Characterization of a new membrane-bound cytochrome *c* of *Rhodopseudomonas capsulata*

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Received 13 December 1982; revision received 4 January 1983

A cytochrome *c* (cyt. *c*) was solubilized with Triton-X-100 and co-purified with cytochrome *c* oxidase from membranes of chemotrophically grown cells of *Rhodopseudomonas capsulata*. Cyt. *c* and cytochrome oxidase were separated on Sephadex G-50 columns. Antibodies against cytochrome c_2 from the same bacterium did not cross react with the membrane-bound cyt. *c*. The IEP of the membrane-bound cyt. *c* was found to be pH 8.2, the midpoint potential was 234 ± 11 mV at pH 7.0. This cyt. *c* binds CO. The native cyt. *c* is a dimer with an apparent M_r of 25 000 containing 2 mol heme per mol dimer, which is believed to function as an electron donor for the high-potential cytochrome *c* oxidase.

Rhodopseudomonas capsulata *Cytochrome* c *Cytochrome oxidase*

1. INTRODUCTION

The role of cytochrome c_2 as a redox carrier in the light driven electron flow [1,2] and in the branched respiratory electron transport chain of *Rhodopseudomonas capsulata* as an electron donator for the high potential cytochrome oxidase has been demonstrated in [3–5].

During isolation of the high potential *b*-type cytochrome oxidase of *R. capsulata* [6], a closely attached cytochrome *c* was observed to co-purify with the cytochrome oxidase. It was spectroscopically similar to cytochrome c_2, but did not cross react with antibodies against cytochrome c_2 of *R. capsulata*. This paper describes the purification and characterization of this membrane-bound cytochrome *c*.

2. MATERIALS AND METHODS

2.1. *Purification of cytochrome* c

Cytochrome *c* was co-purified with the cytochrome *c* oxidase from membranes of chemotrophically grown cells of *R. capsulata* strain 37b4 (DSM 938) as described in [6]. After DEAE-chromatography in TRIPE-buffer (50 mM Tris–HCl (pH 8), 0.1 mM phenylmethylsulfonylfluoride, 0.1 mM ethylenediaminetetraacetate) with 0.1% Triton X-100 (w/v) fractions eluted at 0.29 M KCl were pooled. Concentration, desalting and buffer exchange to 5 mM sodium phosphate buffer (pH 8) with 0.1% Triton X-100 was done on Sephadex G25 (Pharmacia) as in [7]. The concentrated samples were applied to a preequilibrated column of hydroxyapatite (0.6 × 4.5 cm, HTP-Gel, Biorad). Cytochrome *c* oxidase and cytochrome *c* were eluted by 55 mM sodium phosphate buffer (pH 8) and 0.1% Triton X-100. The sample was concentrated again and was applied to a column of Sephadex G50 (2 × 8 cm, Pharmacia). Cytochrome *c* was eluted shortly behind the void volume, where cytochrome *c* oxidase was found. For determination of the native M_r the column was calibrated with the following marker proteins (2 mg/ml): ribonuclease (M_r 13700), chymotrypsinogen (M_r 25000) and ovalbumin (M_r 43000). The latter eluted in the void volume, which was determined by dextran blue (1 mg/ml).

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Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

2.2. *Spectroscopic measurements*

Pyridine hemochromes were prepared as in [8]. Samples for difference spectra were reduced with sodium dithionite (0.2 mM). The concentration of the purified cytochrome *c* was determined at 549–540 nm using an extinction coefficient of 19.1 $mM^{-1}.cm^{-1}$ [9]. Reduced-plus-CO minus reduced spectra and the enzymatic inhibition of cytochrome *c* by CO were obtained as in [19].

2.3. *Electron transfer from cytochrome* c *to oxygen via cytochrome* c *oxidase*

Fractions of the purified components or after ion exchange chromatography were used in TRIPE buffer (pH 8) plus 0.1% Triton X-100 (cytochrome *c* oxidase 0.1 mg and cytochrome *c* 0.4 mg [11]). Ascorbat (0.5 μM) was used as electron donor and diaminodurene (40 μM) as mediator in a 3 ml cuvette. The decrease of oxygen concentration was measured polarographically with a YSI 53 biological oxygen monitor (Yellow Springs Instruments, OH). The decrease of absorption at 551 and 560 nm was measured with a Perkin-Elmer split beam spectrophotometer model 330 (Überlingen). Isoelectric focussing and redox titrations were performed as in [10]. Sodium dodecylsulfate polyacrylamide gel electrophoresis was performed as in [12].

