cytochrome, viz. cytochrome o . Cytoplasmic membranes of *A. calcoaceticus* cells grown under oxygen-limiting conditions also show the presence of the cytochrome b_{554} and the cytochrome o -containing oxidase. In addition a protein has been solubilized with the spectral characteristics of a cytochrome d -containing oxidase. The cytochrome o - and d -containing oxidases appear to be similar to those reported for *Escherichia coli* and *Proteus mirabilis*. On the other hand, cytochrome b_{554} has no counterpart in these organisms since the cytochrome b_{556} described for *E. coli* is quite dissimilar.

Introduction

The components of the cytoplasmic-membrane-bound electron-transport chain of *Acinetobacter calcoaceticus* include flavin, ubiquinone-9, b -type cytochromes (including cytochrome o) and cytochrome d (Asperger *et al.* 1978, 1981; Ensley & Finnerty, 1980). Cytochrome o and cytochrome d are potentially capable of functioning as terminal oxidases. Cytochrome o is present after growth in the presence of high oxygen concentrations, whereas both cytochrome o and cytochrome d are present after oxygen-limited growth (Ensley & Finnerty, 1980). In addition it has been shown that the composition of the electron-transport chain does not affect the efficiency of

the electron-transport-coupled synthesis of ATP (Ensley *et al.*, 1981).

By virtue of the reaction glucose $\rightarrow$ gluconolactone + 2H⁺ + 2e⁻, catalysed by pyrroloquinoline quinone (PQQ)-containing glucose dehydrogenase (GDH), reducing equivalents can be donated to the electron-transport chain. Recently, evidence has been presented for the existence of two different GDHs in *A. calcoaceticus* (Cleton-Jansen *et al.*, 1988; Matsushita *et al.*, 1988): a membrane-bound GDH (83 kDa, one subunit), presumably related to the GDHs in *Escherichia coli* and *Pseudomonas* sp. (Ameyama *et al.*, 1986; Matsushita *et al.* 1986), and a soluble GDH (approx. 100 kDa, two subunits) located in the periplasm and closely associated with the soluble cytochrome b -562 (Dokter *et al.*, 1985, 1986, 1988; Geiger & Görisch, 1986).

It has been suggested by Ameyama and co-workers that the membrane-bound GDH donates reducing equivalents directly to ubiquinone (Matsushita *et al.*, 1987; Ameyama *et al.*, 1986). Also Beardmore-Gray & Anthony (1986) suggested a direct electron transport

Abbreviations: A_m^* , absorbance maximum at full reduction; λ_m , wavelength (nm) at the absorbance maximum; ω_λ , band width (nm) at $A = 0.5 A_m$; cytochrome b_{554} , cytochrome b with an α -band maximum in the reduced form at 77 K at 554 nm (cytochromes b_{558} and b_{556} have been defined in the same way); cytochrome $b_{555/563}$, cytochrome b with a split α -band in the reduced form at 77 K (maxima at 555 and 563 nm); E_o' , midpoint potential at pH 7.0; GDH, glucose dehydrogenase; TMBZ, tetramethylbenzidine.

from the membrane-bound GDH to ubiquinone. Moreover, they argued that the 'glucose oxidase' system of *A. calcoaceticus* is kinetically distinct from the 'NADH oxidase' system, although both the GDH and the NADH dehydrogenase were assumed to donate electrons via ubiquinone-9, the *b*-type cytochromes and the cytochrome *o*-type oxidase to oxygen. In contrast to this direct electron transfer to ubiquinone, for the soluble GDH it has been suggested that the soluble cytochrome *b*-562 functions between the GDH and ubiquinone (Hauge, 1960, 1961; Dokter *et al.*, 1988).

To elucidate the bioenergetic significance of the GDHs in *A. calcoaceticus*, insight into the identity of the components and the structure of its respiratory chain is necessary. The present paper describes the haem-containing proteins of the cytoplasmic membrane, investigated by means of coupled spectrum deconvolution and potentiometric analysis. In addition, two haem-containing proteins, viz. the cytochrome *o*-containing oxidase and the cytochrome *b*₅₅₄, were purified and characterized. Their resemblances to those of *E. coli* and *Proteus mirabilis* are discussed.

Methods

Cell growth. *Acinetobacter calcoaceticus* LMD 79.41 was grown either in an acetate-limited continuous culture (cells provided by Dr B. J. van Schie: Van Schie *et al.*, 1984), or in (oxygen-limited) fed-batch cultures with acetate as a carbon and energy source (Dokter *et al.*, 1985). The cells were harvested and subsequently stored at -20°C .

