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# D-Lactate Oxidation and Generation of the Proton Electrochemical Gradient in Membrane Vesicles from *Escherichia coli* GR19N and in Proteoliposomes Reconstituted with Purified D-Lactate Dehydrogenase and Cytochrome *o* Oxidase

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ABSTRACT: The respiratory chain in the cytochrome *d* deficient mutant *Escherichia coli* GR19N is a relatively simple, linear system consisting of primary dehydrogenases, ubiquinone 8, cytochrome *b*-556, and cytochrome *o* oxidase. By use of right-side-out and inside-out membrane vesicles from this strain, various oxidase activities and the generation of the $H^+$ electrochemical gradient were studied. Oxidation of ubiquinol 1 or *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine, which donate electrons directly to the terminal oxidase, generates a $H^+$ electrochemical gradient comparable to that observed during D-lactate oxidation. In contrast, D-lactate/ubiquinone 1 or D-lactate/ferricyanide oxidoreductase activity does not appear to generate a membrane potential, suggesting that electron flow from D-lactate dehydrogenase to ubiquinone is not electrogenic. Moreover, proteoliposomes reconstituted with purified D-lactate dehydrogenase, ubiquinone 8, and purified cytochrome *o* catalyze D-lactate and ubiquinol 1 oxidation and generate a $H^+$ electrochemical gradient similar to that observed in membrane vesicles. Strikingly, in inside-out vesicles, NADH oxidation generates a $H^+$ electrochemical gradient that is very significantly greater than that produced by either D-lactate or ubiquinol 1; furthermore, NADH/ubiquinone 1 and NADH/ferricyanide oxidoreductase activities are electrogenic. It is suggested that the only component between D-lactate dehydrogenase or ubiquinol and oxygen in GR19N membranes that is directly involved in the generation of the $H^+$ electrochemical gradient is cytochrome *o*, which functions as a "half-loop" (i.e., the oxidase catalyzes the scalar release of 2 $H^+$ from ubiquinol on the outer surface of the membrane, vectorial transfer of 2 $e^-$ from the outer to the inner surface, and scalar utilization of 2 $H^+$ on the inner surface to reduce oxygen). In contrast, between NADH and oxygen, generation of the $H^+$ electrochemical gradient occurs at two sites, one between NADH dehydrogenase and ubiquinone by an unspecified mechanism and the other at cytochrome *o* oxidase.

A compelling body of evidence has now accumulated demonstrating that many energy-coupling membrane systems generate a large $H^+$ electrochemical gradient ($\Delta\bar{\mu}_{H^+}$)[1] that is the immediate driving force for a wide array of phenomena [cf. Skulachev and Hinkle (1981), Nichols (1982), and Kaback (1986) for recent reviews]. However, the mechanism by which $\Delta\bar{\mu}_{H^+}$ is generated, particularly during respiration, is still conjectural and often controversial.

In aerobically growing *Escherichia coli*, like mitochondria, $\Delta\bar{\mu}_{H^+}$ is generated primarily by substrate oxidation via a membrane-bound respiratory chain with oxygen as terminal electron acceptor. Even in this relatively simple respiratory system, neither the site(s) of $\Delta\bar{\mu}_{H^+}$ generation nor the mechanism has been resolved [cf. Ingledew and Poole (1984) for a review]. In general, three types of mechanisms have been proposed to explain respiration-driven $\Delta\bar{\mu}_{H^+}$ generation: (i) "Loops". According to this notion, the electron and $H^+$ carriers that comprise the respiratory chain are disposed alternatively and asymmetrically across the membrane in such a manner that $H^+$ moves from one side of the membrane to the other with a net flux of electrons in the opposite direction (Mitchell, 1966, 1968). (ii) "Pumps". By this means, a specific intermediate in the respiratory chain (e.g., the mitochondrial terminal oxidase) acts as a primary $H^+$ pump during oxida-

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[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, the proton electrochemical gradient; $\Delta\Psi$, membrane potential; $\Delta$pH, pH gradient; octyl glucoside, octyl $\beta$-D-glucopyranoside; PMS, phenazine methosulfate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $TPP^+$, tetraphenylphosphonium; diS-$C_3$-(5), 3,3'-diisopropylthiodicarbocyanine; diBA-$C_2$-(5), bis(1,3-diethylbarbituric acid) pentamethine oxonol; TMPD, *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine; DCI, dichlorophenolindophenol; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; *p*CMBS, *p*-(chloromercuri)benzenesulfonate; BAL, 2,3-dimercapto-1-propanol; RSO, right side out; ISO, inside out; $KP_i$, potassium phosphate; $Q_1H_2$, ubiquinol 1; DTT, dithiothreitol; D-L-DH, D-lactate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide.

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tion/reduction by an unknown mechanism involving conformational changes (Wikström et al., 1981). (iii) "Protonmotive uniquinone cycle" (i.e., "Q cycle"). This mechanism has been proposed by Mitchell (1976) to explain $H^+$ translocation in the absence of $H^+$ carriers after cytochrome *b* in the mitochondrial respiratory chain. With regard to *E. coli*, schemes have been proposed based on both the loop concept (Poole & Haddock, 1975) and the Q cycle (Downie & Cox, 1978; Bragg, 1979).

The *E. coli* aerobic respiratory chain contains two terminal oxidases, cytochrome *o* and cytochrome *d* (Ingledew & Poole, 1984). Recently, each of these oxidases has been purified, reconstituted into proteoliposomes, and shown to generate both a membrane potential ($\Delta\Psi$) and a pH gradient ($\Delta$pH) during turnover (Kita et al., 1982; Matsushita et al., 1983, 1984, 1986; Koland et al., 1984; Hamamoto et al., 1985; Miller & Gennis, 1985). Furthermore, the results as a whole are consistent with the idea that oxidase turnover generates a $\Delta\Psi$ due to vectorial electron flow, while $\Delta$pH results from scalar reactions at the surfaces of the membrane [cf. Matsushita et al. (1984)]. That is, both of these terminal oxidases appear to function as half-loops rather than $H^+$ pumps.

