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Properties of the Two Terminal Oxidases of *Escherichia coli*†

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ABSTRACT: Proton translocation coupled to oxidation of ubiquinol by O_2 was studied in spheroplasts of two mutant strains of *Escherichia coli*, one of which expresses cytochrome *d*, but not cytochrome *bo*, and the other expressing only the latter. O_2 pulse experiments revealed that cytochrome *d* catalyzes separation of the protons and electrons of ubiquinol oxidation but is not a proton pump. In contrast, cytochrome *bo* functions as a proton pump in addition to separating the charges of quinol oxidation. *E. coli* membranes and isolated cytochrome *bo* lack the Cu_A center typical of cytochrome *c* oxidase, and the isolated enzyme contains only 1Cu/2Fe. Optical spectra indicate that high-spin heme *o* contributes <10% to the reduced minus oxidized 560-nm band of the enzyme. Pyridine hemochrome spectra suggest that the hemes of cytochrome *bo* are not protohemes. Proteoliposomes with cytochrome *bo* exhibited good respiratory control, but H^+/e^- during quinol oxidation was only 0.3-0.7. This was attributed to an "inside out" orientation of a significant fraction of the enzyme. Possible metabolic benefits of expressing both cytochromes *bo* and *d* in *E. coli* are discussed.

The branched respiratory chain of *Escherichia coli* contains two different terminal oxidases, viz., cytochrome *bo* and cytochrome *d* [see Anraku and Gennis (1987) for a review]. These two ubiquinol oxidases are expressed differently in different growth conditions. Mutants able to express only one of the two nevertheless appear to be capable of normal growth (Au et al., 1985).

Both enzymes are located in the plasma membrane where their activity generates an electrochemical gradient of protons, which can subsequently be used to drive synthesis of ATP and transport of nutrients (Anraku, 1988).

Both oxidases of *E. coli* have been isolated, purified, and characterized (Kita et al., 1984ab; Matsushita et al., 1984; Miller & Gennis, 1983). Cytochrome *bo* contains two cytochrome *b* type heme groups, suggested to be protohemes, and has been reported to contain two copper ions (Kita et al., 1984a). The protein, as deduced from its gene structure in *E. coli*, strongly resembles that of cytochrome aa_3 (Saraste et al., 1988; Chepuri et al., 1990). The carbon monoxide action spectrum of cytochrome *bo* (Castor & Chance, 1955) showed that one of the two hemes (here called *o*) reacts with CO and

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with O_2. Moreover, it was recently shown that cytochrome *bo* has a bimetallic heme–copper center analogous to that of mitochondrial cytochrome *c* oxidase (Salerno et al., 1990). We show here that cytochrome *bo* lacks the Cu_A center, typical of cytochrome *c* oxidase.

Both terminal oxidases of *E. coli* have been reconstituted into proteoliposomes and were reported to generate a membrane potential, but neither was found to pump protons (Carter & Gennis, 1985; Matsushita & Kaback, 1986; Miller & Gennis, 1985; Koland et al., 1984). This and other findings led to the previously generally accepted conclusion that both oxidases of *E. coli* simply function vectorially to separate the protons and electrons of quinol oxidation ($H^+/e^- = 1$) without true proton pumping. In some other bacteria, as well as in mitochondria, ubiquinol oxidation is catalyzed by the proton-translocating cytochrome bc_1 and aa_3 complexes, and by cytochrome *c*, and the H^+/e^- ratio of overall proton translocation is near 3.0 [see Wikström et al. (1984)]. Thus, oxidation of ubiquinol in *E. coli* would be energetically far inferior to this, and independent of the terminal oxidase utilized.

Puustinen et al. (1989) showed recently, however, that cytochrome *bo* of both *Paracoccus denitrificans* and *E. coli* in fact functions as a proton pump in spheroplasts ($H^+/e^- = 2$). This is interesting in view of the structural similarity to cytochrome aa_3. Cytochrome *d* is, however, considerably different (Anraku & Gennis, 1987). Therefore, it was of interest to clarify whether the latter might function as a proton pump in cells.

We further report results with cytochrome *bo* containing proteoliposomes, which provide a clue to the failure to demonstrate proton translocation by this enzyme in the reconstituted system. New spectroscopic and metal analysis data are also reported on isolated cytochrome *bo*, which help to better define the redox centers as well as their nature.

Materials and Methods

The *E. coli* strains used for spheroplast preparations were GV-102 (cyd^+cyo^-, Oden et al., 1990) and GO-103 (cyd^-cyo^+). They were grown aerobically in succinate-containing medium, and spheroplasts were prepared as before (Puustinen et al., 1989).

