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Purification, characterization and reconstitution of cytochrome *o*-type oxidase from *Gluconobacter suboxydans*

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The *Gluconobacter suboxydans* respiratory chain has a cytochrome *o* as a terminal oxidase. The cytochrome *o*-type oxidase was solubilized with octyl glucoside after washing the membranes with Triton X-100, and was purified by one-step ion-exchange chromatography. The purified oxidase contains four polypeptides, two *b*-type cytochromes (*b*-558 and *b*-562), and 2 mol of heme / mol of enzyme. The oxidase was shown to be a typical cytochrome *o* and to have two CO-binding sites in the molecule. The enzyme catalyzes the oxidation of ubiquinol, and the activity is inhibited with KCN or quinone analogues. The purified cytochrome *o* can be reconstituted with phospholipids prepared from *G. suboxydans* into proteoliposomes by octyl glucoside dilution. The proteoliposome generates a proton electrochemical gradient (inside negative and alkaline) of about −140 mV during ubiquinol oxidation. The generation of membrane potential and pH gradient was determined by fluorometric methods using carbocyanine and dansylglycine, respectively. Thus, cytochrome *o* of *G. suboxydans* was shown to be an ubiquinol oxidase functioning as an energy-generator.

Introduction

Gluconobacter strains are obligate aerobes and well known as the oxidative bacteria which have highly active oxidase activity for sugar or sugar alcohol [1]. Sugar oxidation is mediated by an electron transport system located in the cytoplasmic membrane of the organisms [2]. The respiratory chain of *G. suboxydans* consists of ubiquinone-10 (Q_{10}), cytochromes *c* and cytochrome *o* [3,4]. Cytochrome *o* constitutes the end of the branched respiratory chain which may also be terminated by an alternative oxidase [4]. Thus, it is important to know the function of the cytochrome *o*, especially its electron donor and whether or not it can conserve energy.

A variety of terminal oxidases, including aa_3-type, *d*-type and *o*-type cytochromes, have been identified in bacteria [5]. Cytochrome *o* appears to be the most widely distributed in bacteria and to be divided into two subgroups, *bo*-type and *co*-type cytochrome. Recently, both types of cytochrome *o* have been purified from *Escherichia coli* [6–8], and from *Pseudomonas aeruginosa* [9] and *Methylophilus methylotrophus* [10]. Cytochrome *o* purified from *E. coli* has been shown to catalyze ubiquinol oxidation and those from *Pseudomonas* and *Methylophilus* to oxidize reduced cytochrome *c*. Furthermore, *E. coli* cytochrome *o* has been

Abbreviations: dansylglycine, *N*-[(5-dimethylamino)naphthalene-1-sulfonyl]glycine; diS-C_3-(5), 3,3′-diisopropylthiodicarbocyanine; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; $\Delta\psi$, membrane potential; octyl glucoside, octyl β-D-glucoside; ΔpH, pH gradient; $\Delta\tilde{\mu}_{H^+}$, proton electrochemical gradient; Q, ubiquinone; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; UHNQ, 3-undecyl-2-hydroxy-1,4-naphthoquinone.

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demonstrated to generate a proton electrochemical gradient ($\Delta\tilde{\mu}_{H^+}$) [6,7], and also shown to function as a 'half-loop' (i.e., the oxidase catalyzes the scalar release of protons from ubiquinol outside, vectorial translocation of electrons to inside and scalar proton uptake inside to reduce oxygen) [7,11]. Thus, cytochrome *o* has been shown to conserve energy.

In this communication, we report a relatively simple purification and characterization of the cytochrome *o*-type oxidase from *G. suboxydans* and demonstrate that turnover of the purified enzyme generates $\Delta\tilde{\mu}_{H^+}$ (inside negative and alkaline) when it is reconstituted into proteoliposomes. The results indicate that the cytochrome *o* is an ubiquinol oxidase functioning to generate energy.

Materials and Methods

Materials

Phospholipids were extracted from *G. suboxydans* or *E. coli* cells with isopropanol/hexane, washed with acetone, dissolved in diethyl ether, and stored as described [12]. Octyl β-D-glucoside (octyl glucoside), valinomycin and nigericin were purchased from Calbiochem-Behring. 3,3′-Diisopropylthiodicarbocyanine (diS-C_3-(5)) was from Molecular Probes. Dansylglycine and HQNO were obtained from Sigma, UHNQ from Aldrich Chemical Co. Q_1, Q_2 and Q_6 were kindly supplied by Eizai Co. and Nissin-seifun Co. Reduced forms of these quinones were prepared as described by Rieske [13]. DEAE-Toyopearl, which can be used as a medium-performance anion exchanger, was purchased from Toyo Soda Co. All other materials were of reagent grade and obtained from commercial sources.

