Na^+-Translocating Cytochrome *bo* Terminal Oxidase from *Vitreoscilla*: Some Parameters of Its Na^+ Pumping and Orientation in Synthetic Vesicles†

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ABSTRACT: *Vitreoscilla* cytochrome *bo* ubiquinol oxidase is similar in some properties to the *Escherichia coli* enzyme, but unlike the latter, the *Vitreoscilla* oxidase functions as a primary Na^+ pump. When purified *Vitreoscilla* cytochrome *bo* is incorporated into liposomes made from *Vitreoscilla* phospholipids and energized with a quinol substrate, it translocates Na^+, not H^+, across the vesicle membrane. Since protonophores CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and DTHB (3,5-di-*tert*-butyl-4-hydroxybenzaldehyde) stimulated the Na^+ pumping, it is unlikely that it is a secondary effect due to the presence of Na^+/H^+ antiporter activity in the preparations. The efficiency of the Na^+ pumping was 3.93 Na^+ pumped per O_2 consumed when ascorbate/TMPD was used as the substrate. The cytochrome has a K_m and k_{cat} for Na^+ of 2.9 mM and 277 s^{-1}, respectively. When ferricytochrome *c* was entrapped within liposomes prepared from *Vitreoscilla* phospholipids, it was reduced by Q_1H_2 (ubiquinol-1) but not by ascorbate/TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine). Although Q_1H_2 was oxidized by cytochrome *bo* in solution at a rate approximately 14 times that of the latter substrate, the rate of accumulation of Na^+ within cytochrome *bo* vesicles driven by the membrane impermeable ascorbate/TMPD was 1.23 times that of the membrane permeable ubiquinol. These data allowed a calculation that in these synthetic proteoliposomes the cytochrome *bo* molecules are only 51% directed inward; a value of 61% inward-directed was estimated by measuring the ascorbate/TMPD oxidase activity of the proteoliposomes before and after disrupting them with Triton X-100. A random orientation of the *E. coli* cytochrome *bo* oxidase in proteoliposomes has also been reported.

In most aerobic bacteria, the free energy change of respiration is conserved in the form of a H^+ electrochemical gradient ($\Delta\mu_{H^+}$) which is generated by scalar and vectorial H^+-translocating activities of the respiratory enzymes. The $\Delta\mu_{H^+}$ is used to drive ATP synthesis or other endergonic processes. Na^+ can also serve as the energy-transducing ion; for example, *Propionigenium modestum* employs a Na^+-transporting decarboxylase to generate a Na^+ electrochemical gradient ($\Delta\mu_{Na^+}$) which in turn drives ATP synthesis via a Na^+-motive F_oF_1 ATPase (Dimroth, 1987; Hilpert et al., 1984). Some marine and moderately halophilic aerobic bacteria have Na^+-pumping respiratory chains. For example, *Vibrio alginolyticus* couples the free energy of respiration to Na^+ pumping via a Na^+-transporting NADH:quinone oxidoreductase and uses the resulting $\Delta\mu_{Na^+}$ to drive nutrient uptake (Tokuda & Unemoto, 1982, 1984; Unemoto et al., 1990) and ATP synthesis (Dibrov et al., 1986, 1989). *Vitreoscilla* is also an aerobic bacterium that is Na^+-motive even though it is neither marine nor halophilic (Efiok & Webster, 1990a, 1992). The cytochrome *bo* terminal oxidase from *Vitreoscilla* is both structurally and functionally homologous to the *Escherichia coli* cytochrome *bo* (Georgiou et al., 1988). One significant difference, however, is that the *Vitreoscilla* enzyme pumps Na^+ instead of H^+ during terminal oxidation, which was shown using liposomes into which the purified cytochrome *bo* had been incorporated (Efiok & Webster, 1990b). The latter study used liposomes prepared from *E. coli* phospholipids. We report here a continuation of those preliminary studies using liposomes prepared from *Vitreoscilla* phospholipids which show improved pumping rates. Orientation of the protein in these membranes and other parameters of the system were also investigated for this study.

