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Reconstitution of active transport in proteoliposomes containing cytochrome *o* oxidase and *lac* carrier protein purified from *Escherichia coli*

(chemiosmotic hypothesis/proton electrochemical gradient/carbocyanine/octyl glucoside/detergent dilution)

KAZUNOBU MATSUSHITA*, LEKHA PATEL*, ROBERT B. GENNIS†, AND H. RONALD KABACK*‡

*Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110; and †Department of Chemistry, University of Illinois, Urbana, Illinois 61801

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ABSTRACT **Most active transport across the bacterial cell membrane is driven by a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$, interior negative and alkaline) generated via electron transfer through a membrane-bound respiratory chain. This phenomenon is now reproduced *in vitro* with proteoliposomes containing only two proteins purified from the membrane of *Escherichia coli*. An *o*-type cytochrome oxidase was extracted from membranes of a cytochrome *d* terminal oxidase mutant with octyl β-D-glucopyranoside after sequential treatment with urea and cholate and was purified to homogeneity by ion-exchange chromatography. The purified oxidase contains four polypeptides (M_rs 66,000, 35,000, 22,000, and 17,000), two *b*-type cytochromes (*b*558 and *b*563), and 16–17 nmol of heme *b* per mg of protein, and it catalyzes the oxidation of ubiquinol and other electron donors with specific activities 20- to 30-fold higher than crude membranes. The *lac* carrier protein was purified as described. Proteoliposomes were formed in the presence of the oxidase and *lac* carrier protein by detergent dilution, followed by freeze-thaw/sonication. The system generates a $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) with ubiquinol as electron donor and the magnitude of $\Delta\bar{\mu}_{H^+}$ is dependent on the concentration of cytochrome *o* in the proteoliposomes. Furthermore, the proteoliposomes transport lactose against a concentration gradient to an extent that is commensurate with the magnitude of $\Delta\bar{\mu}_{H^+}$ generated. The results provide powerful additional support for the "chemiosmotic hypothesis" and demonstrate that purified *lac* carrier protein retains the ability to function in a physiological manner.**

Translocation of β-galactosides across the cytoplasmic membrane of *Escherichia coli* is mediated by an intrinsic membrane protein, the *lac* carrier protein or *lac* permease, that is encoded by the *lac y* gene (see ref. 1 for a recent review). The *lac* carrier protein catalyzes the coupled transport of substrate with protons in a symport (cotransport) reaction. Thus, in the presence of a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$, interior negative and alkaline), downhill translocation of protons in response to $\Delta\bar{\mu}_{H^+}$ drives the uphill translocation of substrate (i.e., active transport). Conversely, downhill translocation of substrate under nonenergized conditions drives the uphill translocation of protons with generation of $\Delta\bar{\mu}_{H^+}$, the polarity of which reflects the direction of the substrate concentration gradient.

Recently, by using a strain of *E. coli* with amplified levels of the *lac y* gene, a highly specific photoaffinity label for the *lac* carrier protein and reconstitution of transport activity in proteoliposomes, the *lac* carrier protein was purified to homogeneity in a functional state (2, 3). Proteoliposomes containing purified *lac* carrier exhibit counterflow, proton influx and efflux with appropriately directed lactose concentration gradients, and accumulate lactose against a concentration gradient when $\Delta\bar{\mu}_{H^+}$ (interior negative or alkaline or both) is imposed (2–5). Furthermore, the turnover number of purified *lac* carrier in proteoliposomes is similar to that observed in right-side-out membrane vesicles, as is the K_m for lactose (1). In addition, a secondary structure model for the *lac* carrier protein has been proposed (6), monoclonal antibodies against the purified protein have been prepared and characterized (7), and it has been demonstrated directly that the protein spans the bilayer (8).

In aerobically growing *E. coli*, as in mitochondria, $\Delta\bar{\mu}_{H^+}$ is generated primarily by substrate oxidation via a membrane-bound respiratory chain with oxygen as terminal electron acceptor. Although the precise mechanism of proton ejection is unknown, it has been demonstrated that purified cytochrome oxidases from a number of sources have the capacity to generate $\Delta\bar{\mu}_{H^+}$. Thus, mitochondrial cytochrome oxidase (see ref. 9 for a review) and terminal oxidases from *Paracoccus denitrificans* (10), the thermophile PS-3 (11), and *E. coli* (12) all manifest the ability to translocate electrons or protons (or both) vectorially when incorporated into proteoliposomes.