In this communication, generation of $\Delta\bar{\mu}_{H^+}$ during D-lactate oxidation in membrane vesicles from a cytochrome *d* deficient mutant of *E. coli* is investigated. In addition, D-lactate oxidase activity and D-lactate-dependent $\Delta\bar{\mu}_{H^+}$ generation are reconstituted into proteoliposomes with purified components of the respiratory chain. The results suggest that this electron-transfer pathway contains a single site at which $\Delta\bar{\mu}_{H^+}$ is generated, cytochrome *o*, and that the only vectorial reaction in the pathway involves transfer of electrons through the membrane via the oxidase.

## Experimental Procedures

### *Materials*

Phospholipids were extracted from *E. coli* B with isopropanol/hexane, washed with acetone, dissolved in ether, and stored as described (Viitanen et al., 1986). Cytochrome *o* oxidase (Matsushita et al., 1983, 1984, 1986) and D-LDH (Kaczorowski et al., 1978) were purified as described. Octyl $\beta$-D-glucopyranoside (octyl glucoside), valinomycin, nigericin, phenazine methosulfate (PMS), and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Calbiochem. [$^3$H]Tetraphenylphosphonium ($TPP^+$) and coenzymes $Q_1$ and $Q_8$ were provided by Hoffmann-La Roche, Inc. 3,3′-Diisopropylthiodicarbocyanine [diS-$C_3$-(5)] and bis(1,3-diethylbarbituric acid) pentamethine oxonol [diBA-$C_2$-(5)] were generously donated by Dr. Alan Waggoner. *N*,*N*′,*N*′-Tetramethyl-*p*-phenylenediamine (TMPD), dichlorophenolindophenol (DCI), 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), *p*-(chloromercuri)benzenesulfonate (*p*CMBS), and 2,3-dimercapto-1-propanol (BAL) were obtained from Sigma. Yeast alcohol dehydrogenase was from Boehringer-Mannheim. All other materials were reagent grade and were obtained from commercial sources.

### *Methods*

*Bacterial Strains and Preparation of Membrane Vesicles.* *E. coli* GR19N (*cyd*$^-$) (Green & Gennis, 1983) was grown aerobically into late logarithmic phase in minimal medium A (Kaback, 1971) containing 0.5% sodium lactate and 0.15% casamino acids (pH 7.0). Right-side-out (RSO) membrane vesicles were prepared by osmotic lysis (Kaback, 1971; Short et al., 1975), and inside-out (ISO) vesicles were prepared by passage of cells through a French pressure cell at low shear forces (Hertzberg & Hinkle, 1974; Reenstra et al., 1980). ISO membrane vesicles containing entrapped ferricyanide were prepared by shearing the cells in the presence of 1 mM ferricyanide.

*Enzyme Assays.* Each oxidoreductase activity of the D-lactate oxidase system was measured at room temperature by following the reduction of electron acceptors spectrophotometrically. Reaction mixtures (1 mL total volume) contained 50 mM potassium phosphate ($KP_i$) (pH 7.5), 20 mM D-lactate, 1 mM potassium cyanide, a given electron acceptor, and membrane vesicles. DCI reductase was measured at 600 nm with 125 $\mu$M PMS and 50 $\mu$M DCI, $Q_1$ reductase at 275 nm with 80 $\mu$M $Q_1$, and ferricyanide reductase at 420 nm with 1 mM ferricyanide; activity was calculated by using millimolar extinction coefficients of 16.5, 12.25, and 1.0, respectively.

Oxidase activities were measured with an oxygen electrode at 25 °C. Reaction mixtures (1 mL total volume) contained 50 mM $KP_i$ (pH 7.5), membrane vesicles or proteoliposomes, and given electron donors. The oxygen concentration at 25 °C was assumed to be 258 $\mu$M. D-Lactate, ubiquinol 1 ($Q_1H_2$), and TMPD oxidase activities were measured with 20 mM D-lactate, 80 $\mu$M $Q_1$/5 mM dithiothreitol (DTT), and 2 mM TMPD/10 mM ascorbic acid, respectively.

*$\Delta\Psi$ Determinations.* $\Delta\Psi$ (interior negative) in RSO membrane vesicles was determined from the steady-state distribution of [$^3$H]$TPP^+$ as measured by flow dialysis (Ramos et al., 1979). Alternatively, $\Delta\Psi$ (interior negative) in proteoliposomes was determined by measuring fluorescence quenching of diS-$C_3$-(5) by using valinomycin-induced potassium diffusion potentials for quantification (Waggoner, 1979a).

$\Delta\Psi$ (interior positive) in ISO membrane vesicles was monitored by following fluorescence quenching of diBA-$C_2$-(5) (Waggoner, 1979b). Reaction mixtures (1 mL total volume) contained 50 mM $KP_i$ (pH 7.5), 2 $\mu$M diBA-$C_2$-(5), and ISO membrane vesicles (0.2–0.4 mg of protein). Reactions were carried out at 25 °C, and fluorescence emission was measured at 614 nm with excitation at 588 nm.

*pH Measurements.* Changes in external pH in RSO vesicles were measured directly with a Radiometer pH meter in a closed electrode vessel as described previously (Matsushita et al., 1984; Patel et al., 1982). Reactions were initiated by addition of 10 $\mu$L of air-saturated 150 mM KCl (4.98 nmol of O; Mitchell et al., 1979), and calibration was accomplished by adding aliquots of 1 mM HCl at the end of the experiment. Prior to the measurement, RSO membrane vesicles prepared in 50 mM $KP_i$ (pH 6.6) were diluted into a 3.5-fold excess of 150 mM KCl and centrifuged at 37000*g* for 20 min. The supernatant was discarded and the pellet resuspended in 150 mM KCl. Reaction mixtures (2.5 mL total volume) contained 150 mM KCl, about 0.5 mg of membrane protein, given electron donors, and 1 $\mu$M valinomycin. The reactions were continuously flushed with water-saturated argon, and the pH was adjusted initially to 6.8–7.0 with KOH.

