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A SOLUBLE CO-BINDING *c*-TYPE CYTOCHROME FROM THE MARINE BACTERIUM *BENECKEA NATRIEGENS*

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SUMMARY

1. The supernatant fraction derived from high speed centrifugation at 150000 × *g* of sonicated cells of *Beneckea natriegens* was shown to contain a soluble *c*-type cytochrome.

2. Difference spectra of alkaline–pyridine derivatives of haem extracts showed that this fraction was essentially free of other types of cytochrome.

3. The soluble *c*-type cytochrome was shown to be capable of binding carbon monoxide and to have a high redox potential (E_0'(pH 7.0) = 0.32 V).

4. Small quantities of a pigment of unknown function absorbing at 430–435 nm were found in reduced *plus* CO *minus* reduced difference spectra.

5. The results are discussed with reference to previously discovered CO-binding *c*-type cytochromes.

INTRODUCTION

Marine bacteria of the genus *Beneckea* have recently been isolated and characterised[1]. These organisms show great metabolic versatility and rival the pseudomonads in the number and variety of carbon compounds that they can metabolize. The most versatile species is *Beneckea natriegens*[1], which was previously classified as *Pseudomonas natriegens*[2]. *B. natriegens* has been reported[3] to be capable of growing faster than any other known organism, with doubling times as fast as 9.8 min.

In view of the metabolic versatility and rapid growth rates of *B. natriegens*, we have decided to investigate its electron transport system. This paper reports the presence in *B. natriegens* of a soluble, high potential, CO-binding, *c*-type cytochrome.

MATERIALS AND METHODS

B. natriegens strain 111, obtained from Dr W. J. Payne, was maintained on marine agar slopes at room remperature with monthly subculturing. The bacteria were grown in liquid culture on a minimal medium consisting of: 0.4 M NaCl, 0.01 M $MgSO_4$, 0.01 M KCl, 0.025 M NH_4Cl, 3.3 mM K_2HPO_4, 0.1 mM $FeSO_4$,

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride; DCIP, 2,6-dichlorophenolindophenol.

1 mM $CaCl_2$, 2 mM citric acid, 0.025 M sodium DL-lactate and 0.05 M Tris dissolved in distilled water and adjusted to pH 7.5 with HCl.

10 ml of an overnight culture was inoculated into 500 ml of fresh media in 2-l conical flasks and grown at 35 °C with high aeration on a gyrotary shaker. The cells were harvested in the stationary phase (16 h growth) by centrifugation at $5000 \times g$ for 20 min, resuspended in 50 mM $MgCl_2$–50 mM Tris–HCl buffer (pH 7.5), recentrifuged at $10000 \times g$ for 10 min and finally resuspended in Tris–$MgCl_2$ buffer to a concentration of about 30 mg protein per ml.

The cells were sonicated for 5 times at 1 min on a MSE 150 W ultrasonic disintegrator at maximum output, with cooling in an ice bath between bursts. The sonicate was centrifuged at $12000 \times g$ for 10 min at 4 °C and the sedimented debris discarded. The cell free extract was centrifuged at $150000 \times g$ for 90 min to give a brown particulate fraction and a clear golden supernatant fraction. The particles were washed in 10 mM $MgCl_2$–50 mM Tris–HCl buffer (pH 7.5), recentrifuged and resuspended in 10 mM $MgCl_2$–50 mM Tris–HCl buffer (pH 7.5).

Cytochrome spectra were measured at room temperature and at liquid N_2 temperature (77 °K). Oxidised *minus* reduced spectra were obtained by reducing one cuvette with a few grains of $Na_2S_2O_4$, 1 mM NADH, 30 mM succinate or 1.5 mM ascorbate *plus* 4 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) and oxidising the other cuvette by vigorous aeration, a few grains of $K_3Fe(CN)_6$ or one drop of H_2O_2 (10 vol.). Reduced *plus* CO *minus* reduced spectra were obtained by reducing the cuvettes as described above, and bubbling a steady stream of CO for 30 s through one cuvette. The CO-treated cuvette was left in the dark for 10 min before recording the spectra. Difference spectra at liquid N_2 temperature were recorded on an Aminco–Chance split-beam spectrophotometer using the single freezing technique[4]. Room temperature spectra were measured in the Aminco–Chance or in a Perkin–Elmer 356 spectrophotometer.