Cytochrome *bo* was isolated and purified from the *E. coli* strain RG145 (cyd^-, Au & Gennis, 1987), which overexpresses this cytochrome complex. The bacteria were grown aerobically at 37 °C in a 400-L fermentor using 200 L of medium described elsewhere (Georgiou et al., 1988). Cells were harvested in the late-exponential phase of growth. Alkaline-washed membranes were prepared and extracted with dodecyl maltoside according to Berry and Trumpower (1985). The resultant supernatant was applied to a column of DEAE-Sepharose CL-6B. The column was equilibrated and washed with 0.1% (w/v) dodecyl maltoside, 50 mM potassium phosphate, and 50 mM NaCl, pH 7.5. It was then eluted with a linear gradient of 50–200 mM NaCl in 50 mM potassium phosphate and 0.1% (w/v) dodecyl maltoside, pH 7.5. Combined cytochrome *bo* containing fractions were concentrated by pressure filtration (Amicon YM 10 membrane) and stored in 0.5-mL aliquots in liquid nitrogen.

Polyacrylamide gel electrophoresis of the enzyme preparation in the presence of sodium dodecyl sulfate (not shown) revealed that it was of comparable purity to previously published preparations (Kita et al., 1984a; Matsushita et al., 1984; Georgiou et al., 1988).

For incorporation of cytochrome *bo* into proteoliposomes, sucrose gradient centrifugation of the enzyme was performed using a 5–25% (w/v) sucrose gradient in the presence of 2% (w/v) potassium cholate, as described by Finel and Wikström (1986). After centrifugation, a reddish band in the middle of the gradient was carefully collected and passed through a Sephadex G-50 "centricolumn" (Penefsky, 1977) to change the buffer to 100 mM K-Hepes, pH 7.4 with 2% potassium cholate. Then the enzyme was concentrated to 7.7 μM with a Centricon 30 tube (Amicon).

Proteoliposomes were prepared by mixing equal volumes of 7.7 μM cytochrome *bo* and a sonicated suspension containing 95 mg/mL purified soybean lecitin, 5 mg/mL *E. coli* cardiolipin (Sigma), 2% potassium cholate, and 100 mM K-Hepes, pH 7.4, and dialyzed as described before (Finel & Wikström, 1986). Because the respiratory control ratio (RCR) was low (approximately 2–3) after dialysis, the vesicles were loaded on 10% (w/v) sucrose, 100 mM KCl, and 0.5 mM K-Hepes, pH 7.4. After centrifugation for 2 h at 200000*g*, 0.5-mL fractions were collected from the top of the tubes, and the RCR was determined from each by measuring quinol oxidation spectrophotometrically. Two to three fractions from the top of the tube exhibited reasonable RCR values between 3.5 and 6.

Respiration of spheroplasts was measured polarographically with a Clark-type electrode in a closed stirred glass vessel of 1.6-mL volume at 25 °C. The reaction medium contained 50 mM KCl and 50 mM K-Hepes, pH 7.2. Quinol oxidation of proteoliposomes was measured spectrophotometrically in a medium containing 0.17 mM reduced UQ-1, 50 mM KCl, and 50 mM K-Hepes, pH 7.4, in a 1-cm light-path quartz cuvette at 325–275 nm at room temperature [see Redfearn (1967)]. Valinomycin (200 ng/mL) and nigericin (20 ng/mL) were added to measure the uncoupled rate of quinol oxidation.

Proton translocation in spheroplasts and proteoliposomes was assayed essentially as described previously (Puustinen et al., 1989; Wikström & Penttilä, 1982). One hundred microliters of spheroplasts were added to 3 mL of medium containing 100 mM KCl, 100 mM KSCN, 100 mM sucrose, 3 mM $MgCl_2$, and 0.5 mM K-Hepes pH 7.4, at 25 °C. The suspension was supplemented with 8 μmol of dithiothreitol, 1 μmol of ubiquinone-5 (UQ-1), and 5 μg of valinomycin. When indicated, 100 ng of nigericin was added. With proteoliposomes, the reaction medium was 1.2 mL of 100 mM KCl and 0.5 mM K-Hepes, pH 7.4, supplemented with 0.1 μmol of UQ-1, 4 μmol of dithiothreitol, and 2.5 μg of valinomycin, plus 0.4 mL of proteoliposomes (approximately 125 pmol of cytochrome *bo*).

Reduction kinetics of the *b*-type cytochromes of cytochrome *bo* were measured at 427–460 nm at 25 °C with a DBS-1 dual-wavelength spectrophotometer (Johnson Research Foundation Workshops, University of Pennsylvania, Philadelphia, PA). All other spectrophotometric measurements were performed with a Shimadzu UV-3000 instrument. The wavelength scale of the latter was calibrated by using a didymium filter provided by the manufacturer.

