RAPID OXYGEN-INDUCED REDUCTION OF *b*-TYPE CYTOCHROMES IN *PARACOCCUS DENITRIFICANS*

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1. Introduction

When oxygen is added to an anaerobic suspension of mitochondria supplied with both antimycin and a respiratory substrate, the *b* cytochromes become rapidly reduced; and then, when the oxygen is exhausted they become reoxidised (review [1]). A widely accepted explanation for this curious behaviour of the mitochondrial *b* cytochromes is that, in the presence of oxygen, cytochrome c_1 can oxidise semiubiquinone to ubiquinone thereby promoting the reduction of cytochrome b_{566} by the ubiquinol/semiubiquinone couple [2–5]. In the present work we describe a similar, rapid, antimycin-dependent, oxygen-induced reduction of *b*-type cytochromes in membranes isolated from aerobically and anaerobically grown cells of *Paracoccus denitrificans*. Hitherto this phenomenon has not been demonstrated in bacteria. Our present observations extend the similarities [6] between the respiratory chain of *P. denitrificans* and the mitochondrial respiratory chain. In addition, changes are shown to occur in the cytochrome system of *P. denitrificans* in adapting to anaerobiosis.

2. Materials and methods

Paracoccus denitrificans (*Micrococcus denitrificans* NCIB 8944) was grown anaerobically with succinate as substrate and nitrate as the added terminal electron acceptor as in [7] but in 5 litre volumes. Cells were grown aerobically at 30°C in 10 litre volumes in the same medium as that used for the anaerobic cultures except that KNO_3 was omitted, air was supplied through a glass sparger, and the culture was stirred magnetically. Membrane vesicles were prepared from cells which were harvested during exponential growth, treated with lysozyme, and then broken by osmotic shock, all as described [8]. The membrane vesicles were stored at 1–4°C for up to 7 days. Protein was determined by the Folin method in [9].

The kinetics of oxidation and reduction of the cytochromes were followed using a dual-wavelength spectrophotometer equipped with a regenerative stopped-flow apparatus [10]. The amplifier output was displayed in parallel on a storage oscilloscope and on a strip-chart pen recorder. The oscilloscope displayed the absorption changes which occurred during the first 0.5 s or 1 s after mixing the anaerobic suspension of membranes with oxygenated buffer. The pen recorder followed the complete cycle of absorption changes which occurred after mixing: attainment of aerobiosis, the aerobic steady state, and the return to the anaerobic state on depletion of the added oxygen. Details of the equipment used are in [11]. In the present work the slit-width was 0.5–1.0 mm, the time constant was 5 ms, and the reaction time at the start of observation (the continuous-flow phase) was 12–15 ms.

3. Results and discussion

Reduced minus oxidised difference spectra [12–14] have provided evidence for the presence in

Abbreviation: HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide

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aerobically grown *P. denitrificans* of two *b*-type cytochromes with absorption maxima at 560 nm and 566 nm at room temperature. When an anaerobic suspension of membranes, isolated from aerobically-grown cells, was mixed with oxygen these two *b*-type cytochromes were oxidised in the absence of antimycin but reduced in the presence of antimycin (fig.1). Measurements made over the first 0.5 s of the reaction (fig.1e,f) show that the antimycin-dependent reduction was complete within the period of the flow (15 ms). Measurements over a longer period (fig.1g,h) show that, in the presence of antimycin, the *b*-type cytochrome measured at 560 nm was slowly oxidised during the aerobic phase, while the *b*-type cytochrome measured at 566 nm remained largely reduced until the reaction mixture became anaerobic, at which time it was reoxidised. The antimycin-dependent, oxygen-induced reduction of the two *b*-type cytochromes in the membranes from aerobically grown *P. denitrificans* is consistent with the previous observation [14] that in the steady state these two *b*-type cytochromes are reduced to a greater extent aerobically in the presence of antimycin than anaerobically in the absence of antimycin.

Figure 2 shows the results of similar experiments performed with membranes from cells grown anaerobically with nitrate as the added terminal electron acceptor. As with membranes from aerobically grown cells, the introduction of oxygen resulted in an antimycin-dependent reduction of the *b*-type cytochrome measured at 566 nm (fig.2f,h). Unlike the membranes from aerobically grown cells, there was no antimycin-dependent reduction of the *b*-type cytochrome measured at 560 nm (fig.2e,g); and the *b*-type cytochrome measured at 566 nm, which was reduced on addition of oxygen in the presence of antimycin,

