Biochimica et Biophysica Acta 933 (1988) 179–183
Elsevier

BBA 42735

Relationships between membrane-bound cytochrome *o* from *Vitreoscilla* and that of *Escherichia coli*

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(Received 26 August 1987)

Key words: Cytochrome *o* oxidase; *(Vitreoscilla)*; *(E. coli)*

The cytochrome *o* terminal oxidases from the bacteria *Vitreoscilla* and *Escherichia coli* are structurally and functionally related. They have similar optical spectra, both exhibit ubiquinol-1 oxidase activity and are inhibited similarly. Both enzymes contain four subunits by SDS-polyacrylamide gel electrophoresis analysis and contain protoheme IX and Cu^{2+} prosthetic groups. Antibodies raised against the oxidase purified from *E. coli* crossreact with the *Vitreoscilla* oxidase.

Introduction

Cytochrome *o* is a major component of the electron transport system of many phylogenetically diverse bacteria. It acts as a terminal oxidase by transferring electrons to oxygen to form water. There appear to be two general types of *o*-type terminal oxidases: oxidases of the *co*-type, like the one purified from *Azotobacter vinelandii* [15], and terminal oxidases of the *bo*-type. Of the latter, cytochrome *o* from *Escherichia coli* has been the most extensively characterized [2,4]. More recently, another cytochrome *o* terminal oxidase of the *bo*-type has been purified and partially characterized from the bacterium *Vitreoscilla* sp. C1 [3,13]. Here, we report on the structural and enzymatic similarities between the *o*-type oxidases from *Vitreoscilla* and *E. coli*, and on a new purification method of the cytochrome *o* from *E. coli*. The results indicate that the two enzymes are closely related.

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Materials and Methods

Growth conditions and membrane preparation

The growth of *Vitreoscilla* sp. strain C1 and the preparation of respiratory membrane fragments by osmotic lysis have been described elsewhere [13]. *E. coli* strain RG145, containing plasmid pRG101 that carries the cloned gene *cyo* encoding cytochrome *o* [1], was grown aerobically in a 200-liter fermenter until early stationary phase in a medium consisting of 25 mM potassium phosphate (pH 7.0)/60 mM NaCl/0.15% (w/v) beef extract/0.15% (w/v) yeast extract/0.5% (w/v) peptone/5.5 mM dextrose/30 μM vitamin B-1/80 μM nicotinic acid/50 μg/ml ampicillin. Approximately 600 g (wet weight) of cells were harvested from 200 liters of culture.

The harvested cells (100 g wet weight) were washed in 10 mM Tris buffer (pH 8.0) and were suspended in 500 ml of the same buffer containing 5 mM magnesium sulfate/4 mg pancreatic

DNAase (type I)/1 mM phenylmethylsulfonyl fluoride, freshly prepared in ethanol. The cell suspension was passed once through a French pressure cell (SLM-Amico, Urbana, IL, U.S.A.) at 138 MPa, and the remaining intact cells were removed by centrifugation at $10000 \times g$ for 10 min at 4°C. The membrane fraction in the supernatant was pelleted at $150000 \times g$ for 1 h. Approximately 2 g of membrane protein was prepared from 100 g cells.

Purification of cytochrome o from Vitreoscilla

The deoxycholate-solubilized, membrane-bound cytochrome *o* from *Vitreoscilla* was purified by using a modification of the method of Georgiou and Webster [3]. For the gel filtration purification step, a column of Bio-Gel A-0.5 M (200-400 mesh, Bio-Rad) (95 × 2.2 cm) was used instead of the Sephadex G-200 superfine column.

Purification of cytochrome o from Escherichia coli

The method used in this work is a modification of two methods described elsewhere [3,4]. All purification steps were performed at 4°C unless stated otherwise. The *E. coli* membranes (from 100 g cells) were suspended in solubilizing buffer A (20 mM Tris buffer (pH 8.5) containing 0.25 M sucrose, 1.0 M KCl and 1% (w/v) sodium deoxycholate), with a protein-to-detergent ratio of 1:2.5 (w/w). The suspension was incubated for 30 min on ice and centrifuged for 30 min at $100000 \times g$. The supernatant was dialyzed overnight against 50 volumes of 20 mM sodium phosphate (pH 6.0). The cloudy dialysate was centrifuged at $100000 \times g$ for 30 min and the red pellet was washed once with 20 mM Tris buffer (pH 8.5) containing 0.05% (w/v) sodium deoxycholate. The red pellet was suspended in buffer A with a protein-to-detergent ratio of 1:1 (w/w), and incubated on ice for 30 min. The suspension was centrifuged at $150000 \times g$ for 1 h and the resulting pellet was resolubilized and centrifuged, and both supernatants obtained from the two solubilization steps were combined.

