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THE BRANCHED RESPIRATORY SYSTEM OF PHOTOSYNTHETICALLY GROWN *RHODOPSEUDOMONAS CAPSULATA*

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SUMMARY

Various respiratory electron transport activities of *Rhodopseudomonas capsulata* were studied in membrane fragments prepared from photosynthetically grown cells of a parental strain and two terminal oxidase-defective mutant strains. The NADH and succinate oxidase activities of the mutant having a functional *N,N,N*^{1},*N*^{1}-tetramethyl-*p*-phenylenediamine oxidase, M6, were considerably more sensitive to inhibition by either antimycin A or cyanide than the corresponding activities of the mutant lacking a functional *N,N,N*,^{1}*N*^{1}-tetramethyl-*p*-phenylenediamine oxidase, M7. The parental strain, Z-1, but not the mutants, showed biphasic inhibitory responses of NADH and succinate oxidase activities with either antimycin A or cyanide. In certain reactions no differences in inhibitor susceptibility were found among the strains tested, implying that the pathways involved were unaffected in the mutants. In this category were the actions of rotenone on NADH oxidase, antimycin A on cytochrome *c* reductase and, in M6 and Z-1, cyanide on *N,N,N',N'*-tetramethyl-*p*-phenylenediamine oxidase. These results suggest that the respiratory chain of the parental strain branches at the ubiquinone-cytochrome *b* region into two pathways, each branch goes to a distinct terminal oxidase, and either may be blocked independently by genetic mutation.

INTRODUCTION

Most nonsulfur purple photosynthetic bacteria can obtain energy for growth by either respiration or photophosphorylation, and, depending upon the oxygen tension and the light intensity to which a growing culture is exposed, the energy transducing systems of these organisms become relatively specialized for one mode or the other. Cells grown in the presence of excess oxygen are virtually devoid of any photosynthetic capability, however, cells grown anaerobically in light always retain a reduced but significant respiratory activity [1]. We have adopted a biochemical genetics approach to investigate the relationship between the photosynthetic and respiratory electron transport systems of *Rhodopseudomonas capsulata*. In previous

Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

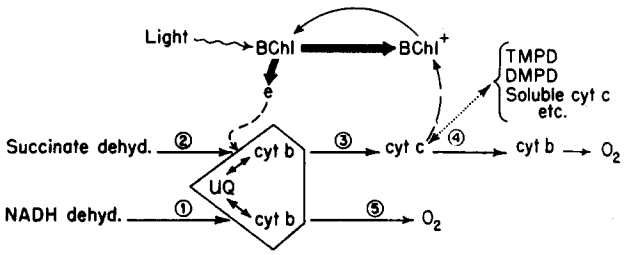

Fig. 1. A model of electron transport pathways present in membranes of photosynthetically grown *R. capsulata*. The numbered arrows represent steps in respiratory electron-transport pathways; some steps may involve more than one electron carrier. The box enclosing ubiquinone and cyt b represents the branching point and symbolizes the interchange of reducing equivalents from the different dehydrogenases to either branch. Strain M6 is blocked in step 5; M7 is blocked in step 4 by virtue of lacking the associated cytochrome *b*. BChl, bacteriochlorophyll; cyt, cytochrome; UQ, ubiquinone; e, electron; DMPD, *N,N*-dimethyl-*p*-phenylenediamine. Modified from Marrs and Gest [2].

studies [2, 3] we have described the isolation and characterization of a variety of mutants possessing altered respiratory electron transport systems. Biochemical analysis of these mutants led to the formulation of a branched model for respiratory electron transport in photosynthetically grown *R. capsulata* [2]. This model, extended to include the identity of one terminal oxidase, is presented as a working hypothesis in Fig. 1. One class of mutants in particular, of which strain M7 is representative, pointed to the branched nature of the respiratory system. These mutants lack virtually all cytochrome *c* oxidase and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) oxidase activities, yet they retain the capacity for vigorous aerobic dark growth, and thus an alternate route to oxygen must exist. A similar branched system was independently proposed by Baccarini-Melandri et al. [4] on the basis of conventional inhibitor studies of the respiratory chain present in aerobically grown *R. capsulata*, and Zannoni et al. [5] subsequently demonstrated that strain M7 lacks a high potential cytochrome with a *b*-type spectrum.

In this report we present the results of further biochemical analyses of the residual respiratory systems of mutant strains deficient in one or the other terminal oxidase activities. These studies support the model previously proposed for the respiratory chain of photosynthetically grown *R. capsulata*. A comparison of our studies with the results concerning the electron transport system of aerobic cells of these mutants, presented by Zannoni et al. in the accompanying paper [6], suggests that there is no qualitative difference between the respiratory chains found in cells specialized for these alternative energy transducing modes.

