Isolation of a *b*-Type Cytochrome Oxidase from Membranes of the Phototrophic Bacterium *Rhodopseudomonas capsulata*

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A cytochrome oxidase (EC 1.9.3.1) was solubilized from the membrane fraction of aerobically grown cells of *Rhodopseudomonas capsulata* by treatment with Triton X-100. The enzyme was purified 160 fold by chromatography on DEAE-Sepharose CL – 6B and affinity chromatography on cytochrome *c*-thiol activated Sepharose 4B.

The purified enzyme has a pH-optimum at 8.5 and a temperature optimum at 35 °C. The apparent K_m for reduced horse cytochrome *c* is 24 μM (at pH 8 and 30 °C). The purified cytochrome oxidase was 50% inhibited by 1.5 μM KCN and 10 μM NaN_3. The purified enzyme contained one polypeptide of m_r 65,000 and *b*-type cytochrome.

Introduction

Rhodopseudomonas capsulata and other facultative phototrophic bacteria produce ATP under chemotrophic conditions in the dark by oxidative phosphorylation [1–3]. The terminal oxidase of the respiratory chain, which catalyzes the reduction of molecular oxygen to water is the cytochrome *c* oxidase (E.C. 1.9.3.1). The existence of a cytochrome oxidase containing a *b*-type cytochrome in bacteria was first demonstrated by Chance *et al.* [4, 5]. Many photosynthetic bacteria contain cytochrome *o*, a *b*-type cytochrome, functioning in the terminal oxidase [6]. The *b*-type cytochrome, active in the cytochrome oxidase of *Rps. capsulata*, has a mid-point potential of about + 410 mV at pH 7.0 [7], a high sensitivity to KCN (K_i: 5×10^{-6} M) and is not inhibited by CO. The respiratory chain of *Rps. capsulata* is branched [7, 8]. Besides the high potential cytochrome oxidase a low potential cytochrome oxidase has been described. It has a midpoint potential of about + 270 mV at pH 7.0 [9], a low sensitivity to KCN (K_i: 5×10^{-4} M) and is inhibited by CO. Antimycin A inhibits preferentially the branch of the chain affected by low concentrations of cyanide [7].

Abbreviations: EDTA, ethylene-diaminetetraacetate; DDT, dithiothreitol; DEAE, diethylaminoethyl; LDAO, N,N-dimethyllauryl amine oxide; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate; Tripe buffer, 50 mM Tris-HCl, pH 8.0, 0.1 mM PMSF, 0.1 mM EDTA; Triton X-100 polyoxyethylene *p-t*-octylphenol.

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An active cytochrome oxidase with a functional cytochrome *c* has been isolated recently from *Rps. palustris* [10]. This preparation contained three polypeptides having apparent m_r of 30,000, 25,000 and 9,500 and a fourth component of m_r 12,200 which is thought to be cytochrome c_2.

In the present study we report on the isolation and characterization of the cytochrome *c* oxidase from *Rps. capsulata*, strain 37b4.

Materials and Methods

Preparation of membrane fraction

Rhodopseudomonas capsulata wild type strain 37b4 (German collection of microorganisms, Göttingen, strain 938) was grown aerobically in the dark at 30 °C as described recently [11]. The cells were harvested at an optical density of 0.6 (0.5 cm light path at 660 nm). Biosynthetic processes were stopped by addition of chloramphenicol (100 μg/ml), NaN_3 (2 mM and phenylmethylsulfonylfluoride (PMSF, 1 mM final concentration). Cells were washed with phosphate buffer (50 mM, pH 7.5) and stored at −70 °C. Three *g* of packed cells were resuspended in 10 ml of 50 mM Tris-HCl buffer (pH 8) plus 0.1 mM PMSF and 0.1 mM EDTA (TRIPE buffer) and disrupted at 112 MPa in a French pressure cell. DNase was added and the extract was centrifuged at 27,000 × *g* for 30 min. The supernatant was centrifuged at 160,000 × *g* for 90 min. The sediment was washed once in Tris buffer (50 mM, pH 8.0) and used immediately for enzyme isolation.