Bacterial strains and preparation of membranes

G. suboxydans IFO 12528 was grown aerobically into the late logarithmic phase in sugar-rich medium with rotary shaking or in a 50-liter jar fermentor at 30 °C [4]. Cells were suspended in 50 mM potassium phosphate buffer (pH 6.5) at a concentration of 1 g wet weight of cells per 5 ml, and passed through a French pressure cell press at 16000 lb/in^2. Cell debris was removed by centrifugation at 10000 × *g* for 10 min, and the supernatant was centrifuged at 68000 × *g* for 90 min in order to sediment the membrane fraction.

Purification of cytochrome o-type oxidase

All steps were performed at 0–6 °C unless noted otherwise. Membranes prepared as described were suspended to a final protein concentration of 10 mg/ml in 50 mM potassium phosphate buffer (pH 6.5)/5 mM $MgSO_4$, and Triton X-100 was added to a final concentration of 1%. The suspension was incubated on ice for 30 min and centrifuged at 68000 × *g* for 60 min, and the supernatant was discarded. The pellet was homogenized to a final protein concentration of about 10 mg/ml in 50 mM potassium phosphate buffer (pH 6.5), and octyl glucoside was added to a final concentration of 1.25%. The suspension was incubated on ice for 20 min and centrifuged at 68000 × *g* for 60 min. The supernatant was applied to a DEAE-Toyopearl column (about 1-ml bed volume per 2 mg of protein applied), which had been equilibrated with 50 mM potassium phosphate buffer (pH 6.5) and washed with 1 bed volume of the same buffer containing 1% octyl glucoside. After application of the sample, the column was washed with 50 mM potassium phosphate buffer (pH 6.5) containing 1% octyl glucoside at a flow rate of about 30 ml/h. After the elution of nonadsorbed major impurity, the enzyme appeared at about 4 bed volumes elution of the buffer. Fractions exhibiting Q_2H_2 oxidase activity were pooled, concentrated by an ultrafiltration and stored in liquid nitrogen. Alternatively, the active fraction was used directly for reconstitution experiments described below.

Reconstitution of cytochrome o into proteoliposomes

Reconstitution of cytochrome *o* into proteoliposomes was performed essentially as described previously [14]. The purified oxidase was mixed with sonicated *G. suboxydans* phospholipids (100 mg per mg of oxidase protein), and octyl glucoside was added to a final concentration of 1.25% (total volume was adjusted with 50 mM potassium phosphate buffer, (pH 6.5), the octyl glucoside : phospholipid ratio being 1.4). The mixture was incubated on ice for 30 min and then diluted into over 30 vol. of 50 mM potassium phosphate buffer (pH 6.5) that had been equilibrated to 25 °C.

After stirring and standing for 10 min, proteoliposomes were collected by centrifugation at 120000 $\times g$ for 3 h. The pellet was suspended in 50 mM potassium phosphate buffer (pH 6.5) to a protein concentration of about 0.2 mg/ml and frozen rapidly in liquid nitrogen. Just prior to use, the samples were thawed and sonicated for 10–20 s with a bath-type sonicator.

Measurement of membrane potential ($\Delta\psi$) and pH gradient (ΔpH)

$\Delta\psi$ (inside negative) was measured by following the fluorescence quenching of carbocyanine dye, diS-C_3-(5); emission was recorded at 670 nm with excitation at 622 nm [7,15]. Reaction mixture contained 1 μM diS-C_3-(5), proteoliposomes and 50 mM potassium phosphate buffer (pH 6.5) in total volume of 1 ml. The reaction was initiated with 2 mM dithiothreitol and 50 μM Q_1H_2 or 5 mM ascorbate and 1.8 mM TMPD, followed by addition of nigericin and valinomycin.

ΔpH (inside alkaline) was measured by following the fluorescence intensity of dansylglycine as described by Bramhall [16]. Reaction mixture consisted of 5 μM dansylglycine, proteoliposomes, 1 μM valinomycin and 50 mM potassium phosphate buffer (pH 6.5) in total volume of 1 ml. The reaction was initiated with 2 mM dithiothreitol and 35 μM Q_1H_2 or with 5 mM ascorbate and 1.8 mM TMPD, and the fluorescence emission was monitored at 510 nm with excitation at 350 nm.

Other analytical procedures

Oxidase activity for Q_1H_2, Q_2H_2, Q_6H_2 or TMPD was measured spectrophotometrically at 25°C by following the increase in absorbance at 275 nm or 520 nm, respectively. The reaction mixture (total volume, 2.5 ml) contained 50 mM potassium phosphate buffer (pH 6.5), 50 μM quinol or 3.6 mM TMPD, 0.04% Tween 20 and the enzyme. Activity was calculated by using a millimolar absorption coefficient of 12.25 for quinol or 6.1 for TMPD.