MATERIALS AND METHODS

Vitreoscilla sp. strain C1 was grown in PY medium: 1% peptone (Fidco) and 1% yeast extract (Hy-Case SF) with the pH adjusted to 7.8 with 1 N NaOH. Each 2.8 L Fernbach flask, containing 1.5 L of PY medium, was inoculated with approximately 100 mL of a culture in log phase, and the cells were grown on a shaker at 200 rpm for 22−24 h. The cells were concentrated using an Amicon DC10L concentrator with a type H5MP01-43 Amicon Hollow Fiber Cartridge. The thick cell slurry obtained from this was centrifuged at 4 °C in a DuPont Sorvall RC-5B refrigerated centrifuge for 30 min. The harvested cells were washed with 0.01 M Tris-HCl buffer (pH 7.5), and the cell paste was stored at −20 °C.

The spheroplasts and membrane fragments were prepared using a published procedure (Georgiou & Webster, 1987) with the single modification that Tris-HCl buffer (pH 7.5), replaced the potassium phosphate buffer (pH 7.2), throughout. The membrane fragments were stored at −20 °C. Cytochrome *bo* was purified from the membrane fragments using modifications of a previous procedure (Georgiou & Webster, 1987). The Sephadex G-200 chromatography step

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was omitted and replaced by chromatography on Bio-Gel A-0.5 which was performed between the two DEAE-Sepharose CL-6B chromatographic steps described previously. Again, Tris-HCl buffers replaced potassium phosphate buffers throughout the purification procedure. The spectral properties of the purified cytochrome *bo* obtained by this modified procedure were indistinguishable from those reported earlier, but the final yield was 3 times that of the original procedure. Four bands were visible on SDS gels stained with Coomassie brilliant blue R-250 as observed previously (Geogiou et al., 1988), although a fifth band was often visible with silver staining. The heme/protein ratio was also comparable to that of the original procedure, approximately 20 nmol/mg.

Crude phospholipids were prepared from *Vitreoscilla* cells by following a published procedure (Clark & Switzer, 1977). The phospholipid was further purified according to the method of Newman and Wilson (1980) and stored in 2 mM 2-mercaptoethanol at a concentration of 50 mg/mL at −80 °C. Some experiments were done using both the crude and purified phospholipids with no notable differences in the results. The lipid content of *Vitreoscilla* has been analyzed by Nicols et al. (1986).

Proteoliposomes containing cytochrome *bo* were prepared using a protein/lipid ratio of 1/125 by a direct incorporation method that initially was used to yield proteoliposomes with cytochrome *c* oxidase predominantly in the inside-out orientation (Eytan et al., 1975) but modified for cytochrome *bo* (Matsushita et al., 1983; Efiok & Webster, 1990b). Direct incorporation reputedly results in the unidirectional incorporation of a membrane protein (Rigaud et al., 1995). Purified cytochrome *bo* (0.5 mg of protein) was mixed with 62.5 mg of sonicated *Vitreoscilla* phospholipids in 25 mM Tris-HCl (pH 7.7), and octyl glucoside was added to a final concentration of 1.25% (w/v). The mixture was incubated on ice for 20 min with slow stirring and then diluted into 30 volumes of the same Tris-HCl buffer at room temperature. After they were stirred for 10 min, the proteoliposomes were collected by centrifugation at 120000*g* for 2 h. The proteoliposomes were resuspended in the same buffer at a protein concentration of 200 μg/mL and stored at −80 °C. The incorporation of the cytochrome *bo* into the liposomes was 88%.

The substrate permeability to liposomes prepared from *Vitreoscilla* phospholipids was tested using liposomes containing entrapped ferricytochrome *c*, which were prepared as follows. *Vitreoscilla* phospholipid (50 mg) and 50 mg of cytochrome *c* in 2 mL of 25 mM Tris-HCl (pH 7.7) were sonicated at 80 W in 10 s on/20 s off cycles for 5 min at 0 °C. The phospholipid vesicles were purified by centrifugation at 35 000 rpm for 12 h at 4 °C on a continuous sucrose density gradient (33 to 44%) and collection of the red band which sedimented at about 36% sucrose.