MATERIALS AND METHODS

The studies were performed on *R. capsulata* strains Z-1, M6, and M7. M6 and M7 are spontaneous revertants of strain M5, a double mutant of Z-1 which has lost the ability to grow aerobically. M6 has regained TMPD oxidase activity and M7 has not (see Fig. 1), however, both grow aerobically. Cultures were grown photosynthetically in RCV, a malate minimal medium [3]. Precultures were grown in screw capped tubes in a 35 °C water bath and used to inoculate approx. 1 l cultures in Blake bottles

grown in a 30 °C light box. Saturating illumination under both conditions was provided by banks of Lumiline lamps.

Cells were harvested in early stationary phase and membrane fragments were prepared by disruption in a French pressure cell and differential centrifugation as previously described [2]. Protein was assayed by the Lowry method [7].

NADH, succinate, and TMPD oxidase activities were measured polarographically at 25 °C using a Yellow Springs Model YSI 53 oxygen meter with a jacketed Gilson Oxygraph reaction chamber (modified to have about a 1.6 ml volume) and a Clark type 0_2 electrode to monitor 0_2 consumption [2]. Membrane fragments were resuspended to about 1 to 2 mg protein/ml in 0.05 M KPO_4 (pH 7.5) buffer and preincubated about 5 min with a 1 to 10 μl aliquot of inhibitor (plus 25 mM Na L-ascorbate for TMPD oxidase assays). The reaction was then initiated by injecting substrate to give a final concentration of 5 mM β-NADH, 40 mM sodium succinate, or 0.5 mM TMPD (except 1.25 mM TMPD was used for M7 assays).

NADH- and succinate-driven cytochrome *c* reductase activities were measured spectrophotometrically [3] and rates were calculated from the absorbance increase at 550 nm using a Heath EU707 ratio recording double-beam spectrophotometer. Membrane fragments were resuspended in cuvettes to about 0.05 mg protein/ml in 0.05 M KPO_4 buffer (pH 7.5) to give a final volume of 3.0 ml. 0.08 mM horseheart cytochrome c, 3.33 mM NaN_3, and inhibitor were added and the reaction mixture was preincubated about 10 min before the reaction was initiated by addition of substrate, 0.2 mM β-NADH or 5.33 mM sodium succinate.

Rotenone and antimycin A were dissolved in dimethylsulfoxide (Me_2SO). Effects from addition of Me_2SO alone were consistently negligible. In all assays, reaction rates were corrected for endogenous activity, that is, the rate with inhibitors but without substrate.

β-NADH, succinate, cytochrome *c* (horseheart, type III), rotenone, and sodium L-ascorbate were obtained from Sigma Chemical Co., St. Louis. TMPD was obtained from K and K Labs, Inc., Plainview, New. York. Antimycin A was a generous gift from Mr. B. Mollov of Ayerst Labs., New York (Ayerst trade name, Fintrol).

RESULTS

Titration of oxidase activities by KCN

Baccarini-Melandri et al. [4] demonstrated the biphasic nature of the inhibition of NADH oxidation by KCN in membrane fragments from aerobically grown cells and concluded that alternative terminal oxidases with different sensitivities to cyanide exist. The results shown in Fig. 2 demonstrate that NADH oxidase activity of membrane fragments from photosynthetically grown cells of strain Z-1 also gives a biphasic KCN inhibition curve. Furthermore, mutant strains M6 and M7 each show only one inhibition constant for KCN. The NADH oxidase activity of the cytochrome *c* oxidase-containing pathway, functional in M6, shows sensitivity to KCN (50 per cent maximal inhibition, $I_{50\,\%}$, at $2 \cdot 10^{-6}$ M) very similar to that of the more sensitive component of the dual system in Z-1 ($I_{50\,\%}$ at $3 \cdot 10^{-6}$ M) and to the TMPD oxidase from both M6 and Z-1 (Fig. 3; $I_{50\,\%}$ at $3 \cdot 10^{-6}$ M and $4 \cdot 10^{-6}$ M, respectively). The alternate oxidase, functional in strain M7 mediates an NADH