Absorption spectrophotometry was performed with a Hitachi 557 dual-wavelength spectrophotometer. Low-temperature difference spectra were recorded in liquid nitrogen with the same photometer using a cuvette of 2 mm light-path. Cytochrome *o* content was calculated from the absorbance difference between 416 and 430 nm in the CO difference spectrum using a millimolar absorption coefficient of 170 [3]. CO-titration was done by anaerobic addition of CO-saturated buffer to the reduced cytochrome in a Thunberg-type cuvette as described [17]. Heme *b* content was determined by measuring pyridine hemochrome using a millimolar absorption coefficient of 20.7 (reduced − oxidized) [18].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed in 10–14% polyacrylamide gels as described by Laemmli [19]. Samples were treated with 3% SDS at 30–40°C for about 30 min prior to electrophoresis.

Protein was assayed as described [20].

Results

Purification of cytochrome o-type oxidase

Cytochrome *o* oxidase of *G. suboxydans* was partially purified by solubilizing the membranes differentially. Triton X-100 (1%) extracted about 30% of the membrane proteins, but almost all cytochrome *o* oxidase activity was retained in the residual membranes, from which the oxidase was solubilized with octyl glucoside. The differential solubilization exhibits a 4- to 8-fold increase in specific activity over the starting membrane with a high recovery. From the octyl glucoside extract, the oxidase was purified by a single-step column chromatography of DEAE-Toyopearl. Relative to the membranes, an overall purification of about 50-fold is obtained with respect to the specific activity of Q_2H_2 oxidase (Table I). As shown in Fig. 1, this chromatographic elution was unex-

TABLE I

PURIFICATION OF CYTOCHROME *o*-TYPE OXIDASE FROM *GLUCONOBACTER SUBOXYDANS*

One unit expresses the activity to oxidize 1 μmol of Q_2H_2 per min.

Fractions	Protein (mg)	Q_2H_2 oxidase activity	
		units	units/mg
Membrane	123	379	3.1
Octyl glucoside extract	19.2	281	14.7
DEAE-Toyopearl	1.6	259	165

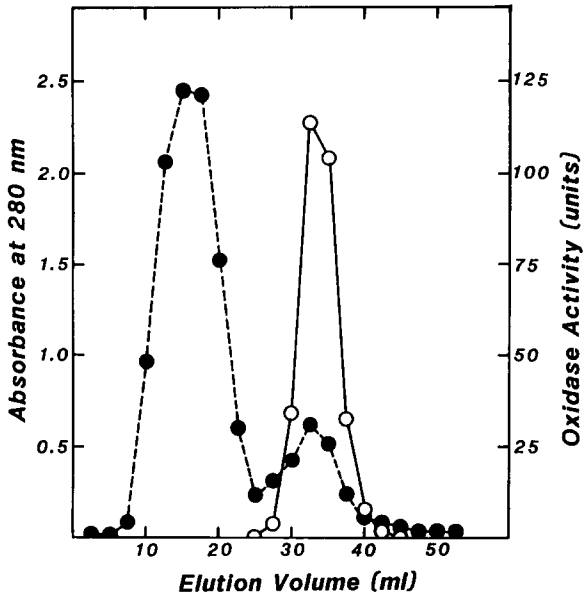

Fig. 1. Elution pattern of cytochrome *o* in DEAE-Toyopearl column chromatography. The octyl glucoside extract (19.2 mg of protein) was applied onto a DEAE-Toyopearl column (2× 3.2 cm) and the oxidase eluted with 50 mM potassium phosphate buffer (pH 6.5) containing 1% octyl glucoside at a flow rate of 30 ml/h. ●------●, Absorbance at 280 nm; ○——○, oxidase activity (units/ml) measured as Q_2H_2 oxidase.

pectedly effective for the purification. The peculiar chromatographic behavior of the oxidase may be due to the hydrophobic nature of the column resin, though the precise reason is not known. Temperature and ionic strength in this chromatography are critical for the separation of the oxidase from other impurities. The chromatography should be performed at less than 6–8°C and in 50 mM potassium phosphate buffer (pH 6 to 6.5). The column resin should not be re-used unless octyl glucoside binding to it is removed by washing the resin with ethanol. Attempts to purify the enzyme further by a DEAE-Toyopearl column chromatography using gradient elution, hydroxyapatite column or by gel filtration with Toyopearl HW 60 column were unsuccessful. These procedures did not affect polypeptide pattern in SDS-gel electrophoresis, or the purity of the oxidase. Thus, cytochrome *o* oxidase is purified relatively easily by differential solubilization of the membranes and column chromatography on DEAE-Toyopearl. Since the purified enzyme aggregates relatively soon after elution from the column, the sample should be concentrated or reconstituted into liposomes. Alternatively, if the enzyme was solubilized together with phospholipids (2 mg/ml), it was relatively stable after column chromatography.

