Characterization of a *b*-type cytochrome *c* oxidase of *Rhodopseudomonas capsulata*

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1. INTRODUCTION

An active preparation of the terminal high-potential cytochrome *c* oxidase (EC 1.9.3.1) of *Rhodopseudomonas capsulata*, isolated from the membrane fraction, contained a *b*-type cytochrome and only one polypeptide (M_r 65 000) [1]. The enzyme was inhibited by KCN (50% at 1.5 μM) and NaN_3 (50% at 10 μM) but not by CO [1]. These features distinguish the enzyme from cytochrome *c* oxidases of other *Rhodopseudomonas* species [2,3]. Here, further properties of the heme and the protein moiety are described.

2 MATERIALS AND METHODS

2.1. *Gel filtration*

Cytochrome *c* oxidase from membranes of chemotrophically grown cells of *Rhodopseudomonas capsulata*, strain 37b4 (DSM 938), was prepared as in [1]; the concentration of Triton X-100 in TRIPE-buffer (50 mM Tris–HCl (pH 8), 0.1 mM phenylmethylsulfonylfluoride, 0.1 mM ethylenediaminetetraacetate) was reduced to 0.1% (w/v). The oxidase preparation from the affinity chromatography was concentrated on Sephadex G-25 (Pharmacia) to a final volume of 0.5 ml containing ~1 mg protein [4]. This preparation was applied to a column of Sephadex G-150 (Pharmacia; 1 × 57.5 cm) equilibrated with TRIPE-buffer and 0.1% Triton. The void volume was determined with 2 mg/0.5 ml Dextran Blue (Pharmacia). The flow rate was 2 $ml \cdot cm^{-2} \cdot h^{-1}$.

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2.2. *Preparation of pyridine hemochrome*

Pyridine hemochromes were prepared as in [5]. Difference spectra were obtained with a Perkin-Elmer split beam spectrophotometer model 330 using 1 cm lightpath cuvettes at room temperature.

2.3. *Heme content*

The content of protoheme of the purified cytochrome oxidase was measured at 555–575 nm using a coefficient of 30 $mM^{-1} \cdot cm^{-1}$ [6].

2.4. *Reaction with CO*

Reduced-plus-CO minus reduced spectra were obtained by bubbling a steady stream of CO (Messer-Griesheim) for 2–5 min through the test cuvette. The CO-treated cuvette was left in the dark for 5–10 min before recording the spectra.

Inhibition of the enzyme by CO was measured after reduction of the purified cytochrome oxidase in the enzyme assay [1] with 100 μM ferrocytochrome *c* and subsequently gassing of the test cuvette for 3 min with CO. The reaction was started by addition of fresh ferrocytochrome *c* and air-saturated buffer.

2.5. *Isoelectric focussing*

Isoelectric focussing was performed as in [7]. Triton X-100 and sucrose were added to a final concentration of 0.1% and 7.6%, respectively. Servalyt AG 2-11 and AG 6-8 (Serva) were used as ampholytes. Gel tracks of 8 cm were cut into 2 mm slices and tested for oxidase activity as in [1]. The pH-gradient was determined with calibration proteins (Serva, mixture 9) or by cutting the gel into 2 mm slices, incubating them in 0.5 ml dis-

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tilled water and measuring the pH of the solution after 3 h.

2.6. *Redox titration*

Redox titrations of the purified cytochrome oxidase were performed at pH 7.0 in 50 mM 2-(morpholino)-propanesulfonic acid (MOPS) according to [8]. Redox mediators were: diaminodurene ($E_{m,7}$ = +240 mV); phenazine methosulfate (+80 mV) and phenazine ethosulfate (+55 mV). Oxidation–reduction potentials were made more positive by adding potassium ferricyanide and more negative by addition of ascorbate or dithionite. Titrations at pH 8.0 and pH 9.0 were performed in 50 mM 4-(2-hydroxyethyl-1-piperazine-ethane) sulfonic acid and in 50 mM tris(hydroxymethyl)aminomethane, respectively. Buffer exchange and concentrations of the samples were done on Sephadex G-25. All buffers contained 0.1% Triton X-100.