Pyridine haemochromes were determined by a combination of the methods of Lanyi[5] and Jacobs and Wolin[6]. 5 to 10 ml of supernatant fraction were washed twice in 40 ml cold acetone. The precipitated protein was washed 4 times with 40 ml cold acetone containing 2% of 2.4 M HCl. The acid–acetone extracts were pooled and evaporated to near dryness, followed by extraction with 25 ml, then 10 ml of diethyl ether and washing with 20 ml distilled water. The diethyl ether extract was evaporated and the powder resuspended in 0.1 M NaOH in 50% pyridine. The residue remaining after acid–acetone extraction was also suspended in alkaline pyridine. The haems present in the extract and residue were identified by their difference spectra[7].

Protein was assayed by the modified biuret method of Gornall *et al.*[8].

NADH and L-ascorbic acid were obtained from Sigma Chemical Co., TMPD from Eastman Kodak Co., and CO from British Oxygen Co. All other chemicals were of the finest grade available and distilled water was used throughout.

RESULTS

Reduced *minus* oxidised difference spectra of the particulate fraction of *B. natriegens* show the presence of large quantities *b* and *c*-type cytochromes and low concentrations of cytochromes a_1 and a_2. (Weston, J. A. and Knowles, C. J., un-

published). Reduced *plus* CO *minus* reduced difference spectra gave Soret peaks at 419 and 414 nm, indicating the presence of CO-binding *b*-type (*i.e.* cytochrome *o*, ref. 9) and *c*-type cytochromes[10–15]. The CO-binding cytochromes are present in low concentrations and represent only a small fraction of the total particulate *b*- and *c*-type cytochromes.

Previously observed bacterial CO-binding *c*-type cytochromes were shown to be soluble[10–15]. Following the discovery of low concentrations of the CO-binding *c*-type in the particulate fraction of *B. natriegens*, we have examined the high speed ($150000 \times g$) supernatant fraction for the presence of this cytochrome. Fig. 1 shows a $Na_2S_2O_4$-reduced *minus* oxidised difference spectrum of the supernatant fraction; the spectrum is typical of a *c*-type cytochrome. No peaks due to other cytochromes were seen.

If an extinction coefficient for the α-peak of $\Delta\varepsilon_{mM}$ (552−540 nm)=19.0 is assumed[16] then in 23 separate extracts the concentration of the cytochrome was in the range 0.08–0.18 (average=0.12) μmole per g supernatant fraction protein.

That the cytochrome was indeed a *c*-type cytochrome was shown by pyridine haemochrome difference spectra of acid–acetone extracts and residues of the supernatant fraction (Fig. 2). Acid–acetone residues gave spectra with peaks at 551, 522 and 416–418 nm, indicative of a mesohaem group covalently linked to the protein[7].

