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The Presence of Two Cytochromes *b* in the Facultative Methylotroph *Pseudomonas* AM1

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Two cytochromes *b* with absorption maxima at 555 and 562nm and differing in their midpoint redox potentials are synthesized in *Pseudomonas* AM1 during growth on methanol or succinate in batch culture, or in NH_4^+-limited or carbon-limited continuous culture. Both cytochromes *b* were also present in a cytochrome *c*-deficient mutant in all growth conditions.

Pseudomonas AM1 is a facultative methylotroph unable to grow on methane, but able to grow on other C_1 compounds and on a range of multicarbon compounds. Cytochromes *b*, *c* and a/a_3 are present in this bacterium, but the electron-transport chain is unconventional in that cytochrome *c* is not always involved in the oxidation of NADH and succinate (Anthony, 1975; Widdowson & Anthony, 1975; O'Keeffe & Anthony, 1978; Keevil & Anthony, 1979). Proposed schemes for electron transport, proton translocation and coupled ATP synthesis during oxidation of NADH and succinate all involve an antimycin-sensitive cytochrome *b* that is not involved in methanol oxidation (see references cited above and Netrusov & Anthony, 1979).

Schemes proposed for coupling electron transport and proton translocation in other bacteria have usually two cytochromes *b* with different midpoint redox potentials and it might be expected that this generalization would be true for *Pseudomonas* AM1 (see Haddock & Jones, 1977). However, the first demonstration of a cytochrome *b* in this bacterium included no evidence on this point and, because of technical difficulties, relied on the use of mutants lacking either carotenoids (PCT7) or cytochrome *c* (PCT76) or both (PCT761) (Anthony, 1975).

The work described in the present paper demonstrates that there are at least two cytochromes *b* in wild-type *Pseudomonas* AM1 differing in their absorption maxima and in their midpoint redox potentials.

Materials and Methods

Methods for maintenance, growth and harvesting of bacteria were as previously described (Anthony, 1975), except that the liquid growth medium was that of Maclennan *et al.* (1971). Continuous culture of bacteria was as described by Keevil & Anthony (1979). Cell suspension and membrane fractions were prepared by the method of Widdowson & Anthony (1975), all final suspensions being in 20mM-potassium phosphate buffer, pH7.0, for recording the spectra.

All spectra were recorded at room temperature (20°C) in a Cary 118C dual-beam spectrophotometer (Varian Associates, Walton on Thames, U.K.), cuvettes being placed as close to the photomultiplier as possible. When redox potentials were being measured the membranes were suspended in 25mM-4-morpholinepropanesulphonic acid buffer, pH7.0, containing 100mM-KCl and the mediator 2,3,5,6-tetramethylphenylenediamine (50μM); potentials were measured in the cuvette with a platinum electrode coupled to a reference calomel electrode by a KCl bridge. O_2-free N_2 was passed over the membrane suspension, which was stirred throughout the titration with ferricyanide.

Results and Discussion

Observation of the cytochromes *b* in wild-type *Pseudomonas* AM1 in the present work has been facilitated by removal of cytochrome *c* from membranes by repeated washing with phosphate, by the slow 'endogenous' reduction of cytochromes in membrane preparations occurring when membranes were left at room temperature in phosphate buffer, by the use of a spectrophotometer cuvette modified for measurement and adjustment of redox potentials during titration with oxidant, and by the use of bacteria from NH_4^+-limited continuous cultures that had no detectable cytochrome *c* bound to their membranes.