Because purified *lac* carrier protein catalyzes active transport in proteoliposomes when either a membrane potential ($\Delta\Psi$, interior negative) or a pH gradient (ΔpH, interior alkaline) is imposed nonphysiologically (1–5) and the *o*-type cytochrome oxidase from *E. coli* generates $\Delta\Psi$ (interior negative) (12), it follows that proteoliposomes containing both proteins might exhibit electron transfer-driven lactose accumulation. In this report, we describe a relatively simple purification and partial characterization of the *o*-type cytochrome oxidase from *E. coli* and demonstrate that turnover of the purified enzyme generates $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) when it is incorporated into proteoliposomes. Strikingly, when proteoliposomes are prepared with the oxidase and purified *lac* carrier protein, they transport lactose against a concentration gradient in a fashion that mimics intact cells and right-side-out membrane vesicles. A preliminary report of some of these findings has appeared (13).

MATERIALS AND METHODS

Growth of Cells and Membrane Preparation. *E. coli* GR19N (*cyt*$^-$) (14) was grown aerobically into late logarithmic phase in 20 liters of minimal medium A (15) containing 0.5% sodium lac-

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Abbreviations: $\Delta\bar{\mu}_{H^+}$, proton electrochemical gradient; $\Delta\Psi$, membrane potential; ΔpH, pH gradient; KP_i, potassium phosphate; Q_1H_2, ubiquinol-1; octyl glucoside, octyl β-D-glucopyranoside; diS-C_3-(5), 3,3′-diisopropylthiodicarbocyanine; TMPD, *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine; PMS, phenazine methosulfate.

‡To whom reprint requests should be addressed.

tate and 0.15% Casamino acids (pH 7.0). Cells were harvested by centrifugation and washed once with 50 mM potassium phosphate (KP_i) (pH 7.5). Washed cells were resuspended in 50 mM KP_i (pH 7.5) containing 5 mM magnesium sulfate (4 ml/g of wet weight of cells), and pancreatic DNase (type I; Sigma) was added to a concentration of 10 μg/ml. The suspension was passed through a French pressure cell at 20,000 psi (1 psi = 6.89 kPa), and unbroken cells were removed by centrifugation at 10,000 × *g* for 10 min. The supernatant was then centrifuged at 120,000 × *g* for 2 hr to sediment the membrane fraction that was washed once in 50 mM KP_i (pH 7.5).

Purification of Cytochrome *o*. All steps were performed at 0–4°C unless noted otherwise. Membranes prepared as described were suspended to a final concentration of about 10 mg of protein per ml in 50 mM KP_i (pH 7.5) and mixed with an equal volume of 10 M urea freshly prepared at room temperature. The suspension was incubated on ice for 20 min and centrifuged at 150,000 × *g* for 2 hr, and the supernatant was discarded. The pellet contained two clearly demarcated portions, a lower transparent layer and an upper red layer. The loose, red layer was resuspended by agitation in 50 mM KP_i (pH 7.5) and adjusted to a concentration of about 4 mg of protein per ml, and 20% sodium cholate (pH 7.8) was added to a final concentration of 6%. The suspension was incubated on ice for 20 min and centrifuged at 120,000 × *g* for 1 hr, and the supernatant was discarded.

The red pellet that was almost devoid of a lower transparent layer was resuspended in 50 mM KP_i (pH 7.5) and washed once by centrifugation at 120,000 × *g* for 1 hr. The pellet was dispersed in 50 mM KP_i (pH 7.5), and *E. coli* phospholipid [50 mg/ml in 50 mM KP_i (pH 7.5)] and octyl β-D-glucopyranoside (octyl glucoside) [12.5% in 50 mM KP_i (pH 7.5)] were added to final concentrations of 3.8 mg/ml and 1.25%, respectively (the final protein concentration was 1.5–2.0 mg/ml). The suspension was stirred for 10 min with a magnetic bar and centrifuged at 120,000 × *g* for 1 hr. The supernatant was applied to a DEAE-Sepharose CL-6B column (Pharmacia) (about 1-ml bed vol per mg of protein applied), which had been equilibrated with 50 mM KP_i (pH 7.5) and washed with 1 bed vol of 50 mM KP_i (pH 7.5) containing 1% octyl glucoside. After application of the sample, the column was washed with 1 bed vol of 50 mM KP_i (pH 7.5) containing 1% octyl glucoside, followed by 2 bed vol of 100 mM KP_i (pH 7.5) containing 1% octyl glucoside. Cytochrome *o* was then eluted with a linear gradient of 100–175 mM KP_i (pH 7.5) containing 1% octyl glucoside (each reservoir contained 2 bed vol of the appropriate buffer) at a flow rate of about 20 ml/hr. The enzyme eluted as a single symmetrical activity peak coincident with protein at about the middle of the gradient. Fractions exhibiting ubiquinol-1 (Q_1H_2) oxidase activity were pooled, frozen in liquid N_2, and stored at −180°C. Results from a typical purification are summarized in Table 1.