The heme content of isolated cytochrome *bo* was measured from the dithionite-reduced minus oxidized absorbance change of the pyridine hemochrome (Berry & Trumpower, 1987), using the recommended millimolar absorptivity of 24 cm^{-1}, but at 553 minus 535 nm (see Results). The copper and iron contents of isolated cytochrome *bo* were determined by graphite oven atomic absorption spectroscopy of a purified enzyme preparation that had been dialyzed against 5 mM EDTA.

EPR spectroscopy was performed with a Bruker ESP 300 X-band spectrometer equipped with an Oxford Instruments ESR 900 liquid helium cryostat. The instrumental conditions were as follows: modulation frequency, 100 kHz; modulation

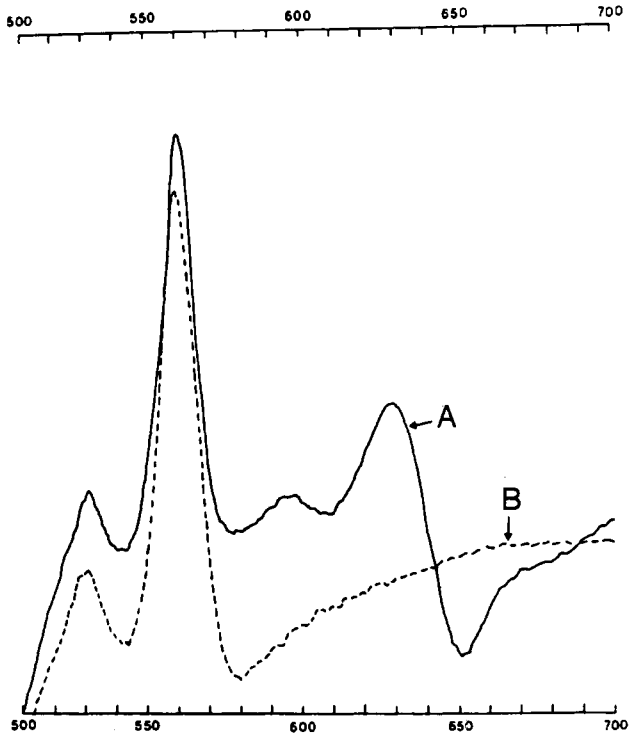

FIGURE 1: Visible reduced minus oxidized absorption spectra of membranes from GV-102 (A) and GO-103 (B) mutants of *E. coli*.

amplitude, 19.8 G; time constant, 41 ms; scanning rate, 11.9 G/s. The microwave frequency was 9.44 GHz, microwave power 10 mW, and sample temperature 12 K.

Ubiquinone-5 (UQ-1) was a generous gift from Hoffmann-La Roche Ltd., Basel, Switzerland. The ubiquinol was prepared by reduction with dithionite according to Rieske (1967). All other reagents were of the highest purity available commercially.

RESULTS

Figure 1 shows optical reduced minus oxidized spectra of membranes from the strains GV-102 and GO-103, which verify that the former expresses only cytochrome *d* (Oden et al., 1990) and the latter only cytochrome *bo*. The isolated spheroplasts from these strains exhibited negligible endogenous respiration. This ascertains that measured proton translocation is indeed associated only with oxidation of the added ubiquinol. Oxygen consumption was initiated in both strains with a combination of 0.13 mM UQ-1 and 2.5 mM dithiothreitol. As expected, the respiration was strongly inhibited by low concentrations of cyanide in the cytochrome *bo* containing GO-103 spheroplasts but was much less sensitive to cyanide for the case of cytochrome *d* (not shown).

Figure 2 (panels A and B) shows the pH responses of anaerobic spheroplasts on addition of aliquots of O_2. The trace for GO-103 (Figure 2B) shows that 1.8 H^+/e^- are initially released before relaxation. In contrast, the cytochrome *d* containing spheroplasts (GV-102; Figure 1A) exhibited H^+/e^- = 1.

In the literature, there has been some uncertainty as to the contribution of the two *b*-type hemes to the optical reduced minus oxidized spectrum (see Discussion). Figure 3 shows a spectral analysis of isolated cytochrome *bo*. Addition of cyanide to the enzyme (as a control, see below) caused little change in the α-band (not shown) at this enzyme concentration but caused a shift in the Soret band with a minimum at 400 nm and a maximum at 418 nm (Figure 3, trace C). This is consistent with cyanide binding to a high-spin ferric heme *o*