The demonstration of two cytochromes *b* in

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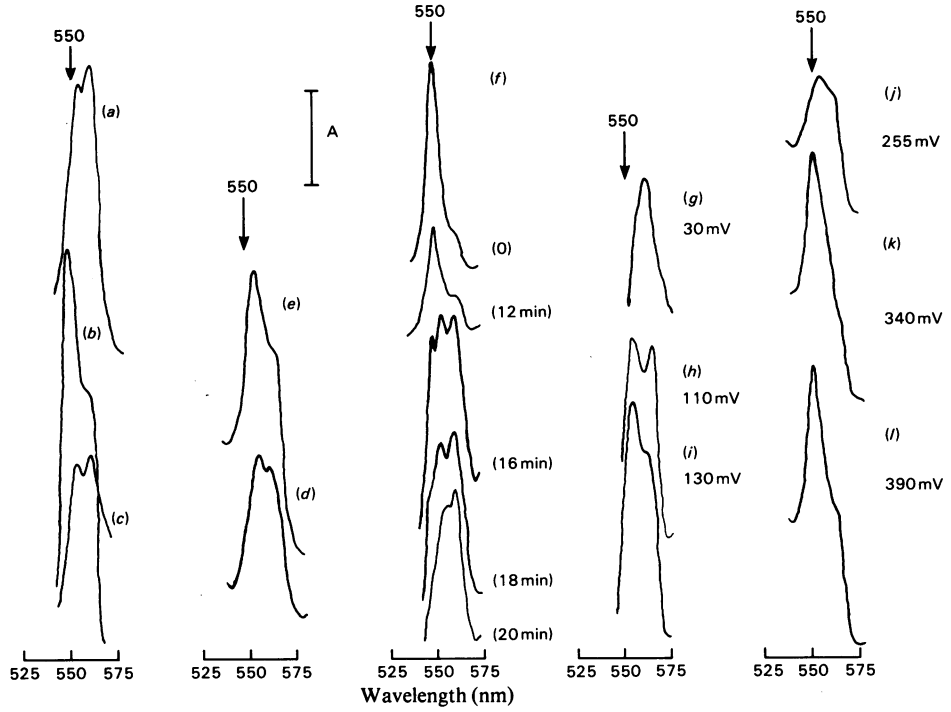

Fig. 1. *Reduced-minus-oxidized difference spectra of Pseudomonas AM1 showing the presence of two cytochromes b*
The reductant added to all cuvettes was $Na_2S_2O_4$ (a few grains) and the amount of oxidation in the reference cuvette was adjusted by a variety of methods. It is noteworthy that the presence of an absorption peak corresponding to a particular cytochrome indicates that it is present in the oxidized form in the reference cuvette. Spectra (*a*), (*b*) and (*c*), membranes of succinate-limited *Pseudomonas* AM1; in (*a*) the reference cuvette contained untreated membranes in which the cytochrome *c* was reduced; in (*b*) all cytochromes were oxidized by addition of a small volume of ferricyanide (100mM) and in (*c*) membranes in the reference cuvette were vigorously aerated in the presence of 0.3mM-KCN. Spectra (*d*) and (*e*), membranes of NH_4^+-limited *Pseudomonas* AM1 (carbon source, succinate); in (*d*) the reference cuvette contained untreated membranes and in (*e*) all cytochromes were oxidized with ferricyanide. Spectrum (*f*), whole cells of NH_4^+-limited *Pseudomonas* AM1 (carbon source, succinate); cells in the reference cuvette were fully oxidized by aeration and then allowed to stand to become fully reduced by endogenous substrate over a 25min period (after 12min the absorption scale changes; see below). Spectra (*g*)–(*l*), membranes of a methanol-limited carotenoid-lacking mutant of *Pseudomonas* AM1 (mutant PCT7); ferricyanide (100mM) was titrated into the reference cuvette to gradually increase the redox potential, which was monitored with platinum/calomel electrodes. The numbers on these spectra correspond to the measured redox potentials in mV; these values are only a rough guide as indicated in the text. The absorbance bar corresponds to the following absorbances (concentrations per ml of protein for membranes or dry wt. for whole cells are given in parentheses): (*a*), (*b*) (13.6mg/ml), $A = 0.005$; (*c*) (13.6mg/ml), (*d*), (*e*) (16.4mg/ml), $A = 0.01$; (*f*) (9.6mg/ml), $A = 0.01$ (0–12min), $A = 0.002$ (16–20min); (*g*)–(*l*) (49.5mg/ml), (*g*)–(*i*), $A = 0.01$; (*j*)–(*l*), $A = 0.05$. The light path was 2mm for spectra (*a*) to (*e*) and 10mm for spectra (*g*) to (*l*).