Purification of cytochrome oxidase

All operations were performed at 4 °C unless stated otherwise. The membrane fraction was resuspended in TRIPE buffer at a concentration of about 6 mg protein per ml. Triton X-100 (10% stock solution) in TRIPE buffer was added dropweise to the suspension under stirring to a final concentration of 1.3% (w/v, protein to detergent ratio of 1 : 2). After incubation for 30 min insoluble material was precipitated at 145,000 × *g*, 90 min, 4 °C. The supernatant contained about 90% of the cytochrome oxidase. The enzyme extract was diluted with TRIPE buffer to a final concentration of 1% Triton X-100.

10 ml of the supernatant (4 mg protein/ml) were applied to a column (1 × 8 cm) of DEAE Sepharose CL-6B (Pharmacia Freiburg) equilibrated with TRIPE buffer plus 1% Triton. The column was washed with 30 ml of TRIPE buffer plus 1% Triton and eluted with a linear gradient of 0 – 1 M KCl (2 × 40 ml). Fractions of 3 ml were collected and tested for cytochrome oxidase activity.

The peak fractions eluted at 0.3 M KCl were pooled and desalted as described in [10]. 9 ml of the desalted enzyme solution (5.6 mg protein) was then applied to a cytochrome *c*-thiol-activated Sepharose 4B column (1 × 4 cm; Pharmacia) equilibrated with TRIPE buffer plus 1% Triton X-100. The coupling of cytochrome *c* from *Saccharomyces cerevisiae* (Sigma, München, type VIII) and the preparation of the cysteine inactivated column were performed as described in [12]. The binding capacity of the column was 7.7 mg cytochrome *c* per ml of packed material. The column was washed with 20 ml of TRIPE buffer and then eluted with a linear gradient of 0 – 0.5 M KCL solution (2 × 40 ml).

Determination of enzymatic activity

Cytochrome oxidase was assayed at 30 °C by following the decrease of absorption at 546 mn in an Eppendorf photometer (type 1101 M) using dithionite reduced horse heart cytochrome *c* as electron donor ($\varepsilon = 21 \times 10^3$ cm²/mmol). The reaction mixture contained 50 mM TRIPE buffer (pH 8), 1 mg ferrocytochrome *c* and enzyme solution in a final volume of 1 ml. The reaction was started by addition of 10 to 100 µl enzyme solution. When inhibitors were used, the enzyme preparation was preincubated for 5 min with the inhibitor. The reaction was then started by addition of reduced cytochrome *c*. Horse heart ferrocytochrome *c* was prepared as described in [10] with the modification that the pH of the ferrocytochrome *c* solution was adjusted to pH 8 with 1 M Tris.

Native polyacrylamide gel electrophoresis (charge- shift-electrophoresis)

Electrophoresis in 5% polyacrylamide slab gels containing 0.1% Triton X-100 was carried out according to the method of Simons *et al.* [13] with the modification that DTT was added to the gel and the buffers in 1 mM concentration. The samples were preincubated in the sample buffer for 15 min at 4 °C. Electrophoresis was performed at 4 °C with 2 mA/gel track. After the run one track was cut into 2 mm slices. The slices were incubated over night with 200 µl of TRIPE buffer plus 0.1% Triton and tested afterwards for activity in the enzyme assay. The slices of the second track were extracted for 4 h with 50 µl of sample buffer (4% SDS) at 60 °C. The extracts were then subjected to SDS-gel electrophoresis. The third track was stained with 0.04% Coomassie brillant blue in 25% isopropanol and 10% acetic acid (v/v) and destained with 10% acetic acid and 10% methanol.

Sodium dodecylsulfate polyacrylamide gel electrophoresis

Slab-SDS-gel electrophoresis was performed according to the method of Laemmli [14] using 11.5 – 16.5% acrylamide gradient gels of 1 mm thickness. The samples were dialyzed for two days, lyophilized and resuspended in sample buffer. All samples were preincubated at 60 °C for 30 min or at 100 °C for 10 min, respectively.