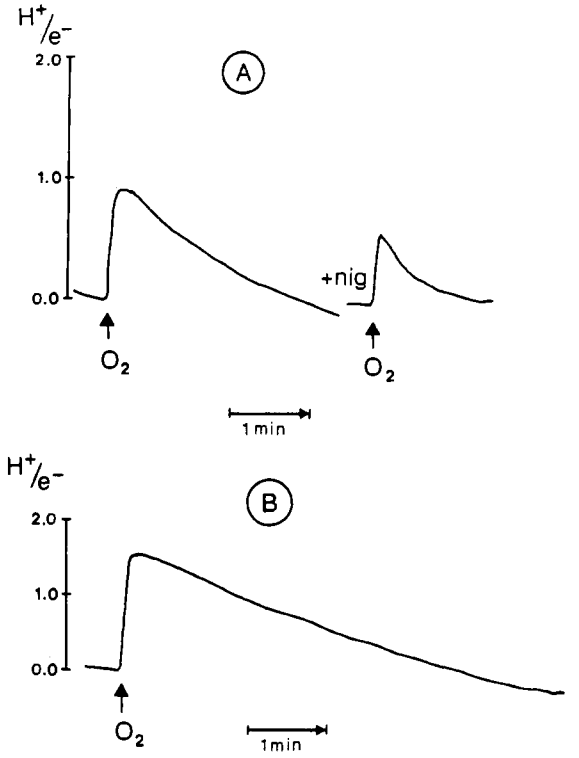

FIGURE 2: Proton translocation in spheroplasts from GV-102 [cyd^+,cyo^- (A)] and GO-103 [cyd^-cyo^+ (B)] mutants of *E. coli*. For conditions, see Materials and Methods. O_2 was added as air-saturated water (25 °C, 1 atm); 5.16 and 2.58 nmol of O_2 in (A) and (B), respectively. nig, 100 ng of nigericin.

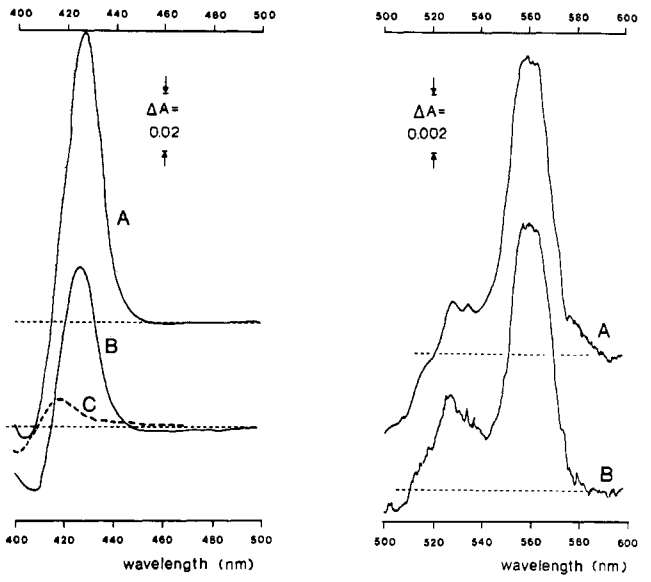

FIGURE 3: Optical spectra of isolated cytochrome *bo* at room temperature. 0.37 μM enzyme was suspended in 100 mM Tris-HCl and 0.01% (w/v) dodecyl maltoside, pH 7.4. Trace A shows the anaerobic fully reduced (with 0.5 mM TMPD plus 5 mM ascorbate) minus fully oxidized difference spectrum. Trace B shows the difference spectrum of aerobic reduced enzyme (ascorbate plus TMPD) in the presence of cyanide (1 mM) minus cyanide-treated enzyme. Trace C shows the difference spectrum in the Soret band of oxidized enzyme treated with 1 mM KCN minus oxidized enzyme.

component, resulting in a high- to low-spin transition.

Figure 3A,B shows reduced minus oxidized difference spectra. In Figure 3A, the enzyme was reduced anaerobically with TMPD + ascorbate (the same result was obtained with reduced UQ-1, not shown). In Figure 3B, it was reduced aerobically in the presence of cyanide, conditions under which

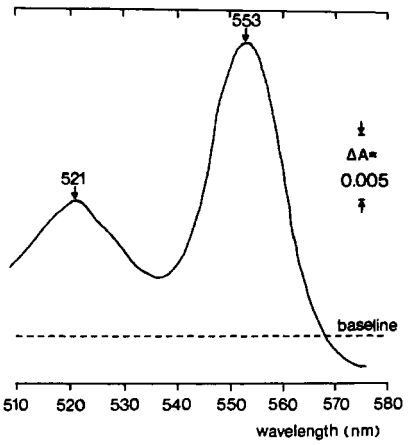

FIGURE 4: Reduced minus oxidized pyridine hemochrome spectrum of cytochrome *bo*. Fifteen microliters of isolated cytochrome *bo* was suspended in 2.3 mL of 50 mM NaOH containing 20% (v/v) pyridine. Five microliters of 0.2 M potassium ferricyanide was added and the base line recorded. A few grains of dithionite were added, and the spectrum was recorded when the spectral change was complete (see Materials and Methods).

