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Cytochrome *c*-550 mediates electron transfer from inducible periplasmic *c*-type cytochromes to the cytoplasmic membrane of *Paracoccus denitrificans*

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Electron transfer from periplasmic cytochromes *c* to the membrane-bound respiratory chain has been studied with the isolated cytochromes and membrane preparations from *Paracoccus denitrificans*. When reduced cytochromes were incubated with spheroplasts only the constitutive cytochrome *c*-550 was rapidly oxidized. The inducible cytochromes *c*-551i and *c*-553i were not oxidized at appreciable rates. Cytochrome *c*-550 was able to mediate the transfer of electrons from either cytochrome *c*-551i or cytochrome *c*-553i to the membrane preparation.

Cytochrome *c*; Electron transfer; Methylotrophic bacteria; (*Paracoccus denitrificans*)

1. INTRODUCTION

Paracoccus denitrificans is capable of growth on methanol or methylamine as a sole source of carbon and energy [1]. Each of these substrates is oxidized to formaldehyde in the periplasm of this Gram negative bacterium by inducible pyrroloquinoline quinone-containing methanol and methylamine dehydrogenases [2,3]. The physiological electron acceptor for methylamine dehydrogenase is an inducible periplasmic type I blue copper protein, amicyanin [4]. Also present in the periplasm of methylamine-grown cells are the constitutive cytochrome *c*-550, and two inducible cytochromes, *c*-551i and *c*-553i, which are present during growth on methanol or methylamine, but absent during heterotrophic growth [5]. Kinetic studies indicate that cytochrome *c*-551i is the physiological electron acceptor for amicyanin [5]. The CO-binding cytochrome *c*-553i is believed to accept electrons from methanol dehydrogenase [5,6]. To determine the manner in which electrons from these inducible cytochromes are donated to the membrane-bound respiratory chain, the redox reactions of these cytochromes with membrane preparations of *P. denitrificans* have been examined.

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2. EXPERIMENTAL

Cytochromes *c*-550, *c*-551i, and *c*-553i were isolated as previously described from *P. denitrificans* (ATCC 13543) which was grown aerobically on methylamine [5]. Cytochromes *c*-551i and *c*-553i were further purified by passage over Sephadex G-100. Spheroplasts were prepared according to Alefounder and Ferguson [2]. The integrity of spheroplast preparations was monitored polarographically with a Clark-type oxygen electrode and a Yellow Springs Instruments model 5300 biological oxygen monitor. Preparations typically exhibited respiration rates with endogenous substrate of 390 nmol $O \cdot min^{-1} \cdot mg^{-1}$, which were stimulated only slightly by addition of succinate or NADH. Rates of 2000 nmol $O \cdot min^{-1} \cdot mg^{-1}$ were attained on addition of 1 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine. These observations are consistent for a preparation of tightly coupled, predominantly right-side out vesicles [8,9].

The reaction of cytochromes *c* with the membrane-bound respiratory chain was assayed with a Uvicon 810 spectrophotometer equipped with a scattered transmission accessory. Spheroplasts were added to a sample of reduced

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cytochrome and changes in absorbance of the α-band of that cytochrome were recorded. After each assay, the complete spectrum of the cytochrome in the presence of spheroplasts was recorded to confirm that any observed decrease in absorbance was due to the oxidation of the cytochrome and not an artifact of the assay. Cytochrome concentrations were determined from previously reported extinction coefficients [5]. Reduced cytochromes were prepared by addition of a few grains of solid $Na_2S_2O_4$. To determine the protein content of spheroplasts, samples were incubated for 2 h in 5 M urea and then assayed by the method of Bradford [7] with Pierce Protein Reagent and bovine serum albumin as a standard.

3. RESULTS AND DISCUSSION

Of the three periplasmic *c*-type cytochromes, cytochrome *c*-550 was clearly the most efficient donor of electrons to the membrane preparation (fig.1c). The rate of oxidation of cytochrome *c*-553i (fig.1b) was approximately one tenth of that observed with cytochrome *c*-550. Cytochrome *c*-551i was unreactive (fig.1a). Given the relatively high rate of reactivity of cytochrome *c*-550, which is known to donate electrons to the cytochrome-aa_3 oxidase of *P. denitrificans*, and the relatively low reactivity of the inducible cytochromes, the ability of cytochrome *c*-550 to mediate the transfer of electrons from cytochromes *c*-551i and *c*-553i to the membrane was examined (fig.2). In this assay, reduced cytochrome *c*-551i or *c*-553i was incubated with spheroplasts as in fig.1, and the absorbance of the α-band of each cytochrome was monitored. A catalytic amount of cytochrome *c*-550 was then added to each sample. After an immediate small increase in absorbance due to the contribution of the added cytochrome *c*-550, a substantial decrease in absorbance was observed for each of the inducible cytochromes. Thus, cytochrome *c*-550 was able to mediate the transfer of electrons from either cytochrome *c*-551i or *c*-553i to the membrane preparation. These experiments were repeated in buffer containing either 150 mM KCl or 20 mM MgCl with essentially the same results. While the absolute rates of cytochrome oxidation varied, the relative reactivities of the cytochromes and ability of cytochrome *c*-550 to mediate electron transfer did not vary.