*Reconstitution of Proteoliposomes with D-Lactate Oxidase Activity.* One milliliter of *E. coli* phospholipids (50 mg) prepared as described above was lyophilized and dissolved in 1 mL of ether. $Q_8$ (500 nmol in ethanol) was added, and the solution was evaporated under a stream of argon. The dried sample was dissolved in ether, evaporated under argon once more, and desiccated overnight to remove ether completely. The dried material was then suspended in 1 mL of 2 mM $\beta$-mercaptoethanol, flushed with argon, and sonicated in a bath-type sonicator (Matsushita et al., 1984, 1986) until a clear yellow solution was obtained.

Proteoliposomes containing cytochrome *o* at a protein: phospholipid ratio of 1:125 (w/w) and $Q_8$ at 10 nmol/mg of

Table I: Enzyme Activities of the D-Lactate Oxidase System in GR19N Membrane Vesicles[a]

| act. | RSO membrane vesicles[b] | ISO membrane vesicles[b] | purified D-LDH[b] | purified cytochrome *o* oxidase[b] |
|---|---|---|---|---|
| D-lactate oxidation | 0.197 | 0.160 | | |
| D-lactate/ferricyanide oxidoreductase | 0.095 | 0.062 | 0.94 | |
| D-lactate/$Q_1$ oxidoreductase | 0.279 | 0.249 | 25.2 | |
| D-lactate/DCI oxidoreductase | 0.282 | 0.289 | 22.4 | |
| $Q_1H_2$ oxidation | 1.134 | 1.215 | | 84.0 |
| TMPD oxidation | 0.143 | 0.091 | | 5.8 |

[a] Enzyme activity was measured as described under Experimental Procedures. [b] Values are in units of μmol of lactate oxidized $min^{-1}$ $mg^{-1}$ or μmol of O uptake $min^{-1}$ $mg^{-1}$.

phospholipid were prepared by octyl glucoside dilution followed by one cycle of freeze–thaw/sonication as described (Matsushita et al., 1986; Viitanen et al., 1986). Ninety microliters of proteoliposomes (180 μg of protein/mL) was diluted into 2 mL of 50 mM $KP_i$ (pH 7.5), and 32 μL of purified D-LDH (15.9 units/mL, containing 0.1% Triton X-100) was added rapidly (final Triton X-100 concentration, 0.0015%). The mixture was incubated at room temperature for 1 h and centrifuged at 120000*g* for 90 min. The supernatant was discarded, and the pellet was resuspended in 100 μL of 50 mM $KP_i$ (pH 7.5).

## RESULTS

*D-LDH, Cytochrome o, and D-Lacate Oxidase Activities in Membrane Vesicles.* Since *E. coli* GR19N membranes contain cytochrome *o* as a sole terminal oxidase, the overall reaction of D-lactate oxidation (i.e., D-lactate oxidase activity) may be considered as a linear series of electron-transfer reactions, some of which can be assayed individually. Thus, the ability of membrane vesicles to oxidize $Q_1H_2$ or TMPD directly reflects cytochrome *o* activity, while D-lactate/DCI and D-lactate/$Q_1$ oxidoreductase activities monitor D-LDH (Table I). This notion is supported by the findings that the ratios of D-lactate/$Q_1$ to D-lactate/DCI oxidoreductase activities and $Q_1H_2$ to TMPD oxidase activities are similar in membrane vesicles and in purified D-LDH and cytochrome *o*, respectively. Ferricyanide, on the other hand, probably accepts electrons from a respiratory component located between D-LDH and cytochrome *o* (e.g., ubiquinol), since the electron acceptor is impermeant and D-LDH is present on the cytoplasmic face of the membrane (Short et al., 19755; Owen & Kaback, 1979). Furthermore, the ratio of D-lactate/ferricyanide oxidoreductase activity to D-lactate/$Q_1$ or D-lactate/DCI oxidoreductase activity is significantly higher in membrane vesicles relative to purified D-LDH.

HQNO and thiol reagents such as *p*CMBS and BAL act as respiratory inhibitors in *E. coli* (Barnes & Kaback, 1971; Kaback & Patel, 1978) and are useful for delineating specific electron-transfer reactions (Table II). HQNO, which was shown previously (Matsushita et al., 1984) to inhibit the activity of purified cytochrome *o*, markedly inhibits D-lactate oxidation and $Q_1H_2$ or TMPD oxidase activity with no effect or slight stimulation of D-lactate/DCI, D-lactate/ferricyanide, and D-lactate/$Q_1$ oxidoreductase activities. Conversely, *p*CMBS or BAL inactivates the latter reactions with essentially no effect on terminal oxidase activity. Moreover, purified D-LDH is insensitive to thiol reagents [data not shown; cf. Kaczorowski et al. (1978)]. Therefore, D-lactate oxidation

Table II: Effect of Inhibitors on Enzyme Activities of the D-Lactate Oxidase System in GR19N ISO Membrane Vesicles[a]

| enzyme act. | remaining act. (%) HQNO | *p*CMBS | BAL |
|---|---|---|---|
| D-lactate oxidation | 26 | 8 | |
| D-lactate/ferricyanide oxidoreductase | 112 | | |
| D-lactate/$Q_1$ oxidoreductase | 131 | 9 | |
| D-lactate/DCI oxidoreductase | 97 | 18 | 5 |
| $Q_1H_2$ oxidation | 0 | 96 | 82 |
| TMPD oxidation | 5 | | |

[a] Enzyme activity was measured in ISO membrane vesicles as described under Experimental Procedures. HQNO or *p*CMBS was added directly to the reaction mixture in a final concentration of 33 or 25 μM, respectively. For BAL inhibition, ISO membrane vesicles were treated with BAL before enzyme assay: 100 μL of ISO membrane vesicles at a concentration of 8.9 mg of protein/mL in 50 mM $KP_i$ (pH 7.5) was mixed with 1 μL of 500 mM BAL dissolved in ethanol. The mixture was incubated at 25 °C for 45 min under oxygen. The reaction was stopped by flushing the mixture with argon and incubating on ice. A control sample was treated in the same way except for addition of 1 μL of ethanol instead of BAL.