Characterization of cytochrome o oxidase

SDS-gel electrophoresis of the purified oxidase exhibits four polypeptides, which can be visualized with silver stain (Fig. 2). The lower two polypeptides are hard to visualize with Coomassie blue staining. The apparent molecular weights of these polypeptides were estimated to be 55000, 30000, 21000 and 15000 in 12% gel, but that of the larger two polypeptides obtained in SDS-gel electrophoresis varied depending on the gel concentration used. Thus, the molecular weights of the various polypeptides were finally estimated to be 76000, 35000, 21000 and 15000 after correc-

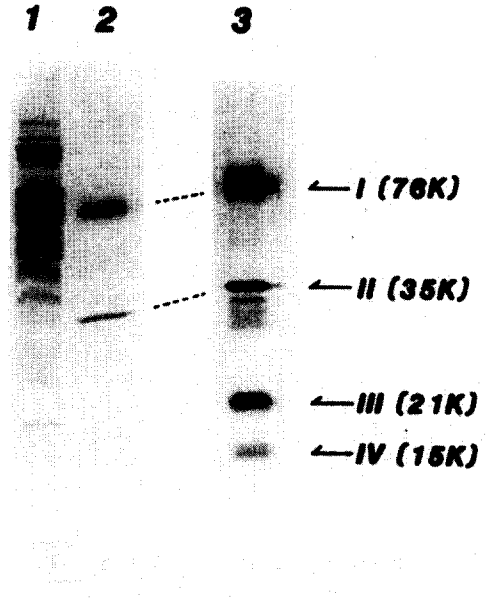

Fig. 2. SDS-polyacrylamide gel electrophoresis of purified cytochrome *o*. SDS-gel electrophoresis was carried out in 12.5% polyacrylamide gel. Lane 1, octyl glucoside extract (10 μg of protein) stained with Coomassie blue; lane 2, purified oxidase (5 μg of protein) stained with Coomassie blue; lane 3, purified oxidase (2 μg of protein) stained with silver. Molecular weights of each subunit were estimated as described in the text from the standard curve prepared with marker proteins of phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase and lysozyme.

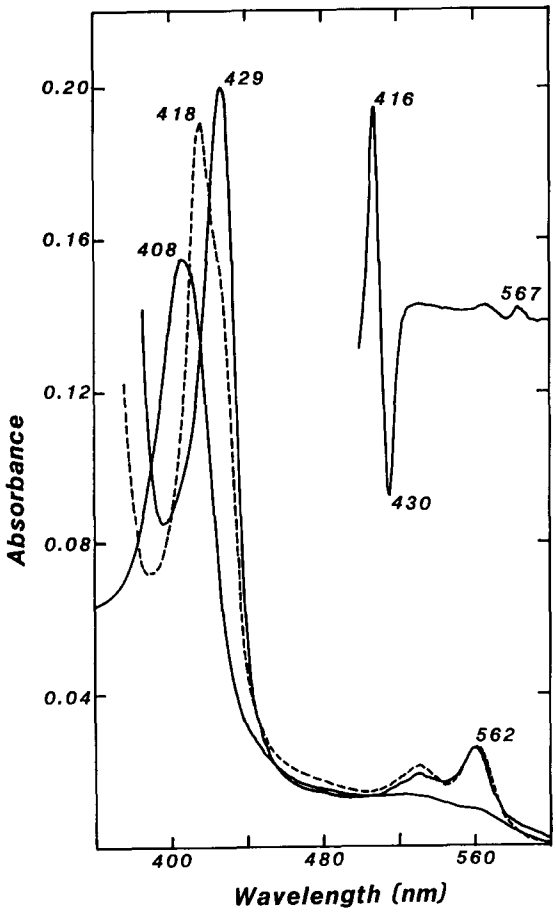

Fig. 3. Absorption spectra of purified cytochrome *o*. The purified sample was used at a protein concentration of 84.3 μg/ml in 50 mM potassium phosphate buffer (pH 6.5) containing 1% octyl glucoside. The sample was reduced with dithionite, and then CO was bubbled through the solution for 1 min. ———, oxidized or reduced forms; - - - - - -, CO+ reduced form. The inset shows the CO+reduced minus reduced difference spectrum with the same absorbance scale but with half scale of wavelength.