3. RESULTS AND DISCUSSION

The pyridine hemochrome difference spectrum of the acid/acetone extract of the purified cytochrome oxidase showed peaks at 555, 523 and 423 nm, characteristic of the *proto*-heme of cytochrome *b*-type (fig.1). A very similar spectrum was described for the protoheme of the cytochrome *o* from *Rhodopseudomonas palustris* [2]. The spectrum of the acid/acetone residue of the purified enzyme did not indicate a *meso*-heme of cytochrome *c*. In contrast to the *b*-type cytochrome oxidase of *Rps. palustris* [2] and *Vitreoscilla* [9,10] the enzyme preparation of *Rps. capsulata* did not show a carbon monoxide difference spectrum (not shown). In addition the cytochrome *c* oxidase activity was not inhibited by CO. This lack of CO-inhibition was known for membrane preparations of *Rps. capsulata* [11]. Thus, this enzyme was defined to be an atypical *c*-type cytochrome or a new cytochrome oxidase of the *b*-type [12].

Cytochrome *c* oxidase was eluted from Sephadex G-150 as a dimer (82% of applied protein) and as a monomer (16%). An apparent M_r of 130 000 and 54 000 for the dimer and the monomer, respectively, was determined (fig.2). The heme content of the dimer, determined from the absorption of the α-peak at 555 nm of the pyridine hemochrome preparation, was 7.7 nmol/mg cytochrome oxidase, indicating 1 mol heme/mol dimer. The heme content of the monomer fraction was 6 nmol/mg, indicating 2 mol heme/5 mol monomer. Activity and heme content of the monomer fraction were 20% reduced compared with the dimer fraction of cytochrome oxidase. This 20% reduction of activity in the monomer fraction may be the result of a reaggregation of 80% of monomers with 1 heme/dimer leaving 20% of monomers free of heme and inactive. No cooperative effects were observed for the oxidation of ferrocytochrome *c*. The monomer/dimer ratio was increased from 1:4 to 3:2 when the Triton concentration was increased 5-fold.

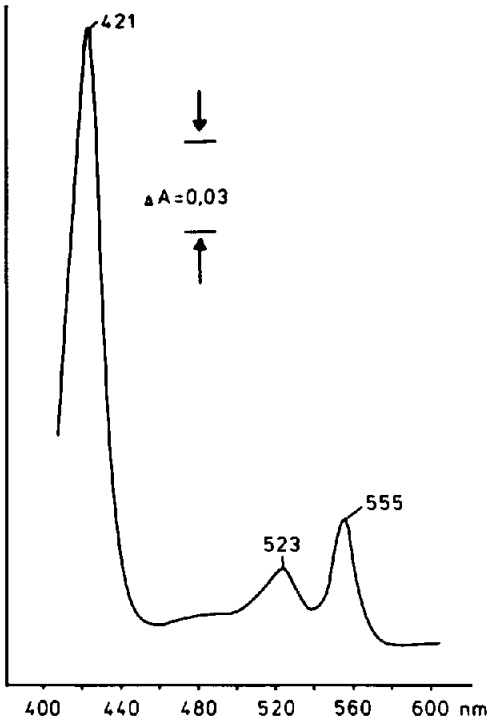

Fig.1. Pyridine hemochrome difference spectrum of acid/acetone extract of the purified cytochrome oxidase of *Rps. capsulata*. The experimental conditions are described in section 2.

A midpoint potential of 385 ± 15 mV was determined by redox titration of the purified cytochrome oxidase at pH 7 and 25°C (fig. 3). This value indicated a functional cytochrome oxidase. The small difference to the value determined in whole membranes [12,13] may be caused by detergent and isolation procedure. The midpoint potential