Determination of protein

Protein concentration was determined by the method of Lowry *et al.* [15]. The presence of Triton X-100 was corrected by addition of 0.5% (w/v) sodium dodecylsulfate to the alkali solution of the Lowry reagent [16]. Bovine serum albumin was used as standard.

Measurement of difference absorption spectra

Difference spectra were obtained with a Perkin-Elmer split beam spectrophotometer model 330 (Überlingen) using 1 cm light-path cuvettes at room

Table I. Purification of cytochrome oxidase from *Rhodopseudomonas capsulata* strain 37 b4. The purification procedure and the enzyme assay are described under Materials and Methods.

	Activity [μmol/min · ml]	Protein [mg/ml]	Specific Activity [μmol/min mg prot.]	Purification (= fold)	Yield [%]
Membranes	2.3	6.15	0.37	1	100
Triton extract	3.4	3.9	0.87	2.4	148
DEAE-Sepharose eluate; peak fraction	1.19	0.19	6.7	17	51
Cytochrome *c*-thiol-activated Sepharose eluate; peak fraction	0.63	0.011	59.7	161	29

temperature. 150 μM potassium ferricyanide was added to the reference cuvette and 100 μM sodium dithionite to the sample. Before recording the spectrum the cuvettes were kept for 2 min in the dark.

Results and Discussion

Solubilization of cytochrome c oxidase

Ninety seven percent of the enzyme was solubilized at a Triton concentration of 1.3% and a protein to detergent ratio of 1 : 2. The activity of the enzyme was enhanced about 30 – 50%. Lowering the Tris concentration from 50 mM to 20 mM reduced the solubilization of the enzyme. An increase of detergent concentration to more than 1.3% resulted in a loss of activity. Addition of KCl up to 1 M stimulated the activity about 10% (in contrast to NaCl) but had no effect on the solubilization of the cytochrome oxidase activity.

The detergent N,N-dimethyllaurylamineoxide (LDAO) solubilized the enzyme activity at a concentration of 1.3% nearly 100% but the half life of the activity dropped to 3 – 5 h. A mixture of LDAO and other detergents or increasing the ionic strength by addition of KCl or NaCl caused a complete loss of the activity. The detergents Brij-58, Tween 20, Lubrol-PX and SDS applied at 4 °C and at room temperature solubilized the enzyme only partially or resulted in inactivation of cytochrome oxidase. Sodium cholate solubilized cytochrome oxidase activity up to 90% but with a lower yield than Triton. In the presence of 1.3% Triton X-100 the cytochrome oxidase had a half-life of 24 h at 4 °C. Freezing of the solubilized enzyme even in the presence of 50% glycerol destroyed the activity. The crude Triton-extract lost approx. 20% of activity at 4 °C in 24 h.

Purification

Triton-extract from membranes was applied to a DEAE-Sepharose CL 6B column. Enzyme activity eluted shortly after a dark red band at 300 mM KCl (Fig. 1). The specific activity was increased 17-fold (Table I).

The enzyme fraction which eluted from the DEAE-Sepharose column contained considerable amounts of cytochrome *c* besides cytochrome *b*. The desalted fraction was loaded on a cytochrome *c*-thiol activated Sepharose 4B column. The cytochrome *c* was covalently linked through its cysteine residue located close to the N-terminus [17] thus leaving free the important lysine residues for binding cytochrome *c* oxidase and reductase [12]. The enzyme was not bound to the column when cystein was inactivated.