3. RESULTS AND DISCUSSION

The cytochrome *c* oxidase fractions from DEAE-chromatography contained a *c*-type cytochrome (fig.1) which was solubilized together with the oxidase from membranes of chemotrophically grown *R. capsulata* by 1.3% Triton X-100 (w/v). Extensive washing of membrane fractions before solubilization did not reduce the amount of this cytochrome *c*. The characteristics of the mesoheme of the pyridine hemochrome preparation seemed to be the same as those of cytochrome c_2 (fig.2), which might be trapped by membrane vesicles during disruption of

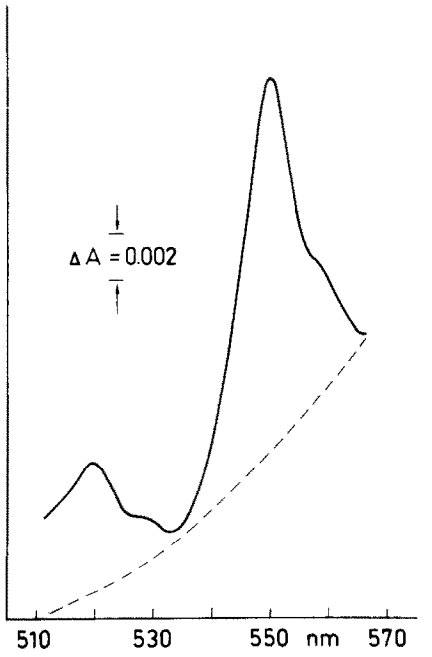

Fig.1. Difference spectrum of the pooled fractions after DEAE-chromatography of Triton-solubilized membranes from *R. capsulata*. Absorption maxima at 551 nm and 520 nm are characteristic for a *c*-type cytochrome. The shoulders at 560 nm and 530 nm belong to the *b*-type cytochrome oxidase.

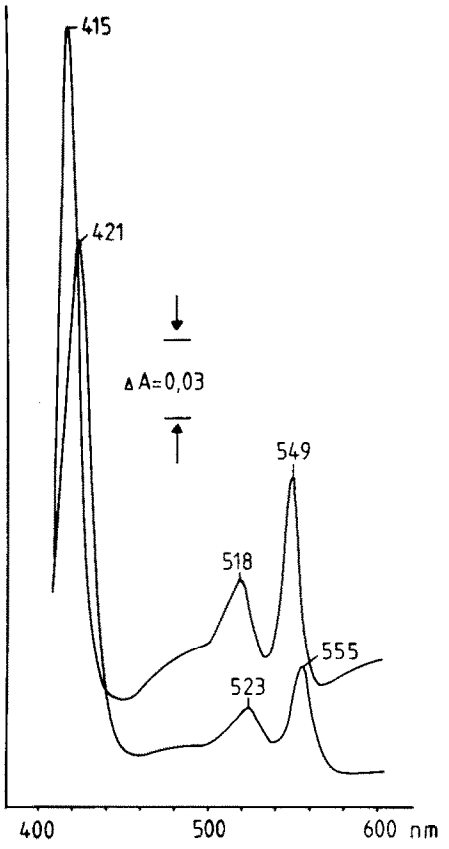

Fig.2. Pyridine hemochrome difference spectra of acid/acetone extract and residue of the sample used for fig.1. Upper line: residue; lower line: extract.

the cells. On the other hand, antibodies against cytochrome c_2 did not cross-react with this cytochrome *c*.

The peak fractions from anion-exchange chromatography at 0.29 M KCl with the highest ratio of the peaks at 551:560 nm eluted shortly before the cytochrome *c* oxidase peak with the highest specific enzyme activity [6]. No cytochrome *c* oxidase fraction was detectable without the associated cytochrome *c*.