Na^+ uptake by the proteoliposomes was measured using a published procedure (Hilpert & Dimroth, 1984) modified for *Vitreoscilla* cytochrome *bo* proteoliposomes (Efiok & Webster, 1990b). The proteoliposomes were incubated with the $^{22}Na^+$ in 1 mL reaction mixtures in 25 mM Tris-HCl buffer (pH 7.7) plus the other reaction components (substrates, protonophores, etc., as described in the figure legends) for the desired time period. Aliquots (100 μL) were then loaded onto Dowex 50W columns in the lithium form to separate free $^{22}Na^+$ from that accumulated by the proteoliposomes. The Dowex-Li^+ columns were in Pasteur pipettes, and each was eluted with 600 μL of 10 mM lithium phosphate (pH 7.7). The radioactivity of the entrapped $^{22}Na^+$ was determined with a Beckman LS1701 liquid scintillation counter using Sigma-Fluor as the cocktail solution and a 1 min counting time. Each uptake experiment was repeated three to five times.

To estimate the stoichiometry of Na^+ pumped/electron transferred, oxygen uptake was measured polarographically using ascorbate/TMPD[1] as substrate essentially as previously described (Efiok & Webster, 1990b) with a YSI model 53 oxygen monitor (Yellow Springs Instrument Co.). The proteoliposomes were preincubated at 25 °C for 1 min in 3 mL of 10 mM NaCl/22.5 mM Tris-HCl (pH 7.6), and then the reaction was started by the addition of 0.8 mM ascorbate/0.1 mM TMPD. Na^+ uptake by the proteoliposomes was measured under the same conditions using the procedure described in the previous paragraph. Initial rates were used to estimate the stoichiometry.

The leakage test used proteoliposomes preloaded with $^{22}Na^+$ by incubating them with $^{22}Na^+$ and ascorbate/TMPD substrate in the Tris-HCl buffer for 10 min, and the free $^{22}Na^+$ was removed using Dowex-Li^+ columns as described for the Na^+ uptake assay. The eluants were pooled and used immediately for the leakage test. Each experiment for this was done in triplicate, three 100 μL aliquots being removed for each time point, and the radioactivity of the entrapped ^{22}Na remaining determined as described above for the uptake procedure.

The procedures for the enzyme assays used in this study have been described previously: menadiol oxidase (Georgiou & Webster, 1987; Efiok & Webster, 1990b), ascorbate/TMPD oxidase (Efiok & Webster, 1990b), and Q_1H_2 oxidase (Georgiou et al., 1988). Q_1 for the latter assay was synthesized from Q_0 and 3-methyl-2-buten-1-ol and purified on a Florisil column.

RESULTS

A typical experiment demonstrating Na^+ pumping by proteoliposomes containing native lipids and cytochrome *bo*, both purified from *Vitreoscilla*, is shown in Figure 1. This includes controls (e.g., minus substrate, liposomes prepared without the cytochrome). In a separate experiment (data not shown), the Na^+-pumping activity was found to be maximal at pH 7.7. By varying the Na^+ concentration (Figure 2), we were able to obtain a K_m of 2.9 mM and an apparent k_{cat} of 137 s^{-1}. To estimate the stoichiometry, ascorbate/TMPD-driven Na^+-pumping and ascorbate/TMPD oxidase activity were assayed under the same conditions. For a Na^+-pumping rate of 342 nmol of Na^+ min^{-1}, the oxygen uptake rate was 87 nmol of O_2 min^{-1}, an efficiency of 3.93 Na^+ pumped per oxygen molecule reduced (average of three experiments). For Q_1H_2, an efficiency of 1.72 was determined (average of five experiments).

If Na^+/H^+ antiporter activity were present in our preparations, the observed Na^+ pumping could be a secondary effect

[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DPIP, 2,6-dichlorophenolindophenol; DTHB, 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde; PMS, phenazine methosulfate; Q_1H_2, ubiquinol-1; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine.