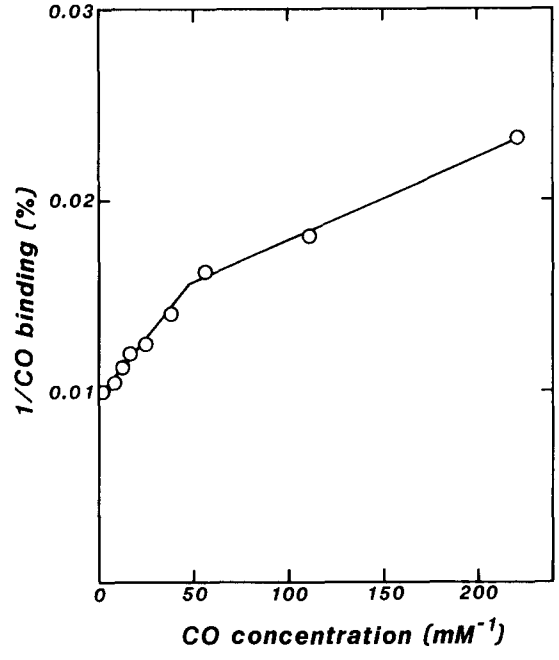

Fig. 4. Lineweaver-Burk plots of CO titration with purified cytochrome *o*. CO titration was carried out at a protein concentration of 79 μg/ml as described in Materials and Methods.

tion by Furgson plot (data not shown). Therefore, the molecular weight of the oxidase complex appears to be 147000 if it consists of subunits at a 1:1 ratio. The purified oxidase contained 14.8 ± 1.9 (11.9–17.3) nmol heme *b*/mg of protein. Cytochrome *o* content was estimated to be 7.1 nmol/mg of protein by using a millimolar absorption coefficient of 170, at a wavelength (λ) of 416–430 nm.

As shown in Fig. 3, absorption spectrum of the purified enzyme exhibits a typical *b*-type cytochrome, as evidenced by the α band at 562 nm, which splits into two peaks at 558 and 562 nm in liquid nitrogen (data not shown). Furthermore, exposure of the reduced oxidase to CO causes a γ band-shift from 429 to 418 nm and yields a CO difference spectrum typical for cytochrome *o* (Fig. 3). The binding of the reduced oxidase to CO was titrated and its Lineweaver-Burk plot was obtained (Fig. 4). The result indicates that the cytochrome *o* has two CO-binding sites with K_m values of 3.0 and 13 μM.

The purified oxidase catalyzes the oxidation of ubiquinol homologues and TMPD (Table II). As shown, Q_2H_2 is a better substrate than Q_1H_2 for the enzyme, and also Q_6H_2 can be oxidized by the enzyme at efficiency of 10% relative to Q_2H_2 when Q_6H_2 is solubilized with octyl glucoside (the maximal activity is obtained at 1.5% octyl glucoside, data not shown). TMPD is also oxidized by the enzyme, but at relatively low rates. Interestingly, reconstitution of the oxidase into proteoliposomes decreases the enzyme activity for ubiquinol; the activity is decreased to 15% for Q_1H_2 oxidase, and to 42% for Q_2H_2 oxidase in

TABLE II

KINETIC PROPERTIES OF PURIFIED CYTOCHROME *o*

Electron donors	K_m (μM)	V_{max} (μmol/min/mg)	Inhibitors	K_i
Q_1H_2	76	250	KCN	2.8 μM
Q_2H_2	22	333	NaN_3	5.3 mM
TMPD	1500	17.8	HQNO	0.4 μM
			UHNQ	2.5 nM

proteoliposomes prepared at protein-to-lipid ratio of 1 : 3. In addition, the purified enzyme is intensively inhibited by cyanide and azide, and also by quinone analogues HQNO and UHNQ (Table II).

$\Delta\tilde{\mu}_{H^+}$ generation in the proteoliposomes reconstituted with purified oxidase

Cytochrome *o* oxidase can be reconstituted into proteoliposomes with phospholipids prepared from *G. suboxydans* or *E. coli* by octyl glucoside dilution or dialysis [6,7,14]. Similar results were obtained with both lipids and also by both methods. Thus, in this study, the proteoliposomes containing the purified oxidase was prepared with *G. suboxydans* lipids by octyl glucoside dilution, for which an octyl glucoside concentration of 1.25% is the best, as in the case of *E. coli* cytochrome *o* [7,14].