Table III: $\Delta\Psi$ Generation in GR19N RSO Membrane Vesicles[a]

| electron donor | $\Delta\Psi$ (interior negative) (mV) −nigericin | +nigericin |
|---|---|---|
| D-lactate | 113 ± 5 (6) | 128 ± 5 (7) |
| $Q_1H_2$ | 109 ± 5 (4) | 113 ± 9 (4) |
| TMPD | 87 ± 10 (4) | 95 ± 6 (4) |

[a] $\Delta\Psi$ was measured by flow dialysis by using [$^3$H]$TPP^+$ as described under Experimental Procedures. The reaction mixtures (200 μL) contained RSO membrane vesicles, 50 mM $KP_2$ (pH 6.6), and 20 μM [$^3$H]$TPP^+$ (2.5 Ci/mmol). To start the reactions, 10 mM D-lactate, 5 mM DTT/16 μM $Q_1$, or 10 mM ascorbate/2 mM TMPD were added as electron donors. Where indicated, 0.125 μM nigericin was added. To terminate the reaction, 5 μM valinomycin was added. Data are given ±SD with tne number of experiments in parentheses.

in this system requires a minimum of three proteinaceous components in the following order—D-LDH, a thiol-containing component that probably precedes ubiquinone, and the *o*-type cytochrome oxidase.

*Site and Mechanism of $\Delta\bar{\mu}_{H^+}$ Generation during D-Lactate or NADH Oxidation.* In RSO vesicles from *E. coli* GR19N, D-lactate oxidation produces a $\Delta\Psi$ of approximately −128 mV in the presence of nigericin (Table III), a value comparable to that observed previously (Ramos et al., 1976) under similar conditions. $Q_1H_2$ or TMPD, which donate electrons directly to cytochrome *o* oxidase (Matsushita et al., 1984), also generate $\Delta\Psi$ values of similar magnitudes. On the other hand, electron flow from D-lactate to ferricyanide does not appear to generate a significant $\Delta\Psi$, an observation that will be documented below.

Generation of $\Delta\Psi$ was also studied in ISO vesicles by using fluorescence quenching of diBA-$C_2$-(5), a voltage-sensitive probe that responds to $\Delta\Psi$, interior positive (Waggoner, 1979b) (Figure 1). As shown, the extent of fluorescence quenching observed with D-lactate and $Q_1H_2$ are comparable but significantly less than that observed with NADH, an electron donor that has been postulated to provide electron flow through an additional "coupling site" (Poole & Haddock, 1975). The differences are compared quantitatively in Figure 2, where the extent of fluorescence quenching of diBA-$C_2$-(5) is related to the rate of oxygen uptake in the presence of the appropriate electron donors. At the same rates of oxidation, D-lactate and $Q_1H_2$ produce similar extents of fluorescence quenching, while NADH is significantly more effective.

Potassium cyanide is an effective inhibitor of cytochrome *o* (Matsushita et al., 1984) that completely blocks $\Delta\Psi$ generation in the presence of $Q_1H_2$ (Figure 1–4). By the study

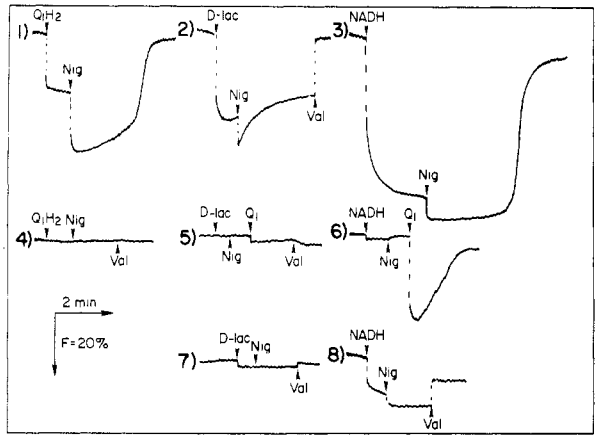


FIGURE 1: Fluorescence quenching of diBA-$C_2$-(5) in ISO vesicles from *E. coli* GR19N. Reactions 1–3 were carried out as described under Experimental Procedures with 0.22 mg of membrane protein. To initiate the reactions, 2.5 mM DTT and 16 $\mu$M $Q_1$ ($Q_1H_2$) or 10 mM D-lactate (D-lac) or 1 mM NADH were added to the reaction mixtures, as indicated. Reactions 4–6 were carried out in the same manner except that 2.5 mM potassium cyanide was added to each of the reaction mixtures and 80 $\mu$M $Q_1$ was added to reactions 5 and 6 during the course of the experiments as indicated. Reactions 7 and 8 were performed with ISO vesicles containing entrapped ferricyanide (0.42 mg of membrane protein) in the presence of 2.5 mM potassium cyanide. Where indicated, nigericin (Nig) and valinomycin (Val) were added to final concentrations of 0.025 and 1.0 $\mu$M, respectively. Fluorescence was monitored continuously as described under Experimental Procedures.

