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The Dual Role of Cytochrome c_2 in the Facultative Phototrophic Bacterium *Rhodopseudomonas capsulata*

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Succinate oxidation is stimulated by addition of cytochrome c_2 in cytochrome c_2-deficient spheroplasts from the M6-mutant and from the wild type strain of *Rhodopseudomonas capsulata*. Inhibition of the alternative oxidase in the wild type by CO facilitates this observation. The finding confirms a dual role of cytochrome c_2, in photosynthetic and in respiratory electron transport.

(Keywords: Bacterial respiration; Cytochrome c_2; Photoherterotrophy; Rhodospirillaceae)

Die doppelte Rolle von Cytochrom c_2 in Rhodopseudomonas capsulata

Die Oxidation von Succinat wird durch Zugabe von Cytochrom c_2 zu Cytochrom c_2-defizienten Sphäroplasten der Mutante M6 und des Wildtyps von *Rhodopseudomonas capsulata* stimuliert. Die Hemmung der alternativen Oxidase durch CO im Wildtyp erleichtert diese Beobachtung. Der Befund bestätigt die Doppelrolle von Cytochrom c_2 im photosynthetischen und respiratorischen Elektronentransport.

Introduction

The purple non-sulfur bacteria (Rhodospirillaceae) are the only organisms which are able to respire and carry out photosynthesis at the same time. Thus combining certain features of chloroplasts as well as of mitochondria these bacteria are most interesting objects to study[1], also for evolutionary aspects[2]. They possess a respiratory and a photosynthetic electron transport chain in their continuous membrane system, the relative amount present depending on the growth conditions, which also affect the degree of membrane differentiation[3]. The interrelation of these two electron transport pathways is under current investigation and a picture like in Fig. 2 might be considered. It holds that the two

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pathways share certain components like ubiquinone, cytochromes b and c_2[1].

The role of cytochrome c_2 as the immediate electron donor for the reaction center bacteriochlorophyll (P870) is firmly established, but conclusive evidence for the participation of this cytochrome in the respiratory pathway was much more difficult to obtain. These studies had been complicated by the occurrence of a branched respiratory chain in Rhodospirillaceae[1], and only recently was it possible to demonstrate an obligatory role of cytochrome c_2 in one of the respiratory branches of *Rhodopseudomonas capsulata*. This was possible with a specific antibody against cytochrome c_2 from photosynthetic cells, and spheroplasts obtained from the mutant M6, which had lost the alternative oxidase (b_{260} in Fig. 2) in the side path[4]. However, to observe a clear inhibition of respiration by the antibody it was necessary to select a spheroplast preparation with a high content on cytochrome c_2 (50-60%; 4). So far it was not possible to control the cytochrome c_2 content and usually 80-90% are lost.

The present paper shows that respiration of spheroplasts deficient in cytochrome c_2 can be stimulated by readdition of this cytochrome. This is demonstrated not only for the mutant M6, but also for the wild type strain of *Rhodopseudomonas capsulata*.

Methods and Materials

Rhodopseudomonas capsulata, strain KB1 and mutant strain M6 were grown from agar stab cultures in the medium of *Ormerod* et al. as previously described[4, 8]. A 100 ml inoculum was first grown anaerobic in the light. This was then transferred to a 1 l *Fernbach* flask, fitted for aeration and shaking, and the culture was grown at 27 °C in the dark to mid logarithmic phase (ca. 20 h). For the M6-mutant 0.5 mg/ml ascorbate was included in the growth medium.

Spheroplasts were obtained from washed bacteria by lysozyme/*EDTA*-treatment essentially as described before[5]. After centrifugation of the incubation mixture for 20 min at 10,000 × g, only the loosely packed part of the sediment was homogenized with the aid of cotton wool in 30 mM Tris-HCl, *pH* 8.0, 50 mM Mg SO_4 and 20% sucrose, and was centrifuged again. The final sediment was taken up concentrated in the same buffer, and a grain each of *DNA*'se and *RNA*'se was added. This spheroplast suspension was kept on ice for immediate use, or was frozen in liquid nitrogen after addition of glycerol to 30% (*v*/*v*), without loss of activity. Protein was determined by the method of *Lowry*[6]. Respiration was measured in a stirred thermostated reaction vessel at 30 °C, following O_2-uptake with an oxygen electrode (Hansatech). The reaction mixture[4] contained in 1 ml, 30 mM Tris-HCl, *pH* 7.5, 50 mM NaCl, 5 mM *EDTA*, 10% sucrose, spheroplasts equivalent to 1 mg protein and the amounts of cytochrome c_2 and CN^- specified in Fig. 1 and Table 2. The reaction was started by the addition of 10 μmoles succinate in 10 μl, through the bore of the ground glass stopper of the vessel with the help of a syringe.