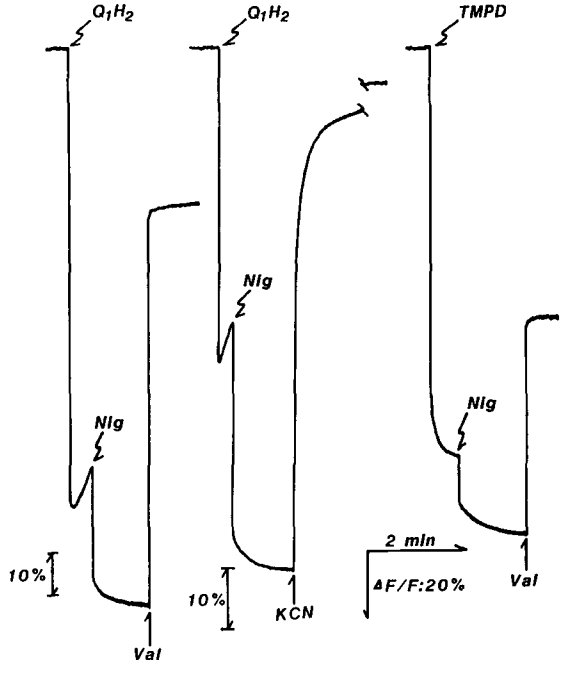

Fig. 5. Fluorescence quenching of diS-C_3-(5) in proteoliposomes containing cytochrome *o*. Reactions were performed as described in Materials and Methods with proteoliposomes containing cytochrome *o* (0.4 μg of protein). As indicated, 0.025 μM nigericin (Nig), 1 μM valinomycin (Val) or 2.5 mM KCN was added.

Generation of $\Delta\psi$ was examined in the proteoliposomes reconstituted with the oxidase purified from *G. suboxydans* by measuring the fluorescence quenching of the carbocyanine dye, diS-C_3-(5) [7,14]. As shown in Fig. 5, addition of Q_1H_2 to the proteoliposomes quenches the fluorescence of diS-C_3-(5). The quenching is enhanced with nigericin, an ionophore that catalyzes electroneutral exchange of proton for K^+, and diminishes rapidly with addition of valinomycin, an ionophore that collapses $\Delta\psi$ in the presence of K^+. As shown, KCN, an inhibitor of the oxidase, disturbs the fluorescence quenching and TMPD, another electron donor for the oxidase, induces the quenching. Q_1H_2-induced quenching of diS-C_3-(5) is dependent on the concentration of Q_1H_2, and the maximal quenching is obtained around 50 μM (Fig. 6). The magnitude of the $\Delta\psi$ generated during oxidation of Q_1H_2 or TMPD can be estimated by comparing with standard curves constructed from experiments in which fluorescence quenching is induced by a valinomycin-mediated K^+ diffusion gradients of known magnitude (Fig. 6). Thus, the values of -140 and -60 mV were obtained with 70 μM Q_1H_2 and 1.8 mM TMPD, respectively, in the presence of nigericin.

Generation of ΔpH was exmained in the proteoliposomes by using an amphiphilic fluorescence dye dansylglycine [16]. When the fluorescence change of dansylglycine was induced by diluting liposomes containing 50 mM potassium phosphate buffer (pH 7.5) into 50 mM potassium phosphate buffer (pH 5.5), it was dependent linearly on the concentration of dansylglycine up to 5 μM (data not shown). Furthermore, when the ΔpH was changed by changing the outside buffer, the fluorescence intensity was dependent exponentially on ΔpH, which can be used for the quantitation (see Fig. 7). Thus, the experiments were carried out with 5 μM dansylglycine in 50 mM potassium phosphate buffer (pH 6.5) that was used for pre-

