ISOLATION AND CHARACTERIZATION OF A CYTOCHROME c_2-DEFICIENT MUTANT OF *RHODOPSEUDOMONAS CAPSULATA*

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1. Introduction

The role of cytochrome c_2 as a functional redox carrier in light-driven electron flow has been clearly demonstrated [1,2], and its involvement in the respiratory electron transport system of *Rhodopseudomonas capsulata* has been confirmed [3]. *Rps. capsulata* is thought to have a branched respiratory electron transport pathway with cytochrome c_2 (cyt. c_2) functioning in only one of the branches [4–7]. This latter suggestion and other aspects of the physiological functions of cyt. c_2 can be examined in mutants lacking this heme protein.

Here we describe tetracycline suicide, a new method for the isolation of mutants with deranged energy metabolism, and the properties of a mutant of *Rps. capsulata* obtained by this method are presented. The mutant, TL11, lacks cyt. c_2, a phenotype not yet described. The properties of this mutant and membranes isolated from it provide the first clear and direct evidence that one of the branches of the respiratory electron transport system of *Rps. capsulata* lacks cyt. c_2, and we independently confirm several important features of cyt. c_2 function, namely:

(1) Cytochrome c_2 is the only physiologically significant reductant of the photosynthetic reaction center;
(2) Cytochrome c_2 functions in both respiration and photosynthesis;
(3) The second and third phases of the single-turnover flash-induced carotenoid bandshift are dependent upon the presence of ferrocytochrome c_2 [8].

2. Materials

Tetracycline suicide selection for mutants with deranged light-dependent energy metabolism was performed as follows. *Rhodopseudomonas capsulata* strain SB1003 [9] was grown under photosynthetic growth conditions in RCV medium as in [10]. Tetracycline–HCl (Sigma Chemical Co.) was added at 1.0 μg/ml final conc. to an exponentially growing culture of SB1003. Incubation in the light was continued for 2 days, then samples of the culture were spread on RCV plates to detect survivors capable of aerobic dark colony formation. One such colony, designated TL11, was normally pigmented but incapable of photosynthetic growth, and it was selected for further study. A green derivative of TL11 that is named MT113 and accumulates a mixture of neurosporene, hydroxyneurosporene and methoxyneurosporene in place of the wild-type carotenoids was constructed by gene transfer from strain R121 [11]. An isogenic control strain (MT1131) that regained photosynthetic competence was constructed from MT113 by a second round of treatment with gene transfer agent from R121.

Semiaerobic cultures were grown in the dark and membrane fragments prepared as in [12]. Redox titrations of cytochromes were performed at (pH 7.0) in a medium containing 50 mM KCl and 50 mM 2(*n*-morpholino)ethane sulphonic acid (MES) by the technique introduced in [13]. Rapid spectrophotometry of cytochromes, reaction center bacteriochlorophyll dimer and carotenoid bandshift have

been described [14,15]. Protein content was estimated by the Lowry method [16] and bacteriochlorophyll was measured by extraction using $\epsilon = 75\ mM^{-1} . cm^{-1}$ [17].

3. Results and discussion

Strain TL11 was chosen for study because it is a normally pigmented, non-photosynthetic survivor of tetracycline suicide that shows a negative Nadi reaction in the colony staining assay for cytochrome oxidase activity [4]. Membrane fragments obtained from cells of *Rps. capsulata* TL11 grown semiaerobically in the dark contain significant amounts of carotenoids corresponding to spheroidene and spheroidenone. Because the absorption of these pigments is very strong in the cytochrome region, a green derivative of TL11 (MT113) that accumulates only the neurosporene family of carotenoids and an isogenic strain (MT1131) that has regained the capacity for photosynthetic growth have been constructed and used for all the experiments described (see section 2). Fig.1 shows the reduced minus oxidized spectra of cytochromes in chromatophores from MT113 and MT1131. The absence of the peaks at 552 and 518 nm, corresponding to the α and β bands of cyt. c_2 is conspicuous in MT113. Redox titrations of MT113 membranes indicated that the normal *b*-type cytochromes were present but no traces of cyt. c_2 (also known as cyt. c_{340}) could be detected (fig.2,3). A second *c*-type component, described in both photosynthetic and aerobic membranes of *Rps. capsulata* St Louis [2,7] and characterized by a mid-point potential of ~+115 mV at pH 7.0, is present in membrane vesicles from both MT113 and MT1131. However, the amount of cyt. c_{115} in MT113 is less than in wild-type membranes (0.02 nmol/mg protein in MT113 and 0.06 nmol/mg protein in MT1131). Redox titrations of the *b*-type cytochromes (561–570 nm) in these two strains reveal only one significant difference, namely the amount of cyt. b_{145} in MT1131 is ~0.05 nmol/mg protein, while the same component is MT113 amounts to only 0.03 nmol/mg protein. The concentration of cyt. c' is also lower in MT113 than in MT1131 as estimated by the absorbance of the Soret bands in the high-speed supernatant of broken cells in the presence of carbon monoxide [18]. In view of the fact that cyt. c' has a broad absorption maximum extending from 550–563 nm in the reduced state, it may be possible that the roughly paralleled concentration decreases of cyt. c_{115}, cyt. b_{145} and cyt. c' in MT113 relative to MT1131 may be understood in terms of one cytochrome being responsible for all 3 signals.