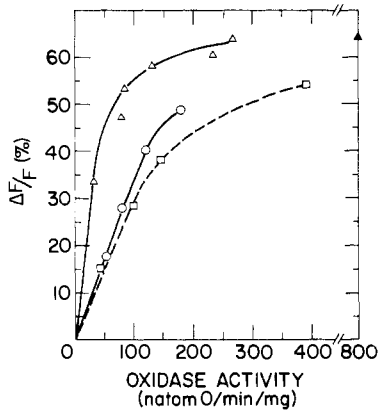


FIGURE 2: Relationship between diBA-$C_2$-(5) quenching and oxidase activity in ISO vesicles from *E. coli* GR19N. Fluorescence quenching and oxidase activity were measured independently with varying concentrations of given electron donors as described under Experimental Procedures. With NADH as electron donor, low concentrations (2–200 $\mu$M) were maintained with an NADH regenerating system consisting of NAD, 50 $\mu$g/mL yeast alcohol dehydrogenase, and 100 mM ethanol. (△) NADH; (▲) 1 mM NADH without the regenerating system; (○) D-lactate; (□) $Q_1H_2$.

of diBA-$C_2$-(5) quenching in the presence of the inhibitor, $\Delta\Psi$ generation during electron flow from D-lactate or NADH to $Q_1$ or ferricyanide can be measured. As shown in parts 5 and 7 of Figure 1, electron flow from D-lactate to $Q_1$ or ferricyanide, respectively, does not lead to diBA-$C_2$-(5) quenching, suggesting that electron flow over this portion of the respiratory chain is not electrogenic. In contradistinction, significant quenching is observed during electron flow from NADH to either $Q_1$ (Figure 1–6) or ferricyanide (Figure 1–8).

Although $H^+$ translocation has been measured in *E. coli* with D-lactate or $Q_1H_2$ as electron donors, and $H^+/O$ ratios of 2 were reported (Poole & Haddock, 1975; Jones et al., 1980), a *d*-type terminal oxidase was probably present in addition to cytochrome *o*. Furthermore, studies of this nature with intact cells or spheroplasts are complicated by high rates

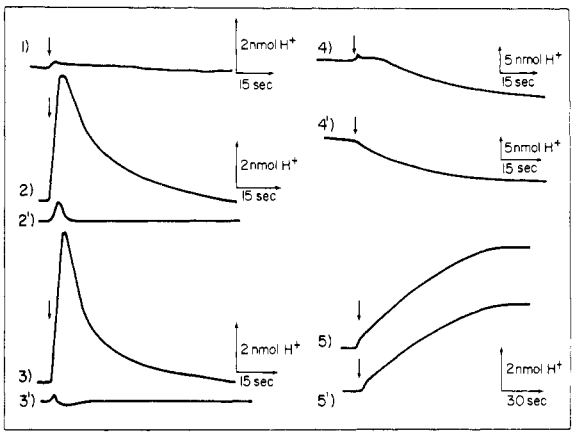


FIGURE 3: External pH changes in GR19N RSO vesicles. Experiments were performed as described under Experimental Procedures. Reaction 1 was carried out in the absence of exogenous electron donors. Reactions 2 and 2′ were carried out in the presence of 2.5 mM DTT and 16 $\mu$M $Q_1$ without and with 0.2 $\mu$M nigericin, respectively. Reactions 3 and 3′ were carried out in the presence of 10 mM D-lactate without and with 2 $\mu$M CCCP, respectively. Reactions 4 and 4′ were carried out with 1 mM TMPD in the absence and presence of 2 $\mu$M CCCP, respectively. Reactions 1–4 were initiated by addition of 10 $\mu$L of air-saturated 150 mM KCl (4.98 nmol of O). Reactions 5 and 5′ contained 10 mM D-lactate and 1 mM potassium cyanide without and with 2 $\mu$M CCCP, respectively, and were initiated by adding 5 nmol of ferricyanide which was adjusted to the same pH as the reaction mixture and flushed with argon.

Table IV: $H^+/2e^-$ Stoichiometries[a]

| act. | $H^+/2e^-$ ratio −CCCP | $H^+/2e^-$ ratio +CCCP |
|---|---|---|
| D-lactate oxidation | 1.78 ± 0.26 (7) | 0 |
| $Q_1H_2$ oxidation | 1.78 ± 0.13 (6) | 0 |
| TMPD oxidation | | −1.66 ± 0.16 (4) |
| D-lactate/ferricyanide oxidoreductase | 1.90 ± 0.24 (5) | 1.78 ± 0.28 (5) |

[a] Reactions were performed as described in Figure 3. $H^+/2e^-$ ratios for D-lactate and $Q_1H_2$ oxidation were calculated as described by Mitchell et al. (1979). $H^+/2e^-$ ratios for TMPD oxidation and for D-lactate/ferricyanide oxidoreductase were calculated from the steady-state levels of alkalinization and acidification, respectively. Where indicated, CCCP was added to 2 $\mu$M final concentration. Data are given ±SD with the number of experiments in parentheses.

of endogenous respiration. For these reasons, we carried out similar measurements with RSO vesicles from GR19N. As shown (Figure 3-1), the vesicles do not exhibit $H^+$ translocation due to endogenous respiration. When a limited amount of oxygen is introduced in the presence of $Q_1H_2$ (Figure 3-2) or D-lactate (Figure 3-3), however, the medium rapidly becomes acidic until oxygen is depleted, at which point the extruded $H^+$ flows back into the vesicles, and the phenomena are blocked by addition of appropriate ionophores (Figure 3-2′,3′). As shown previously (Matsushita et al., 1984), TMPD, an unprotonated electron carrier, causes slow alkalinization of the medium, the rate of which is accelerated by CCCP (Figure 3-4,4′). Finally, electron transfer from D-lactate to ferricyanide results in acidification of the external medium (Figure 3-5), but the process is unaffected by CCCP (Figure 3-5′). Comparison of the extent of acidification or alkalinization with the amount of oxygen added permits calculation of $H^+/2e^-$ ratios under the conditions described (Table IV). In each instance, the stoichiometry approximates a $H^+/2e^-$ ratio of 2.