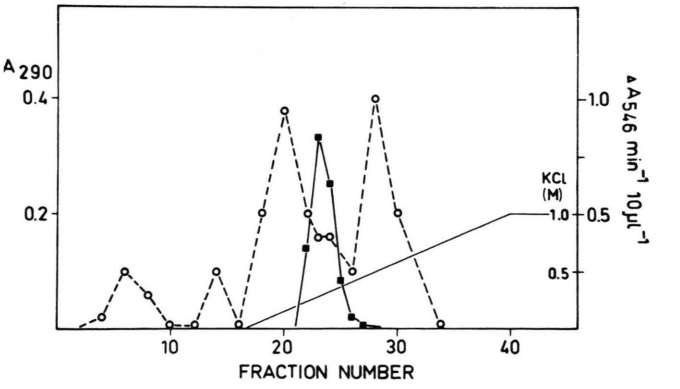

Fig. 1. Ion exchange chromatography on DEAE-Sepharose Cl-6B-column (1 × 8 cm; Pharmacia) in presence of 1% Triton X-100 and standard buffer (0.05 M Tris-HCl pH 8, 0.1 mM PMSF, 0.1 mM EDTA). —■—■— Cytochrome oxidase-activity; --○---○--- A_{290}; ——— KCl-gradient 0 – 1 M KCl, 2 × 40 ml; fractions containing 3 ml.

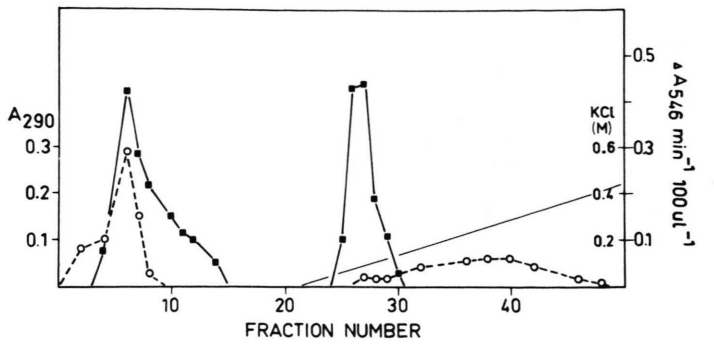

Fig. 2. Affinity chromatography on cytochrome *c*-thiolactivated Sepharose 4B-column (1 × 4 cm; in presence of 1% Triton X-100 and TRIPE buffer, see Materials and Methods). —■—■— Cytochrome oxidase-activity; ---○---○--- A_{290}; ——— KCl-gradient 0–0.5 M, 2×40 ml; fractions of 3 ml.

Cytochrome oxidase showed no affinity for cytochrome-CNBr-activated Sepharose 4B, even when a spacer of succinylamino-dipropylamine was employed.

At a Triton concentration of more than 1% the affinity of the solubilized enzyme to the cytochrome *c* column was completely lost. The cytochrome oxidase activity was eluted from the affinity column at 100 mM KCl (Fig. 2). The column absorbed 54% of the solubilized enzyme. The remaining activity was eluted in the void volume.

The degree of purification is demonstrated by the protein patterns in SDS polyacrylamide gels (Fig. 3).

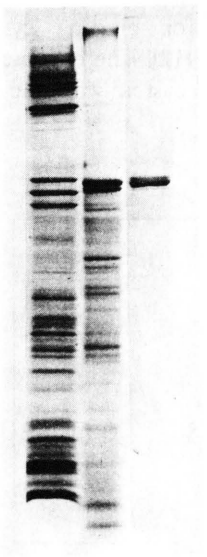

Fig. 3. Slab polyacrylamide gradient (11.5–16.5% acrylamide) gel electrophoresis (0.1% SDS). 1) Crude membrane fraction of cells grown aerobically in the dark; 2) cytochrome oxidase fraction from DEAE-Sepharose chromatography; 3) purified cytochrome *c* oxidase after affinity chromatography. The preperation of samples is described in Materials and Methods.

