THE RESPIRATORY SYSTEM OF THE MARINE BACTERIUM *BENECKEA NATRIEGENS*. OXIDATION–REDUCTION POTENTIALS OF THE CYTOCHROMES

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1. Introduction

Room temperature, reduced *minus* oxidised, difference spectra of respiratory membranes of the marine bacterium *Beneckea natriegens* have an α-peak at 552 mm due to *c*-type cytochromes, with a shoulder at 559.5 nm due to *b*-type cytochromes [1]. Small amounts of cytochromes a_1 and a_2 (=*d*) are also observed. At 77°K the peaks are resolved to cytochromes b_{562}, b_{557}, *b* or c_{554}, $c_{549.5}$ and c_{547} (subscripts refer to the positions of the α-peaks at 77°K). Reduced *plus* CO *minus* reduced difference spectra indicate that some of the *b* and *c*-type cytochromes bind CO (cytochromes *o* and c_{CO} respectively). Also present in *B. natriegens* is a soluble CO-binding *c*-type cytochrome [2].

In order to gain more insight into the cytochrome species present in *B. natriegens* we have used the potentiometric titration technique [3–6] to measure the redox potentials of the cytochromes. The results given in this paper indicate that the absorbance peak at 559.5 nm (at room temperature) is due to a mixture of two cytochromes, whilst the peak at 552 nm is due to three cytochromes.

2. Materials and methods

B. natriegens strain 111 was grown, harvested, disrupted and fractionated into particulate and supernatant fractions as described previously [1,2].

The oxidation–reduction potentials of the cytochromes were determined essentially as described by Wilson and Dutton [3–6] by the stimultaneous measurement of the absorbance changes of the cytochromes and their redox potentials in the presence of mediator dyes under strictly anaerobic conditions. The redox potential was measured with a combined platinum electrode (Pye-Unicam Ltd.) connected to a pH meter/millivolt meter (model 290, Pye-Unicam Ltd.). Absorbance changes were monitered in a Hitachi–Perkin Elmer model 356 dual-wavelength spectrophotometer. The oxidation–reduction mediators used were potassium ferricyanide (E_m = + 430 mV, n=1), phenazine ethosulphate (PES) (E_m = + 55 mV, n=1), phenazine methosulphate (PMS) (E_m = + 80 mV, n=2), diaminodurol (DAD) (E_m = + 240 mV, n=2), and duroquinone (DQ) (E_m = + 5 mV, n=2). The particles or supernatant were suspended in 10mM $MgCl_2$–50mM Tris–HCl buffer (pH 7.0). Aliquots of 24 mM NADH were added as a reductant and 100 mM ferricyanide as an oxidant. The determinations were carried out at room temperature.

3. Results and discussion

The redox potentials of cytochromes *b* in the particulate fraction were measured at 559.5 nm *minus* 575 nm. Fig. 1A shows the logarithm of the ratio of the oxidised to reduced cytochrome *b* plotted against the measured oxidation-reduction potential (E_h). The resulting curve is sigmoid, indicating that the absorbance change is due to a mixture of two components [6]. Resolution of the curve (fig. 1B) shows the presence of a high potential component of 220 mV (25% of the total) and a low potential component of 5 mV (75%).