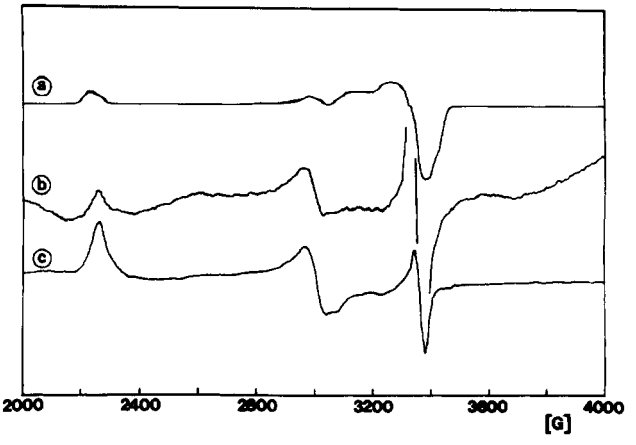

FIGURE 5: EPR spectra of (a) approximately 0.12 mM bovine heart cytochrome aa_3, (b) bacterial membranes of *E. coli* RG145 (cyd$^-$), corresponding to 29 μM cytochrome *bo*, and (c) 0.135 mM isolated cytochrome *bo*, both in 50 mM K-Hepes–2 mM EDTA, pH 7.4. Note that the gain is different in the different spectra. For EPR conditions, see Materials and Methods.

the cyanoheme *o* remains oxidized. The extent of the Soret band is decreased by the presence of cyanide and air to approximately 57% of fully reduced minus oxidized. In contrast, the α-band is decreased by less than 10%. This finding is very similar to that with cytochrome aa_3, where the contribution of the high-spin heme a_3 to the reduced minus oxidized α-band is minimal, as compared to its 50% contribution to the Soret band [see Wikström et al. (1976, 1981)].

The activity of the purified enzyme with reduced UQ-1 was similar to that reported previously [see, e.g., Kita et al. (1984a)]. The iron and copper contents were determined by atomic absorption after dialysis against 5 mM EDTA to remove adventitious heavy metals. The Cu/Fe ratio was found to be 0.50 after correction for the Fe and Cu contents of the suspending medium. Thus, it seems clear that the enzyme contains only one copper in addition to the two hemes. We ascribe the Cu/Fe ratio of 0.75 reported by Kita et al. (1984a) to adventitious copper in the preparation. The reduced minus oxidized $\epsilon_{560-580}$ for the cytochrome *bo* unit was found to be about 21–22 mM^{-1} cm^{-1}, based on the copper content, and 24.2 mM^{-1} cm^{-1}, based on the pyridine hemochrome measurement. Kita et al. (1984a) reported a value of 18.7 mM^{-1} cm^{-1} based on pyridine hemochrome. In part, this difference may be due to different millimolar absorptivities in the pyridine hemochrome measurement (and see below).

Figure 4 shows the reduced minus oxidized pyridine hemochrome spectrum of the isolated enzyme, assayed as described by Berry and Trumpower (1987). An identical spectrum was obtained after initial extraction of the heme prior to pyridine treatment (Falk, 1964). The spectrum is not typical for protoheme, which has α- and β-peaks at 556.5 and 526 nm, respectively (Falk, 1964; Berry & Trumpower, 1987), as we also confirmed using myoglobin as an internal standard. The single pyridine hemochrome of cytochrome *bo* has peaks at 553 and 521 nm, respectively, in the difference spectrum (Figure 4). This has not been previously reported and indicates a difference in the side chains of the porphyrin ring from those of protohemes (see Discussion). Distribution of the isolated hemes from myoglobin, *Paracoccus* cytochrome *c* oxidase, and cytochrome *bo* between pyridine–ether and aqueous hydrochloric acid–pyridine (Lemberg & Benson, 1959) also showed that the hemes of cytochrome *bo* behaved differently from the protoheme of myoglobin. The *bo* hemes exhibited more hydrophobic character than protoheme.

Due to the high degree of similarity between primary protein structures of cytochromes *bo* and aa_3, it was of interest to determine whether the Cu_A center typical of the latter is present in cytochrome *bo*. Figure 5 (traces b and c) shows the g_z and g_y resonances of one of the heme irons [cf. Hata et al. (1985)], but in the $g = 2.0$ region (near 3400 G), there is only a free radical signal both in *E. coli* membranes (trace b) and in the isolated enzyme (trace c), in both cases without any indication of the signal typical for Cu_A. The oxidized state of the low-spin heme iron ensures also that Cu_A should have been oxidized and EPR-visible, if present. The broad resonance near $g = 2.0$ reported by Hata et al. (1985) at 1-mW microwave power and 11 K, and ascribed to Cu^{2+}, was not found in our EPR conditions, which were optimized for detection of Cu_A. Figure 5 (trace a) shows, for comparison and in the same EPR conditions, the spectrum of Cu_A together with the low-spin heme iron signals of ferricytochrome *a*, for a cytochrome *c* oxidase preparation from bovine heart. We conclude that cytochrome *bo* contains two hemes, but not Cu_A. The copper ion indicated by the metal analysis is EPR-invisible in the oxidized enzyme, presumably due to spin coupling to heme iron in a bimetallic oxygen reduction center (Salerno et al., 1990).

