PERIPLASMIC CO-BINDING *C*-TYPE CYTOCHROME IN A MARINE BACTERIUM

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Received 4 September 1974

1. Introduction

Studies on extracts of the marine bacterium *Beneckea natriegens* have shown that it contains some CO-binding cytochrome *c* [1–3]. Spectra of intact cells show that CO-binding is not due to denaturation of native cytochrome *c* during cell fractionation [1,2]. Furthermore, action spectra of intact cells show that CO-binding cytochrome *c* acts as an oxidase [2]. Unlike the other, membrane bound, cytochromes of *B. natriegens* the CO-binding cytochrome *c* is located mainly in the supernatant fraction after centrifugation of sonic extracts at 150 000 *g* [1,2]. CO-binding *c*-type cytochromes have also been found in the supernatant fractions of sonic or French press extracts of methylotrophic bacteria [4], sulphur bacteria [5,6], *Pseudomonas denitrificans* [7] and a cyanide-evolving strain of *Chromobacterium violaceum* (D. F. Niven and C. J. Knowles, unpublished observations).

Recent studies on the marine pseudomonad B-16 have shown that a cytochrome *c* can be easily leached from the cell envelope [8,9], whilst the other cytochromes are firmly bound to the cytoplasmic membrane [10]. Preliminary experiments showed that the released cytochrome *c* binds CO and is therefore probably related to the cytochromes *c* mentioned above. The cell wall layers may be selectively stripped from this bacterium by suitable washing procedures [8,9] and it therefore offers a unique opportunity to study cytochrome distribution in the cell envelope of Gram-negative bacteria. This paper presents data on cytochrome composition and localisation in this organism, with particular reference to the CO-binding *c*-type cytochrome.

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2. Materials and methods

The organism used in this study was marine pseudomonad B-16 (ATCC 19855, NCMB 190, varient 3 [11]. This bacterium has recently been classified as *Altermonas haloplanktis* [12].

The organism was grown at 25°C with high aeration on a gyrotary shaker in a medium containing 0.8% nutrient broth (Difco), 0.5% yeast extract (Difco), 0.3 M NaCl, 0.026 M $MgSO_4 \cdot 7H_2O$, 0.01 M KCl, and 0.1 mM $FeSO_4(NH_4)_2SO_4$. Nine ml of an overnight culture were inoculated into 300 ml of fresh medium in a 2 litre conical flask and the cells grown for 2.5 hr for mid-logarithmic phase cells or 15 hr for stationary phase cells. Following harvesting, cells were either washed three times in complete salts (0.03 M NaCl plus 0.05 M $MgCl_2$ plus 0.01 M KCl) or once in complete salts and the wall layers selectively removed by various washing procedures.

Washing marine pseudomonad B-16 three times in 0.5 M NaCl removes the loosely bound outer layer (LBOL) and subsequent incubation for 30 min at 25°C in 0.5 M sucrose followed by a cold 0.5 M sucrose wash removes both the outer double track membrane (ODT) and the underlying (periplasmic) layer (ULL) [8,13]. The ODT and ULL can be separated by centrifugation at 73 000 *g* for 2 hr, which causes sedimentation of the ODT [9]. The stripped cells, devoid of LBOL, ODT and ULL but retaining the murein layer have been called mureinoplasts [8].

In this study the stripping procedure was followed as described previously [8,9] using 20–25% and 70–80% of the growth volume of washing solutions for logarithmic and stationary phase cells respectively. The pooled NaCl and pooled sucrose washes were dialyzed overnight against three changes of 5 mM Tris–HCl (pH 7.0) buffer and concentrated 10- to 20-fold in a rotary evaporator under vacuum at 32°C.

Cells and mureinoplasts, suspended in complete salts (10–20 mg dry wt per ml), were disrupted in a French press at 20 000 lb per sq in. Debris was removed by centrifugation at 18 000 *g* for 12 min and the cell free extract fractionated into particulate and supernatant fractions by centrifugation at 180 000 *g* for 3 hr. Only the top half of the supernatant fraction was retained for use. The particulate fraction was rinsed with a little 20 mM Tris–HCl (pH 7.0) buffer followed by resuspension in the same buffer.