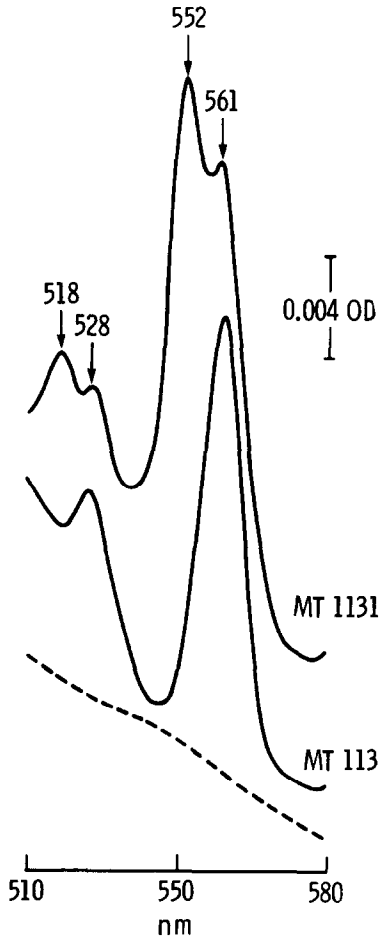


Fig.1. Dithionite-reduced minus ferricyanide-oxidized difference spectra, recorded at +25°C, of membrane fragments from *Rps. capsulata* MT1131 and MT113. Particles were suspended at 2.25 mg/ml in 50 mM MES (pH 7.0) Upper trace, MT1131; lower trace, MT113.

The respiratory electron transport system activities of membranes from MT113 and MT1131 are shown in table 1. The overall NADH oxidase activity/mg protein is reduced in MT113 compared to MT1131, but in the presence of a low [KCN], which has been shown to preferentially inhibit the cyt. *c* oxidase-containing pathway [19], both strains exhibit similar activities. High [KCN] inhibit the alternate oxidase

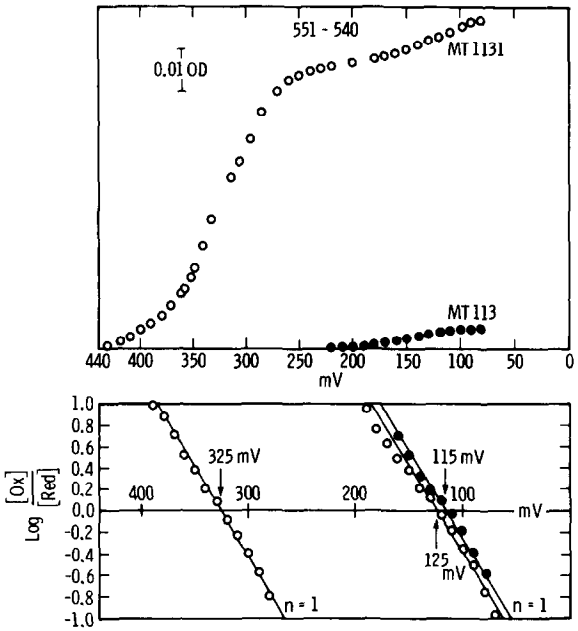


Fig.2. Potentiometric titrations at 551 minus 540 nm in particles prepared from *Rps. capsulata* MT1131 and MT113. Particles were suspended in 50 mM MES plus 50 mM KCl (pH 7.0) at 10 mg/ml and 12 mg/ml for MT1131 and MT113, respectively. Open circles, MT1131; closed circles, MT113.