The cytochrome *c* was separated from the oxidase by gel filtration on Sephadex G50. The isoelectric point of the purified cytochrome *c* was determined to be at pH 8.2, indicating the compensation of most of its positive charges by the interaction with the negatively charged cytochrome oxidase (isoelectric point at pH 6.5 [10]) during DEAE-chromatography. The purified cytochrome *c* had a midpoint potential of 234 ± 11 mV at pH 7 (fig.3), which was different from that of cytochrome c_2 at 342 mV [13] and from that of purified cytochrome c_1 of *R. sphaeroides* at 285 mV [14].

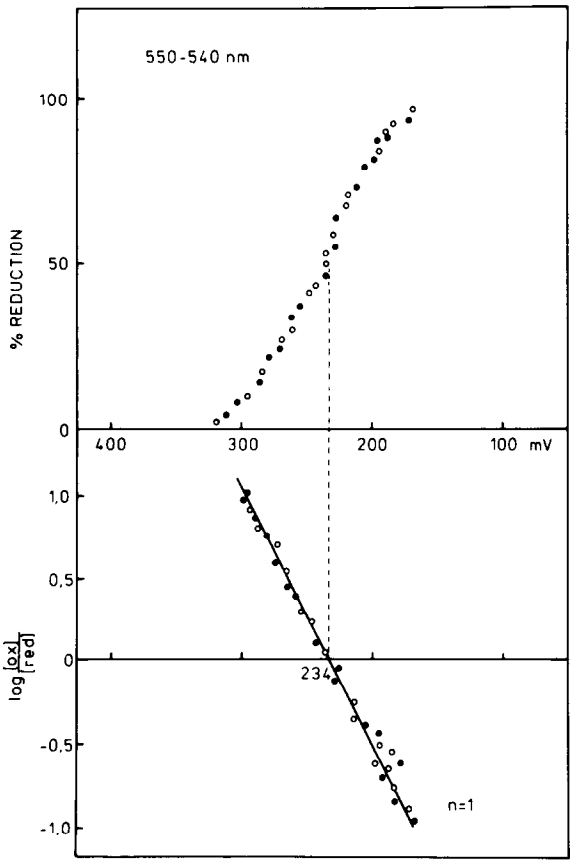

Fig.3. Potentiometric titrations at 550–540 nm of the purified cytochrome *c*. 0.4 mg protein/ml was resuspended in 50 mM MOPS buffer (pH 7) plus 0.1% Triton X-100 in the presence of 20 μM diaminodurene, 20 μM phenazine methosulfate and 20 μM phenazine ethosulfate. Values are means of 6 redox titrations: (●) reductive phase; (○) oxidative phase. A theoretical n = 1 line is drawn through the points.

The electron transport by the purified cytochrome *c* could be completely inhibited by CO, whereas the oxidase as an atypical cytochrome *b* did not react with CO [10]. Reduced-plus-CO minus reduced spectra showed peaks at 567, 525 and 415 nm and troughs at 552, 520 and 425 nm (fig.4). The CO-inhibited cytochrome *c* showed reduced binding to the cytochrome oxidase resulting in a higher oxidizing activity of added

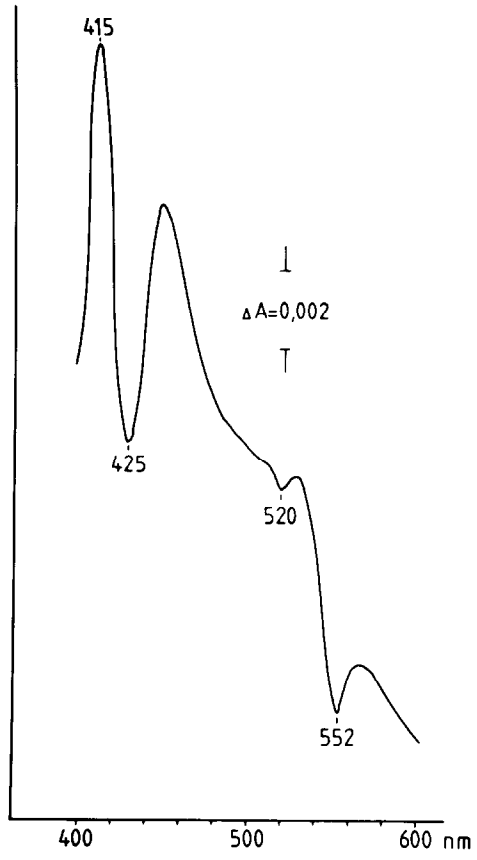

Fig.4. Dithionite-reduced-plus-CO minus reduced difference spectrum of the membrane-bound cytochrome *c* of *R. capsulata*.