Purification of *lac* Carrier Protein. *lac* carrier protein was prepared from *E. coli* T206 as described (2, 3).

Reconstitution of Proteoliposomes Containing Cytochrome *o* and *lac* Carrier Protein. Bath-sonicated *E. coli* phospholipids [0.56 ml of a solution containing 28 mg of phospholipid in 50 mM KP_i (pH 7.5)] prepared as described (18) were mixed with octyl glucoside [0.16 ml of 12.5% octyl glucoside dissolved in 50 mM KP_i (pH 7.5)], purified cytochrome *o* [1.0 ml of a solution containing 170 μg of protein and 1% octyl glucoside in 50 mM KP_i (pH 7.5)], purified *lac* carrier protein [2.0 ml of a solution containing 68 μg of protein and 1.25% octyl glucoside in 10 mM KP_i (pH 6.0)], and 50 mM KP_i (pH 7.5) (0.68 ml containing 1 mM dithiothreitol) (total volume, 4.4 ml). The sample was mixed by hand, incubated on ice for 20 min, and then diluted rapidly into 132 ml of 50 mM KP_i (pH 7.5) containing 1 mM dithiothreitol at room temperature. After stirring with a magnetic bar for 10 min, proteoliposomes were collected by centrifugation at 120,000 × *g* for 2 hr. The supernatant was discarded and the proteoliposomes were resuspended in 50 mM KP_i (pH 7.5) containing 1 mM dithiothreitol to a final volume of 0.75 ml (300 μg of protein per ml). The suspension was then rapidly frozen in liquid N_2, thawed at room temperature, and sonicated in a plastic tube for 10–20 s with a bath-type sonicator (80 W, 80 Hz, generator model G80-80-1, tank model T80-80-1-RS from Laboratory Supplies, Hicksville, NY). Judging from the recovery of *p*-nitro[2-^{3}H]phenyl-α-D-galactopyranoside-photoaffinity-labeled *lac* carrier (2, 3) and Q_1H_2 oxidase activity, proteoliposomes prepared in this manner contained 223 μg of cytochrome *o* per ml and 77 μg of *lac* carrier per ml (i.e., a molar ratio of oxidase to *lac* carrier of 1:1, by using M_rs of 130,000 and 45,000, respectively). Where indicated, either cytochrome *o* or *lac* carrier protein was omitted or the cytochrome *o* content was varied to give different ratios of oxidase to *lac* carrier. The octyl glucoside concentration used in each preparation was the same, however.

Table 1. Purification of cytochrome *o* oxidase from *E. coli* GR19N

Fraction	Protein, mg	Q_1H_2 oxidase* Units	Q_1H_2 oxidase* Units/mg	Cytochrome *o*, nmol/mg†
Membrane	828	3,458	4.18	0.21
Octyl glucoside extract from urea/cholate-treated membranes	32.4	729	22.5	2.1
DEAE-Sepharose pooled fractions	5.27	466	88.4	11.5

* A unit of Q_1H_2 oxidase activity is defined as 1 μmol of Q_1H_2 oxidized per min.

† Calculated from CO difference spectra by using a millimolar extinction coefficient of 80 at 415–430 nm (16). The specific heme *b* content was determined by the pyridine hemochromogen method (17) to be 16–17 nmol/mg for the final preparation.

Analytical Procedures. Q_1H_2 oxidase activity was measured at room temperature by following absorbance at 275 nm in a Beckman DU-8 recording spectrophotometer. Reaction mixtures contained (in final concentrations) 160 μM Q_1H_2, 0.1% Tween 20, 50 mM KP_i (pH 7.5), and enzyme in a total volume of 1.0 ml. Activity was calculated by using a molar extinction coefficient of 12.25 for Q_1H_2 (19).