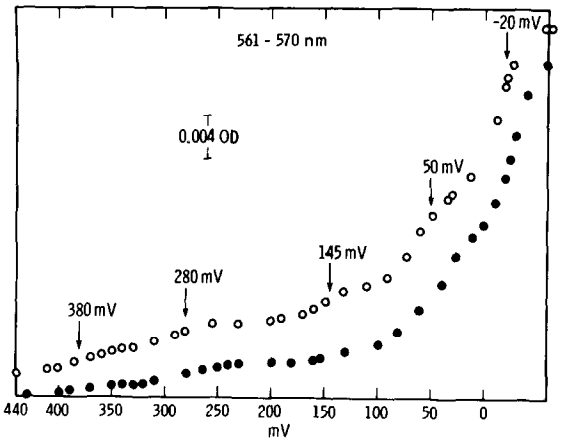


Fig.3. Potentiometric titrations at 561 minus 570 nm in particles prepared from *Rps. capsulata* MT1131 and MT113. Conditions as in fig.2. Chromatophores were suspended at 4 mg/ml for both strains. Open circles, MT1131; closed circles, MT113.

pathway in both strains. Similarly, carbon monoxide inhibits each strain to the same extent when cyt. *c* oxidase activity is eliminated by the addition of low [KCN].

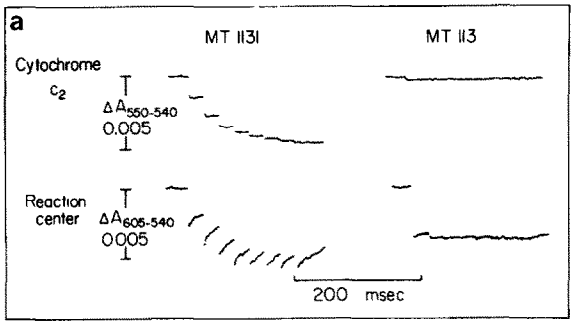
Assays of the activities attributable to the cyt. *c* oxidase-containing pathway alone reveal more striking differences between MT113 and MT1131. NADH-driven horse heart cyt. *c* reductase activity is 4.3 μeg . h^{-1} . mg protein^{-1} for MT1131, whereas the same activity is almost undetectable (0.2 μeg . h^{-1} . mg protein^{-1}) in MT113. Cytochrome *c* oxidase activity, measured as the ability of the membranes to catalyze the oxidation of reduced DCPIP, is greatly reduced in MT113, and the slight residual activity is KCN-sensitive (table 1). These results indicate an obligatory role for cyt. c_2 in these respiratory activities, whereas the alternate oxidase pathway is unimpared by the absence of that cytochrome.

Table 1
NADH and cyt. *c* oxidase activities[a] in membrane vesicles from aerobically grown cells of *Rps. capsulata* MT1131 and MT113

Electron donors	Inhibitors added	MT1131		MT113	
		Act.[a]	% Inhib.	Act.[a]	% Inhib.
NADH	–	16.5	0	7.9	0
NADH	KCN (2×10^{-5} M)	7.0	68	6.2	22
NADH	KCN (2×10^{-3} M)	0.2	98	0.0	100
NADH	KCN (2×10^{-5} M) CO (1200 μM)	2.6	84	0.9	89
Asc (3 mM) +DCPIP (100 μM)	–	31.0	0	2.0	0
Asc (3 mM) +DCPIP (100 μM)	KCN (2×10^{-5} M)	2.0	96	0.0	100

[a] Activities expressed as μeq O_2 h^{-1} mg protein^{-1}

To verify the interaction between cyt. c_2 and photosynthetic reaction centers, we exmained electron transport induced by a train of single-turnover flashes of light. MT113 shows no light-induced oxidation of cyt. c_2, appropriate for its absence. Furthermore, it is quite evident that the reaction center of MT113 is functional, and is not re-reduced for ⩾200 ms (fig.4(a)), consistant with the role proposed for cyt. c_2 as the immediate reductant for the reaction center during light-induced electron flow. It is interesting that no other reductant interacts with the reaction center on this time scale.