horse heart ferro cytochrome *c*. The K_m of the purified cytochrome *c* for the cytochrome *c* oxidase was 3.4 μM at pH 8 and 30°C, 7-fold lower than that of reduced horse heart cytochrome *c* [6].

The apparent M_r of the membrane-bound cytochrome *c* in sodium dodecylsulfate polyacrylamide gel electrophoresis was determined to be 12600. The native cytochrome *c* eluted from Sephadex G50 in 0.1% Triton X-100 as a dimer with an apparent M_r of 25000 containing 2 mol heme/mol dimer.

Fig.5 shows the in vitro reaction of the purified cytochrome *c* with the *b*-type cytochrome oxidase. Cytochrome *c* did not seem to react directly with oxygen although it bound CO.

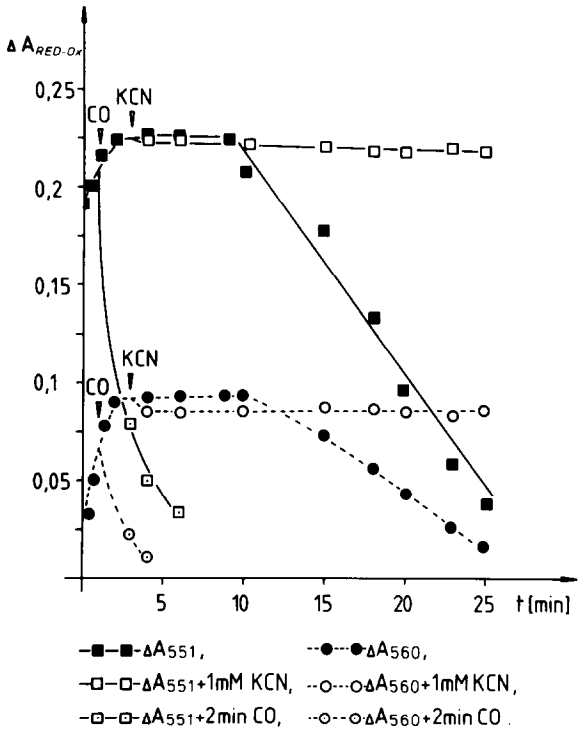

Fig.5. Electron transfer from membrane-bound cytochrome *c* to oxygen via the high potential cytochrome *c* oxidase. At $t = 0$, 0.5 μM ascorbate was added to the sample. The fig. shows the results of 3 different experiments: (1) Oxidation of cytochrome *c* via cytochrome *c* oxidase by O_2: (■—■) ΔA_{551}; (●---●) ΔA_{560}; (2) Inhibition of the oxidation by addition of 1 mM KCN to the sample: (□—□) ΔA_{551}; (○---○) ΔA_{560}; (3) Inhibition of the oxidation by bubbling a steady stream of CO through the test cuvette for 2 min: (⊡—⊡) ΔA_{551}; (⊙---⊙) ΔA_{560}.

When we inhibited the oxidase with an excess of KCN, cytochrome *c* remained in the reduced form and no reduction of O_2 could be detected.

This membrane-bound, CO-binding cytochrome *c* is different from the already described cytochrome *cc'* [4,5,15], which is situated in the periplasm and has a midpoint potential of 0 mV (*R. capsulata*) and + 150 mV (*R. palustris*), respectively [16]. The purified cytochrome *c* reacts with CO but does not seem to function as an oxidase. In the absence of cytochrome c_2 the membrane-bound ferrocytochrome *c* reduces oxygen in vitro via direct interaction with the high potential cytochrome *c* oxidase of *R. capsulata*. These data support the idea that the membrane-bound, high-potential cytochrome *c* functions as an electron donor for the high potential cytochrome oxidase.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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