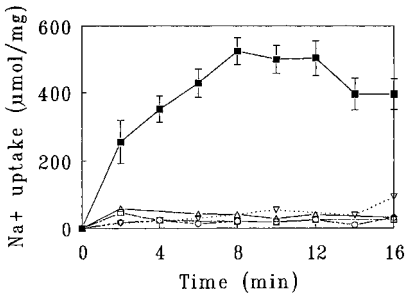

FIGURE 1: Uptake of ^{22}Na by inside-out cytochrome *bo* proteoliposomes. The ^{22}Na uptake was measured as described in Materials and Methods. The medium for the complete assay (total volume of 1 mL) contained 25 mM Tris-HCl (pH 7.7), proteoliposomes (0.38 nmol of cytochrome *bo*), 0.2 M $^{22}NaCl$ (specific activity of 2229 cpm/μmol of Na^+), and 0.8 mM ascorbate/0.1 mM TMPD: (■) complete system, (△) minus ascorbate/TMPD, (▽) minus NaCl, (○) minus cytochrome *bo*, and (□) minus cytochrome *bo* and minus ascorbate/TMPD. The standard deviation is shown for the complete system ($n = 4$). The mg on the ordinate is milligrams of purified cytochrome *bo* protein.

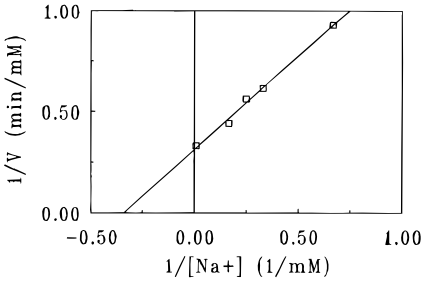

FIGURE 2: Effect of Na^+ concentration on the rate of Na^+ uptake by inside-out cytochrome *bo* proteoliposomes. The mixture for each assay (total volume of 1 mL) contained 25 mM Tris-HCl (pH 7.7), proteoliposomes (0.38 nmol of cytochrome *bo*), 1.5, 3, 4, 6, or 200 mM $^{22}NaCl$, and 0.8 mM ascorbate/0.1 mM TMPD. From the intercepts, $K_m = 2.9$ mM and $V_{max} = 3.13$ μmol/min under these conditions.

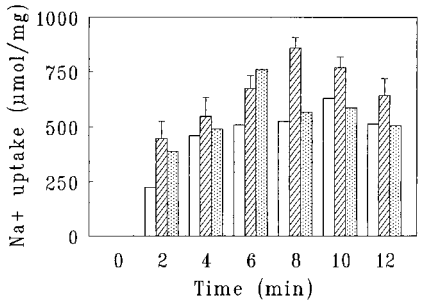

FIGURE 3: Effect of CCCP and DTHB on Na^+ uptake by inside-out cytochrome *bo* proteoliposomes. Experimental conditions were the same as described in the legend for Figure 1 except for the addition of the protonophore: plain bar, control; striped bar, + 80 μM CCCP; and stippled bar, + 80 μM DTHB. The standard deviation is shown for CCCP ($n = 3$).

of H^+ pumping, being driven by the resultant H^+ electrochemical gradient. In this case, protonophores should inhibit Na^+ pumping. In fact, the protonophores, CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and DTHB (3,5-di-*tert*-butyl-4-hydroxybenzaldehyde), both stimulated Na^+ translocation (Figure 3). This result is consistent with *Vitreoscilla* cytochrome *bo* being a primary Na^+ pump but not with the Na^+ translocation being driven by a Na^+/H^+ antiporter associated with the cytochrome.