$\Delta\Psi$ (interior negative) was determined by measuring fluorescence quenching of 3,3′-diisopropylthiodicarbocyanine [diS-C_3-(5)] in a Perkin–Elmer MPF-4 spectrophotofluorimeter (12). Experiments were performed at 25°C; excitation was at 622 nm, and emission was recorded at 670 nm. Reaction mixtures contained (in final concentrations) 50 mM KP_i (pH 7.5), 1 μM DiS-C_3-(5), and proteoliposomes as indicated in a total volume of 2.0 ml. Data were quantitated by comparison to valinomycin-mediated potassium diffusion potentials ($K^+_{in} \rightarrow K^+_{out}$).

Uptake of [1-^{14}C]lactose by proteoliposomes was measured by filtration as described (2–5).

$NaDodSO_4$/polyacrylamide gel electrophoresis was performed in 12% polyacrylamide gels (5 cm) with 5% polyacrylamide (1 cm) stacking gels as described by Laemmli (20). Samples were incubated in 3% $NaDodSO_4$/100 mM dithiothreitol at room temperature for 30 min prior to electrophoresis.

Heme *b* content was determined by measuring pyridine hemochromogen by using a millimolar extinction coefficient of 20.7 (reduced-oxidized) (17).

Protein was assayed as described (2, 3).

Materials. Valinomycin, nigericin, cholic acid, and octyl glucoside were purchased from Calbiochem. Ubiquinone-1 was obtained from Hoffmann–La Roche, and Q_1H_2 was prepared by the method of Rieske (21). Phospholipids were prepared from *E. coli* B as described (18). All other materials were reagent grade purchased from commercial sources.

RESULTS

Purification of Cytochrome *o*. Sequential extraction of *E. coli* membranes with urea and cholate provides a significant *in situ* purification of the *lac* carrier protein (2, 3) and octyl glucoside has been shown to extract *N,N,N',N'*-tetramethylphenylenediamine (TMPD) oxidase activity (22). These procedures were combined to partially purify and solubilize cytochrome *o* oxidase from the membrane of a cytochrome *d*-deficient mutant (14) (Table 1). As shown, the octyl glucoside extract from urea/cholate-treated membranes exhibits a 5-fold increase in specific activity over the starting material with 20% recovery of total activity.§ The octyl glucoside extract was then chromatographed on DEAE-Sepharose, which provides another 4-fold increase in specific activity with over 50% recovery. Relative to the starting material, an overall purification of 20- or 50-fold was obtained with respect to the specific activity of Q_1H_2 oxidase activity or cytochrome *o* content (i.e., CO binding; last column in Table 1), respectively. Although not shown, the Q_1H_2 oxidase activity of the purified material is stimulated ≈50% by either *E. coli* or soybean phospholipids at concentrations of 0.25 mg/ml. Furthermore, after passage of the enzyme through Sephacryl S-200, activity is completely dependent upon exogenous phospholipid.

$NaDodSO_4$/polyacrylamide gel electrophoresis of the pooled DEAE-Sepharose fractions demonstrates that the purified oxidase contains two subunits that stain with Coomassie blue, one at M_r 55,000 and the other at M_r 34,000 (data not shown). However, the use of a more sensitive silver stain suggests the presence of two additional lower molecular weight subunits (M_rs 22,000 and 17,000) in the terminal oxidase complex, a finding that is consistent with the recent immunological characterization of cytochrome *o* (23). Furthermore, the mobility of the M_r 55,000 subunit is dependent on the concentration of polyacrylamide used during electrophoresis, and quantitation (24) yields a M_r of about 66,000 (data not shown).

Purified oxidase contains two *b*-type cytochromes, as evidenced by the α bands in the reduced spectrum (not shown). Furthermore, exposure of the reduced oxidase to CO yields a CO-difference spectrum that is typical of cytochrome *o* (16, 25). Notably, moreover, the purified enzyme has a heme *b* content of 16–17 nmol/mg of protein (*ca.* 2.0 mol/mol of enzyme).