Inhibition of respiration by CO was achieved by flushing the whole reaction

mixture including the spheroplasts in a 5 ml *Erlenmeyer* flask, closed by a rubber septum, for 10 min with a mixture of CO/O_2 of 80/20 (*v/v*) via injection needles with the aid of a gas mixing pump (Wösthof O.H.G., Typ M3/a-F). Then the mixture was transferred with a syringe to the vessel with the oxygen electrode, and the reaction was started by addition of succinate.

Cytochrome c_2 was isolated from 140 l anaerobic, phototrophic culture to near homogeneity by the procedure of *Bartsch*[7].

The content of suspensions in cytochrome c_2 was estimated by redox-difference spectroscopy with the split-beam mode of an Aminco spectrophotometer (DW-2 UV-VIS). After splitting the suspension into the two cuvettes, a grain of ferricyanide was added into each and a baseline was recorded between 500 and 600 nm. Then a small excess of ascorbate was added to the sample cuvette, and the difference spectrum was recorded on top of the baseline. For the difference in absorption at 551 nm an ε of 20 mM^{-1} cm^{-1} was taken.

Lysozyme, *DNA*'se and *RNA*'se were purchased from Sigma.

Results

Table 1 summarizes the relative amounts of cytochrome c_2 found in spheroplasts of the wild type strain (3 experiments) and of the M6-mutant (2 experiments), after the first and second sedimentation, and in the corresponding supernatants. Only 5-24% of the original cytochrome c_2 was retained in the washed spheroplasts. This amount varies considerably, as noticed before[5].

Succinate respiration in these cytochrome c_2 deficient spheroplasts can be stimulated by addition of cytochrome c_2 (Table 2). The almost

Table. 1. *Liberation of cytochrome c_2 from aerobically grown Rhodopseudomonas capsulata by spheroplast formation*

Spheroplast were prepared from cells grown aerobically in the dark and isolated by centrifugation as described under Methods. The contents of cytochrome c_2 were estimated by redox-difference-spectroscopy as also described. 100% corresponds to 1.2 and 1.8 μmoles cytochrome c_2 per g protein in the wild type (wt) and in the M6-mutant strain, respectively.

	% cytochrome c_2				
	wt			M6	
Exp.	1	2	3	1	2
Incubation mixture	100	100	100	100	100
Sediment 1	14	26	10	26	16
Supernatant 1	86	74	90	74	84
Sediment 2	5	21	9	19	24
Supernatant 2	9	5	1	7	2

Table 2. *Stimulation of succinate respiration in spheroplasts from Rhodopseudomonas capsulata by cytochrome* c_2

The preparation of spheroplasts, the assay for O_2-uptake and the incubation with CO is described under Methods. M6 stands for M6-mutant, wt for wild type and c_2 for cytochrome c_2.

	IEU O_2-uptake	
	—	$+10^{-5}$ M CN^-
M6	0.012	0.003
M6 + 12 μM c_2	0.031	0.003
wt	0.057	0.057
wt + 12 μM c_2	0.080	0.061
wt + CO	0.022	0.020
wt + CO + 12 μM c_2	0.047	0.020

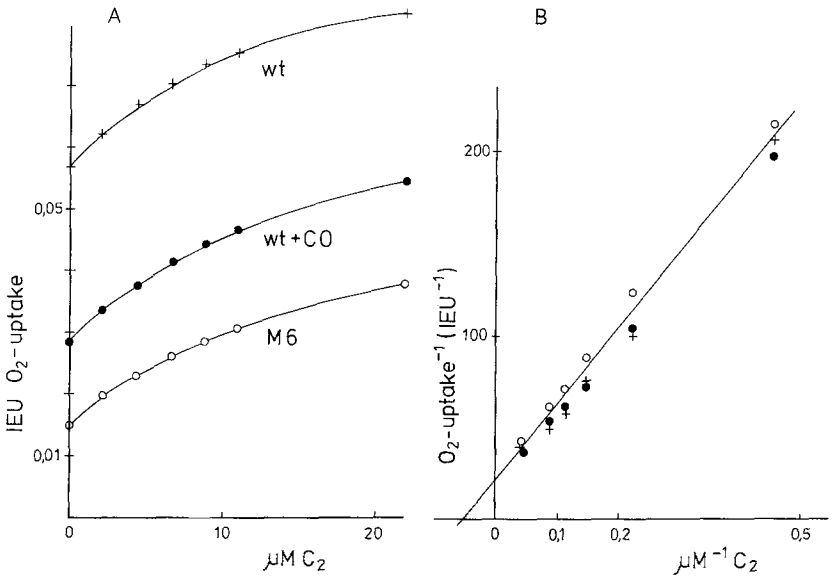

Fig. 1. Titration of succinate respiration in spheroplasts of *Rhodopseudomonas capsulata* with cytochrome c_2. *A* shows the titration of O_2-uptake by spheroplasts from the M6-mutant and from the wild type (wt), +/— CO. *B* shows the double-reciprocal plots of the cytochrome c_2-stimulated increments of O_2-uptake. Preparation of spheroplasts, assay of O_2-uptake and incubation with CO is described under Methods