The rate of leakage of Na^+ across the membranes was about 3%/min when it was tested using vesicles preloaded

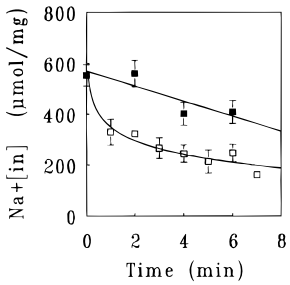

FIGURE 4: Leakage of cytochrome *bo* proteoliposomes preloaded with $^{22}Na^+$. The liposomes were preloaded with $^{22}Na^+$ for 10 min and passed through a Dowex-Li^+ column as described in Materials and Methods. The preloaded liposomes were incubated with and without monensin (10 μM), and the ^{22}Na remaining entrapped was counted as described in Materials and Methods: (■) proteoliposomes alone and (□) + monensin. Standard deviations are shown.

with $^{22}Na^+$ by substrate oxidation coupled to Na^+ pumping (Figure 4). This leakage was inhibited in the presence of either ubiquinol or ascorbate/TMPD (not shown). In contrast, the leakage was stimulated in the presence of 10 μM monensin, a Na^+-carrying ionophore (Figure 4). Monensin is not an electrogenic carrier but partially replaces the Na^+ electrochemical gradient with a H^+ electrochemical gradient. The generation of the latter would then inhibit further monensin-mediated Na^+ translocation. The data are consistent with this explanation; in the presence of monensin, there was an initial rapid rate of Na^+ release which then decreased. Monensin also had a stimulatory effect on the oxygen uptake by the vesicles, which leveled off similarly as the Na^+ uptake leveled off as shown in Figure 1 for ascorbate/TMPD. Adding monensin resulted in a renewed burst of oxygen uptake before it again leveled off (data not shown). The internal volume of the vesicles was estimated to be 0.8% of that of the phospholipid from the data in Figure 5. This would give an internal Na^+ concentration of 0.50 M for the vesicles in Figure 1.

Information on the orientation of the molecules is important for accurate determinations of rates and stoichiometry of transport. A preliminary estimate of the orientation of *Vitreoscilla* cytochrome *bo* in proteoliposomes was done by measuring the ascorbate/TMPD oxidase activity of the proteoliposome suspension before and after disruption with Triton X-100. These proteoliposomes, reconstituted using the direct incorporation procedure reported to favor an inside-out orientation for cytochrome *c* oxidase (Eytan et al., 1975; Rigaud et al., 1995), showed a 63% increase in activity after disruption, which computes to 61% of the cytochrome *bo* molecules being in this orientation (B. J. S. Efiok, unpublished). However, the activity of the enzyme is affected by both the type of detergent and its concentration, so this method of estimating orientation can be unreliable.

Ascorbate/TMPD has been used as an impermeable substrate for lipid vesicles. Ascorbate alone was used as the membrane impermeable reductant for a study of phospholipid flip-flop in synthetic vesicle membranes containing spin-labeled phosphatidylcholine (Kornberg & McConnell, 1971). TMPD has been used as an electron donor to the *E. coli* cytochrome *bo* on the outer surface of membrane vesicles (Matsushita et al., 1984). However, there are contradictory reports regarding the membrane permeability of TMPD in the literature. For example, one study of cytochrome *c* oxidase proteoliposomes used TMPD at high