Generation of $\Delta\bar{\mu}_{H^+}$ in Proteoliposomes. By measuring fluorescence quenching of the carbocyanine dye diS-C_3-(5), Kita *et al.* (12) concluded that proteoliposomes containing purified cytochrome *o* generate $\Delta\Psi$ (interior negative). The conclusion is confirmed and extended by the data presented in Fig. 1, which demonstrate that addition of Q_1H_2 to proteoliposomes containing cytochrome *o* (Fig. 1, curve C) or cytochrome *o* and *lac* carrier protein (Fig. 1, curve B) causes quenching of diS-C_3-(5) fluorescence. Moreover, quenching is enhanced considerably when nigericin is added. Nigericin is an ionophore that catalyzes electrically neutral exchange of protons for K^+ (or Na^+), thereby collapsing ΔpH with a compensatory increase in $\Delta\Psi$ (26–28). In contrast, immediately after addition of valinomycin, the signal returns rapidly to the baseline, an observation

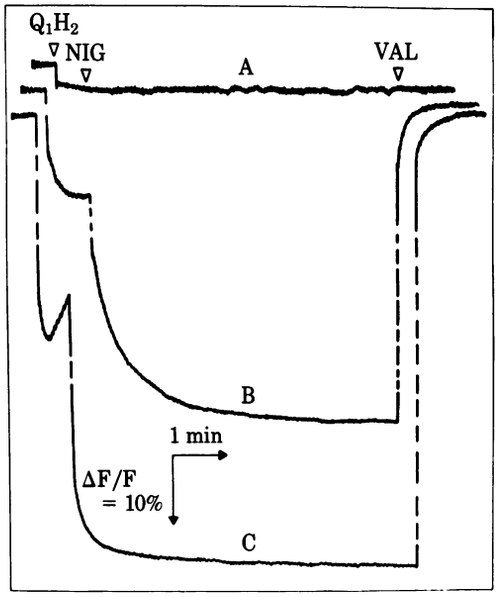

FIG. 1. diS-C_3-(5) fluorescence quenching induced by Q_1H_2 in proteoliposomes containing *lac* carrier protein (curve A), cytochrome *o* oxidase (curve C), and *lac* carrier protein with cytochrome *o* oxidase (curve B). Reaction mixtures contained 50 mM KP_i (pH 7.5), 1 μM diS-C_3-(5), 5 mM dithiothreitol, and proteoliposomes prepared in a total volume of 2.0 ml. Where indicated (▽), 2 μl of a solution of 16 mM Q_1H_2 (in ethanol), 1 μl of a solution of 0.25 mM nigericin (NIG) (in ethanol), and 1 μl of a solution of 10 mM valinomycin (VAL) (in ethanol) were added sequentially. For curve A, the proteoliposomes contained 1.8 μg of *lac* carrier protein; for curve C, 1.2 μg of cytochrome *o* oxidase; and for curve B, 1.12 μg of cytochrome *o* and 0.38 μg of *lac* carrier protein (i.e., a molar ratio of 1:1). The $\Delta\Psi$s generated were estimated by comparison to standard curves (not shown) constructed from experiments in which fluorescence quenching was induced by imposition of K^+ diffusion gradients of known magnitude in the presence of valinomycin. Calibration was performed independently for the proteoliposomes used in curves B and C.

that is consistent with the known ability of this ionophore to collapse $\Delta\Psi$ in the presence of K^+ (26–28). The findings provide a strong indication that during turnover, cytochrome *o* oxidase generates a significant ΔpH (interior alkaline) in addition to $\Delta\Psi$. As expected, proteoliposomes containing purified *lac* carrier alone do not exhibit fluorescence quenching on addition of Q_1H_2 (Fig. 1, curve A).

The magnitude of the $\Delta\Psi$ generated during oxidation of Q_1H_2 can be estimated by comparison to standard curves constructed from experiments in which fluorescence quenching is induced by imposition of K^+ diffusion gradients of known magnitude in the presence of valinomycin. With proteoliposomes containing cytochrome *o* only (Fig. 1, curve C), $\Delta\Psi$s of about −80 mV and −115 mV are calculated before and after addition of nigericin, respectively. In proteoliposomes containing both the oxidase and *lac* carrier protein at a molar ratio of 1:1 (Fig. 1, curve B), $\Delta\Psi$s of about −50 mV and −100 mV are observed under analogous conditions. Notably, Q_1H_2-induced quenching of diS-C_3-(5) fluorescence is a linear function of the concentration of proteoliposomes used, and similar values for $\Delta\Psi$ are obtained by measuring the steady-state distribution of [^{3}H]tetraphenylphosphonium by flow dialysis (29)¶ (data not shown).

Lactose Transport in Proteoliposomes Containing Cytochrome *o* and *lac* Carrier Protein. Proteoliposomes containing purified oxidase and *lac* carrier at a molar ratio of 1:1 catalyze lactose transport when $\Delta\Psi$s of −90 mV and −135 mV are im-

§ Because Q_1H_2 is probably oxidized through a number of different pathways in crude membranes, it is difficult to determine the yield of cytochrome *o* from measurements of Q_1H_2 oxidase activity.