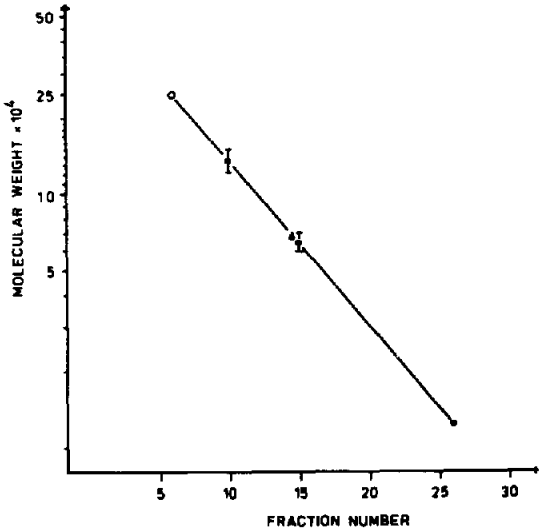

Fig.2. Gel filtration of the purified cytochrome oxidase on Sephadex G-150 in 50 mM TRIPE-buffer plus 0.1% Triton X-100. The marker proteins are: (○) catalase (Boehringer Mannheim, M_r 240 000; (△) bovine serum albumin (Sigma, M_r 68 000); (□) horse heart cytochrome *c* (Sigma, M_r 12 200; (●) cytochrome oxidase activity. Values are means of 8 column runs. Standard deviations are indicated. For further details see section 2.

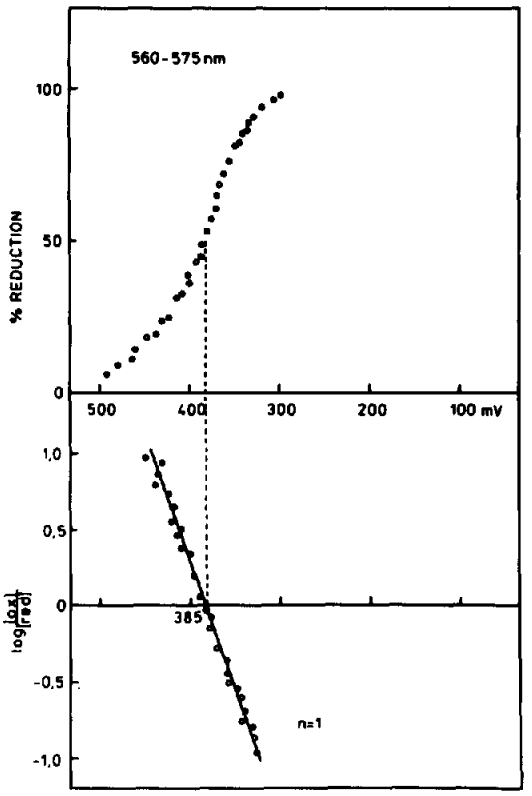

Fig.3. Potentiometric titration at 560–575 nm of the purified *b*-type cytochrome oxidase: 0.4 mg protein/ml was resuspended in 50 mM MOPS buffer (pH 7) plus 0.1% Triton in the presence of 20 μM diaminodurene, 20 μM phenazine methosulfate and 20 μM phenazine ethosulfate. Values are means of 6 redox titrations: (●) reductive phase; (○) oxidative phase. A theoretical $n = 1$ line is drawn through the points.

was strongly pH-dependent. When the pH was increased to pH 9 the potential decreased ~180 mV/pH. The oxidase precipitated when the pH was lowered to 6.

This *b*-type cytochrome oxidase was not inhibited by a 10-fold excess of antimycin concentration inhibiting completely the cytochrome-*b,c*-oxidoreductase [13].

The isoelectric point of the purified cytochrome oxidase was determined to be at pH 6.5. The pH-optimum of the oxidase activity was at pH 8.5 [1]. Below pH 7 the enzyme precipitated and the activity was lost completely.

In contrast to the results of the charge shift electrophoresis in 0.1% Triton, where much activity was lost [1], the recovery of the enzyme activity after electrofocussing was ~95%.

The active form of the purified high-potential cytochrome *c* oxidase of *Rps. capsulata* seemed to be a dimer of M_r 130 000 containing one molecule of heme, which might be sandwiched between two copies of the monomer. However, a loss of heme during the isolation and purification must also be considered. The low isoelectric point of the enzyme supports the idea that the polypeptide interacts with the lysine residues of a membrane-bound cytochrome *c*, which seems to be different from the soluble cytochrome c_2.

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