The specificity of the membrane-bound cytochrome oxidase for cytochrome *c*-550 is noteworthy. Cytochromes *c*-551i and *c*-553i are of lower potential than cytochrome *c*-550 [10] and like cytochrome *c*-550 have relatively acidic

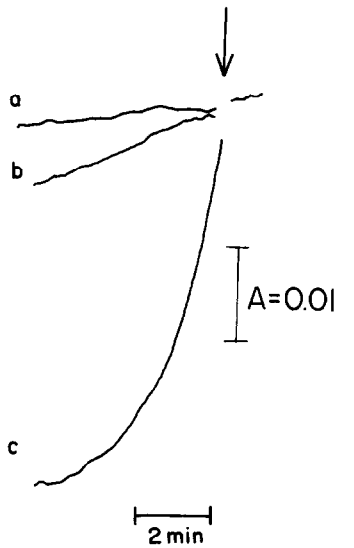

Fig.1. The oxidation of cytochromes *c* by spheroplasts of *P. denitrificans*. The assay mixtures contained (a) 2.9 nmol of reduced cytochrome *c*-551i, (b) 3.3 nmol of reduced cytochrome *c*-553i, and (c) 2.0 nmol of reduced cytochrome *c*-550. Each was present in 1 ml of 10 mM potassium phosphate, pH 7.2. The arrow indicates addition of 20 μl of spheroplasts containing 16 μg of protein to both the sample and reference cuvettes. Changes in absorbance were monitored at (a) 551 nm, (b) 553 nm, or (c) 550 nm.

isoelectric points [5]. Thus, no thermodynamic or electrostatic barrier should prevent the inducible cytochromes from interacting directly with the oxidase. That the relative reactivities of these cytochromes with the spheroplasts and with each other was not altered by the presence of 150 mM KCl or 20 mM MgCl further suggests that these phenomena are caused by specific protein-protein interactions and not simply a thermodynamically favorable reaction between non-specifically associated redox proteins.

It is well documented that the components of the membrane-bound respiratory chain and periplasmic cytochrome *c*-550 of *P. denitrificans* bear strong similarities to their mitochondrial counterparts [11]. When grown on methylamine and methanol, several additional periplasmic redox proteins are synthesized that participate in the dissimilation of these C-1 compounds [2–5]. The point at which the electrons derived from the oxidation of these substrates leave the periplasm and are donated to a membrane-bound electron carrier was not known. It is now possible to pro-

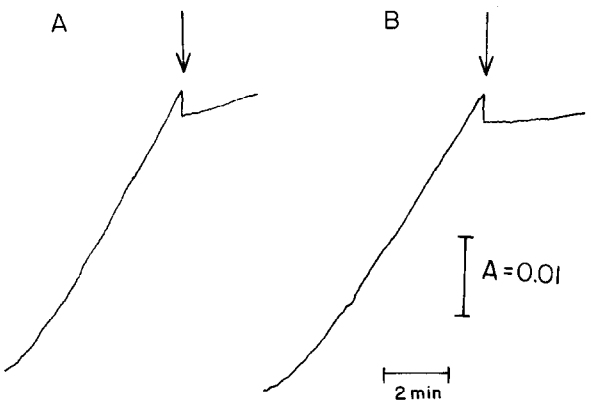

Fig.2. Cytochrome *c*-550 mediated oxidation of cytochromes *c*-551i and *c*-553i. The assay mixtures initially contained (A) 3.3 nmol of reduced cytochrome *c*-553i and (B) 2.9 nmol of reduced cytochrome *c*-551i. Each also contained spheroplasts containing 16 μg of protein and 1 ml of 10 mM potassium phosphate, pH 7.2. Each arrow indicates the addition of 0.2 nmol of cytochrome *c*-550. Changes in absorbance were monitored at (A) 553 nm and (B) 551 nm.

pose a scheme by which this most likely occurs (fig.3). These data (figs 1,2) clearly demonstrate that the constitutive cytochrome *c*-550 can mediate the transfer of these electrons from the inducible periplasmic cytochromes *c*-551i and *c*-553i to the membrane. Previous work in this laboratory has shown that amicyanin is the immediate electron acceptor for methylamine dehydrogenase and that it mediates the transfer of electrons most efficiently to cytochrome *c*-551i [4,5,12]. No electron transfer in vitro from methanol dehydrogenase to any of the periplasmic cytochromes *c* has been observed. However, the induction pattern of cytochrome *c*-553i and its ability to bind CO suggest that it functions in methanol-dependent respiration [5,6]. Thermodynamic considerations suggest that electron transfer from cytochrome *c*-553i to cytochrome *c*-551i may also occur.

Cytochrome *c*-550 plays a central role in respiration by *P. denitrificans*. As in mitochondria, it mediates the transfer of electrons from the cytochrome bc_1 complex to the cytochrome-aa_3 oxidase. During anaerobic growth, it can mediate the transfer to alternate terminal electron acceptors such as nitrite via reaction with nitrite reductase. These data establish that during methylamine- and

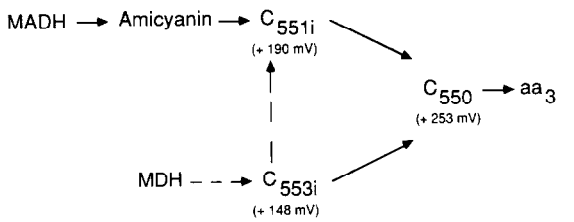

Fig.3. Proposed pathway of electron transport from methylamine and methanol to the cytoplasmic membrane in *P. denitrificans*. Each of these components is a soluble periplasmic protein except for cytochrome aa_3 which is an integral membrane protein. Solid arrows denote documented reactions. Dashed arrows denote reactions which are possible or likely but have not been proven to occur. The midpoint potential values for each cytochrome [10] are given in parentheses. MADH, methylamine dehydrogenase; MDH, methanol dehydrogenase; aa_3, cytochrome aa_3.

methanol-dependent respiration, cytochrome *c*-550 also mediates the entry of the electrons that are derived from these substrates from inducible periplasmic redox proteins to the membrane.

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