¶ With proteoliposomes containing cytochrome *o* and *lac* carrier protein at a molar ratio of 1:1, $\Delta\Psi$s of −68 mV and −83 mV, respectively, were obtained before and after addition of nigericin.

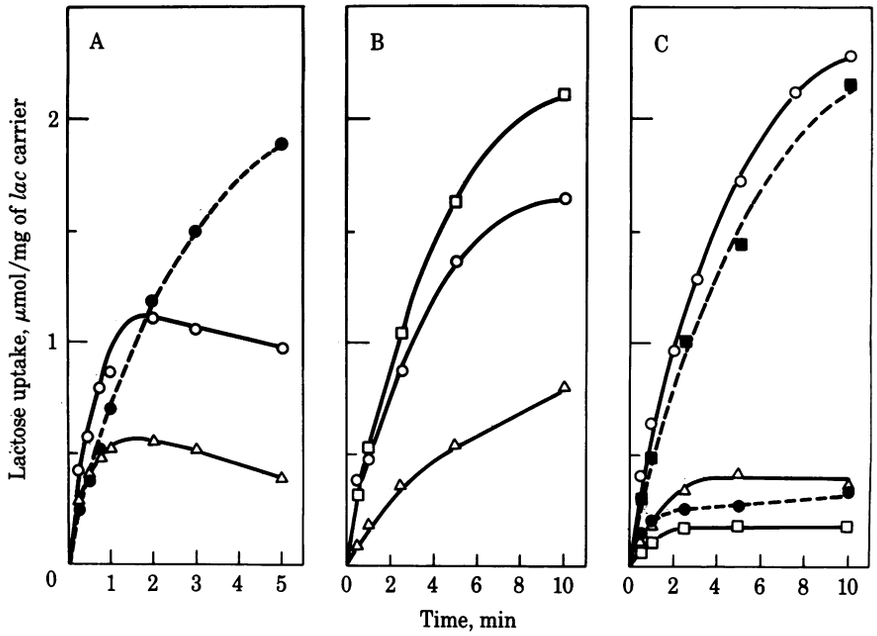

FIG. 2. Lactose transport in proteoliposomes containing purified cytochrome *o* oxidase and *lac* carrier protein. Oxidase and *lac* carrier protein were purified and reconstituted into proteoliposomes at a 1:1 molar ratio. (*A*) Lactose transport induced by K^+ diffusion potentials ($K^+_{in} \rightarrow K^+_{out}$) of −90 mV (△) and −135 mV (○) or by addition of Q_1H_2 (●). For diffusion potential-driven transport, valinomycin was added to the proteoliposomes to a final concentration of 200 μM, and either 1 μl (−135 mV) or 3 μl (−90 mV) was diluted rapidly into 200 μl or 100 μl, respectively, of 50 mM NaP_i (pH 7.5) containing 0.33 mM [1-^{14}C]lactose (19 mCi/mmol; 1 Ci = 3.7×10^{10} Bq). For Q_1H_2-driven transport, 5 μl of proteoliposomes was added to 100 μl of a solution containing 50 mM KP_i (pH 7.5), 5 mM dithiothreitol, 16 μM Q_1H_2, and 0.33 mM [1-^{14}C]lactose (19 mCi/mmol). (*B*) Lactose transport in the presence of Q_1H_2 (○), reduced PMS (□), or TMPD (△). The experiments were performed as described for Q_1H_2-driven transport in *A*, except that, where indicated, the reaction mixtures contained either 20 mM ascorbate and 75 μM PMS or 2.5 mM TMPD in place of dithiothreitol and Q_1H_2. (*C*) Effect of cyanide (●), nigericin (■), valinomycin (△), or valinomycin with nigericin (□) on Q_1H_2-driven lactose transport (○). Experiments were performed as described for Q_1H_2-driven transport in *A*, except that, where indicated, 0.5 μM nigericin, 20 μM valinomycin, or 0.5 μM nigericin with 20 μM valinomycin were present in the reaction mixtures. Alternatively, the proteoliposomes were incubated with 10 mM potassium cyanide for 10 min prior to addition to the reaction mixtures (final cyanide concentration, 0.5 mM). Samples incubated in the absence of electron donor (□) yielded values identical to those obtained in the presence of Q_1H_2 and valinomycin with nigericin. The experiments were performed at 25°C, and the reactions were terminated at the times indicated and assayed by filtration as described (2–5).