Chemicals. All chemicals were obtained from commercial sources and were of reagent grade.

Membrane isolation. Cytoplasmic membranes were prepared by the method of Widdowson & Anthony (1975).

Solubilization of membrane-bound haem-containing proteins. To solubilize cytochrome-containing complexes from the cytoplasmic membranes, the membranes (5 mg protein ml^{-1}) were incubated overnight at 4°C in 10 mM-Tris/HCl buffer, pH 8.0, containing 10 mM-MgCl₂ and 5% (w/v) Triton X-100 (Kita *et al.*, 1986). The suspension was centrifuged for 2 h at 100 000 *g*. The supernatant, referred to as the 'Triton X-100 extract', contained the solubilized proteins.

Purification of the solubilized haem-containing proteins. The Triton X-100 extract (215 ml, 3.2 mg protein ml^{-1}) was applied to a DEAE-Sephacrose CL-6B Fast Flow column (2.2 cm $\times$ 24 cm) equilibrated with TE buffer (10 mM-Tris/HCl buffer, pH 8.0, containing 1 mM EDTA and 1%, w/v, Triton X-100). The column was washed with TE buffer, and the proteins were then eluted with a linear gradient of 0–0.2 M-NaCl in TE buffer. Fractions (8 ml) with a significant difference in absorbance at 408 and 440 nm were pooled, concentrated by pressure filtration (Millipore, membrane type PTGC 047), and stored at -70°C . In the case of the cytochrome *o*-containing oxidase, the concentrate (10 ml, 7.3 mg protein ml^{-1}) was diluted with TE buffer to lower the NaCl concentration (end volume about 40 ml). The diluted sample was applied to a DEAE-Sephacrose column (1.1 cm $\times$ 8.4 cm) and chromatography was performed as described above.

Molecular mass determinations. Molecular mass determinations of native cytochrome complexes were made on polyacrylamide gradient gels (Pharmacia, PAA 4/30), using proteins of high- and low-molecular-mass electrophoresis calibration kits as references [method described in the Pharmacia manual *Laboratory Techniques* (1986)]. Electrophoresis under denaturing conditions (in the presence of 1%, w/v, SDS) and cytochrome staining (with tetramethylbenzidine: TMBZ) were carried out according to Thomas *et al.* (1976). Protein staining was performed with Coomassie Brilliant Blue R250.

Protein determinations. Protein concentrations were determined by the Lowry method, with bovine serum albumin as a standard. To remove the Triton X-100, which would interfere in the protein determinations, a column of Bio-beads (Bio-Rad) was used (Holloway, 1973).

Cytochrome analysis. During the purification procedure, cytochrome *b* concentrations were estimated from reduced-minus-oxidized difference spectra, by using a molar difference absorption coefficient ($\epsilon_{560-575}$) of $22 \text{ mM}^{-1} \text{ cm}^{-1}$ (Chance, 1957). Absorption spectra of cytoplasmic membranes were recorded with a Beckman UV 5260 spectrophotometer, placing the cuvette holder just in front of the photomultiplier; those of optically clear cytochrome preparations were recorded with a Hewlett-Packard model HP 8450 A spectrophotometer.

Pyridine haemochrome spectra were measured in aqueous alkaline pyridine solutions and the haem content was determined by the method of Fuhrhop & Smith (1975).

For detailed characterization of the cytochromes, spectra were recorded with a DW-2a spectrophotometer (American Instrument Co.) equipped with a low-temperature accessory (J4-9603, American Instrument Co.). To improve the signal-to-noise ratio of 77 K spectra, the averages of nine sequential scans were used (Van Wielink *et al.*, 1982). To determine CO-spectra, CO ($\text{O}_2 \leq 3 \text{ p.p.m.}$) was flushed over the solution. Reduction of the cytochromes was accomplished by dithionite. After flushing for 90 min at room temperature, an absorption spectrum was recorded and a sample was taken for a 77 K spectrum. To prevent light-induced dissociation of the CO-liganded complexes, freezing and storage of these samples took place in the dark.

Flash-photolysis. Light-induced dissociation of the CO-liganded complexes in the frozen samples was accomplished by four flashes of a Rollei E22C flash lamp. After each flash the cuvette was submerged in liquid nitrogen. Since the crystal structure did not change, the spectra recorded before and after the treatment could be compared without corrections.