$Na_2S_2O_4$ reduced minus $K_3Fe(CN)_6$ or H_2O_2 oxidized and $Na_2S_2O_4$ reduced plus CO minus $Na_2S_2O_4$ reduced difference spectra were measured as described previously [1,2]. Room temperature spectra were measured in 1 cm cuvettes and liquid nitrogen temperature (77°K) spectra were measured in 2 mm cuvettes in the presence of 0.8 M sucrose.

Alkaline phosphatase was assayed at 22°C by the hydrolysis of *p*-nitrophenyl phosphate (PNPP) [13]. Isocitrate dehydrogenase was assayed at 22°C by the formation of NADPH [14]. Protein was estimated by the modified biuret method [15].

3. Results and discussion

Reduced minus oxidized difference spectra of the particulate fraction derived from complete salts washed stationary phase cells are shown in fig. 1a. Room temperature spectra have peaks at 559 nm and 527 nm due to cytochrome *b* and at 552 nm and 522 nm due to cytochrome *c*. The Soret peak is at 425 nm (not shown). There are no peaks in the 570–650 nm region indicating the complete absence of any *a* or *d*-type cytochromes. This spectrum confirms previous spectra of membrane vesicles of this organism [10]. Low temperature spectra further resolve the peaks, with α-peaks at 558.5 nm and 556.5 nm due to *b*-type cytochromes and a peak and shoulder at 549 nm and 547 nm respectively due to *c* type cytochromes.

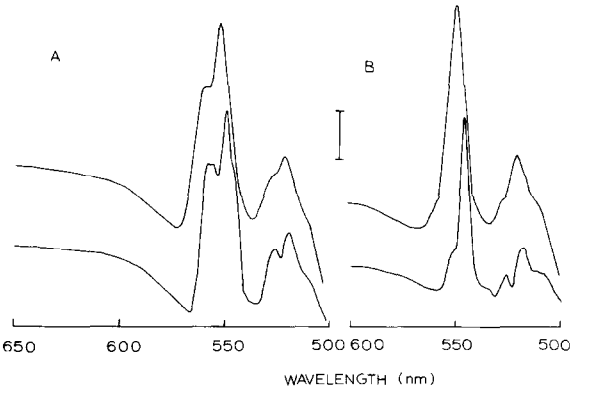

Fig. 1. $Na_2S_2O_4$ reduced minus $K_3Fe(CN)_6$ oxidized difference spectra of particulate (A) and supernatant (B) fractions derived from stationary phase cells of marine pseudomonad B-16. In each case the upper curve is the room temperature spectrum in 1 cm cuvettes and the lower curve is the liquid nitrogen temperature spectrum in 2 mm cuvettes and in the presence of 0.8 M sucrose. The protein concentrations used were 2 mg per ml for the particulate fraction, 16 mg per ml for the room temperature spectrum of the supernatant fraction and 8 mg per ml for the low temperature spectrum of the supernatant fraction. The vertical bar represents 0.01 absorbance units for the upper curves and 0.03 absorbance units for the lower curves.

Room temperature reduced minus oxidised difference spectra of the supernatant fraction derived from stationary phase cells (fig. 1b) have peaks at 549, 521 and 416 nm (the Soret peak is not shown in fig. 1b) indicative of a *c*-type cytochrome. Pyridine haemochrome spectra after acid–acetone treatment [1] show that there is only *c*-type cytochrome present in the supernatant fraction.

The α-peak at 549 nm in room temperature spectra of the supernatant fraction splits in 77°K spectra (fig. 1b) to give a peak at 546 nm, a prominent shoulder at 550 nm and a very small shoulder at 530 nm. The splitting could be due to the presence of either more than one cytochrome *c* or splitting of the α-peak of a single cytochrome *c*. Mammalian mitochondrial cytochrome *c* gives a similar pattern of α-peak splitting in low temperature spectra [16]. The 550 nm shoulder is a constant 22–26% of the 546 nm peak in supernatant fractions derived from logarithmic or stationary phase cells as well as in sucrose washes (c.f. below) of pseudomonad B-16, suggesting that there is only one cytochrome present.