The combined supernatants were brought to 5% (v/v) Triton X-100, dialyzed overnight against buffer B (10 mM Tris (pH 8.0) containing 1% (v/v) Triton X-100), and loaded on a DEAE-Sepharose CL-6B column (2.5 × 40 cm) that had been previously equilibrated with buffer B. The column was washed with one bed volume of buffer B and the cytochrome *o* was eluted at 0.2 M NaCl with a 500 ml linear gradient from 0 to 0.3 M NaCl in buffer B at a flow rate of 25 ml/h. The cytochrome fractions that had an absorbance at 415 nm greater than 0.1 units were combined and, after dialysis against buffer B, were rechromatographed using the same conditions as stated above.

Fractions that had an absorbance (at 415 nm) greater than 0.1 units were pooled and loaded on a hydroxylapatite column (1.5 × 10 cm). They were washed with 2 bed volumes of buffer C (10 mM potassium phosphate (pH 7.5) containing 0.05% (w/v) sarcosyl), and cytochrome *o* was eluted with 0.7 M sodium phosphate buffer (pH 7.5) containing 0.05% (w/v) sarcosyl, and was dialyzed overnight against buffer C (Table I).

Immunological procedures

The polyclonal antibodies against the *E. coli* cytochrome *o* terminal oxidase used in this work have been described previously [2]. For Western immunoblotting [6], the ProtoBlot immunoscreening system (Promega Biotec) was used. This system employs antibody-alkaline phosphatase conjugates and a color development substrate [7].

TABLE I

PURIFICATION OF CYTOCHROME *o* FROM *ESCHERICHIA COLI*

The steps for the purification of cytochrome *o* are described in detail in Materials and Methods.

Purification step	Total protein (mg)	Total heme *b* (nmol)	Specific activity (nmol heme *b*/mg)	Yield (heme *b*) (%)
Membrane fraction	1944	2592	1.3	100
Differential pH precipitation and deoxycholate solubilization	162	1198	7.4	46.2
Second DEAE-Sepharose CL-6B chromatography	56	804	14.4	31
Hydroxylapatite chromatography	42	603	14.4	23.3

Separate SDS-polyacrylamide gel electrophoresis lanes were loaded with 0.01 μg of pure *E. coli* cytochrome *o*, 4 μg of pure *Vitreoscilla* cytochrome *o*, 8 μg pure *E. coli* cytochrome *d*, and 8 μg each of the prestained molecular weight standards, myosin (H-chain) 200000, phosphorylase *b* 97400, bovine serum albumin 68000, ovalbumin 43000, α-chymotrypsinogen 25700, β-lactoglobulin 18400 and lysozyme 14300 (Bethesda Research Labs, Inc.).

Electrophoresis

The SDS-polyacrylamide gel electrophoresis systems used in this work are modifications of the systems of Dispirita et al. [8] and Kadenbach et al. [9]. The stacking gel consisted of 4% acrylamide (w/v) for Western immunoblotting or 6% (w/v) for protein staining, with 0.1 M Tris/0.1% (w/v) SDS (pH 6.8). The separating gel consisted of 12% acrylamide (w/v) for Western immunoblotting or 16% (w/v) for protein staining, with 0.375 M Tris/0.1% (w/v) SDS/3.6 M urea/13% (v/v) glycerol (pH 8.8). The concentration of the cross-linker was 1.5% (w/w) of the total acrylamide. The 2× sample buffer consisted of 0.125 M Tris/28% (v/v) glycerol/16% (w/v) SDS/2% (v/v) β-mercaptoethanol (pH 6.8). Boiling of the samples was avoided because of protein aggregate formation. Instead, samples were incubated for 30 min at room temperature. Gels were stained with 0.1% (w/v) Coomassie brilliant blue R-250 in *n*-propanol/water/acetic acid (1 : 8 : 1, v/v) and destained in the same solution without the dye. Molecular weight markers were used, as described in the previous section.