Purified cytochrome *bo* was reconstituted into proteoliposomes by the dialysis procedure (see Materials and Methods). The total vesicle population had a poor respiratory control ratio (RCR), as also reported previously (Matsushita & Kaback, 1986). However, after centrifugation of the vesicles into 10% (w/v) sucrose, the topmost fractions showed respiratory control ratios between 3.5 and 6, indicating a reasonable degree of membrane reconstitution (Figure 6A). Nevertheless, the observed H^+/e^- ratios were only of the order of 0.3–0.7 in different preparations (Figure 6B). This is in great contrast to the result with spheroplasts [Figure 2; cf. Puustinen et al. (1989)] and is much less than expected even from pure charge separation during ubiquinol oxidation.

It occurred to us that reconstitution might result in a fraction of the enzyme being orientated "inside out", so that proton release and possible proton pumping take place toward the inside of the membrane, and proton uptake from the outside. However, several attempts failed to physically separate such vesicle fractions by gel filtration or ion-exchange chromatography. We also tried to separate the two enzyme orien-

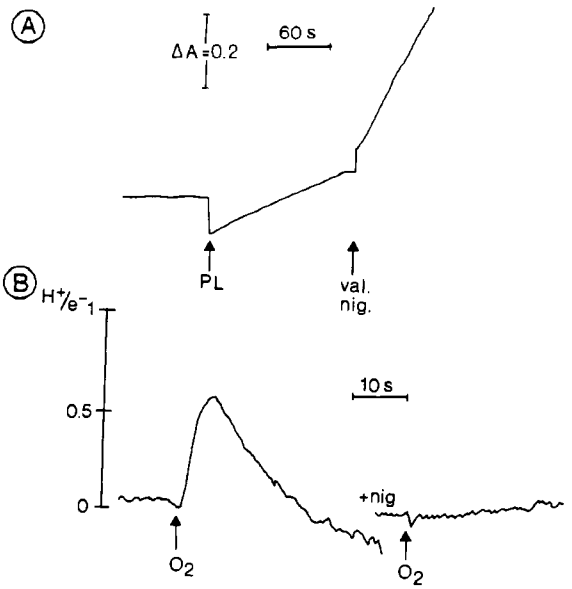

FIGURE 6: Respiratory control and proton translocation in proteoliposomes containing cytochrome *bo*. (A) Measurement of quinol oxidation at 325–275 nm. PL, proteoliposomes; val., valinomycin; nig., nigericin. For conditions, see Materials and Methods. (B) Injection of 0.258 nmol of O_2 in the absence (left) and presence (right) of 100 ng of nigericin.

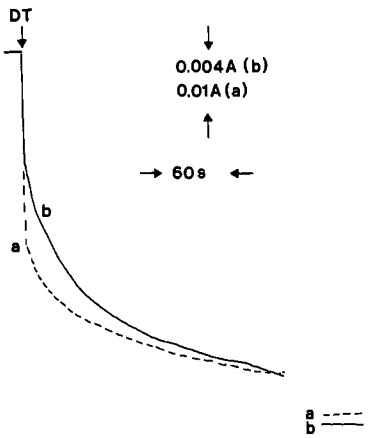

FIGURE 7: Reduction kinetics of the hemes by dithionite in solubilized cytochrome *bo* and *bo*-containing proteoliposomes. Medium: 50 mM KCl–50 mM K-Hepes, pH 7.4. A few grains of solid dithionite were dissolved in this medium to make a stock solution. The reaction was started by addition of 20 μL of this dithionite solution (DT) to a final volume of 1.2 mL, containing either 5 μL of cytochrome *bo* (trace a) or 0.5 mL of proteoliposomes (trace b). Wavelength pair, 427–460 nm; temperature, 25 °C. In both traces, there is a fast increase of the absorbance difference (downward), which occurs within the stirring time, followed by a slow phase. The final equilibrium deflections are also shown. For further details, see Materials and Methods.

tations functionally, by selectively inhibiting the activity of one population only. $ZnCl_2$, protease, and antibody treatment of the proteoliposomes all failed to achieve such selectivity.

With solubilized cytochrome *c* oxidase, it is well-known that the two hemes are reduced by dithionite with widely different kinetics [see, e.g., Brunori et al. (1985)]. Heme *a* is reduced quickly, and heme a_3 much more slowly. As shown in Figure 7 (trace a), the reduction of isolated solubilized cytochrome *bo* is also biphasic when measured in the Soret band. The fast phase, the extent of which is slightly larger than half of the total change (cf. Figure 3), occurs within the stirring time. The slow phase is much slower, making distinction easy between the phases. By analogy to cytochrome aa_3, we may attribute these kinetics to fast reduction of one of the heme groups (heme *b*), which like heme *a* of cytochrome *c* oxidase is located near the outside of the membrane in the physiological orientation. The other heme (heme *o*), which corresponds to heme a_3, is reduced much more slowly. These assumptions are validated by the strong homology between primary structures of the heme binding subunits I of cytochromes aa_3 and *bo* (Saraste et al., 1988; Chepuri et al., 1990), and by the spectral analysis in Figure 3.