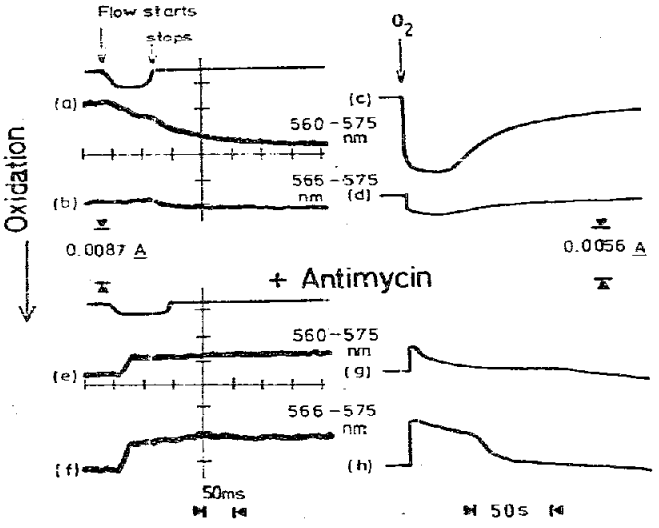

Fig.1. Effect of antimycin on the kinetics of oxidation and reduction of the *b*-type cytochromes in membranes from aerobically grown *P. denitrificans*. The stopped-flow dual-wavelength spectrophotometer measurements were made with the measuring and reference wavelengths indicated and were recorded in parallel on a storage oscilloscope (a,b,e,f) and a chart recorder (c,d,g,h). The major syringe, maintained at 27°C, contained in 20 ml: 75 mM potassium phosphate (pH 7.5), membrane vesicles (30 mg protein), gramicidin D (2 μg), 30 mM NH_4Cl, 0.2 mM NADH, alcohol dehydrogenase (1 mg), ethanol (0.2 ml) and rotenone (4 mg). Where indicated (e–h) 12.5 μM antimycin was included. The minor syringe contained 75 mM potassium phosphate (pH 7.5) continuously bubbled with oxygen at room temperature.

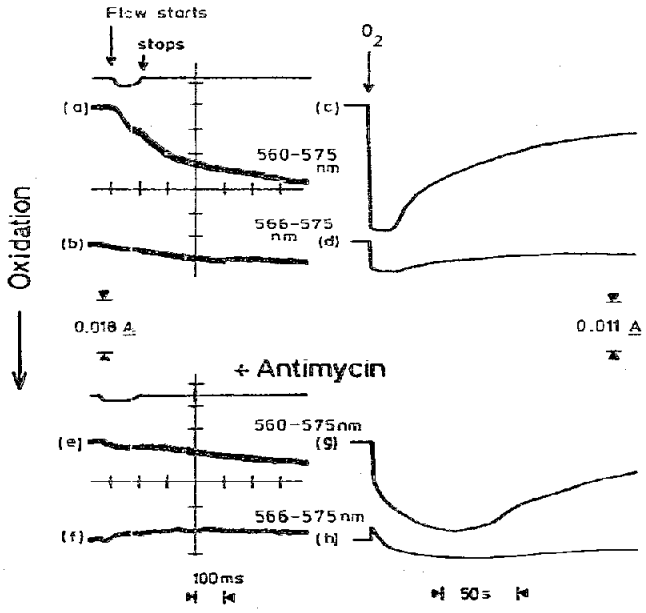

Fig.2. Effect of antimycin on the kinetics of oxidation and reduction of the *b*-type cytochromes in membranes from anaerobically grown *P. denitrificans*. Conditions were as for fig.1, except that the membranes (40 mg protein) were isolated from cells grown anaerobically with nitrate as the terminal electron acceptor, and 1 mg rotenone was present.

was slowly reoxidised during the aerobic phase (fig.2h).

As in mitochondria [15] replacement of the antimycin by HQNO did not result in a rapid, oxygen-dependent reduction of the *P. denitrificans* *b*-type cytochrome measured at 566 nm (fig.3c,g). However, an addition of HQNO did abolish the antimycin-dependent, oxygen-induced reduction of the *b*-type cytochrome measured at 566 nm (fig.3d,h). A similar addition of HQNO did not significantly affect the oxidation or reduction kinetics of the *c*-type cytochromes measured at 550 nm (fig.3i–p), but it increased by about 2-fold the time required for all the oxygen in the major syringe to be consumed, either in the absence (fig.3m,o) or in the presence of antimycin (fig.3n,p). Taken together with the previous observations [16] that both nitrate reduction and oxygen uptake are inhibited by HQNO but only oxygen uptake is inhibited by antimycin, the present data may be taken to imply that HQNO and antimycin can inhibit at different sites in the respiratory chain of anaerobically grown *P. denitrificans*.