posed nonphysiologically by means of K^+ diffusion gradients ($K^+_{in} \rightarrow K^+_{out}$) in the presence of valinomycin, thus demonstrating that the *lac* carrier is functional in the preparation (Fig. 2*A*). Under these conditions, uptake is dependent on the magnitude of $\Delta\Psi$ and, in both cases, increases linearly for about 30 s, reaches a maximum at 1–2 min, and then gradually decreases as the K^+ diffusion potential dissipates. When Q_1H_2 is added to drive turnover of the oxidase, the same proteoliposomes accumulate lactose at a linear rate for about 2 min, and uptake continues to increase for >5 min because $\Delta\bar{\mu}_{H^+}$ is maintained through the continuous activity of the oxidase. Furthermore, it is apparent that the rate of oxidase-driven lactose uptake is similar to that observed with a K^+ diffusion potential of −90 mV, a finding that is entirely consistent with the $\Delta\bar{\mu}_{H^+}$ generated in the presence of Q_1H_2 (i.e., −83 mV to −100 mV; cf. Fig. 1, curve B after addition of nigericin).¶

In the presence of reduced phenazine methosulfate (PMS), transport is similar or slightly enhanced relative to Q_1H_2, whereas much lower activity is observed with TMPD (Fig. 2*B*). Although not shown, this order correlates with the activity of purified cytochrome *o* toward the different electron donors.

Q_1H_2-driven lactose transport is almost completely abolished by cyanide, demonstrating that oxidase turnover is critical for transport (Fig. 2*C*). Moreover, nigericin causes only minor inhibition of lactose transport in the presence of Q_1H_2, whereas valinomycin depresses transport by about 80% and combination of both ionophores yields the same level of activity as that observed in the absence of electron donors. The findings are similar to those obtained with right-side-out membrane vesicles (30), which led to the conclusion that the steady-state level of lactose accumulation is dependent on $\Delta\bar{\mu}_{H^+}$ with a bias toward $\Delta\Psi$. It is also noteworthy that the proteoliposomes accumulate lactose 10- to 20-fold against a concentration gradient in the presence of Q_1H_2 (compare values observed at 10 min in the presence and absence of electron donor), which is consistent with the $\Delta\bar{\mu}_{H^+}$ observed.

With proteoliposomes containing increasing concentrations of cytochrome *o* and increasing ratios of oxidase to *lac* carrier protein, both the magnitude of the $\Delta\bar{\mu}_{H^+}$ developed [i.e., diS-C_3-(5) fluorescence quenching in the presence of nigericin] and the rate of lactose transport increase up to an oxidase/*lac* carrier ratio of 1.5:1 (Table 2). The observations are reasonable because at low oxidase concentrations, proteoliposomes containing *lac* carrier but no oxidase molecules would neither generate $\Delta\bar{\mu}_{H^+}$ nor exhibit Q_1H_2-dependent lactose transport. However, when the ratio is increased to 3.9:1, $\Delta\bar{\mu}_{H^+}$ continues to increase, but transport activity decreases significantly. This effect is largely prevented when superoxide dismutase (but *not* peroxidase or catalase) is added to the reaction mixtures (data not shown). Clearly, therefore, either oxidation of Q_1H_2 or reduction of oxygen by the oxidase give rise to superoxide anion, which has a more deleterious effect on the *lac* carrier than on cytochrome *o*.

DISCUSSION

In addition to describing a relatively simple purification of the *o*-type cytochrome oxidase from *E. coli* and extending previous evidence (12) demonstrating that the oxidase generates $\Delta\Psi$ when incorporated into proteoliposomes, the findings presented here show that an important function of the bacterial cytoplasmic membrane, active transport, can be reproduced with a high de-

Table 2. $\Delta\bar{\mu}_{H^+}$ and lactose transport activity in proteoliposomes containing increasing ratios of cytochrome *o* oxidase to *lac* carrier protein

Oxidase/ *lac* carrier, mol/mol*	Protein/ lipid, mg/mg*	$\Delta\bar{\mu}_{H^+}$, mV†	Initial rate of lactose uptake, μmol/min per mg of *lac* carrier‡
0.08	0.003	−31	0.09
0.39	0.005	−66	0.13
0.77	0.008	−115	0.17
1.54	0.014	−122	0.26
3.85	0.030	−163	0.22