complete inhibition of O_2-uptake by the M6-mutant for low concentrations of CN^- shows once more that the alternative oxidase is not functioning[8]. In the wild type preparation the effect of cytochrome c_2 is seen better if the CN^--insensitive oxidase is suppressed by CO[5]. Oxidation of *NADH* was also stimulated by cytochrome c_2 (not shown).

Fig. 1 shows the titration of respiration with cytochrome c_2 up to 22 μM, which is not saturating yet. From the replot of the cytochrome

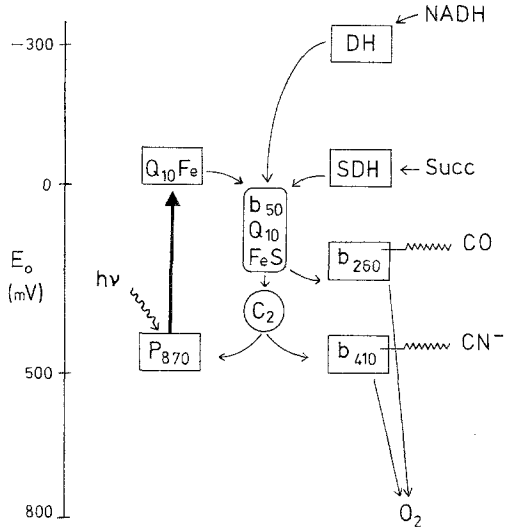

Fig. 2. Electron transport system of *Rhodopseudomonas capsulata* on a redox potential scale. Unusual symbols are: P_{870} reaction center bacteriochlorophyll; Q_{10} ubiquinone; b_{50}, b_{260}, b_{410} b-type cytochromes with midpoint potentials of 50, 260 and 410 mV, respectively; c_2 cytochrome c_2; *Fe—S Rieske*'s iron sulfur protein; *SDH* succinate dehydrogenase; *DH NADH*-dehydrogenase

c_2-dependent increments of O_2-uptake in Fig. 1 B one can estimate a $[S]_{0.5}$ of about 20 μM, for each of the three titrations. This value is at least ten times higher than the one corresponding to the endogenous amount of cytochrome c_2 per mg protein present in the suspension before separation of the spheroplasts (s. Table 1), which suggests that the cytochrome is not firmly bound by the cytoplasmic membrane, but is concentrated in a mobile form in the periplasmic space of the cell.

Discussion

The results of this paper confirm our previous, immunological experiments[4], showing that cytochrome c_2 is an electron transport component shared by the photosynthetic—, and one branch of the

respiratory electron tranport in the membranes of *Rhodopseudomonas capsulata*. This additional independent proof was desirable because the effect of the antibody depended on the condition of the spheroplasts tested. In particular a relatively high cytochrome c_2 content seemed necessary, which could not be reliably reproduced.

Together with the older observations that cytochrome c_2 reversibly changes its redox state in situ during cycles of aerobiosis/anaerobiosis, that it can not be oxidized in a mutant lacking the oxidase, cytochrome b_{410}, and that the decreased content of spheroplasts in cytochrome c_2 is accompanied by a loss of CN^--sensitive respiration[5,8], our results firmly establish the dual role of cytochrome c_2 as depicted in Fig. 2. The same conclusion can be drawn from recent studies with cytochrome c_2-negative mutants of *Rhodopseudomonas capsulata*[9,10]. A similar dual role for ubiquinone and the cytochrome b_{50}-complex seems feasible, but has not been established yet. Another component shared by the respiratory and the photosynthetic pathway of energy conservation is the *ATP*-forming membrane complex[11]. For both cases, the *ATP*-synthetase and cytochrome c_2, it is not clear whether a common pool or two separate portions exist. Preliminary studies with mixotrophically grown *Rhodopseudomonas capsulata* M6 show, however, that most of the cytochrome c_2 contained by the cells can be oxidized either by light or by aeration, suggesting one common pool[12].

We believe that components common to the photosynthetic and respiratory pathways of Rhodospirillaceae like the widely distributed, periplasmically located[13] cytochrome c_2, reflect the economy of the cell and are general phenomena in facultative photosynthetic bacteria, not just specific for *Rhodopseudomonas capsulata*.

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