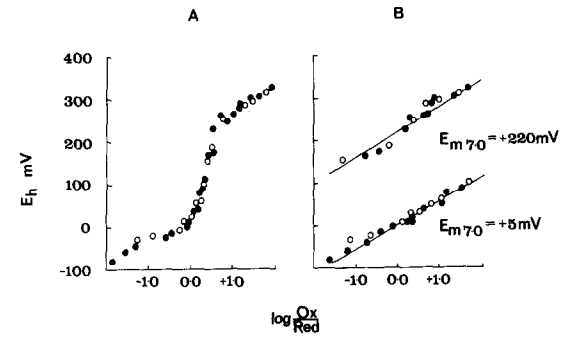

Fig. 1. Graph of measured redox potential (E_h) versus $\log_{10}$ (fraction oxidised/fraction reduced) of cytochromes *b* in the particulate fraction of *B. natriegens* using the wavelength pair 559.5–575 nm. The particles were suspended in 50 mM Tris buffer (pH 7.0)–10 mM $MgCl_2$ (6 mg protein per ml). The suspension contained 100 μM ferricyanide, 40 μM PMS, 40 μM PES and 60 μM DQ. Anaerobiosis was attained by adding aliquots of NADH. After anaerobiosis, the absorbance change was titrated oxidatively by ferricyanide addition (-o-) and then reductively by NADH addition (-•-). In (A) the absorbance change is treated as a single component, whilst in (B) the sigmoid curve is resolved into its two component parts. Theoretical one electron transfer (n=1) lines are drawn through the points.

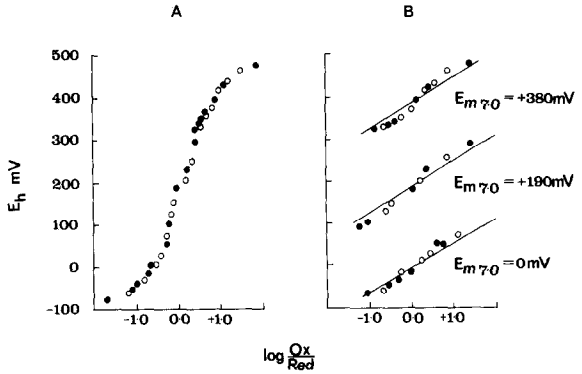

Fig. 2. The oxidation–reduction potential dependence of the 552–538 nm absorbance change of cytochromes in the particulate fraction of *B. natriegens*. The experimental conditions were similar to those in fig. 1, except that 30 μM DAD was also present, and the DQ concentration was reduced to 20 μM. The protein concentration was 4 mg per ml. An oxidative titration with ferricyanide (-o-) was followed by a reductive titration with NADH (-•-). In (B) the sigmoid curve is resolved into three components. Theoretical n=1 lines are drawn through the points.

When the redox potentials of the *c*-type cytochromes of the particulate fraction were measured (at 552 nm *minus* 538 nm) a complex curve was obtained (fig. 2A) which may be resolved (fig. 2B) into three components of redox potentials of 380 mV (20% of the total), 190 mV (40%) and 0 mV (40%).

Potentiometric titrations at a range of wavelengths in the region 548–563 nm indicated that the 0–5 mV component had an absorbance maximum at 560–561 nm and is therefore a *b*-type cytochrome with its α-peak overlapping the peaks of the *c*-type cytochromes; it could correspond to cytochrome b_{557} in 77°K difference spectra [1]. The components with redox potentials at 190 mV (at 552–538 nm) and 220 mV (at 559.5–575 nm) are probably separate cytochromes (cf. below); due to their similar potentials and overlapping spectra in the 554 to 558 nm region we were unable to determine the exact positions of their absorbance maxima. The 380 mV component observed in the titration at 552–538 nm has its peak position at 552–554 nm; this most likely corresponds to cytochrome $c_{549.5}$ found in 77°K difference spectra [1].

The respiratory system of *B. natriegens* is terminally branched (ref. [7] and unpublished observations). One pathway is inhibited by 10 μM cyanide or 2 mM azide and the other by 8 mM cyanide, or, at physiological rates of respiration, by carbon monoxide. NADH and succinate are oxidised via both pathways, whilst ascorbate-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine by the former pathway only. The oxidases include one of the *c*-type cytochromes and possibly cytochrome *o* (from their ability to bind carbon monoxide and from action spectra [1]).

Carbon monoxide (70 % CO: 30 % N_2 gas phase), cyanide (10 μM or 3 mM) and azide (2 mM) had similar effects on the potential of the higher potential *b*-type cytochrome, in each case raising it to 320 mV. Its percentage contribution to the total absorbance change was unaltered, but the total absorbance change was reduced 10–20% by the inhibitors.

The peak at 552 nm titrated as two, rather than three, components in the presence of carbon monoxide, azide or cyanide with potentials of 310 mV (50% of the total) and 10 mV (50%). The total absorbance was reduced 10–20% in the presence of the inhibitors.