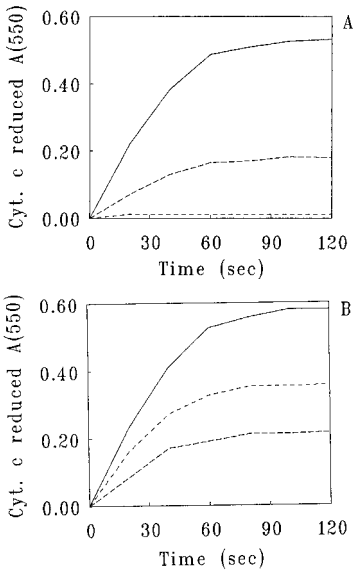

FIGURE 5: Effect of ascorbate/TMPD or Q_1H_2 on ferricytochrome c entrapped within liposomes prepared from *Vitreoscilla* lipids. The preparation of liposomes was as described in Materials and Methods. The assay medium (1 mL final volume) contained 25 mM Tris-HCl (pH 7.7), 1 mg/mL cytochrome *c* or 0.5 mL of cytochrome *c*-entrapped phospholipid vesicles (containing approximately 3 mg of phospholipid), and (A) 0.8 mM ascorbate/0.1 mM TMPD or (B) 150 μM Q_1H_2. (A) Top curve, ascorbate/TMPD + ferricytochrome *c* in solution; bottom curve, ascorbate/TMPD + ferricytochrome *c* in liposomes; and middle curve, ascorbate/TMPD added after sonication of the liposomes. (B) Top curve, Q_1H_2 + ferricytochrome *c* in solution; middle curve, Q_1H_2 + ferricytochrome *c* in liposomes; and bottom curve, Q_1H_2 added after sonication of the liposomes.

concentrations as a membrane permeable substrate (Cooper & Nicholls, 1990). Additionally, the presence of a nonprotein TMPD/H^+ antiport mechanism in these vesicles was postulated (Cooper & Nicholls, 1987). Permeability may also be affected by the lipids used to form the vesicles, and since we were using native *Vitreoscilla* lipids for our reconstitution experiments, it was necessary to test the permeability of ascorbate/TMPD in our system. This was done using vesicles containing entrapped cytochrome *c*. Reduction of the ferricytochrome *c* by this substrate did not occur until the vesicles were sonicated to release the protein (Figure 5A), confirming the impermeability of ascorbate/TMPD to our vesicles. In contrast, when Q_1H_2, which has been reported to be membrane permeable and capable of reacting with oxidase sites on both sides of the membrane (Futami et al., 1979), was added to vesicles containing entrapped cytochrome *c*, the cytochrome *c* within the vesicles was reduced without the need for sonication (Figure 5B). In this last figure, the decrease in the amount of cytochrome *c* observed when Q_1H_2 was added after disruption of the vesicles by sonication was probably due to denaturation and oxidation of the protein by the sonication procedure. In all these experiments, reduction of the cytochrome *c* was complete after about 90 s and the A_{550} was unchanged after 5 min.

These results indicate that ascorbate/TMPD will only react with cytochrome *bo* molecules that have their oxidase sites on the outside of the vesicle which will result in Na^+ being pumped into the vesicle. Since Q_1H_2 can also react with oxidase sites on the inner surface of the membrane, net inward pumping of Na^+ will be reduced by the outward

Table 1: Relative V_{max} and K_m Values of Different Substrates for *Vitreoscilla* Cytochrome *bo*[a]

substrate	relative V_{max}	K_m (μM)
Q_1H_2	1.00	60
Q_0H_2	0.28	71
menadiol	0.033	132
ascorbate/TMPD	0.069	185
ascorbate/DPIP	0.055	246
ascorbate/PMS	0.030	437

[a] The oxidase activities were measured in 2 mL of 60 mM Tris-HCl (pH 7.7) containing 5 μg/mL purified cytochrome *bo* protein and varying amounts of the electron donors added from stock solutions of the following concentrations: 23 mM Q_1H_2 or 114 mM Q_0H_2 (each formed from the quinone by reduction with dithioerythritol), 4.8 mM menadiol (formed from menadione by stoichiometric reduction with sodium borohydride), 16.3 mM TMPD, 3.5 mM PMS, or 11.7 mM DPIP. Assay solutions for the latter three dyes also contained 10 mM ascorbate. In all cases, the reaction was initiated by the addition of the quinol or dye and oxygen consumption was measured with a Yellow Springs Instrument Co. Model 53 oxygen monitor. Corrections were made for the rate of autoxidation of the quinols and dyes in the absence of added enzyme.