Assays

Protein concentration in samples that contained detergents was estimated by a modification of the Lowry method [10] using bovine serum albumin (Fraction V) as protein standard. Protoheme IX was measured by the pyridine hemochromogen method [11]. The ubiquinol-1 oxidase activity of cytochrome *o* from *E. coli* was measured by the rate of oxygen consumption as described elsewhere [12]. The concentration of oxygen in the air-saturated potassium phosphate buffer was assumed to be 250 μM. The ubiquinol-1 oxidase activity of cytochrome *o* from *Vitreoscilla* was measured in a similar manner with one modification. The reaction was initiated with ubiquinol-1 that had been pre-reduced by titrating the ubiquinone-1 with dithioerythritol, because excess dithioerythritol in the assay inactivates the *Vitreoscilla* enzyme. Ubiquinone-1 was synthesized according to a procedure provided by Dr. Chang-An Yu, University of Oklahoma (unpublished data). The K_m and V_{max} of the ubiquinol-1 oxidase activity of cytochrome *o* from *Vitreoscilla* were determined by measuring the initial enzyme velocity (V_o) with the ubiquinol-1 concentration, [S], in the range 10–300 μM, and analyzing the data with the Hanes-Woolf plot ([S]/V_o vs. [S]) [5]. The inhibition of cytochrome *o* from *Vitreoscilla* with KCN and NaN_3 was determined using stock solutions of 1 mM and 1.5 M, respectively, prepared in 100 mM potassium phosphate (pH 7.5). For these assays, the concentrations of ubiquinol-1 and cytochrome *o* were 270 μM and 42 nM, respectively.

Results

To examine the possible similarity of the *o*-type oxidases from *E. coli* and *Vitreoscilla*, the *E. coli* enzyme was purified using the same preparative protocol employed for the *Vitreoscilla* enzyme, which uses different detergents than in the previously reported preparative protocols for the *E. coli* oxidase [2,4]. This procedure involves solubilization of membranes with deoxycholate, a pH-induced precipitation, and ion exchange chromatography (see Table I). The protocol successfully yielded a preparation of the *E. coli* enzyme that had properties similar to those reported previously using different purification protocols. The ubiquinol-1 oxidase specific activity was about the same as for enzyme prepared by the procedure of Matsushita et al. [2], which is several times less than that reported using the preparative protocol of Kita et al. [4]. The current protocol has a disadvantage in that the losses during the critical pH-induced precipitation step varied widely in different preparations and were difficult to control. The final product has similar properties even in preparations where the final yield was low.

In 16% polyacrylamide gels, both the *E. coli* and *Vitreoscilla* enzymes have four polypeptide

subunits with similar molecular weights (Fig. 1). The previously reported apparent molecular weights for the *E. coli* oxidase subunits are 60 000, 36 000, 18 000 and 13 000 [14], whereas the apparent sizes of the *Vitreoscilla* subunits are 55 000, 35 000, 19 000 and 10 000. Efforts to obtain N-terminal sequence data from the isolated subunits of the *E. coli* oxidase have been unsuccessful because the amino termini are apparently blocked. Hence, the possibility that more than one polypeptide are present in each of the bands visualized following SDS-polyacrylamide gel electrophoresis cannot be excluded.

Polyclonal antibodies were raised against the purified *E. coli* oxidase and shown to immunoblot to subunit II (mol. wt., 36 000) (Carter, K. and Gennis, R.B., unpublished data). Fig. 2 shows that this antibody crossreacts with subunit II of the *Vitreoscilla* oxidase, though about 400-fold more protein is required in the SDS-polyacrylamide gel lane to elicit a similar immunoblot response. It is not known whether this is due to a difference in the transfer efficiency to nitrocellulose or to an immunological difference. The latter is more likely. The antibodies do not crossreact with the subunits of the cytochrome *d* oxidase from *E. coli* or the molecular weight standards.

Finally, the *E. coli* cytochrome *o* is a well

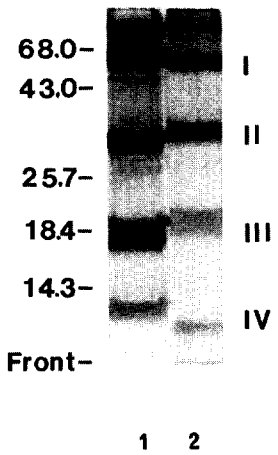

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cytochromes *o*: Lane 1 from *Escherichia coli*; lane 2 from *Vitreoscilla*. The conditions for the electrophoresis are described in Materials and Methods. Subunits are designated by Roman numerals.