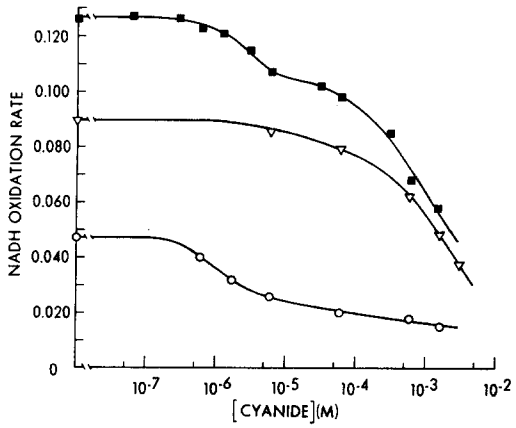

Fig. 2. Inhibition of NADH oxidation activity of membrane fragments as a function of cyanide concentration. Strains: Z-1, ■–■; M6 ○–○; M7 ▽–▽. Membrane fragments were prepared as described in Materials and Methods. Oxidase rates are expressed as μeqO_2 oxidized $\cdot$ min^{-1} $\cdot$ mg^{-1} protein.

oxidase activity which titrates like the KCN resistant portion of the parental strain ($I_{50\,\%}$ at about 10^{-3} M for each).

Succinate oxidase activity of Z-1 photosynthetic membrane fragments also shows a biphasic inhibition by KCN, in contrast to the monophasic profile obtained by Baccarini-Melandri et al. with respiratory membrane fragments. As is the case for NADH oxidation, M6 and M7 each oxidize succinate via pathways which coincide in KCN sensitivity with one of the two pathways in the parental strain (Fig. 4), and M6 is more sensitive.

An examination of the NADH and succinate oxidase activities of the parental type as a function of KCN concentration suggests that a relatively small fraction of

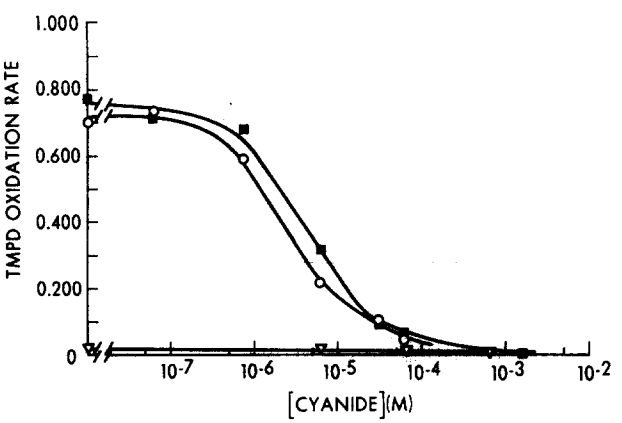

Fig. 3. Inhibition of TMPD oxidation activity of membrane fragments as a function of cyanide concentration. Strain identification symbols and oxidase activity units the same as given in Fig. 2.

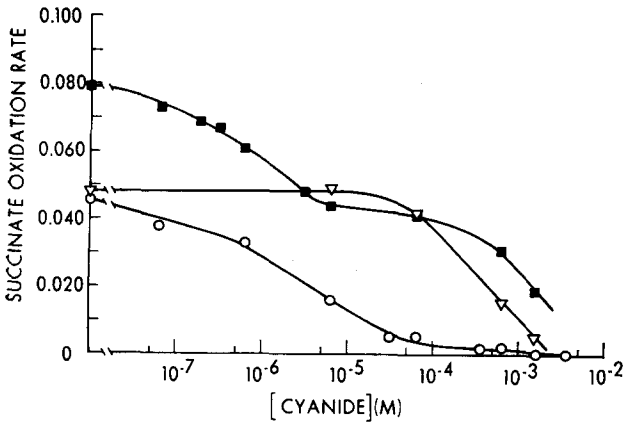

Fig. 4. Inhibition of succinate oxidation activity of membrane fragments as function of cyanide concentration. Strain identification symbols and oxidase activity units the same as given in Fig. 2.

the total NADH oxidase activity is lost when the KCN-sensitive oxidase is blocked, whereas a larger portion of the succinate oxidase activity can be inhibited at low KCN concentrations. This observation is consistent with the concept of channelling developed in our earlier report [2], i.e. electrons originating from either succinate or NADH may be oxidized via either route, but in fact a greater portion of the flux of electrons from NADH go to oxygen via the alternate oxidase, while succinate is oxidized at approximately equal rates by either pathway.