Room temperature CO spectra (fig. 2) of the

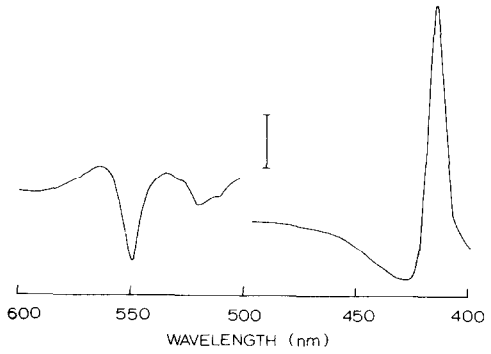

Fig. 2. Room temperature $Na_2S_2O_4$ reduced plus CO minus $Na_2S_2O_4$ reduced difference spectrum of the supernatant fraction derived from stationary phase cells (28 mg protein per ml). The cuvette path-length used was 1 cm. The vertical bar represents 0.01 absorbance units for the visible region of the spectrum and 0.03 absorbance units for Soret region.

supernatant fraction have peaks at 562 nm, 536 nm and 414 nm and troughs at 549 nm and 426 nm, showing that the cytochrome *c* binds CO as reported for other, apparently soluble, bacterial cytochromes *c* [1–7]. CO spectra at 77°K are similar but the peaks are shifted 2 nm towards the ultraviolet region.

Difficulty was experienced in obtaining reproducible CO spectra of the particulate fraction, with reduction by $Na_2S_2O_4$, NADH or succinate. However, a Soret peak at 415–416 nm and a trough in the visible region at 549 nm were usually seen, suggesting that a little CO-binding *c*-type cytochrome remains attached to the membrane. The shoulder at 547 nm seen in 77°K reduced minus oxidized difference spectra of the particles (fig. 1a) may therefore correspond to the cytochrome c_{549} of the supernatant fraction (α-peak at 546 nm in 77°K spectra, fig. 1b). Alternatively these absorbance bands could be due to the presence of some cytochrome *o* (a *b*-type cytochrome) [17], but the positions of the absorbance maxima and minima resemble more closely those of CO-binding *c*-type cytochromes found in other bacteria [1–7] than other cytochromes *o* [17].

It is possible that CO-binding by the *c*-type cytochrome found in the supernatant fraction of marine pseudomonad B-16 is due to denaturation [18,19] of the cytochrome during cell disruption and fractionation. This could be due, for example, to dislocation of the cytochrome from the cytoplasmic membrane. Estimation of CO-binding of intact cells washed with complete salts and the particulate and supernatant fractions derived from them reveal that no increase in CO-binding occurs, and there is good quantitative agreement between the total amount of CO-binding cytochrome found in the cells and that found in the particulate plus supernatant fractions.

Room temperature and low temperature spectra of the particulate and supernatant fractions obtained from cells harvested in the logarithmic phase have spectra similar to those from stationary phase cells, except that the concentrations are lower (table 1).

Washing cells of marine pseudomonad B-16 three times with NaCl to release the LBOL, followed by incubation in sucrose and a subsequent sucrose wash to remove the ODT and ULL [8] causes loss of cytochrome c_{549} from the cell (fig. 3). Cytochrome c_{549} is found almost entirely in the combined sucrose washes (ODT plus ULL). Spectra of the particulate and super-

Table 1
Cytochrome concentrations of the particulate and supernatant fractions derived from marine pseudomonad B-16 harvested in logarithmic and stationary growth phases

Growth phase	Particulate fraction					Supernatant fraction		
	Protein	Cytochrome c_{552}		Cytochrome b_{559}		Protein	Cytochrome c_{549}	
	(mg)	concn (nmoles/mg)	total (nmoles)	concn (nmoles/mg)	total (nmoles)	(mg)	concn (nmoles/mg)	total (nmoles)
Logarithmic	96	0.61	59	0.47	45	225	0.11	25
Stationary	192	0.89	171	0.67	129	426	0.22	94

Concentrations of the cytochromes were estimated from room temperature spectra using published values for extinction coefficients [19].