The disappearence of the 380 mV component at 552 nm indicates that it binds with the inhibitors in

the reduced form [8,9]; its increased potential in the presence of the inhibitors means that it is not oxidised by ferricyanide. The increase in potential of the mid-potential component from 190 mV to 310 mV indicates that it too combines with the inhibitors in the reduced form. Similarly the increase in potential of the 220 mV cytochrome *b* to 320 mV shows that it also combines preferentially with the inhibitors in the reduced form.

The ability of carbon monoxide to bind both *b* and *c*-type cytochromes is unusual, but not unexpected in view of our previous data [1,2]. By analogy with mitochondria, carbon monoxide is likely to bind the reduced form of the cytochrome oxidases (above, and from reduced *plus* carbon monoxide *minus* reduced difference spectra [1,2]. However, unlike the situation found in mitochondria [8], azide and cyanide also bind the reduced form of the cytochromes.

Wilson and coworkers [4,5,9] have reported that cytochromes *b* and a_3 of mitochondria undergo changes of redox potential of 200–300 mV on addition of ATP. He has postulated that the respiratory chain contains four isopotential groups and that cytochromes *b* and a_3 are involved in energy conservation between two of the groups [9], though this interpretation has been criticised [10].

Measurements of the effect of ATP on the redox potentials of the particulate fraction *b* and *c*-type cytochromes of *B. natriegens* show that in the presence of 6 mM ATP* the redox potentials at 559.5–575 nm were 320 mV (25% of the total) and 10 mV (75%) and at 552–538 nm the potentials were 390 mV (20%), 255 mV (40%) and 5 mV (40%). The higher potential cytochrome at 559.5 nm thus had its potential raised from 210 mV to 320 mV and the mid-potential cytochrome at 552 nm had its potential raised form 190 mV to 255 mV by ATP. The differences in the potentials of these components in the presence of ATP (320 mV and 255 mV respectively) show that these are different cytochromes. The relatively small increases in redox potential (110 mV and 65 mV respectively) do not appear to be large enough to postulate a role for these cytochromes in energy transduction.

We have previously measured the redox potential

* Under the conditions of these experiments there is only low ATPase activity in the particulate fraction.

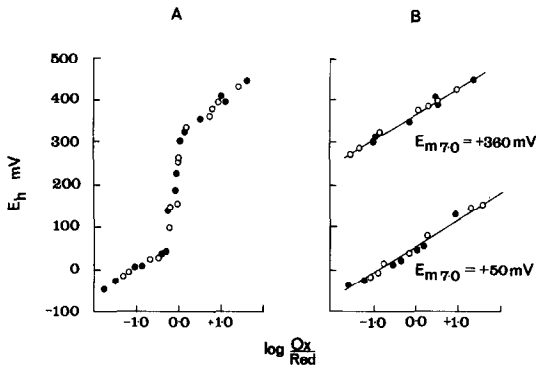

Fig. 3. Potentiometric titration (A) of the 552–538 nm absorbance change of cytochromes *c* of the supernatant fraction of *B. natriegens*. The experimental conditions were similar to those of fig. 2. The protein concentration was 15 mg per ml. In (B) the sigmoid titration curve is resolved into two components. Theoretical n=1 lines are drawn through the points.

of the soluble *c*-type cytochrome of *B. natriegens* [2] by its potential at its half-reduction point using the ferro-ferricyanide couple [11]. However, the values for the standard potential of the ferro-ferricyanide couple that we used could well be incorrect [12]. Furthermore, such a technique is valid only if there is just one component present.

We have therefore measured the redox potential of the cytochrome *c* of the supernatant fraction of *B. natriegens* using Wilson and Dutton's technique [3–6]. Fig. 3A shows that potentiometric titrations of the supernatant fraction at 552–538 nm gave sigmoid curves which can be resolved into two components (fig. 3B) of redox potentials of 360 mV (45% of the total) and 50 mV (55%). The 360 mV cytochrome possibly corresponds to the cytochrome of similar potential in the particulate fraction, but the 50 mV component represents a further cytochrome *c* not seen in the particles.

The soluble *c*-type cytochromes are being purified in order that we may more clearly determine their properties and functions.

Acknowledgements

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