Table I: Proton Translocation and Orientation of Cytochrome *bo* in Proteoliposomes[a]

prepn	$(H^+/e^-)_{obs}$	*f* predicted		*f* found
		no pump	pump	
1	0.3	0.7	0.6	0.57
2	0.5	0.8	0.6	0.60

[a] Two vesicle preparations were analyzed for proton translocation and for kinetics of reduction by dithionite. *f* is the fraction of enzyme correctly oriented "outside out"; the found values are determined by dithionite reduction, as described in the text (see Figure 7). The predicted values for *f* are calculated from eq 1 and 2 on the basis of the observed proton translocation ratio, and for a proton-pumping and a nonpumping enzyme, respectively (see the text).

In enzyme molecules orientated "inside out", the fast-reacting heme *b* is expected to be buried inside the vesicles whereby it becomes unable to react quickly with the membrane-impermeable reductant. Figure 7 (trace b) shows that the fast reduction phase is indeed much diminished in extent in proteoliposomes, as compared with the solubilized enzyme. The decreased extent of the fast phase may thus report the degree by which the enzyme is orientated "inside out".

The following holds ideally for a proton-pumping cytochrome *bo*:

$$(H^+/e^-)_{obs} = 4f - 2 \quad (1)$$

where the observed H^+/e^- ratio is a simple function of the fraction *f* of enzyme in the correct "outside out" orientation, as in the bacterial plasma membrane.

For a nonpumping enzyme that nevertheless separates the charges of quinol oxidation, the corresponding relationship is

$$(H^+/e^-)_{obs} = 2f - 1 \quad (2)$$

Table I presents the experimentally observed *f* values for two proteoliposome preparations using the described dithionite reduction technique. These values may be compared with the *f* values predicted from the observed H^+/e^- ratios, on the basis of eq 1 and 2, respectively. The measured enzyme orientation and proton translocation data fit best with the notion that cytochrome *bo* is a proton pump also in proteoliposomes. However, this still needs to be more definitely demonstrated.

Discussion

We have established here that the two terminal quinol oxidases of *E. coli*, cytochrome *bo* and cytochrome *d*, have different energy conservation efficiencies. Cytochrome *bo* functions as a proton pump exhibiting a H^+/e^- ratio near 2.0 [cf. Puustinen et al. (1989)], while the structurally unrelated cytochrome *d* only catalyzes the separation of the protonic and electronic charges during quinol oxidation, yielding an H^+/e^- ratio of 1.0 [cf. Miller and Gennis (1985)].

Experiments to date with liposomes reconstituted with cytohrome *bo* have failed to show proton pumping. This may have been due to low respiratory control ratios and undetermined orientation of the enzyme (Matsushita & Kaback, 1986). The proteoliposomes of this work were well coupled but yet exhibited H^+/e^- ratios even lower than expected from

charge separation alone. The reason for this is probably a dual orientation of the enzyme in the membrane. A similar phenomenon has been described by cytochrome *c* oxidase proteoliposomes (Casey, 1984), and for proteoliposomes containing the cytochrome *d* complex (Miller & Gennis, 1985). With cytochrome *c* oxidase one of the substrates, ferrocytochrome *c*, is membrane-impermeable, supporting activity only in the "outside out" enzyme population. In contrast, the substrates of cytochrome *bo* (ubiquinol and O_2) are both easily soluble in the membrane, which results in full activity of enzyme molecules of both orientations.

The observed respiratory control in the present cytochrome *bo* proteoliposomes implies that individual liposomes mostly contain equally oriented enzyme molecules, or, more likely, each proteoliposome may contain only one enzyme molecule on average [see Casey (1984)]. Our results are consistent with proton pumping in the reconstituted enzyme when correction is made for "inside out" oriented molecules, but further work is required to prove this more definitely.