Potentiometric titrations. The cytoplasmic membranes and the solubilized cytochrome-containing complexes were titrated in 0.1 M-HEPES, pH 7.0 at 25°C , using the mediator cocktail and equipment as described by Van Wielink *et al.* (1982). Reductive titrations were performed by stepwise addition of an anaerobic solution of NADH or, to achieve low redox potentials, sodium dithionite. In oxidative titrations, the contents of the titration vessel were first reduced with sodium dithionite, then oxidized by stepwise addition of an anaerobic solution of potassium ferricyanide.

Oxidase activity. The oxidase activity was measured polarographically at 30°C in air-saturated 0.1 M-potassium phosphate buffer, pH 7.0, containing 0.05% (w/v) Triton X-100 with duroquinol as electron donor (Matsushita *et al.*, 1984). Initial oxygen consumption rates were calculated from the slopes induced by addition of duroquinol (0.017–0.417 mM). Duroquinol was prepared from duroquinone by a method described by White *et al.* (1978).

Results

Spectral characterization of the *b*-type cytochromes in the membrane

Cytoplasmic membranes from acetate-limited chemostat-grown cells and from oxygen-limited fed-batch-grown cells contained at least three *b*-type cytochromes, as revealed from analysis of series of 77 K spectra of membrane samples poised at different redox potentials (Fig. 1, Table 1). Cytochrome $b_{554-555}$ ($E'_0 = +50$ mV) and cytochrome $b_{555/563}$ (+160 mV), the latter with a split α -band [or cytochrome b_{555} (+165 mV) and cytochrome b_{563} (+160 mV)], contributed equally to the absorption band between 540 and 580 nm in both cytoplasmic membrane preparations. In addition to these cytochromes a low contribution of a cytochrome b_{558} (+170 mV) was necessary to fit the data of membranes from oxygen-limited cells. (In Table 1 the components which may be identical are depicted side by side on one line.)

Solubilization and fractionation of the haem-containing proteins

The solubilization procedure applied to membranes of acetate-limited cells resulted in a preparation giving two chromatographically different haem-containing proteins on the DEAE-Sephacel column (fractions I and II; Fig. 2). Fraction I appeared to be a cytochrome *o*-type oxidase in view of its spectral characteristics and its duroquinol oxidase activity (see below). Fraction II showed no oxidase activity and according to its absorption maximum at 77 K, it was designated as cytochrome b_{554} . Both fractions were also obtained from membranes prepared from oxygen-limited fed-batch-grown cells, although a third haem-containing fraction (eluting before fraction I) was observed in this case. This appeared to be a cytochrome *d*-type oxidase in view of its spectral

Fig. 1. 77 K spectra of cytoplasmic membranes from *A. calcoaceticus* poised at different redox potentials. Cytoplasmic membranes were isolated from (a) cells of an acetate-limited chemostat culture and (b) cells of an O_2 -limited fed-batch culture. The solid lines represent the recorded 77 K spectra. The reticular broken lines represent the fittings based on the assumption of three components (a) and four components (b). For parameter values see Table 1.

Fig. 2. DEAE-Sephacel column chromatography of the solubilized haem-containing proteins from cytoplasmic membranes isolated from cells of an acetate-limited chemostat culture. The pooled fractions are indicated by I and II. For further details see Methods. —, ΔA (408–440 nm); ---, NaCl concentration.

Table 1. Best-fitting parameters for the potentiometric titrations of the cytochromes *b* present in cytoplasmic membranes of cells grown under acetate or oxygen limitation

Acetate-limited, chemostat					Oxygen-limited, fed-batch				
%	A_m^*	λ_m (nm)	ω_λ (nm)	E'_0 (mV)	%	A_m^*	λ_m (nm)	ω_λ (nm)	E'_0 (mV)
29	0.042	562.9	5.8	+168	30	0.038	562.6	5.7	+152
					8	0.017	558.2	3.6	+170
23	0.029	555.7	6.3	+159	16	0.026	554.6	4.3	+168
48	0.031	554.9	12.5	+45	46	0.027	554.2	12.2	+52

Fig. 3. SDS-polyacrylamide gel electrophoresis of the purified cytochrome *o*-type oxidase (lane A) and the cytochrome *b*₅₅₄ (lane B), followed by protein staining.

characteristics (in the reduced form absorption bands at 560, 595 and 635 nm). Due to the instability of the preparation (as observed from the disappearance of the absorption bands), no attempts were made to purify the oxidase.

The purification schemes of the cytochrome *o*-type oxidase and the cytochrome *b*₅₅₄ are given in Table 2.