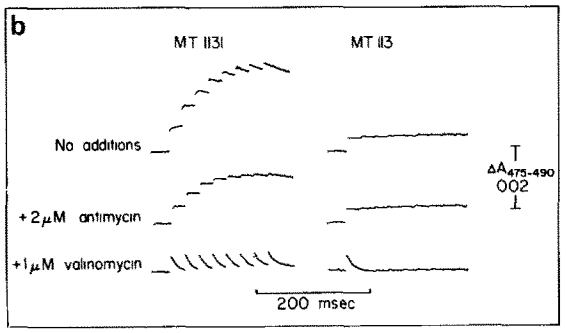


Fig.4. (a) The response of cyt. c_2 and reaction center in membrane particles of MT1131 and MT113 to a train of single-turnover flashes of light. Chromatophores were suspended in 20 mM glycylglycine, 100 mM KCl, 1 mM $MgCl_2$ 2 μM antimycin, 1.5 μM 2-hydroxy 1,4-naphtoquinone, 0.5 μM pyrocyanine, 0.3 μM 2,3,5,6-tetramethyl phenylenediamine, *N*-methyl phenazonium methosulfate and *N*-methyl phenazonium ethosulfate (pH 7.0). The ambient redox potential was adjust near to +150 mV to reduce completely cyt. c_2 (E_h' = +340 mV) and the vesicles were then subjected to a train of 8 single turnover flashes of light.

Fig.4. (b) The response of the carotenoid band-shift to a train of single turnover flashes of light in MT1131 and MT113 chromatophores. Conditions as in fig.4 (a) except the experiment reproduced in the upper trace in which antimycin was absent and the further addition of valinomycin in the experiment of the lower trace, as indicated.

Fig.4(b) shows the carotenoid shift observed in these membranes. The red-shift in the spectrum of the carotenoid complement of the chromatophores consists of 3 kinetically resolvable phases [20,21]. Phases I and II, appear to be coupled to electron transfer from the reaction center $BChl_2$ to a ubiquinone–iron complex associated with the reaction center and from ferrocyt. c_2 to $BChl_2^+$, respectively [21]. Phase III is developed as a response of an antimycin A-sensitive electron transfer in the ubiquinone–cyt. b–c_2 region. As shown in fig.4(b), MT113 membranes present only the first phase of the carotenoid shift, evidence which strongly supports the carotenoid-shift model described above in which phases II and III require the presence of cyt. c_2. The slow decay of the first phase of the carotenoid band shift in the mutant shows that MT113 membranes are capable of maintaining charge separation, a conclusion also supported by the retention of the carotenoid shift in MT113 for ⩾200 ms; appropriate to this conclusion, valinomycin in the presence of potassium greatly speeds up the course of decay.

4. Conclusions

The 'tetracycline suicide' technique has been used to select the first mutant of a photosynthetic bacterium lacking cyt. c_2. This mutant cannot grow photosynthetically, but grows well via respiration mediated by the alternate oxidase. From these data we can conclude that cyt. c_2 is the physiological electron donor both to cyt. c oxidase during dark reactions and to the reaction center in light-induced electron flow. Furthermore, the absence of both NADH-horse heart cyt. c reductase and DCPIP oxidase activities in membranes of MT113 indicates that cyt. c_2 serves as an essential electron carrier in each of these commonly used assays.

We suggest the possibility that the cytochromes identified as cyt. b_{145} and cyt. c_{115} on the basis of redox titrations monitored at different wavelength pairs may in fact represent a single redox component, namely cyt. c'. We note that cyt. b_{145}, c_{115} and c' show parallel concentration changes in the strains of *Rps. capsulata* studied here, however further characterization of these components is clearly required to test this hypothesis.

The presence of the first phase but lack of phases II and III of the carotenoid shift in MT113 membranes lends strong support to the model describing the sources of the different phases proposed [21]. In the model, cyt. c_2 was considered essential because phase II appeared as ferrocyt. c_2 reduced the flash-generated reaction center $BChl_2^+$, while phase III appeared as ferricyt. c_2 was reduced by the component in the Q–b–c_2 region.

MT113 provides a valuable source of material for studies probing the role of cyt. c_2 via reconstitution. For example, preliminary results indicate that NADH-driven horse heart cyt. c reductase activity can be restored to membranes of MT113 by addition of a small amount of purified cyt. c_2 from *Rps. capsulata*. This implies that specific (protein–protein?) interactions occur during normal cyt. c_2 reduction. Further studies in this area are in progress.

Acknowledgements

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