Inhibition by antimycin A

Antimycin A typically blocks electron transport between *b*- and *c*-type cytochromes, and thus we anticipated a differential effect of this inhibitor on strains M6 and M7. This expectation is confirmed by the data presented in Figs. 5 and 6. NADH

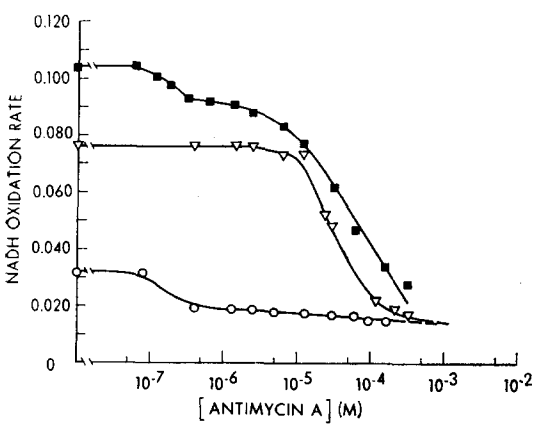

Fig. 5. Inhibition of NADH oxidation activity of membrane fragments as a function of antimycin A concentration. Strain identification symbols and oxidase activity units the same as given in Fig. 2.

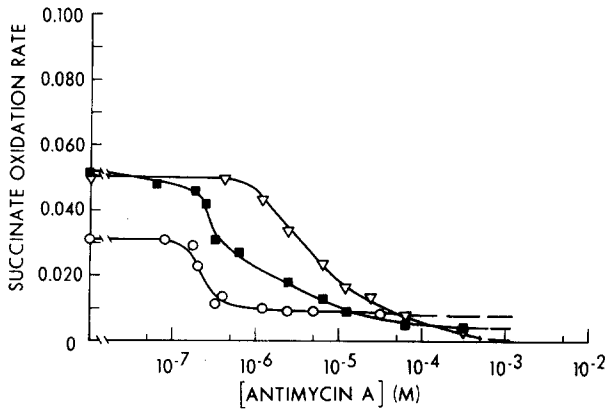

Fig. 6. Inhibition of succinate oxidation activity of membrane fragments as a function of antimycin A concentration. Strain identification symbols and oxidase activity units the same as given in Fig. 2.

oxidase activity of M6 is extremely sensitive to antimycin A ($I_{50\%}$ at $2.5 \cdot 10^{-7}$ M) while the same activity in M7 requires more than two orders of magnitude higher concentration for inhibition ($I_{50\%}$ at $3.3 \cdot 10^{-5}$ M). The parental strain shows a biphasic titration curve with one inflection corresponding to each branch's sensitivity to the inhibitor. M6 demonstrates a sensitivity of succinate oxidase to antimycin A ($I_{50\%}$ at $2.3 \cdot 10^{-7}$ M) which is very similar to that of NADH oxidase in M6, but the succinate oxidase activity of M7 is more sensitive to antimycin A ($I_{50\%}$ at $5.5 \cdot 10^{-6}$) than is NADH oxidase in M7. The titration curve of Z-1 succinate oxidase activity with antimycin A shows no plateau between the two phases of inhibition, consistent with the partial overlap of the inhibitions observed in the strains possessing only one or the other respiratory route. In contrast to the differences in antimycin A sensitivity observed among the oxidases of Z-1, M6 and M7, the NADH and succinate driven cytochrome *c* reductase activities both showed the same sensitivity to antimycin A for

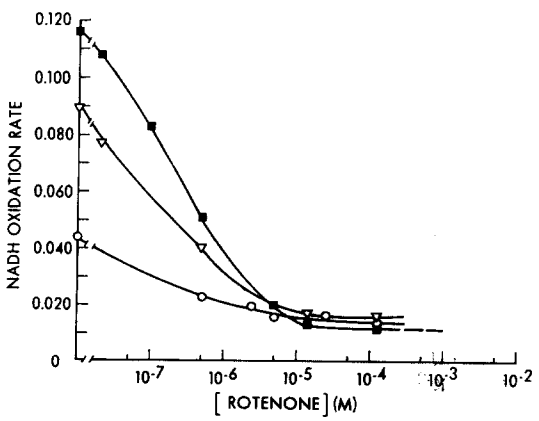

Fig. 7. Inhibition of NADH oxidation activity of membrane fragments as a function of rotenone concentration. Strain identification symbols and oxidase activity units the same as given in Fig. 2.

all three strains (uninhibited rate about 0.2 μmol cytochrome *c* reduced per min mg protein; $I_{50\,\%}$ at approx. $2 \cdot 10^{-8}$ M).

Rotenone inhibition of NADH oxidase activity

NADH-stimulated O_2 uptake catalyzed by membrane fragments from each of the strains examined was partially resistant to cyanide and to antimycin A. The residual activity in all cases was 0.010–0.020 mE $\cdot$ min^{-1} $\cdot$ mg^{-1} protein. A quantitatively similar residual activity is observed in strain M5, the double mutant combining the lesions in M6 and M7. Rotenone had no effect on that fraction of NADH oxidase activity which was resistant to KCN, and rotenone also failed to inhibit a quantitatively similar fraction of total NADH oxidase activity (Fig. 7). Each strain showed the same sensitivity to rotenone in this assay ($I_{50\,\%}$ at about $2 \cdot 10^{-7}$ M).