Characterization of the cytochrome *o*-type oxidase

The maximum rate of oxygen consumption of the purified cytochrome *o*-type oxidase was 2.5 µmol O₂ per nmol cytochrome *o* per minute with duroquinol as

electron donor (the apparent *K_m* for duroquinol was 0.1 mM).

Protein staining after electrophoresis of the cytochrome *o*-type oxidase (after the second DEAE-Sephacrose chromatography step) on polyacrylamide gradient gels revealed only one band, of 150 kDa (results not shown), indicating that the preparation was homogeneous. Under denaturing conditions, bands of 55, 41, 33 and 17 kDa were visible (Fig. 3). As revealed by the TMBZ staining procedure, only the 55 kDa band contained haem.

Pyridine haemochrome spectra of the cytochrome *o*-type oxidase clearly indicated the presence of protohaem (in reduced-minus-oxidized difference spectra, maxima at 418, 526 and 557 nm and a minimum at 541 nm were found). The haem content appeared to be 8.5 nmol haem (mg protein)⁻¹, that is 1.3 haem groups per molecule of cytochrome *o*-type oxidase (150 kDa). This value might indicate either the inaccuracy of the determinations or the possibility that *in vivo* the *o*-type oxidase contains two haem groups and that haem has apparently been detached from the protein during purification.

Spectra recorded at 25 °C of the oxidized cytochrome *o*-type oxidase showed a γ-band at 406 nm, whereas the reduced oxidase preparation showed bands at 426 nm (γ-band), 530 nm (β-band) and 564 nm with a shoulder at 558 nm (α-bands; see Fig. 4a). At 77 K, α-bands were found at 554.5 and 564 nm (Fig. 5a). In the reduced-minus-oxidized spectrum at 25 °C, peaks were found at 428, 530, 558 (shoulder) and 564 nm, and a trough at 406 nm (Fig. 4b). From these spectra molar absorption coefficients of 16.1 (ε₅₆₄₋₅₈₀) and 250 mM⁻¹ cm⁻¹ (ε₄₂₈₋₄₀₆) were calculated (assuming the presence of two haem groups per oxidase molecule).

The cytochrome *o*-type oxidase (the cytochrome *o* part) appeared to bind CO since the CO-difference spectrum (the oxidase reduced in the presence of carbon

Table 2. Purification of the cytochrome *o*-type oxidase and the cytochrome *b*₅₅₄

The proteins were purified from acetate-limited chemostat cells. For further details see Methods.

Preparation	Cyt. <i>b</i> (nmol)	Yield (%)	Protein (mg)	Cyt. <i>b</i> /protein ratio (nmol mg ⁻¹)
Crude extract	1299	100	4292	0.3
Cytoplasmic membranes	947	73	1229	0.8
Triton X-100 extract	573	44	690	0.8
Cytochrome <i>o</i> -type oxidase*	256	20	73	3.5
Cytochrome <i>o</i> -type oxidase†	223	17	45	5.0 (8.5‡)
Cytochrome <i>b</i> ₅₅₄ *	174	13	134	1.3 (3.7‡)

* After the first DEAE-Sephacrose chromatography step.

† After the second DEAE-Sephacrose chromatography step.

‡ Haem content [nmol haem (mg protein)⁻¹] determined by pyridine haemochrome method of Fuhrhop & Smith (1975).

Fig. 4. Absorption spectra of the cytochrome *o*-type oxidase. The purified oxidase preparations contained 0.64 mg protein ml⁻¹ (a and b) 1.0 mg protein ml⁻¹ (c) in 0.1 M-HEPES, pH 7.0, at 25 °C. (a) —, oxidized form; ---, reduced form (after reduction by dithionite). (b) Reduced minus oxidized. (c) CO-difference spectrum (reduced by dithionite in the presence of CO minus reduced in the presence of argon). The vertical bars represent a ΔA of 0.5.

monoxide minus the oxidase reduced in the presence of argon) showed maxima at 417.5 and 569 nm and troughs at 435.5 and 554 nm (Fig. 4c). The molar absorption coefficient ($\epsilon_{417.5-435.5}$), based on two haems per oxidase molecule, was 218 mM⁻¹ cm⁻¹. At 77 K, peaks were found at 417 and 566.5 nm and troughs at 438.5 and 553 nm (Fig. 5b).

By applying light flashes to the CO-treated, reduced sample, part of the CO could be dissociated from the complex. Since reassociation at 77 K is slow, the effect of four light flashes could be visualized by subtraction of a 77 K spectrum of a reduced sample treated with CO and flash light from a spectrum of the same sample not treated with flash light. Peaks were detected at 413.5 nm and 566 nm, and troughs at 431 nm and 533 nm (Fig. 5c).