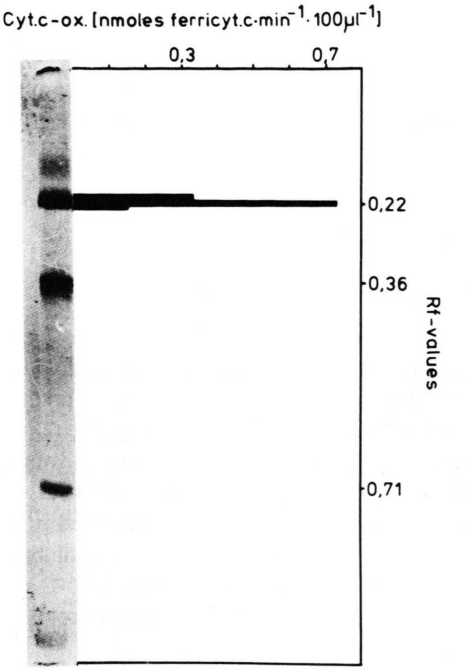

Fig. 4. Charge-shift electrophoresis in 0.1% Triton X-100 of the peak activity fraction after DEAE-Sepharose chromatography. The preparation of the samples and the gels is described under Materials and Methods.

The enzyme preparation from DEAE sepharose chromatography contained numerous proteins. Affinity chromatography removed most of the nonenzyme proteins (Fig. 3).

Native polyacrylamide gel electrophoresis in 0.1% Triton showed one protein band with low oxidase activity in the gel, probably because of partial loss of the hem group or denaturation of the enzyme during electrophoresis (Fig. 4). The protein band of the native PAGE was subjected to SDS-gel electrophoresis resulting in one protein band with a molecular weight of 65,000 (Fig. 5).

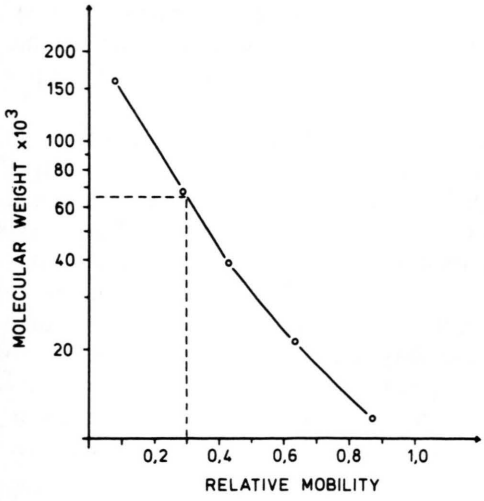

Fig. 5. Detemination of the apparent molecular weight of the cytochrome oxidase polypeptide. Polyacrylamide electrophoresis was performed as described under Materials and Methods (11.5–16.5% acrylamide step gradient). The marker proteins (o) are RNA-polymerase (*E. coli*) core-enzyme β,β′-unit (mol wt. 160,000), bovine serum albumin (68,000). RNA-polymerase α-unit (39,000), typsin-inhibitor (21,500) and horse heart cytochrome *c* (11,700). The position of the cytochrome oxidase is indicated by the intersection of broken lines.

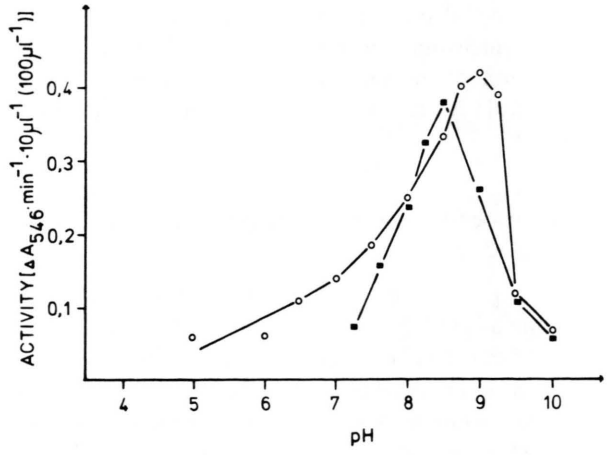

Fig. 7. Effect of pH on cytochrome oxidase activity. The assay of the enzyme activity is performed as described under Materials and Methods. —○—○— Membrane fraction; —■—■— purified enzyme.