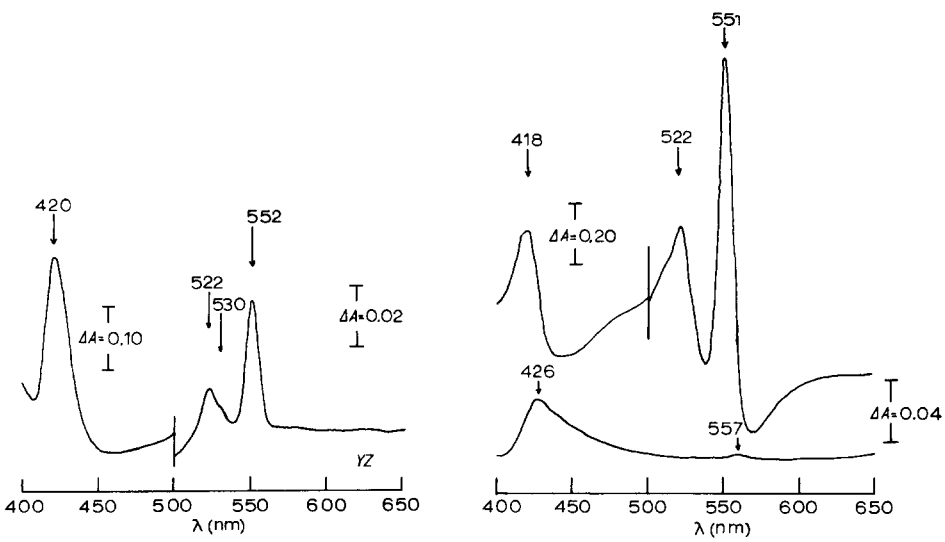

Fig. 1. $Na_2S_2O_4$-reduced *minus* oxidised difference spectrum of the supernatant fraction. 14 mg protein per ml was suspended in 10 mM $MgCl_2$–50 mM Tris–HCl buffer (pH 7.5); one cuvette was reduced by a few grains of $Na_2S_2O_4$ and the other oxidised by vigorous aeration. The light path was 1 cm and the temperature 21 °C.

Fig. 2. Pyridine–haemochrome spectra of acid–acetone extract and residue of supernatant fraction. Preparation of the acid–acetone extract and residue is given in Materials and Methods. The pyridine haemochromes were reduced with a few grains of $Na_2S_2O_4$ and oxidised by one drop of H_2O_2 (10 vol.); the original protein concentration was 15 mg per ml and the extract and residue were each made up to a final volume equivalent to one quarter of the initial supernatant fraction volume. The light path was 1 cm and the temperature 21 °C. Upper line, residue; lower line, extract.

Acid–acetone extracts contained only marginal amounts of *b*- (α-peak at 557 nm), *a*- (580–590 nm) and *d*-type (600–620 nm) haems.

$Na_2S_2O_4$-reduced *plus* CO *minus* $Na_2S_2O_4$-reduced spectra of the supernatant fraction are shown in Fig. 3 at both room temperature (Fig. 3a) and at 77 °K (Fig. 3b). A single large Soret peak at 414 nm (413 nm at 77 °K) was observed. However, the trough at about 425–430 nm, observed for other CO-binding *c*-type cytochromes (Weston, J. A. and Knowles, C. J., unpublished; refs 10–15) was partially obscured by a small peak at 435 nm. In the visible region the spectra are complex, with pronounced peaks at 549 nm (548 nm at 77 °K) and 572 nm (570 nm), a trough at 554 nm (553 nm) and a multiplicity of peaks in the 500–545 nm region (Fig. 3).

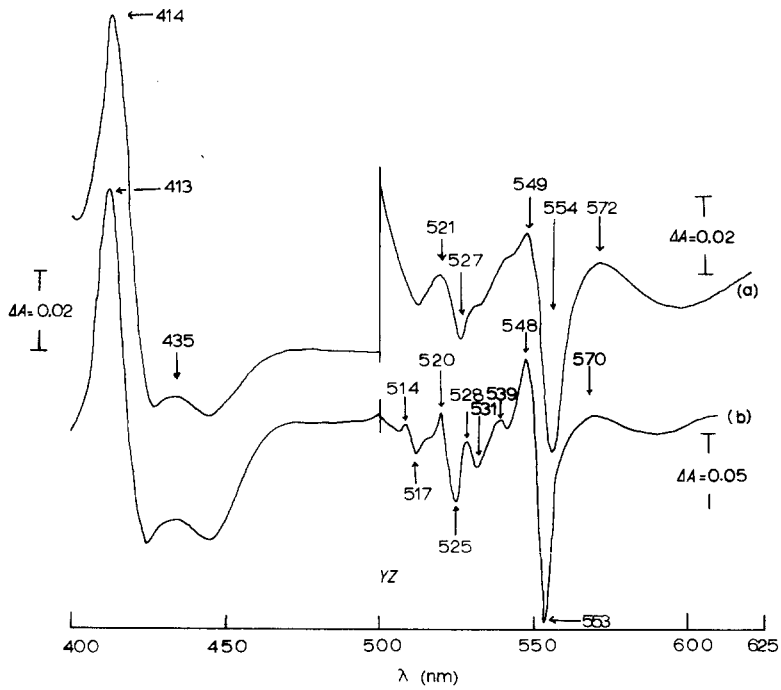

Fig. 3. $Na_2S_2O_4$-reduced *plus* CO *minus* $Na_2S_2O_4$-reduced difference spectra of the supernatant fraction. 7 mg protein per ml was suspended in 10 mM $MgCl_2$–50 mM Tris–HCl buffer (pH 7.5) and each cuvette reduced by solid $Na_2S_2O_4$. CO was bubbled for 30 s through one cuvette and the cuvette incubated for 10 min before recording the spectrum at room temperature (a) or freezing to 77 °K and recording (b). Note the intensification of the peaks in the visible region at low temperature; a similar intensification in the Soret region was not observed (*cf.* ref. 12). The cuvette light path was 2 mm.