Fig. 5. 77 K spectra of the cytochrome *o*-containing oxidase. The spectra were taken of samples containing 1 mg cytochrome *o* oxidase ml⁻¹ under the following conditions: reduced by dithionite (a); reduced in the presence of CO minus reduced in the presence of Ar (b); reduced in the presence of CO minus reduced in the presence of CO and treated with four light flashes (c). The vertical bars represent a ΔA of 0.5.

The small troughs in the CO-difference spectra, recorded at 25 °C (Fig. 4c), might indicate that the contribution of cytochrome *o*(red) to the Soret-band as well as to the α -band, is very small. In contrast, that of cytochrome *o*(red)-CO is more pronounced, as apparent from the peaks in Fig. 4(c) and 5(b).

The midpoint potential of the main cytochrome *b* component (79%, see Fig. 6), contributing to the α -band of the cytochrome *o*-containing oxidase, was +177 mV. The component having a low midpoint potential (-20 mV), occupying 21% of the spectral area in the 540–580 nm region might originate from denatured cytochrome(s) *b*.

Characterization of the cytochrome *b*₅₅₄

The cytochrome *b*₅₅₄ preparation appeared to be homogeneous, as revealed by polyacrylamide gel electrophoresis after protein staining as well as haem staining.

Fig. 6. Potentiometric titration of the purified cytochrome *o*-containing oxidase (●) and the cytochrome *b*₅₅₄ (○). The percentage reduction of the cytochromes in the preparations (calculated from the peak areas of α -bands in the 540–580 nm region) are plotted vs the redox potential. Protein contents: 1.0 mg ml⁻¹ (the cytochrome *o*-containing oxidase) and 0.66 mg ml⁻¹ (the cytochrome *b*₅₅₄). The lines represent best-fit analyses with two components for the titration of the cytochrome *o*-containing oxidase (21% of the area underneath the α -band titrates with a midpoint potential of -20 mV, 79% with a midpoint potential of +177 mV) and with one component for the titration of the cytochrome *b*₅₅₄ (100% of the area underneath the α -band titrates with a midpoint potential of +100 mV).

Fig. 7. Spectra of a cytochrome *b*₅₅₄ preparation at 25 °C. Samples contained 0.66 mg cytochrome *b*₅₅₄ ml⁻¹. (a) Spectra of the oxidized form (—) and the dithionite-reduced form (---). (b) The difference spectrum (reduced minus oxidized). The vertical bars represent a ΔA of 0.5.

This cytochrome is a monomeric protein since a molecular mass of 70 kDa was found for the native as well as for the denatured form (Fig. 3).

Pyridine haemochrome spectra showed the presence of protohaem: 3.7 nmol haem (mg protein)⁻¹ or 0.25 mol

Table 3. Comparison of cytochrome *o*-containing oxidases

For data of *A. calcoaceticus*, see this paper. The ranges of data for the *E. coli* oxidase were compiled from Kranz & Gennis (1983), Matsushita *et al.* (1983, 1984), Kita *et al.* (1984a) and Anraku & Gennis (1987). NM, Not measured.

Property	Cytochrome <i>o</i> -containing oxidase from:	
	<i>A. calcoaceticus</i>	<i>E. coli</i>
Mol. mass (kDa, native)	150	140
Mol. mass (kDa, denatured)	55 (haem)	51–66 (haem)
	41	28–36
	33	18–22
	17	13–17
<i>E</i> ' ₀ (mV)	<i>b</i> _{555/563} : +160	<i>b</i> ₅₆₂ : +150
	<i>o</i> ₅₅₄ : NM	<i>o</i> ₅₅₅ : +150

haem (mol protein)⁻¹ (70 kDa). The latter value strongly suggests that loss of haem occurred during purification.

Spectra of the oxidized cytochrome *b*₅₅₄, recorded at 25 °C, showed a γ -band at 410 nm. The reduced cytochrome showed bands at 422 nm (γ -band), 524 nm (β -band) and 556 nm (α -band; see Fig. 7a). At 77 K, the α -band was found at 554 nm (consequently the cytochrome is designated as cytochrome *b*₅₅₄). In the reduced-minus-oxidized spectrum at 25 °C, maxima were found at 424, 528 and 556 nm, and a trough at 406 nm (Fig. 7b). Molar absorption coefficients calculated from the reduced-minus-oxidized spectra are: $\epsilon_{424-406} = 119 \text{ mM}^{-1} \text{ cm}^{-1}$ and $\epsilon_{556-576} = 13.3 \text{ mM}^{-1} \text{ cm}^{-1}$ (assuming the presence of one haem per cytochrome *b*₅₅₄).