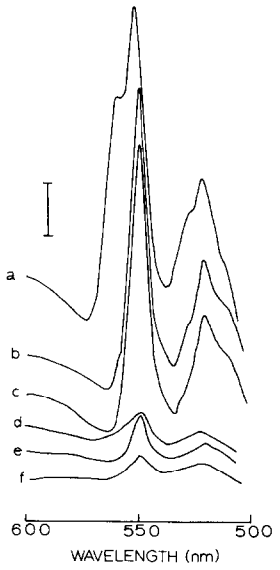

Fig. 3. Room temperature $Na_2S_2O_4$ reduced minus $K_3Fe(CN)_6$ oxidized difference spectra of the fractions of marine pseudomonad B-16 obtained by washing the cells with NaCl and sucrose and then disrupting and fractionating the resulting mureinoplasts. Curve (a) 180 000 *g* particulate fraction of disrupted mureinoplasts (4 mg protein per ml); curve (b) pooled, dialyzed and concentrated crude sucrose washes (ODT plus ULL) (2.35 mg per ml); curve (c) underlying layer (ULL) (1.90 mg per ml); curve (d) outer double track (ODT) (2.70 mg per ml); curve (e) pooled, dialysed and concentrated NaCl washes (loosely bound outer layer, LBOL) (1.95 mg per ml); curve (f) 180 000 *g* supernatant fraction of disrupted mureinoplasts (6.5 mg per ml). The cuvettes pathlength was 1 cm. The vertical bar represents 0.01 absorbance units.

natant fractions of disrupted NaCl and sucrose washed cells (fig. 3) indicate that essentially all the cytochrome c_{549} normally associated with the supernatant fraction is lost, whilst spectra of the particulate fraction are similar to those derived from complete salts washed cells.

Quantitative estimates of the cytochrome released to that found in the supernatant fraction of unextracted cells are good. Cytochrome c_{549} released from logarithmic phase cells is 100% (3 experiments) and from stationary phase cells it is 90–100% (5 experiments). Only 0–16% of the total cytochrome c_{549} is found in the NaCl washes, and 80% or more is found in the sucrose washes. The concentration of cytochrome c_{549} in the sucrose washes from stationary phase cells is 1.2–1.6 nmoles per mg protein.

In addition to the similarity of room temperature reduced minus oxidized difference spectra of the concentrated dialyzed sucrose washes to those of the supernatant fractions derived from complete salts washed cells, low temperature and CO spectra of the sucrose washes are similar to those of cytochrome c_{549} in the supernatant fraction of complete salts washed cells.

Centrifugation of the sucrose washes at 73 000 *g* for 2 hr or 140 000 *g* for 3 hr to separate the ODT (sedimented) and ULL [9], show that 30–55% of the total protein is sedimented but none of the cytochrome c_{549} (fig. 3).

The underlying layer (ULL) has been chemically and morphologically identified as the periplasmic region of marine pseudomonad B-16 [8,9] and contains all the alkaline phosphatase [13]. This enzyme is most likely located in the periplasm of other Gram-negative bacteria [21].

Table 2 shows the distribution of cytochrome c_{549} in the different layers of the cell envelope. Also included are data for the distributions of alkaline phosphatase and isocitrate dehydrogenase. The latter is a soluble enzyme located in the cytoplasm of bacteria and has been assayed as a marker enzyme to show that leaching of cytochrome c_{549} and alkaline phosphatase from the cell is not due to their leakage from the cytoplasm. Cytochrome c_{549} is distributed in the separate fractions in a similar manner to alkaline phosphatase, but there is no release of isocitrate dehydrogenase activity from the cell thus confirming the periplasmic location of cytochrome c_{549}. Cytochrome c_{549} also lies in the periplasm of marine pseudomonad B-16. It is not a soluble enzyme found in the cytoplasm as the initial experiments (fig. 1) suggested.