DISCUSSION

The data presented in this report support the branched chain model for respiratory electron transport catalyzed by membranes of photosynthetically grown *R. capsulata* (Fig. 1). The biochemical analysis of mutants is a valid approach to the study of a normal, wild type, system provided the mutations perturb the system to a limited and thus definable extent. We have chosen to study the respiratory electron transport system of photosynthetically grown cells of respiratory mutants, because under photosynthetic growth conditions the oxidase system is gratuitous, and compensatory responses to partial loss of respiratory functions may be avoided. The mutations involved in this study fulfill the criterion of limited perturbation admirably. The oxidation rates obtained from strain Z-1, containing both arms of the branched chain, most often equalled the sum of the rates of the complementary mutant strains, M6 and M7, which each contain only one functional arm (although the absolute oxidation rates obtained from various membrane preparations of the same strain may vary as much as two-fold). This is taken to indicate that the alterations in the mutant strains are discrete, blocking one branch without changing the characteristics of the rest of the system.

The following features of the model for respiratory electron transport presented in Fig. 1 are supported by the inhibitor data presented here: (a) the pathway is branched, since two terminal oxidases may be distinguished by KCN sensitivity and by mutation; (b) the branch point must occur before the antimycin A sensitive sites, since the oxidase activities mediated by the two branches display grossly different sensitivities to this antibiotic; (c) the pathway functional in M6 is more sensitive to both antimycin A and KCN than the alternate pathway functional in M7; (d) the alternate pathway does not function in the reduction of exogenous cytochrome *c*; (e) both NADH and succinate can be oxidized by either branch, but NADH is oxidized more rapidly by the alternate pathway while succinate is oxidized at comparable rates by both routes; (f) TMPD is oxidized exclusively via the cytochrome *c* oxidase; (g) a low level, rotenone-insensitive NADH oxidase activity is present in membrane preparations from all three strains. This activity is probably not due to the respiratory system.

It was observed in strain M7 that lower concentrations of antimycin A were necessary to achieve 50 per cent of maximal inhibition of succinate oxidation com-

pared to NADH oxidation. This was not observed in strain M6. The different sensitivities of oxidase activities in M7 probably do not reflect the presence of alternative sensitive sites in NADH and succinate oxidation pathways, but rather an effect of substrate on sensitivity, as observed in other systems [8].

The sensitivity of the alternate pathway oxidase activity to antimycin A suggests that it contains a *b*-type cytochrome, and the difference in antimycin A sensitivity between the two branches suggests that the cytochromes *b* in each path are either in very different environments, or they are two distinct species.

Cytochrome *c* reductase activity seems to be mediated entirely by the cytochrome *c* oxidase-containing branch, since this activity is inhibitable by the same low concentration of antimycin A in all strains with either substrate. The concentration of antimycin A needed to achieve 50 % inibition of cytochrome *c* reductase activities is about 10 times lower than that which causes comparable inhibition of the oxidase activities mediated by the same pathway. This may be a result of the lower protein concentrations used in the reductase assays, or the greater specific rate of electron transport activity which the reductase assay exhibits compared to the oxidase assay.

A rough comparison of the published oxidase activities of membranes from photosynthetic and aerobic cells shows that aerobic membranes have greatly enhanced NADH oxidase activity, and a greater portion of that activity is KCN sensitive in aerobic membranes. Succinate and cytochrome *c* oxidase activities, on the other hand, are only slightly higher in aerobic membranes than in photosynthetic. These observations are consistant with a differential increase of NADH dehydrogenase activity in the aerobic membranes, perhaps accompanied by a slight increase in activity of the cytochrome *c* oxidase-containing branch of the system. An enhancement of the cytochrome *c* oxidase-containing branch relative to the alternate pathway may explain why the previous study [4] on aerobic membranes failed to resolve two inflections in the KCN titration of succinate oxidase activity.

The isolation and analysis of respiratory mutants has provided several insights to the electron transport system of photosynthetic bacteria. The model presented portrays the respiratory and photosynthetic systems as sharing or competing for a portion of the electron transport chain. The data presented here do not test this hypothesis, however, we are currently studying a mutant strain blocked in electron transport between cytochrome *b* and cytochrome *c* which we are confident will further our understanding of the relationship between the photosynthetic and respiratory electron transport systems in these organisms.

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