*D-Lactate Oxidation and $\Delta\bar{\mu}_{H^+}$ Generation in Reconstituted Proteoliposomes.* Purified cytochrome *o* oxidase can be reconstituted functionally into proteoliposomes by using octyl glucoside dilution followed by one cycle of freeze–thaw/sonication (Matsushita et al., 1983, 1984, 1986), and proteoli-

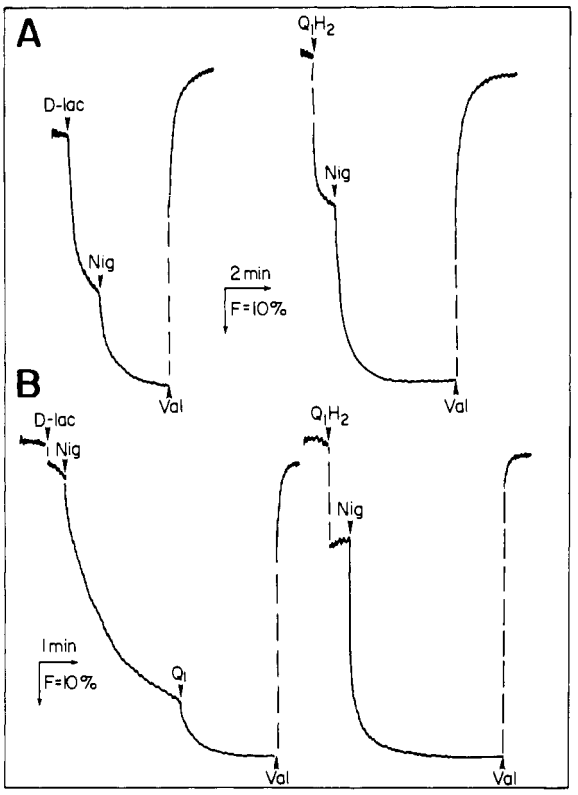


FIGURE 4: Fluorescence quenching of diS-$C_3$-(5) in GR19N RSO membrane vesicles (panel A) and proteoliposomes reconstituted with D-LDH, $Q_8$, and cytochrome *o* (panel B). Reaction mixtures (2 mL total volume) contained 50 mM $KP_i$ (pH 6.6 or pH 7.5 for vesicles or proteoliposomes, respectively), 1 $\mu$M diS-$C_3$-(5), and RSO vesicles (24 $\mu$g of protein) or proteoliposomes (1.45 $\mu$g of protein). Reactions were initiated by adding 10 mM D-lactate (D-lac) or 2.5 mM DTT and 16 $\mu$M $Q_1$ ($Q_1H_2$), followed by addition of 0.025 $\mu$M nigericin (Nig) and 1 $\mu$M valinomycin (Val), as indicated. $Q_1$ was added to a final concentration of 16 $\mu$M, as shown in panel B on the left. Fluorescence was measured as described under Experimental Procedures.

posomes formed in this manner are able to bind purified D-LDH in much the same manner as RSO membrane vesicles (Short et al., 1975; Haldar et al., 1982). Addition of $Q_1$ to oxidase proteoliposomes containing bound D-LDH allows D-lactate oxidation and concomitant generation of $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) (data not shown). However, the activities observed under these conditions reflect the ability of $Q_1$ to act as a water-soluble electron carrier in the bulk phase. That is, electron flow occurs from D-LDH to $Q_1$ in the bulk phase and from $Q_1H_2$ to oxygen via cytochrome *o*.

As demonstrated with cytochrome *d* oxidase (Koland et al., 1984), $Q_8$, the physiological ubiquinone in *E. coli*, can be incorporated into proteoliposomes where it functions as an electron-transfer intermediate within the bilayer. Similarly, proteoliposomes containing cytochrome *o*, *E. coli* phospholipids, and $Q_8$ with purified D-LDH bound to the outer surface catalyze D-lactate oxidation (Table V). However, D-lactate oxidase activity in this system, as opposed to RSO vesicles from GR19N, is stimulated by addition of $Q_1$ and is relatively insensitive to *p*CMBS. Furthermore, in the reconstituted oxidase system in the absence of $Q_1$, the ratio of the D-lactate oxidase activity to the D-lactate/DCI oxidoreductase activity is only about 0.1, compared to a value of 0.55–0.88 in membrane vesicles (cf. Table I, in addition).

Like RSO vesicles, proteoliposomes reconstituted with cytochrome *o*, $Q_8$, and D-LDH generate $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) in the presence of D-lactate or $Q_1H_2$, as demonstrated by fluorescence quenching with diS-$C_3$-(5) (Figure

Table V: D-Lactate Oxidation and D-LDH Activity in Reconstituted Proteoliposomes and GR19N RSO Membrane Vesicles[a]

| | | proteoliposomes | | RSO membrane vesicles | |
|---|---|---|---|---|---|
| act. | $Q_1$ | units/mg | % act. with *p*CMBS | units/mg | % act. with *p*CMBS |
| D-lactate/DCI oxidoreductase | | 17.1 | 107 | 0.150 | 49 |
| D-lactate oxidation | − | 1.71 | 84 | 0.132 | 21 |
| D-lactate oxidation | + | 8.42 | 85 | 0.121 | 28 |

[a] Proteoliposomes reconstituted with purified D-LDH, $Q_8$, and cytochrome *o* were prepared as described under Experimental Procedures. D-LDH activity was measured with PMS and DCI as described. Where indicated, $Q_1$ and/or *p*CMBS were/was added to the reaction mixture at concentrations of 16 and/or 25 $\mu$M, respectively.