Cytochrome *b*₅₅₄ did not bind CO (spectra not shown). The midpoint potential of cytochrome *b*₅₅₄ was +100 mV.

Discussion

Cytochrome *b*₅₅₄ and cytochrome *o*-type oxidase are very major components of the cytoplasmic membranes of *A. calcoaceticus*, as can be deduced from the purification schemes (Table 2). After growth under oxygen-limiting conditions cytochrome *d*-type oxidase is also found.

Comparison of the properties of the cytochrome *o*-type oxidase from *A. calcoaceticus* with those of the *E. coli* oxidase (Table 3), reveals similarity (although there are significant differences in the subunit molecular masses). Based on the absorbance changes induced by CO, it is concluded that the reduced cytochrome *o* of *A. calcoaceticus* only slightly contributed to the absorbance between 540 and 580 nm [main absorbance assigned to cytochrome *b*_{555/563} (*E*'₀ approx. +160 mV)]. For the cytochrome *o*-type oxidase of *E. coli*, Hackett & Bragg (1983) came to the same conclusion. Lorence *et al.* (1984),

however, attributed both the 555 and the 562 nm bands to cytochrome *o*, while Kita *et al.* (1984a, 1986) attributed the band at 555 nm to cytochrome *o*. The band at 562 nm was assigned to the other cytochrome in the oxidase (see also Anraku & Gennis, 1987). The midpoint potentials of cytochrome *o* and cytochrome b_{562} were found to be identical ($E'_0 = +150$ mV).

Under oxygen-limited conditions the synthesis of cytochrome *d* is induced in *A. calcoaceticus* (Ensley & Finnerty, 1980). The presence of cytochrome b_{558} ($E'_0 = +170$ mV) in *A. calcoaceticus* LMD 79.41 suggests a similar cytochrome b_{558} -*d*-containing oxidase as reported for *E. coli* (Miller & Gennis, 1983; Hata *et al.*, 1985). This view is in agreement with the finding of Kranz & Gennis (1985), who showed that the cytochrome *d* complex of *Acinetobacter* HO1-N is immunologically related to the cytochrome *d* complex of *E. coli*.

The third haem-containing protein purified from the membranes of *A. calcoaceticus* is cytochrome b_{554} (E'_0 approx. +100 mV). The protein consists of one polypeptide chain (70 kDa) with at least one haem group. It may be identical to cytochrome $b_{554-555}$ (E'_0 approx. +50 mV) detected during titrations of the cytoplasmic membranes (see Table 1). Cytochrome b_{554} is different from the cytochrome b_{556} that Kita *et al.* (1978) solubilized from cytoplasmic membranes of *E. coli*, since the latter is an oligomer composed of polypeptides of 17.5 kDa, with a much lower midpoint potential (−45 mV according to Kita *et al.*, 1978; +35 mV according to Lorence *et al.*, 1984). Initially it was suggested that this cytochrome is required for electron transfer to the cytochrome *o*-type oxidase (Haddock & Jones, 1977; Kita & Anraku, 1981) and, possibly, the cytochrome *d*-type oxidase (Kita *et al.*, 1984b). For aerobically grown *P. mirabilis*, it has been suggested that a cytochrome b_{557} ($E'_0 = +50$ mV) may function (together with cytochrome $b_{563/556}$, $E'_0 = +140$ mV) in a Q- or *b*-cycle (Van Wielink *et al.*, 1983). On the other hand, the cytochrome *d*-containing oxidase of *E. coli* (Koland *et al.*, 1984) and the cytochrome *o*-containing oxidase of both *E. coli* (Carter & Gennis, 1985) and *A. calcoaceticus* (this paper) are ubiquinol oxidases and can function in the absence of cytochrome b_{556} or cytochrome b_{554} , respectively. Recently Murakami *et al.* (1985) provided evidence that cytochrome b_{556} of *E. coli* is a product of the *sdhC* gene (succinate dehydrogenase cluster) of *E. coli*, so that it may have a function in electron transfer from this dehydrogenase. Accordingly, investigations on the membrane-bound dehydrogenases of *A. calcoaceticus* may be rewarding in the assignment of a function to cytochrome b_{554} of *A. calcoaceticus*.

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