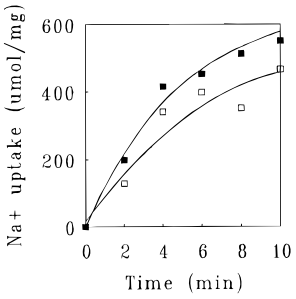

FIGURE 6: Na^+ uptake by cytochrome *bo* proteoliposomes driven by ascorbate/TMPD or Q_1H_2 as substrates. The ^{22}Na uptake was measured as described in Materials and Methods. The medium for the complete assay (total volume of 1 mL) contained 25 mM Tris-HCl (pH 7.7), proteoliposomes (0.30 nmol of cytochrome *bo*), 0.2 M $^{22}NaCl$ (specific activity of 2229 cpm/μmol of Na^+), and 0.8 mM ascorbate/0.1 mM TMPD (■) or 0.74 mM Q_1H_2 (□).

pumping of these sites. Thus, by comparison of the rates of Na^+ pumping by the two substrates, an estimate of the orientation of the molecules in the vesicles can be made. The equation for this method is

$$F_i = \frac{N_R/P_R}{(2N_R/P_R - \alpha)} \quad (1)$$

where F_i = the fraction of cytochrome *bo* molecules oriented inward, N_R = the observed rate of inward Na^+ pumping for nonpermeable substrate N, P_R = the observed rate of inward Na^+ pumping for permeable substrate P, and α = (the rate of oxidation of N)/(the rate of oxidation of P), i.e., the ratio of oxidation of substrates N and P by the cytochrome *bo* in solution. The experimental value of α for the substrate pair ascorbate/TMPD−Q_1H_2, estimated using the solubilized, purified enzyme, for *Vitreoscilla* cytochrome *bo* was 0.069 (Table 1). This value is identical to that reported in an earlier study of the *E. coli* enzyme in which TMPD was used as an electron donor to the cytochrome *bo* on the outer surface of membrane vesicles (Matsushita et al., 1984). The observed rate of inward Na^+ pumping for ascorbate/TMPD was 90 μmol/min and for Q_1H_2 was 73 μmol/min, which are N_R and P_R, respectively (Figure 6). These are initial rates estimated from the initial part of the curves shown in Figure 6 and

were obtained under saturating conditions of the substrates, Na^+, and oxygen. Substitution of these values in eq 1 gives $F_i = 0.51$. Thus, only 51% of the cytochrome *bo* molecules are oriented inward in these proteoliposomes according to this method of determination, which is in general agreement with the value of 61% determined by measuring ascorbate/TMPD oxidase activity of the proteoliposomes before and after disrupting them with detergent.

DISCUSSION

It has generally been observed that when membrane proteins are assimilated into preformed liposomes they are incorporated unidirectionally (Rigaud et al., 1995). It is presumed that this occurs because the most hydrophobic region of the protein is inserted first. Additionally, the distribution of charges in the protein, which may interact with the head groups of the phospholipids at the lipid/water interface, may also influence membrane protein insertion. However, if a protein has a fairly uniform surface with respect to hydrophobicity, for example, or if it has approximately symmetrical exposed hydrophobic regions, there is no reason to expect a unidirectional insertion. Instead, the insertion and final orientation of the protein would probably be roughly 50% inward- and 50% outward-directed. Our results for *Vitreoscilla* cytochrome *bo* indicate such a random incorporation and orientation, but this is not a unique protein in this regard. The experiments that first showed that cytochrome *bo* in *E. coli* was a proton pump were done using spheroplasts (Puustinen et al., 1989), but in a later study on *E. coli* cytochrome *bo* proteoliposomes, the low rates of H^+ pumping led to the conclusion that the *E. coli* enzyme also shows a relative lack of specific inward or outward orientation in synthetic vesicles (Puustinen et al., 1991). Similarly, the Na^+-translocating F_1F_o ATPase of *P. modestum* was shown to be oriented 50% to the outside in reconstituted proteoliposomes; incorporation in this case was achieved by a freeze/thaw/sonication procedure (Laubinger & Dimroth, 1988; Kluge & Dimroth, 1992).