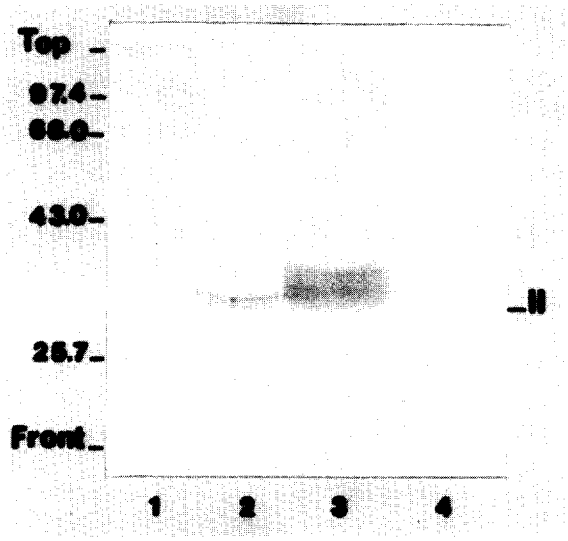

Fig. 2. Western immunoblotting of cytochromes *o*. Cytochromes *o* were blotted with polyclonal antibody raised against the cytochrome *o* from *E. coli*: lane 1, prestained molecular weight markers; lane 2, cytochrome *o* from *E. coli*; lane 3, cytochrome *o* from *Vitreoscilla*; lane 4, cytochrome *d* from *E. coli*. The running conditions are described in Materials and Methods. Subunits are designated by Roman numerals. Note that the molecular weight markers are visible in lane 1 due to pre-attached stain, and not to a response during the immunoblotting procedure.

characterized ubiquinol oxidase, and tests were made to determine whether the cytochrome *o* from *Vitreoscilla* is also a quinol oxidase. The data confirm that the *Vitreoscilla* cytochrome *o* has ubiquinol-1 oxidase activity with $K_m = 65\ \mu M$ and turnover of 10 s^{-1} (mol of molecular oxygen consumed per mol of enzyme) at 37 °C, pH 7.5. The ubiquinol-1 oxidase activity of the *Vitreoscilla* cytochrome *o* is inhibited by potassium cyanide (8 μM for 50% inhibition) and sodium azide (12 mM for 50% inhibition), whereas for the *E. coli* enzyme the corresponding concentrations of the inhibitors are 10 μM and 15 mM, repsectively.

Discussion

Cytochromes *o* from the diverse bacteria *Vitreoscilla* and *E. coli* show evident similarities in their structure and enzymatic activity. Previous studies [3,4,14] have shown that the two enzymes have similar optical properties and prosthetic groups. At 77 °K, the reduced-*minus*-oxidized

spectrum of the *E. coli* enzyme has alpha absorption peaks at 555 nm and 562 nm, whereas the *Vitreoscilla* enzyme absorbs maximally at 558 nm and 561 nm. Both enzymes possess 2 mol of protoheme IX and 2 mol of copper per mol enzyme, and bind to carbon monoxide. The oxidation-reduction potential of the *Vitreoscilla* cytochrome *o* is 165 mV (pH 7.0), identical to that of the *E. coli* enzyme [16]. The cytochrome *o* complex from *E. coli* is a coupling site for oxidative phosphorylation [12] and is a ubiquinol oxidase [2,4,12]. Preliminary work on the enzyme from *Vitreoscilla* is suggestive of a similar function.

The current work has significantly extended the similarities of the two terminal oxidases. On a functional level, both the *Vitreoscilla* and *E. coli* enzymes exhibit similar affinities for ubiquinol-1 (K_m values were 65 μM and 48 μM, respectively), although the turnover number at pH 7.5 of the *Vitreoscilla* cytochrome *o* (10 s^{-1}) is 25-times slower than that of the *E. coli* enzyme [4]. Possibly, this reflects partial denaturation of the *Vitreoscilla* oxidase during purification. The *Vitreoscilla* oxidase has a K_m for menadiol (38 μM) which is approximately 4-fold lower than that of the *E. coli* enzyme [3,14]. The concentrations of cyanide and azide required for 50% inhibition of the ubiquinol-1 oxidase activity of the enzymes are approximately the same.

On a structural level, a high resolution SDS-polyacrylamide gel electrophoresis system has shown that each enzyme contains four subunits with similar molecular weights. The crossreactivity of the antibodies raised against the *E. coli* enzyme with that from *Vitreoscilla* strongly suggests an evolutionary relationship between these two enzymes.

Acknowledgements

This work was supported by Public Health Service grants HL 16101 (R.B.G.) and GM27085 (D.A.W.) from the National Institutes of Health.

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