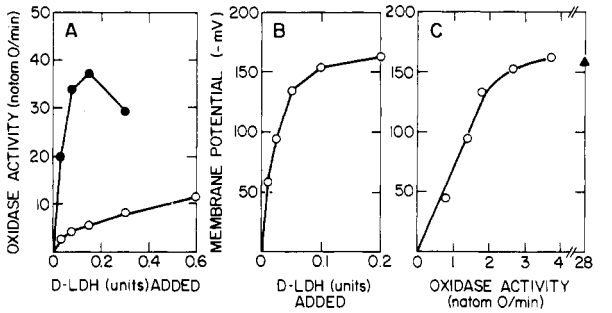


FIGURE 5: D-Lactate oxidation and D-lactate-dependent $\Delta\Psi$ generation in proteoliposomes reconstituted with D-LDH, $Q_8$, and cytochrome *o*. Proteoliposomes containing $Q_8$, cytochrome *o*, and given amounts of purified D-LDH were prepared as described under Experimental Procedures. Under the conditions described, all of the D-LDH added was bound to the proteoliposomes. (A) D-Lactate oxidation was measured with an oxygen electrode in reaction mixtures (1 mL total volume) containing 50 mM $KP_i$ (pH 7.5) and proteoliposomes containing given amounts of D-LDH, $Q_8$, and 2.75 $\mu$g of cytochrome *o* in the presence (●) and absence (○) of 16 $\mu$M $Q_1$. Reactions were initiated by addition of 10 mM D-lactate. (B) Fluorescence quenching of diS-$C_3$-(5) was measured as described in Figure 4. Reaction mixtures (2 mL total volume) contained 50 mM $KP_i$ (pH 7.5), 1 $\mu$M diS-$C_3$-(5), 0.025 $\mu$M nigericin, and proteoliposomes containing given amounts of D-LDH, $Q_8$, and 0.92 $\mu$g of cytochrome *o*. D-Lactate (10 mM final concentration) was added to initiate the reactions. The $\Delta\Psi$ generated in each case was determined from a calibration curve derived from experiments in which potassium diffusion potentials of known magnitude were imposed in the presence of valinomycin. (C) Relationship between D-lactate oxidase activity and $\Delta\Psi$ generation. Data were taken from panels A and B. (▲) $Q_1H_2$ oxidase activity and $\Delta\Psi$ generation in proteoliposomes containing the same amounts of $Q_8$ and cytochrome *o* but no D-LDH.

4). Although D-lactate-induced quenching in proteoliposomes is slow relative to that observed with $Q_1H_2$, the extent of fluorescence quenching is similar with either electron donor (Figure 4B). However, the rate of D-lactate oxidation and the extent of fluorescence quenching of diS-$C_3$-(5) (i.e., the magnitude of the $\Delta\Psi$ generated) is dependent upon the amount of D-LDH bound to the proteoliposomes over a certain range (Figure 5). As shown in Figure 5A, D-lactate oxidase activity in the reconstituted system continues to increase even when 0.6 units of D-LDH is bound. In the same type of experiments with pyruvate oxidase (Koland et al., 1984), the rate of pyruvate oxidation is saturated when 4 units of enzyme are bound by the proteoliposomes (data not shown). However, since pyruvate oxidase is soluble in aqueous solution in the absence of detergent, very high concentrations can be added to the proteoliposomes. In other words, the rate of D-lactate oxidation in the reconstituted system might increase to that observed during $Q_1H_2$ oxidation if sufficient amounts of D-

LDH were added. The magnitude of the $\Delta\Psi$ achieved during D-lactate oxidation increases to about −150 mV over a range of 0–0.1 units of D-LDH added and increases slightly between 0.1 and 0.2 units of enzyme (Figure 5B). Therefore, relatively low rates of oxidation (2–3 nmol of O/min) are sufficient to generate a maximal $\Delta\Psi$ (Figure 5C). It is also noteworthy that incorporation of partially purified (ca. 80%) cytochrome *b*-556 into proteoliposomes with cytochrome *o*, $Q_8$ and D-LDH makes no significant difference in either D-lactate oxidase activity or D-lactate-induced $\Delta\Psi$ generation (data not shown).

## Discussion

The results presented here provide a strong indication that the respiratory chain of *E coli* GR19N that catalyzes electron transfer from D-lactate to oxygen contains a single site at which $\Delta\bar{\mu}_{H^+}$ is generated across the membrane. This conclusion is consistent with the following observations: (i) D-lactate as well as $Q_1H_2$ oxidation in RSO vesicles exhibits a "protonmotive stoichiometry" that approximates 1 $H^+/e^-$. (ii) In RSO vesicles, D-lactate oxidation generates a $\Delta\bar{\mu}_{H^+}$ of the same magnitude and polarity as $Q_1H_2$ oxidation. (iii) In ISO vesicles, oxidation of D-lactate or $Q_1H_2$ generates similar $\Delta\Psi$ values, as judged by quenching of diBA-$C_2$-(5) fluorescence, while the same rates of NADH oxidation are much more effective. (iv) Electron transfer from D-lactate to $Q_1$ or ferricyanide does not appear to generate $\Delta\Psi$ in ISO vesicles, while electron transfer from NADH to the same electron acceptors leads to $\Delta\Psi$ generation. (v) Proteoliposomes reconstituted with purified D-LDH, $Q_8$, and purified cytochrome *o* generate a $\Delta\bar{\mu}_{H^+}$ of the same polarity and similar magnitude as RSO vesicles during D-lactate or $Q_1H_2$ oxidation.

In addition, these observations in conjuction with previous experiments (Matsushita et al., 1984) suggest that the pathway of electron transfer from either D-lactate or $Q_1H_2$ to oxygen utilizes the same site for $\Delta\bar{\mu}_{H^+}$ generation, cytochrome *o*. Thus, it has been demonstrated that proteoliposomes reconstituted with purified cytochrome *o* establish a very significant $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) during turnover in the presence of $Q_1H_2$. Apparently, the $\Delta$pH generated in the reconstituted system results from scalar (i.e., nonvectorial) reactions that consume and release $H^+$ at the inner and/or outer surface of the membrane, respectively, while $\Delta\Psi$ is due to vectorial flow of electrons from the outer to the inner surface of the membrane through the oxidase. Such a $H^+$ translocation mechanism is confirmed here with GR19N RSO membrane vesicles. As shown in Figure 3, during $Q_1H_2$ oxidation, $H^+$ is released outside and consumed inside, while with unprotonated TMPD, little change in external pH is observed until CCCP is added, at which point the medium becomes alkaline. On the other hand, in the case of D-lactate oxidation, the membrane vesicles may extract $H^+$ from inside and release them outside, unlike $Q_1H_2$ oxidation. However, electron flow from D-lactate, which is oxidized at inner surface of the membrane by D-LDH, to ferricyanide outside translocates 1 $H^+$, per $e^-$ from the inside to outside by a process that is not electrogenic. This implies that $H^+$ must be translocated with $e^-$ across the membrane. A carrier that is able to carry both $H^+$ and $e^-$ and to transverse the membrane is ubiquinol, which also reacts with cytochrome *o*. Thus, it seems highly likely that cytochrome *o* represents the only site in the respiratory chain between D-LDH or ubiquinone and oxygen at which $\Delta\bar{\mu}_{H^+}$ is generated.