Pseudomonas AM1 has depended on measurement of reduced-minus-oxidized difference spectra (Fig. 1). In all these experiments the contents of one cuvette were reduced with dithionite while the redox potential in the reference cuvette was adjusted by various means. The spectra recorded are thus the spectra of those cytochromes that are in the oxidized state in the reference cuvette.

When bacteria were grown in NH_4^+-limited continuous culture on succinate the cytochrome *c* was readily washed from membranes with 20mM-phosphate buffer, pH7.0, whereas after growth under succinate limitation some of the cytochrome *c* could not be removed by repeated washing; this is seen by comparing spectra in Figs. 1(*b*) and 1(*e*). Even with cytochrome *c* present, two cytochromes *b* were observable when the cytochrome *c* on membranes in the reference cuvette was allowed to become reduced (Fig. 1*a*). In the same preparation both cytochromes *b* could be readily seen when the reference cuvette

was aerated in the presence of 0.3mM-KCN, which was sufficient to prevent oxidation of cytochromes *c* and a/a_3, but insufficient to prevent oxidation of both cytochromes *b* (Fig. 1*c*). This result indicates that either both cytochromes *b* can be directly oxidized by O_2 or that, more likely, one reacts both with O_2 and with the other cytochrome *b*. This observation does not necessarily suggest that either of the cytochromes *b* is a physiological oxidase (see Widdowson & Anthony, 1975; O'Keeffe & Anthony, 1978). The presence of the two cytochromes *b* was also demonstrated in suspensions of whole bacteria by a procedure illustrated in Fig. 1(*f*). In whole cells all of the cytochromes were reducible by endogenous substrate and when incubated without aeration they became anaerobic; the cytochromes of more positive midpoint potential were the first to become reduced and thus not detectable in the difference spectra recorded here. Hence in Fig. 1(*f*) at zero time only cytochrome *c* is clearly seen; by 16min this was almost fully reduced and both cytochromes *b* and a little cytochrome *c* are visible. By 25min the reference cells were completely reduced. The first cytochrome *b* to be reduced was that with an absorption maximum at 555nm, suggesting that this cytochrome has a more positive midpoint potential than that with an absorption maximum at about 562nm. This was confirmed by using membranes from a methanol-limited culture of mutant PCT7, lacking carotenoid pigments. In this experiment ferricyanide solution (100mM) was slowly added to an anaerobic suspension of membranes in the reference cuvette and the redox potential was measured. Because the potentiometer was not designed for use with turbid suspensions and because equilibrium could not be ensured at each measured redox potential, the potentials recorded on Fig. 1 cannot be used for accurate assessments of midpoint potentials of the cytochromes, but only as a confirmation of the sequence in which the cytochromes became oxidized. In Figs. 1(*g*) to 1(*l*) the spectra obtained during the titration are given; these show that the sequence of cytochromes in order of increasing midpoint potentials are cytochromes b_{562}, b_{555} and *c*.

Similar spectra to those recorded in Fig. 1 have demonstrated the presence of two cytochromes *b* in *Pseudomonas* AM1 grown on methanol or succinate in batch culture and in continuous culture in which growth was limited by methanol, succinate, NH_4^+ or O_2. The cytochrome *c*-deficient mutant of *Pseudomonas* AM1 (mutant PCT76) also contained two cytochromes *b* during growth on succinate in batch culture and in carbon- or NH_4^+-limited continuous culture. The bacteria used in all the experiments described in the present paper were exactly the same as those used in the measurements of $\rightarrow H^+/O$ ratios and cell yields described elsewhere (Keevil & Anthony, 1979).

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