Proteoliposomes containing given ratios of cytochrome *o* oxidase to *lac* carrier protein were prepared. Each preparation utilized the same amount of *E. coli* phospholipid (7 mg), *lac* carrier protein (18 μg), octyl glucoside (15 mg), and various amounts of cytochrome *o* oxidase (4–200 μg) in 50 mM KP_i (pH 7.5) containing 1 mM dithiothreitol in a final volume of 1.48 ml. Proteoliposomes were formed by diluting each mixture into 50 ml of 50 mM KP_i (pH 7.5) containing 1 mM dithiothreitol. After harvesting, the proteoliposomes were resuspended to 0.25 ml in 50 mM KP_i (pH 7.5) containing 1 mM dithiothreitol, and all assays were performed after freeze-thaw/sonication.

* Values given represent ratios present during detergent dilution, and it is assumed that all of the protein was recovered in the proteoliposomes. Molar ratios were estimated based on M_rs 130,000 and 45,000 for the oxidase and *lac* carrier protein, respectively.

† $\Delta\bar{\mu}_{H^+}$ was estimated from fluorescence quenching of diS-C_3-(5) in the presence of Q_1H_2 and nigericin as described in the legend to Fig. 1. Independent calibration curves were constructed for each proteoliposome preparation.

‡ Initial rates of Q_1H_2-dependent lactose transport were measured over the first 30 s of the reaction as described in the legend to Fig. 2.

gree of fidelity in proteoliposomes reconstituted with only two proteins. Thus, proteoliposomes containing cytochrome *o* and *lac* carrier protein generate $\Delta\bar{\mu}_{H^+}$ during turnover of the oxidase, and $\Delta\bar{\mu}_{H^+}$ generated in this manner drives active transport in a fashion that closely resembles intact cells and right-side-out membrane vesicles (1). Conceptually, the experiments complement the well-known studies of Racker and Stoeckenius (31), who reconstituted photophosphorylation in proteoliposomes containing bacteriorhodopsin and the H^+-ATPase complex. However, in this system the orientation of the components in the proteoliposomes is similar to that observed in the intact membrane, and importantly, the system functions in a highly efficient manner—that is, the rate and steady-state level of lactose accumulation during oxidase turnover reflect the magnitude of $\Delta\bar{\mu}_{H^+}$ to a reasonable approximation. Finally, it should be emphasized that lactose transport activity in this system responds to a given $\Delta\bar{\mu}_{H^+}$, regardless of whether the driving force is generated by a bulk-phase K^+ diffusion potential or by oxidase turnover. In other words, the data provide strong, if not incontrovertible, support for one of the central postulates of the "chemiosmotic" hypothesis (1, 32, 33), that active transport is driven by a transmembrane, bulk-phase $\Delta\bar{\mu}_{H^+}$.

Enhancement of Q_1H_2-induced fluorescence quenching of diS-C_3-(5) by nigericin clearly suggests that oxidase turnover generates a significant ΔpH in addition to ΔΨ. Other findings (not shown) are also consistent with this interpretation. During flow dialysis, the proteoliposomes accumulate permeant weak acids on addition of Q_1H_2 to an extent that is indicative of a ΔpH of −40 mV to −50 mV. Furthermore, pH measurements with lightly buffered suspensions of proteoliposomes demonstrate that oxidase turnover results in acidification of the medium, and experiments with an entrapped pH probe indicate that the internal space becomes alkaline during Q_1H_2 oxidation. However, whether generation of ΔpH occurs by a vectorial or scalar mechanism is an open question.

Although the *lac* carrier protein purified to homogeneity and incorporated into proteoliposomes catalyzes all of the translocation reactions typical of the β-galactoside transport system in the membrane of *E. coli* with similar turnover numbers and K_ms (1–5), earlier studies imply that active lactose accumulation might require more than a single polypeptide species (34–37). Furthermore, Wright *et al.* (38), using *lac* carrier partially purified and reconstituted by techniques different from those described (2, 3), have reported recently that their preparations catalyze counterflow but do not exhibit $\Delta\bar{\mu}_{H^+}$-driven lactose accumulation. However, in view of previous evidence from this laboratory (1–5) and the findings documented here, it seems readily apparent that all of the reactions catalyzed by the β-galactoside transport system, which now include electron transfer-driven lactose accumulation against a gradient, are catalyzed by a single polypeptide species, the product of the *lac y* gene.

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