The α-band of reduced minus oxidized cytochrome *bo*, which is split at 77 K, has usually been ascribed to equal contributions from the two hemes *b* and *o* [see, e.g., Kita et al. (1984a) and Salerno et al. (1989)]. However, the extent of the reduced minus oxidized 560-nm α-band is decreased only by <10% in conditions where the low-spin heme *b* component is reduced, while the heme *o* component remains oxidized and liganded to cyanide (Figure 3). Thus, the spectral contribution of reduced minus oxidized heme *o* is very small in the α-band (<10%), but of the order of 40–50% in the Soret band. This is highly reminiscent of cytochrome aa_3 (Wikström et al., 1976, 1981) and is expected from the reduced minus oxidized spectrum of a high-spin heme. Moreover, the reduced minus oxidized $\epsilon_{560-580}$ of the order of 20 mM^{-1} cm^{-1} (see Results) is typical for a low-spin *b*-type heme. Since this value is based on the content of cytochrome *bo* units (Kita et al., 1984a; this paper), this also indicates that only one of the two hemes contributes significantly to this band. The splitting of the α-band is then due either to asymmetry of the optical transition of the low-spin heme *b* and/or to interactions of this heme with the binuclear center. Further work is required to resolve this question.

Our pyridine hemochrome spectra of isolated cytochrome *bo* are surprising in view of the generally accepted view that the enzyme contains two protohemes. Although the hemes in situ are spectrally of cytochrome *b* type, the 3–4-nm blue shift in the pyridine hemochrome spectrum strongly suggests modification of substituents on the porphyrin ring from those of protoheme. Comparison to spectra of several pyridine hemochromes (Falk, 1964) reveals that modification of the vinyl group in position 2 (or 4) of protoheme to hydroxyethyl would, for example, cause such a blue shift. An intriguing possibility presently under investigation is that the hemes of cytochrome *bo* might have a hydroxyalkyl chain in position 2, as in heme A, and that this may be another common feature of *bo*- and aa_3-type oxidases. The more hydrophobic character of the *bo* hemes as compared to protoheme observed here supports this possibility.

Our EPR data show that cytochrome *bo* does not contain the Cu_A center typical of cytochrome *c* oxidase. This is supported by the finding that the isolated enzyme contains only one Cu per oxidase molecule. This is most probably the Cu_B-like copper of the bimetallic oxygen reduction center, which is magnetically coupled to heme iron (Salerno et al., 1990). Cu_A has been suggested to be bound to a specific site in subunit II of cytochrome *c* oxidase, in which two conserved cysteines and one histidine were suggested to be ligands to the copper (Stevens et al., 1982; Martin et al., 1988; Wikström et al., 1985; Holm et al., 1987). The corresponding subunit of cytochrome *bo* is clearly related to subunit II of cytochrome aa_3 although sequence identity is scarce (Saraste et al. 1988; Chepuri et al., 1990). However, the putative ligands of Cu_A are conspicuously lacking. This picture, together with our finding that cytochrome *bo* indeed lacks the Cu_A center, strongly supports the proposed location of Cu_A in subunit II. However, it stands in contrast to the claim by the Azzi group [see Azzi and Müller (1990)] that the Cu_A center is in subunit I.

It is interesting that the protein and prosthetic group homologies between cytochromes *bo* and aa_3 extend to the functional homology of proton pumping. There is good reason, then, to assume that also the proton-pumping mechanisms are homologous. Therefore, mechanisms directly involving Cu_A (Gelles et al., 1987), or a hydrogen bond to the formyl group of heme *a* (Babcock & Callahan, 1983), seem unlikely. Recent experimental data have suggested that the binuclear dioxygen reduction center, which is analogous in the two enzymes, may be involved in proton pumping (Wikström, 1989). However, the strong anticooperativity between the redox centers in both enzymes (Salerno et al., 1990; Wikström et al., 1981) may indicate a more global mechanism, not necessarily confined to a single site.

The two ubiquinol oxidases of *E. coli* catalyze the same redox reaction, but differ by a factor of 2 in their energy-coupling efficiency. By controlling the relative expression of the two enzymes, the bacteria can switch between states of low and high energy conservation efficiency of ubiquinol oxidation. The very high oxygen affinity of cytochrome *d* as compared to *bo* (Kita et al., 1984b) enables cells to sustain aerobic metabolic flux at extremely low oxygen pressures.

Efficient coupling to energy conservation may have adverse kinetic consequences for operation at very low pO_2. At a high energy state of the cell, the apparent K_M for O_2 is raised considerably in mitochondrial respiration [see Petersen et al. (1974)]. This effect can be attributed to a lowered steady-state occupancy of the O_2 binding fully reduced state of the binuclear center. The probable reason for this is that the reactions in the catalytic O_2 reduction cycle leading to this state are slowed down at high protonmotive force due to their linkage to proton translocation [see Wikström (1989)]. Such an effect is expected to be less prominent the less the reaction is coupled to proton translocation. This might be one rationale for the existence of cytochrome *d*. Its low energy coupling efficiency combined with its high oxygen affinity may make it ideally adapted for aerobic life at very low oxygen pressures.

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Registry No. H, 12408-02-5; Cu, 7440-50-8; Fe, 7439-89-6; cytochrome *d*, 9035-36-3; ubiquinol 5, 52590-98-4; oxidase, 9035-73-8.

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