The small peak at 430–435 nm in the CO spectra is possibly due to contaminating *a*-type cytochrome, though peaks in the 580–650 nm region were not observed (Fig. 3). The differential rate of CO-binding by the 430–435-nm absorbing material and the *c*-type cytochrome shows that this peak is due to a different component to the *c*-type cytochrome (Fig. 4). The small peak at 430 nm is fully developed within the 30 s that it takes to record the spectra after CO flushing, whilst the peaks of the *c*-type cytochrome are not fully developed until about 30–45 min after CO

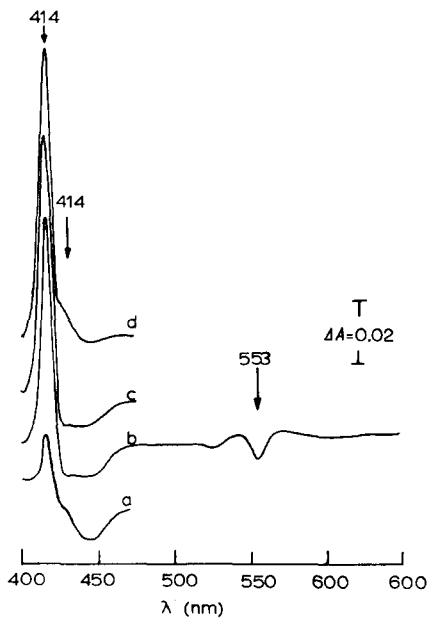

Fig. 4. The time dependency of CO binding by the soluble *c*-type cytochrome. Supernatant fraction (14 mg protein per ml) was reduced by solid $Na_2S_2O_4$. One cuvette was bubbled for 30 s with CO and spectra recorded immediately (a), after 10 min (b), after 30 min (c) and after 90 min (d). For the sake of clarity only the 10 min spectrum is recorded in the visible region. The light path was 1 cm and the temperature 21 °C.

flushing. Studies of the rate of appearance of the *c*-type cytochrome Soret peak at 414 nm showed that the cytochrome was 78–83% saturated with CO after 10 min development.

That the *c*-type cytochrome was fully soluble was confirmed by centrifugation of the supernatant fraction at $300000 \times g$ for 3 h; no sedimentation occurred.

Over 95% of NADH, succinate, ascorbate *plus* TMPD, or ascorbate *plus* 2,6-dichlorophenolindophenol (DCIP) oxidase activities were found to occur in the particulate fraction. However, addition of any of these substrates to the supernatant fraction caused almost complete reduction of the soluble *c*-type cytochrome. The redox potential E_0' (pH 7.0) was measured using ferro–ferricyanide[17] and found to be +0.32 V.

DISCUSSION

The high speed supernatant fraction of *B. natriegens* has been shown to contain a soluble *c*-type cytochrome. Alkaline–pyridine spectra of the haems showed this fraction to be essentially free of other cytochromes.

The soluble cytochrome *c* of *B. natriegens* is of interest as it is able to combine with CO (Fig. 3). It is becoming increasingly clear that CO-binding soluble *c*-type