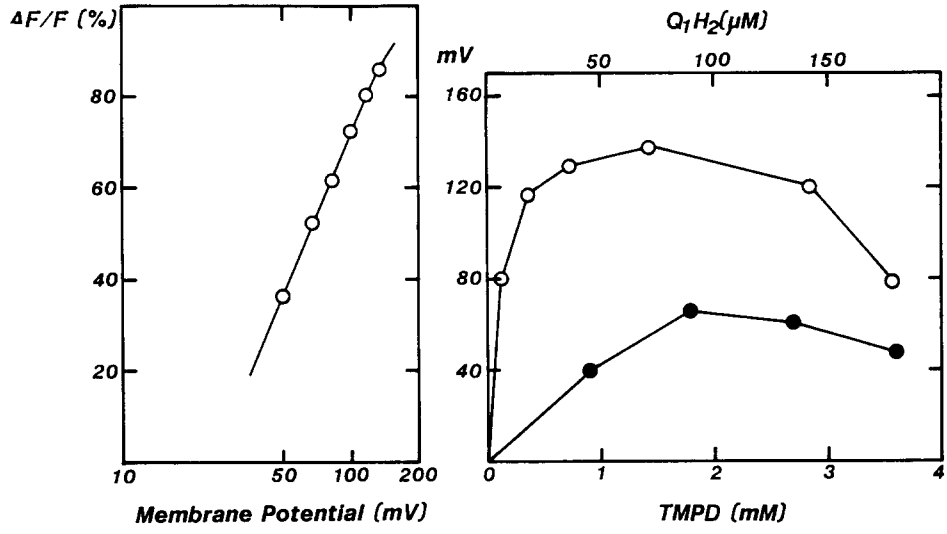

Fig. 6. Calibration curve of $\Delta\psi$ measurement with diS-C_3-(5) and effect of substrate concentration on $\Delta\psi$ generation. (Left panel) Calibration curve was obtained using proteoliposomes containing 0.4 μg of protein as described in the text. (Right panel) The experiments were performed as described in Fig. 5 with given concentrations of Q_1H_2 (○) or TMPD (●). $\Delta\psi$ values were calculated from the calibration curve.

paring proteoliposomes. As seen in Fig. 7, the increase of fluorescence intensity with proteoliposomes is observed following the addition of Q_1H_2 in the presence of valinomycin. Although the reason is not clear, the fluorescence change due to enzyme turnover is slow relative to the change

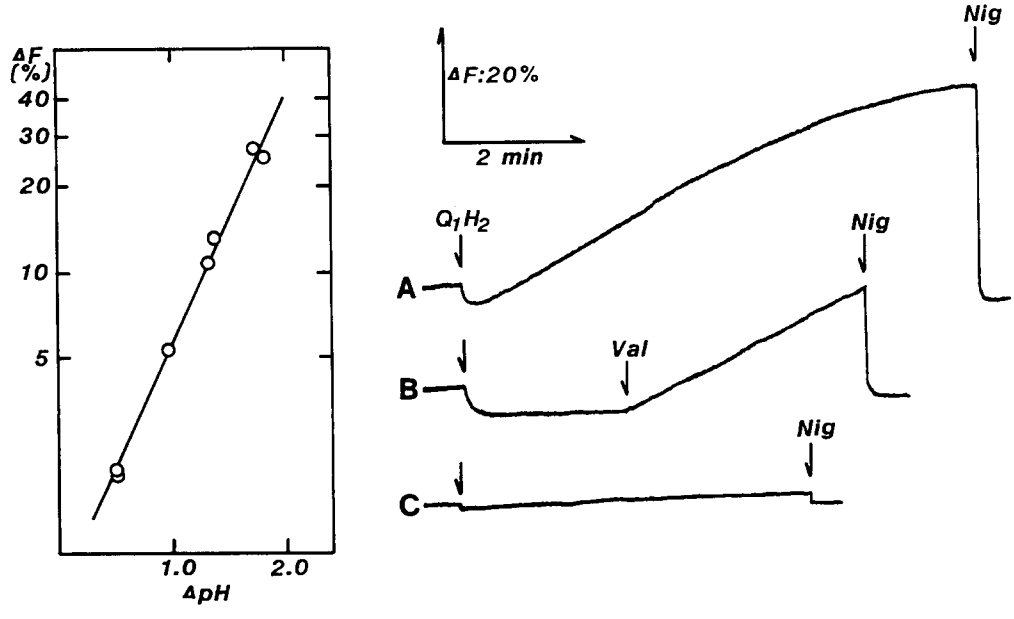

Fig. 7. Fluorescence increase of dansylglycine in proteoliposomes containing cytochrome *o*. Reactions were carried out using proteoliposomes containing cytochrome *o* (5.8 μg of protein) as described in Materials and Methods. (Left panel) Standard curve was prepared as described in the text by measuring the fluorescence increase induced by putting the proteoliposomes into 50 mM potassium phosphate buffer of different pH values. (Right panel) Fluorescence change due to oxidation of Q_1H_2 was monitored. Reactions A and B were done in the presence and absence, respectively, of 1 μM valinomycin (Val). Reaction C was carried out in the presence of Val and 2.5 mM KCN. As indicated, 0.025 μM nigericin (Nig) or Val was added.

induced by an imposed ΔpH. The fluorescence increase of dansylglycine was not induced in the absence of valinomycin or in the presence of KCN, and collapsed rapidly upon the addition of nigericin. The magnitude of ΔpH generated during enzyme turnover was estimated to be 1.94 and 1.74 (inside alkaline) with 35 μM Q_1H_2 and 1.8 mM TMPD, respectively, by comparing with the standard curve (Fig. 7).