Our results indicate that, in the *Vitreoscilla* cytochrome *bo* proteoliposomes, the substrate oxidizing sites are randomly oriented, about half inside and half outside. Even though Q_1H_2 is oxidized faster by the soluble oxidase than ascorbate/TMPD, when the cytochrome is embedded in the liposomal membrane, the fact that the ubiquinol can function as a substrate on both sides of the membrane results in Na^+ being pumped both inward and outward, with a net reduction in the rate of Na^+ accumulation inside the vesicles. If ascorbate/TMPD were equally permeable, less Na^+ would have been pumped with this substrate and even less Na^+ would have accumulated inside than would have with the ubiquinol because of its smaller turnover number with the enzyme. What was observed was that the rate of inward Na^+ pumping by ascorbate/TMPD relative to that of the ubiquinol was 1.23. Although this result by itself is evidence that TMPD is less permeable than ubiquinol to the membranes used in this study, the different permeabilities of the two substrates to these membranes was confirmed by an independent procedure, namely their ability to reduce ferricytochrome *c* entrapped within the vesicles (Figure 5). Ascorbate/TMPD was not capable of the reduction of cytochrome *c* until its release from the vesicles by sonication, whereas ubiquinol was capable of reducing the cytochrome *c* within the vesicles. Using these observations plus the relative rates of Na^+ pumping, the orientation of the *Vitreoscilla* cytochrome *bo* in the proteoliposomes was estimated to be roughly half inside-out and half outside-in.

This method for determining orientation of membrane proteins should be generally applicable if suitable substrates are available, but it does have some limitations. First, because of the differences in permeability found here for TMPD (Figure 5A) and that reported by others (Cooper & Nicholls, 1990), the permeability of each substrate should be independently tested for every system. In the case of cytochrome *c* oxidase proteoliposomes, it was reported that oxidase sites on the inner surface of the vesicles could function equally well as proton pumps as those with sites facing outward when high concentrations of TMPD were used (Cooper & Nicholls, 1990). Additionally, it was proposed that $TMPD^+$/TMPD transport across the membrane under some conditions was further facilitated by a TMPD/H^+ antiport mechanism mediated by free fatty acids present in the vesicles (Cooper & Nicholls, 1987). Under the conditions of our experiments, however, ascorbate/TMPD was impermeable to *Vitreoscilla* lipid vesicles, which was shown by direct experimentation (Figure 5A). Ideally, the experiment should be done with two structurally similar substrates with similar affinities for the enzyme, here, for example, a synthetic quinol containing a charged group in a position that would not drastically affect its K_m and V_{max} but would make it membrane impermeable. Actually, the rate of ascorbate oxidation by solubilized, purified *Vitreoscilla* cytochrome *bo* was relatively independent (varying only by a factor of about 2) whether TMPD, DPIP, or PMS was used as the electron carrier (Table 1), but the latter two were not tested for Na^+-pumping ability or membrane permeability.

One of the assumptions in the derivation of eq 1 is that the diffusion of P into the vesicles is not rate-limiting, and for Q_1H_2, this assumption appears to be valid. The native ubiquinol undergoes reduction and subsequent reaction with ubiquinol oxidase sites while remaining within the membrane. Thus, the Q_1H_2 presumably first dissolves in the membrane and then reacts with the oxidase sites of the cytochrome *bo* in either orientation. Another limitation of the method is that, if the relative rates of pumping for the permeable and nonpermeable substrates are similar, as they were here, then F_i in eq 1 becomes somewhat sensitive to α in the denominator.

The experiments described above used *Vitreoscilla* cytochrome *bo* in liposomes made from native phospholipids which is probably the reason for the improved pumping rates and stoichiometry observed compared to our earlier studies which used *E. coli* phospholipids. The turnover number (k_{cat}) for Na^+ pumping using these proteoliposomes and ascorbate/TMPD as substrate was 141 s^{-1}, corrected for leakage; the true turnover number would be 277 s^{-1}, assuming that only 51% of the enzyme molecules are oriented inward. This rate is comparable to turnover numbers (for ubiquinol oxidation, not H^+ pumping) of 550 and 263 s^{-1} for the purified *E. coli* cytochrome *bo* (Kita et al., 1984) and the purified *Vibrio alginolyticus* cytochrome *bo* (Miyoshi-Akiyama et al., 1993), respectively.

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