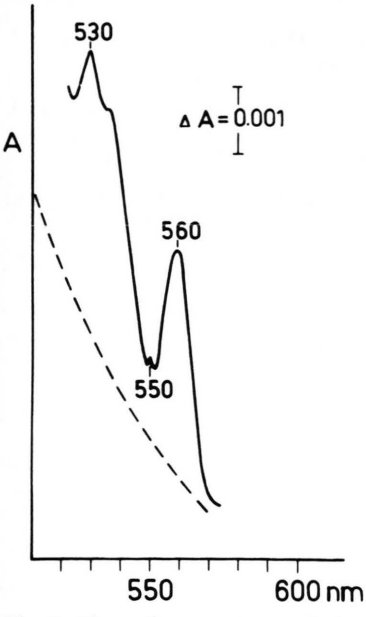

Fig. 6. Absorption spectrum of the purified cytochrome oxidase (after affinity chromatography) at 235 K. Absorption maxima at 560 nm and 530 nm are characteristic for a *b*-type cytochrome. The small peak at 550 nm indicates trace amounts of *c*-cytochrome.

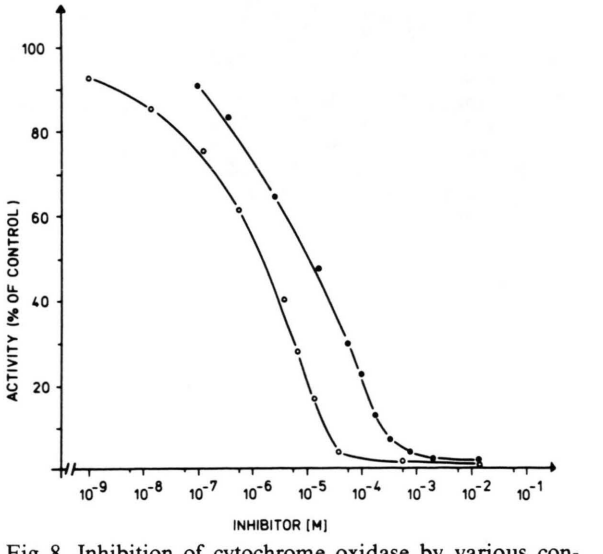

Fig. 8. Inhibition of cytochrome oxidase by various concentrations of KCN and NaN_3. 100% of activity is 0.63 and 0.64 µmol ferrocyt. *c* formed × min^{-1} × mg protein $^{-1}$, respectively. The assay of the enzyme activity is performed as described under experimental procedures. —○—○— KCN; —●—●— NaN_3.

During affinity chromatography most of the *c*-type cytochrome has been removed. The absorption spectrum of the purified enzyme showed peaks at 530 and 560 nm characteristic for cytochrome *b* (Fig. 6).

pH Dependence, substrate specifity and effect of inhibitors

The purified cytochrome oxidase has a pH optimum at pH 8.5, the pH optimum of the membrane bound enzyme, however, was at pH 9 (Fig. 7).

The optimum temperature for enzyme activity of crude membranes as well as of purified oxidase preparations was 35 °C. The purified cytochrome oxidase preparation catalyzed the oxidation of horse heart cytochrome *c* and yeast cytochrome *c*. The apparent K_m for reduced horse cytochrome *c* is 24 μM (at pH 8 and 30 °C). The K_m for yeast ferrocytochrome *c* is 31 μM. The K_m-values were constant when pH or temperature were changed but V_{max} varied. At infinite horse cytochrome *c* concentration, the most active preparation catalyzed the oxidation of 66.9 μmol horse ferrocytochrome *c* per min per mg of protein.

Purified cytochrome oxidase was 50% inhibited by 1.5 μM KCN and 10 μM NaN_3 (Fig. 8). The low K_i of KCN together with the characteristics mentioned above are indicative of the high potential cytochrome oxidase localized in the branched respiratory chain of *Rps. capsulata* [7, 11]. In contrast to the eukaryotic and some prokaryotic cytochrome oxidases this *b*-type cytochrome oxidase seems to contain only one type of polypeptide. Under the experimental conditions employed we were unable to detect the low potential cytochrome oxidase activity.

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