The location of the CO-binding cytochrome c_{549} in the periplasm of marine pseudomonad B-16 suggests that the CO-binding cytochromes *c* found in other bacteria [1–7] may also be located in the periplasmic region of the cell. There are precedents for the location of cytochromes in the periplasm of Gram-negative bacteria, since *c*-type cytochromes (not CO-binding) associated with nitrate reductase activity in *Escherichia coli* [22] and *Spirillum itersonii* [23] appear to be present in the periplasmic region.

In both *B. natriegens* [24] and *C. violaceum* (D. F. Niven and C. J. Knowles, unpublished observations) respiration of the particulate fraction is highly active

Table 2
The distribution of cytochrome c_{549}, alkaline phosphatase and isocitrate dehydrogenase in the different fractions of marine pseudomonad B-16 harvested in the stationary phase

Fraction	Composition	Protein	Cytochrome c_{549} [c]		Alkaline phosphatase		Isocitrate dehydrogenase	
			concn	total	specific activity	total activity	specific activity	total activity
		(mg)	(nmoles/mg)	(nmoles)	(μmoles/min/mg)	(μmoles/min)	(μmoles/min/mg)	(μmoles/min)
NaCl washes[a]	loosely bound outer layer (LBOL)	132	0.23	30.4	0.021	2.78	$\leqslant 10^{-4}$	–
Sucrose washes[a]	outer double track (ODT) plus underlying layer (ULL)	118	1.31	154	0.382	45.0	$\leqslant 10^{-4}$	
Sucrose washes, sediment at 140 000 *g* for 3 hr	ODT	32	0.13	4.2	0.063	2.08	–	–
Sucrose washes, supernatant from 140 000 *g* for 3 hr	ULL (periplasm)	85	1.56	133	0.468	39.8	–	–
Particulate[b] fraction	Cytoplasmic membrane plus murein	220	n.d.[d]	n.d.	0.022	4.84	0.0059	1.30
Supernatant[b] fraction	Cytoplasm	468	0.032	15.0	0.016	7.48	0.228	107.0

[a] After dialysis and concentration (c.f. Materials and methods section). [b] The values given for these fractions are corrected for the 20% loss of material resulting from centrifugation at 18 000 *g* for 12 min to remove the debris and whole cells following cell discruption in a French press. [c] Assayed from $Na_2S_2O_4$ reduced minus $K_3Fe(CN)_6$ oxidized difference spectra at room temperature, using a published extinction coefficient [19] to estimate its concentration. [d] n.d. = not determined, but probably a small quantity present as seen in low temperature spectra (c.f. text).

despite the loss of 80–90% of the total CO-binding cytochrome *c*, and there is no stimulation of activity on re-addition of the supernatant fraction. The membrane fractions of these bacteria also contain small quantities of cytochromes a_1, d ($= a_2$) and possibly *o* in addition to the residual CO-binding cytochrome *c*. In the case of marine pseudomonad B-16 the particulate fraction contains absolutely no *a* and *d*-type cytochromes and only low, variable concentrations of the CO-binding cytochrome *c* (or, less probably, cytochrome *o*), yet there is active respiration of NADH, succinate and ascorbate-TMPD (0.12, 0.012 and 0.10 μg atoms oxygen uptake per min per mg protein respectively at 25°C). Re-addition of the crude supernatant fraction containing CO-binding cytochrome *c* does not cause any stimulation of activity.

The residual CO-binding cytochrome associated with the membrane could therefore be sufficient for maximal activity. Alternatively, following dislocation of the cytochrome *c* from the membrane, it is not able to reassociate with the membrane at the correct site or in an active form. Further possibilities are that it does not have a role in regular, aerobic respiration, but is associated with a side branch of respiration, oxidation of a single, specific substrate, linkage of respiration to active transport, etc. Such speculation can only be satisfied by purification and characterization of the CO-binding *c*-type cytochrome.

Studies are in progress to see the effect of selective stripping of the wall layers of marine pseudomonad B-16 and depletion of the CO-binding cytochrome *c* on respiration and active transport.

Acknowledgements

This work was supported by a National Research Council of Canada grant (to R.A.M.) and a Royal Society travel grant (to C.J.K.). Mrs Margaret Smith provided excellent technical assistance.

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