The kinetic spectra of fig.4 show the extent of the absorption changes measured between 550 nm and 566 nm which occurred during the first 15 ms of the reaction. With membranes from both aerobically and anaerobically-grown cells, either in the absence or in the presence of antimycin, there was an absorption maximum at about 552 nm which can be attributed to the *c*-type cytochromes of *P. denitrificans* [12]. The kinetic spectra of the two types of membrane differed in two respects:

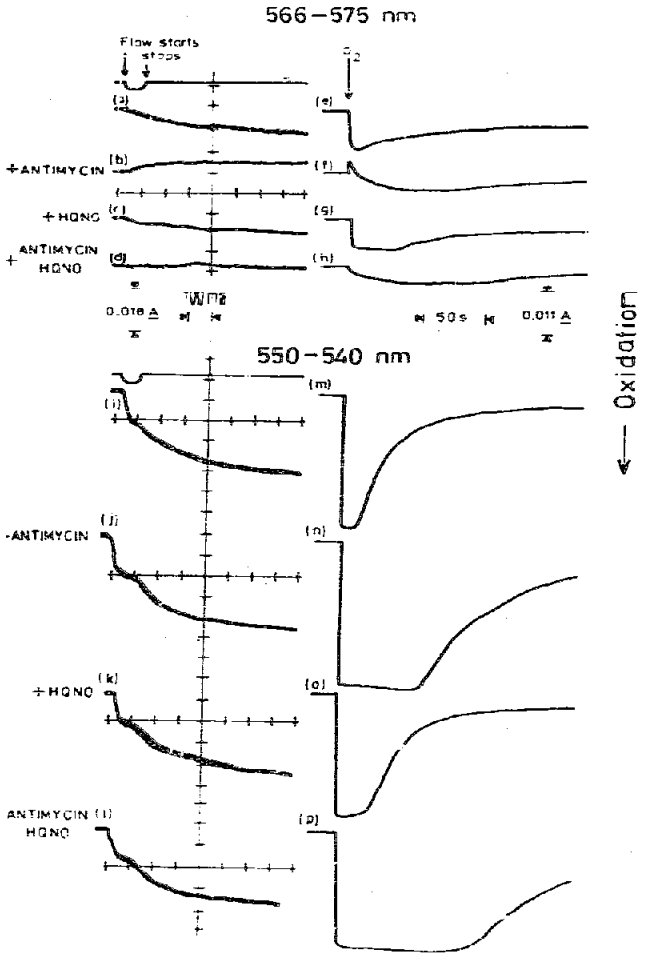

Fig.3. Effect of HQNO and antimycin on the oxidation and reduction kinetics of the *b*-type and *c*-type cytochromes in membranes from anaerobically grown *P. denitrificans*. Reaction conditions were as for fig.2. Where indicated the reaction mixture also contained 12.5 μM antimycin (b,f,j,n), 50 μM HQNO (c,g,k,o), or both 12.5 μM antimycin and 50 μM HQNO (d,h,l,p). Measurements were made either at 566–575 nm (a–h) or at 550–540 nm (i–p).

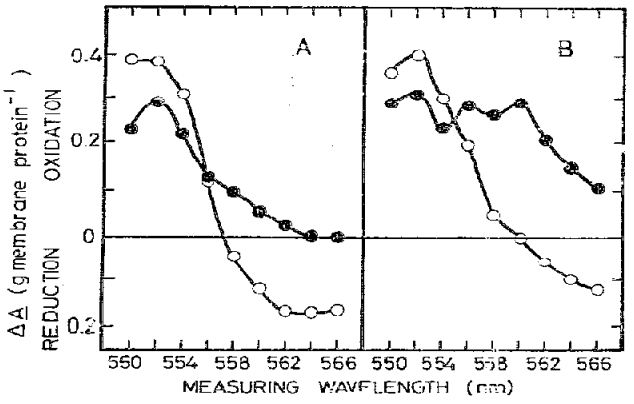

Fig.4. Kinetic spectra of the absorbance changes recorded during the first 15 ms after mixing oxygen with anaerobic membranes from (A) aerobically and (B) anaerobically grown *P. denitrificans*. The stopped-flow, double-beam spectrophotometer measurements were recorded on a storage oscilloscope and made as described in fig.1 (for A) and fig.2 (for B). The reference wavelength was maintained at 575 nm. Antimycin (12.5 μM) was omitted (–●–) or included (–○–) as indicated.

(i) In the absence of antimycin, membranes from anaerobically grown cells showed greater absorption changes at wavelengths higher than 556 nm than did the membranes from aerobically-grown cells.

(ii) In the presence of antimycin, membranes from aerobically grown cells had a more extensive absorption band attributable to the *b*-type cytochromes reduced on addition of oxygen than did membranes from anaerobically grown cells.

These differences between the kinetic spectra obtained with the two types of membrane may be due to the cytochrome *o*, which, from reduced +CO minus reduced difference spectra, has been shown [15,17] to be synthesised in increased amounts when *P. denitrificans* is grown anaerobically. However the significance of cytochrome *o* for the present study must remain uncertain until its absorption spectrum and its location in the respiratory chain are known (see [18]).

In conclusion, our observation of an oxygen-induced reduction of *b*-type cytochrome in membranes from both aerobically and anaerobically grown *P. denitrificans* is compatible with the previous proposition [19] that, in adapting to an anaerobic existence, *P. denitrificans* adds new components to a retained, aerobic respiratory chain, which closely resembles the mitochondrial respiratory chain.

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