TABLE I

THE PROPERTIES OF SOME CO-BINDING *c*-TYPE CYTOCHROMES

Organism	*Remarks*	*Difference spectra maxima (nm)*		*Redox potential, E'_0 (pH 7.0) (V)*	*Ref.*
		Oxidised minus reduced	*Reduced plus CO minus reduced*		
Anacystis nidulans	—	549, 521, 419*	560, 525–530, 414, trough 550*	−0.26	18
Navicula pelliculosa	—	554, 523, 418*	560, 532, 415, trough 550*		19
Chromatium Strain D	In supernatant fraction cytochrome *c* binds CO, in chromatophores no CO binding	553, 524, 422	565, 535, 414, trough 552	+0.01	10, 20, 21, 25
Desulphovibrio vulgaris Miyazaki	—	552, 523, 419*	564, 536, 416, trough 552	−0.29	12
Desulphovibrio spp.	Soluble, also 20% in particulate fraction**	552, 523, 419	565, 535, 412, trough 552		11
Rhizobium japonicum	Soluble, also 50% in particulate fraction	552	556, 538, 413.5, trough 551		13
Pseudomonas denitrificans	—	553, 524, 417*	555, 526, 414*	0 to −0.09	14
Escherichia coli	% CO binding increases on cell breakage	552, 523, between 408 and 419			15
Beneckea natriegens	Soluble, also 10–20% in particulate fraction	552, 521, 420	572, 549, 414, trough 554	+0.32	This paper

* Redrawn from published absolute spectra.

** Derived from absorbance values and quoted protein concentrations.

cytochromes occur widely in microorganisms (Table I). They have been discovered in algae, diatoms, photoautotrophic bacteria, symbiotic bacteria, anaerobic bacteria, aerobic bacteria and facultatively anaerobic bacteria. The spectrally variant cytochromes *c'* and *cc'* are also known to combine with carbon monoxide[10,21].

Spectrally all these cytochromes are similar (Table I), with peaks in oxidised *minus* reduced spectra typical of *c*-type cytochromes. In reduced *plus* CO *minus* reduced difference spectra the positions of the maxima are remarkably similar. Of particular note are the positions of the Soret peaks (412–416 nm) and the troughs in the visible region (550–554 nm), which are 0–10 nm nearer the ultraviolet region than the values for the otherwise similar CO spectra of cytochromes *o* (refs 9, 22). However, cytochromes *o* are usually membrane bound whilst CO-binding *c*-type cytochromes are, partially at least, soluble.

The CO-binding *c*-type cytochromes have diverse properties with widely varying redox potentials, isoionic points, haem contents and molecular weights. *B. natriegens* cytochrome *c* was fully reduced by ascorbate *plus* TMPD or DCIP and has a high redox potential (0.32 V) in contrast to the redox potentials of other CO-binding *c*-type cytochromes, suggesting a different function for this cytochrome. However, no single function has been assigned to these cytochromes. In *D. vulgaris* it has been suggested[12] that cytochrome c_3 acts as an electron carrier for hydrogenase. In *Chromatium* cytochrome c_{553} undergoes light-induced oxidoreduction[23]. Cytochrome c_{552} of *R. japonicum* is maximally synthesised in bacteriods whilst aerobically grown cells in free culture contain minimal concentrations of the cytochrome[13]. In bacteriods the conditions approach anaerobiosis[24]. Though the cytochrome *c* of *B. natriegens* binds only slowly with CO, its high redox potential tempts one to speculate that it might act as a terminal oxidase: such a function cannot be presumed without action spectra of the release of CO inhibition.

In *B. natriegens* we have been unable to determine the concentration of the CO-binding *c*-type cytochrome in the particulate fraction due to overlap of the Soret peak with that of cytochrome *o*. At best it is only 10–20% of that found in the supernatant fraction. CO spectra do not permit us to distinguish between the Soret peaks of the *c*-type cytochrome and cytochrome *o* in intact cells, but the total concentrations of these two cytochromes is very much greater than can be accounted for by the cytochrome *o*, as found from its concentration in the particulate fraction, and is similar to the value found for the soluble *c*-type cytochrome. The CO-binding ability of the *c*-type cytochrome is not therefore due to modification during aqueous extraction and occurs in the native cytochrome within the intact cell.

The small peak at 430–435 nm in CO spectra is not due to an *a*-type cytochrome as we were unable to find peaks in the visible region, nor in oxidised *minus* reduced spectra or in haem extracts. Appleby[13] has noticed CO-binding pigments P-420 and P-428 in *R. japonicum*, and Jones and Hughes[26] have implicated pigment P-425 of *Pseudomonas ovalis* Chester with a function as a cytochrome *c* peroxidase.

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