As a simple scheme that can account for the data obtained here with *E. coli* GR19N, we propose a model (Figure 6) similar to that suggested by Mitchell (1966) and by Poole and Haddock (1975). As shown, the only component in the pathway between D-LDH or ubiquinol and oxygen that is directly involved in the generation of $\Delta\bar{\mu}_{H^+}$ is cytochrome *o*, which catalyzes the scalar release of 2 $H^+$ from ubiquinol on the outer surface of the membrane, vectorial transfer of 2 $e^-$ from the outer to the inner surface, and scalar utilization of 2 $H^+$ on the inner surface to reduce oxygen. On the other hand, between NADH and oxygen, $\Delta\bar{\mu}_{H^+}$ generation occurs at two sites, one between NADH dehydrogenase and ubiquinone by an unspecified mechanism and the other at cytochrome *o*, as described above.

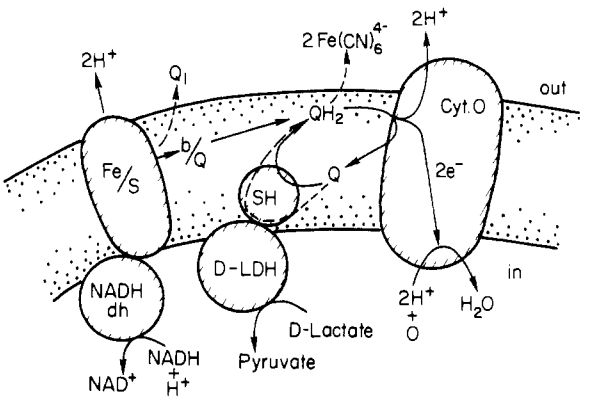


FIGURE 6: Postulated pathways for electron transfer from NADH and D-lactate to oxygen in the *E. coli* GR19N respiratory chain with sites at which $\Delta\bar{\mu}_{H^+}$ is generated. As indicated, the only component in the pathway between D-LDH or ubiquinol that is directly involved in the generation of a proton electrochemical gradient is cytochrome *o*, which catalyzes the scalar release of 2 $H^+$ from ubiquinol on the outer surface of the membrane, vectorial transfer of 2 $e^-$ from the outer to the inner surface, and scalar utilization of 2 $H^+$ on the inner surface to reduce oxygen. Between NADH and oxygen, generation of a proton electrochemical gradient is postulated to occur at two sites, one between NADH dehydrogenase (NADH dh) and ubiquinone by an unspecified mechanism and the other at cytochrome *o*. Fe/S, non-heme iron sulfur; b, cytochrome *b*-556; SH, thiol-sensitive component between D-LDH and ubiquinone (cf. text); Cyt. O, cytochrome *o*.

At face value, the results are also inconsistent with the notion that either a protonmotive ubiquinone cycle or a primary $H^+$ pump is involved in D-lactate oxidation in this system. The so-called Q cycle translocates 2 $H^+$ during transfer of a single $e^-$; however, during D-lactate oxidation, only 1 $H^+$ seems to be translocated per $e^-$. Similarly, the activity of a primary $H^+$ pump would be expected to yield a $H^+/e^-$ stoichiometry that is greater than unity. Such a pump should also be electrogenic because positive charge is transported across the membrane, but neither D-lactate/$Q_1$ nor D-lactate/ferricyanide oxidoreductase activity leads to generation of $\Delta\Psi$.

Previous studies (Barnes & Kaback, 1971; Kaback & Patel, 1978) demonstrate that D-lactate oxidation in *E. coli* membrane vesicles is inhibited by thiol reagents. The finding is confirmed here. Furthermore, by comparing several electron-transfer activities in membrane vesicles and purified D-LDH, it is shown that the site of inhibition is near the primary dehydrogenase. Proteoliposomes reconstituted with purified D-LDH appear to lack the thiol-sensitive component and to catalyze electron transfer at a relatively low rate. However, the reconstituted system is clearly able to generate $\Delta\bar{\mu}_{H^+}$. Therefore, as suggested (Kaback & Patel, 1978; Haldar et al., 1982), although the thiol-sensitive component is not involved in $\Delta\bar{\mu}_{H^+}$ generation, it may be the rate-limiting step in electron transfer from D-LDH to oxygen.

Finally, it is noteworthy that previous experiments from this laboratory with RSO vesicles from *E. coli* ML 308-225 (Barnes & Kaback, 1971; Konings et al., 1971), which is presumably wild-type with respect to the respiratory chain, demonstrate that a variety of electron donors, NADH in

particular, reduces all species of cytochromes to the same extent as D-lactate or ascorbate/PMS without generating $\Delta\bar{\mu}_{H^+}$ (Ramos et al., 1976). RSO vesicles from GR19N are dramatically different in their behavior in that any oxidizable substrate, NADH included, generates $\Delta\bar{\mu}_{H^+}$ and drives transport (unpublished observations). Although this difference may be very important, an explanation is not readily apparent.

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**Registry No.** D-LDH, 9028-36-8; $Q_1H_2$, 52590-98-4; TMPD, 100-22-1; DCI, 956-48-9; $Q_1$, 727-81-1; NADH, 58-68-4; $Q_8$, 2394-68-5; H, 12408-02-5; cytochrome *o* oxidase, 9035-48-7; D-lactate, 10326-41-7; ferricyanide, 13408-62-3.

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