Discussion

The results presented here indicate that cytochrome *o* oxidase purified from *G. suboxydans* contains four polypeptides and two *b*-type cytochromes, like *E. coli* cytochrome *o* [7,14]. Although it cannot be concluded that all four peptides are components of the oxidase, each polypeptide species is retained after further purification by several procedures described in Results. Therefore, it seems highly likely that the oxidase consists of four polypeptides. The purified oxidase has been shown to contain two hemes *b*, and Daniel [3] has shown that the membranes of *G. suboxydans* contain two cytochromes *o*, cytochrome *o* (558) and cytochrome *o* (565), having different O_2 or CO affinity. Therefore, it is likely that the cytochrome *o* oxidase may contain two cytochrome *o* components, distinguished by Daniel, corresponding to two hemes *b* in the molecule. This possibility is supported by the following observations: (i) the oxidase has two CO-reactive sites; (ii) the low-temperature difference spectrum indicates the presence of two *b*-type cytochromes, *b*-558 and *b*-562; (iii) in the absolute CO spectrum, the γ peak of the reduced oxidase shifts almost totally to a new γ peak by binding CO, suggesting that two hemes *b* bind CO, unlike *E. coli* cytochrome *o*, in which split γ peaks are observed upon reaction with CO. However, there is one negative observation, that the cytochrome *o* content is only half of the heme *b* content if calculated using a millimolar absorption coefficient of 170. Therefore, it is not possible to state conclusively at this moment whether the cytochrome *o* oxidase of *G. suboxydans* contains two cytochrome *o* components.

The purified oxidase catalyzes the oxidation of ubiquinol homologues Q_1H_2, Q_2H_2 and Q_6H_2. Activity of the enzyme for these quinol is decreased by reconstitution of the oxidase into proteoliposomes, which may suggest that the reactive site of the enzyme is embedded in phospholipids and thus hard to react with relatively soluble quinol homologues. Furthermore, the quinone analogues, HQNO and UHNQ, are potent inhibitors of the oxidase. These findings suggest that the cytochrome *o* oxidase of *G. suboxydans* is capable of reacting with physiological ubiquinol, $Q_{10}H_2$ in this particular case, in the membranes. Recently, *G. suboxydans* cytochrome *o* has been reconstituted into proteoliposomes together with Q_{10} and glucose or alcohol dehydrogenase, and the proteoliposomes have been shown to reproduce glucose or alcohol oxidase respiratory chain (unpublished results). Thus, cytochrome *o* of *G. suboxydans* appears to be $Q_{10}H_2$ oxidase, analogous to the *E. coli* cytochrome *o*, which has been demonstrated to be Q_8H_2 oxidase [11,21,22].

The results presented here also indicate that cytochrome *o* oxidase of *G. suboxydans* generates a $\Delta\tilde{\mu}_{H^+}$ (inside negative and alkaline) of about -140 mV when the oxidase is incorporated into proteoliposomes. This can be demonstrated by fluorometric measurement of carbocyanine and dansylglycine. In $\Delta\psi$ measurement with carbocyanine, the fluorescence quenching was stimulated with nigericin, an ionophore known to collapse ΔpH with a concomitant increase in $\Delta\psi$ [7,23], suggesting that the oxidase generates ΔpH in addition to $\Delta\psi$. Furthermore, in the measurement of ΔpH with dansylglycine, the fluorescence increase was observed only after addition of valinomycin, able to collapse $\Delta\psi$, suggesting that the oxidase generates a large $\Delta\psi$ which depresses ΔpH. Thus, it is evident that the $\Delta\tilde{\mu}_{H^+}$ generated with the cytochrome *o* oxidase consists of both components, $\Delta\psi$ and ΔpH. As shown previously [7,11], cytochrome *o* oxidase of *E. coli* generates $\Delta\tilde{\mu}_{H^+}$ by catalyzing the scalar release of protons from ubiquinol on the outer surface of the membrane, vectorial transfer of electrons from the outer to the inner surface, and scalar utilization of protons on the inner surface to reduce oxygen. It is possible that the cytochrome *o* oxidase of *G. suboxydans* also generates $\Delta\tilde{\mu}_{H^+}$ by the same 'half-loop' mechanism as mentioned above, although an experiment to demonstrate this has not

been done. In fact, with intact cells of *G. suboxydans,* oxidation of several respiratory substrates yields H^+/O ratio close to 2 (unpublished). Since cytochrome *o* oxidase of *G. suboxydans* oxidizes ubiquinol as shown, an H^+/O ratio of 2 is expected by the 'half-loop' mechanism of the oxidase [7].

Thus, it seems reasonable to conclude that the cytochrome *o*-type oxidase of *G. suboxydans* is an ubiquinol oxidase functioning to generate $\Delta\tilde{\mu}_{H^+}$.

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