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Reconstitution of the Membrane-Bound, Ubiquinone-Dependent Pyruvate Oxidase Respiratory Chain of *Escherichia coli* with the Cytochrome *d* Terminal Oxidase†

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ABSTRACT: Pyruvate oxidase is a flavoprotein dehydrogenase located on the inner surface of the *Escherichia coli* cytoplasmic membrane and coupled to the *E. coli* aerobic respiratory chain. In this paper, the role of quinones in the pyruvate oxidase system is investigated, and a minimal respiratory chain is described consisting of only two pure proteins plus ubiquinone 8 incorporated in phospholipid vesicles. The enzymes used in this reconstitution are the flavoprotein and the recently purified *E. coli* cytochrome *d* terminal oxidase. The catalytic velocity of the reconstituted liposome system is about 30% of that observed when the flavoprotein is reconstituted with *E. coli* membranes. It is also shown that electron transport from pyruvate to oxygen in the liposome system generates a transmembrane potential of at least 180 mV (negative inside), which is sensitive to the uncouplers carbonyl cyanide *p*-(trichloromethoxy)phenylhydrazone and valinomycin. A transmembrane potential is also generated by the oxidation of ubiquinol 1 by the terminal oxidase in the absence of the flavoprotein. It is concluded that (1) the flavoprotein can directly reduce ubiquinone 8 within the phospholipid bilayer, (2) menaquinone 8 will not effectively substitute for ubiquinone 8 in this electron-transfer chain, and (3) the cytochrome *d* terminal oxidase functions as a ubiquinol 8 oxidase and serves as a "coupling site" in the *E. coli* aerobic respiratory chain. These investigations suggest a relatively simple organization for the *E. coli* respiratory chain.

Many of the components of the *Escherichia coli* aerobic electron-transport chain have now been identified. The respiratory chain is branched at both the substrate side and the oxygen side. There are several flavoprotein dehydrogenases, including the pyruvate oxidase flavoprotein, that feed electrons into the chain [see Bragg (1979)]. Ubiquinone 8 is clearly an important component, as demonstrated by the use of ubiquinone-deficient strains in studies of the NADH, succinate, and D-lactate oxidase systems (Cox et al., 1970; Wallace & Young, 1977; Downie & Cox, 1978). The respiratory chain contains two terminal oxidases. Both the cytochrome *d* terminal oxidase complex and the cytochrome *o* terminal oxidase complex have now been purified to homogeneity. The cytochrome *d* complex contains cytochromes b_{558}, a_1, and *d* and has two subunits as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[1] (Miller & Gennis, 1983). The cytochrome *o* complex is a *b* type cytochrome with absorption peaks at 555 and 562 nm, which has four subunits by SDS–PAGE analysis (Kranz & Gennis, 1983; Matsushita et al., 1983) and has been shown to function as a "coupling site" in vitro with ubiquinol 1 (UQ-1) as a reductant (Matsushita et al., 1983; Kita et al., 1982). Together, these two terminal oxidases account for most of the cytochromes that have been identified by spectroscopic and electrochemical methods of analysis of the membranes (Shipp, 1972; Scott & Poole, 1982; Reid & Ingledew, 1979; Van Wielink et al., 1982; Pudek & Bragg, 1976; Hendler et al., 1975). One additional component, cytochrome b_{556}, has also been purified (Kita et al., 1978), and this cytochrome has been suggested to be part of the "*o* branch" (Kita & Anraku, 1981), though this has yet to be rigorously demonstrated.

Pyruvate oxidase is a flavoprotein dehydrogenase that has been isolated from *E. coli* (Williams & Hager, 1966; O'Brien et al., 1976). The enzyme catalyzes the oxidative decarboxylation of pyruvate to yield acetate plus CO_2 and is located on the inner surface of the cytoplasmic membrane (Shaw-Goldstein et al., 1978). Previous work has shown that when *E. coli* membrane vesicles are supplemented with the purified flavoprotein, pyruvate-driven oxygen consumption is observed (Shaw-Goldstein et al., 1978; Deeb & Hager, 1964; Cunningham & Hager, 1975). The pure enzyme does not react rapidly with oxygen, but significant oxidase activity is manifested in the presence of *E. coli* membranes. Pyruvate oxidase flavoprotein, like other *E. coli* flavoprotein dehydrogenases (Jaworowski et al., 1981; Haldar et al., 1982; Schryvers et al., 1978), appears to couple to the aerobic respiratory chain of the cytoplasmic membrane.

The pyruvate oxidase flavoprotein is unique among these dehydrogenases in that it is a peripheral membrane protein and easily dissociates from the membrane in a water-soluble form. This has been an advantage in studying the enzymology and lipid-binding properties of the enzyme (e.g., Schrock &

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[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UQ, ubiquinone; MK, menaquinone; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; CCCP, carbonyl cyanide *p*-(trichloromethoxy)phenylhydrazone; TPP, thiamin pyrophosphate. In UQ and MK, the number of isoprene units in the side chain is indicated by a number following the abbreviation.

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Gennis, 1977; Mather et al., 1982; Koland & Gennis, 1982), but it has hindered efforts to study the endogenous system because of the ease with which the enzyme dissociates from the membrane [see Shaw-Goldstein et al. (1978)]. Previous studies have demonstrated that the affinity of the flavoprotein for lipids is allosterically regulated. The reduced flavoprotein has an altered conformation compared to the oxidized form (Russell et al., 1977a) and has a higher affinity for phospholipids and detergents (Schrock & Gennis, 1980, 1977; O'Brien et al., 1982). The binding of amphiphiles to the flavoprotein has been shown to kinetically activate the enzyme (Blake et al., 1978) as has proteolysis under certain conditions (Recny & Hager, 1982; Russell et al., 1977a,b).

The involvement of quinones in the reconstituted pyruvate oxidase system has been previously indicated (Cunningham & Hager, 1975), but the exact functions and identity of the quinones were not determined. In the present work, an attempt has been made to identify the function of quinone in this system. By reconsititution of the flavoprotein and the cytochrome *d* complex together, the ubiquinone-dependent electron-transport chain from pyruvate to oxygen has been successfully reconstructed in artificial phospholipid vesicles. This electron-transfer reaction generates a transmembrane electrical potential across the bilayer of at least 180 mV (inside negative). The data indicate that ubiquinone 8 is the primary electron acceptor for the pyruvate oxidase flavoprotein and that the cytochrome *d* terminal oxidase complex functions as a ubiquinol 8 oxidase.

Experimental Procedures

Materials. The following materials were supplied by the indicated firms: pyruvate, thiamin pyrophosphate (TPP), cholic acid, phenylmethanesulfonyl fluoride, and crude soybean phosphatidylcholine (type IV-S) (Sigma Chemical Co); octyl β-D-glucoside, ubiquinone 10 (UQ-10), CCCP, valinomycin, nigericin, phosphatidylglycerol (egg), and phosphatidylethanolamine (*E. coli*) (Calbiochem-Behring); sodium dodecyl sulfate (Pierce Chemical Co.); sodium ferricyanide (Pfaltz and Bauer, Inc.). Ubiquinone 1 (UQ-1) was the kind gift of Hoffmann-La Roche. Cyanine dyes and oxonol VI were obtained from Molecular Probes, Inc. Cholic acid was recrystallized from 70% ethanol before use.

Enzyme Preparations. The purification of the flavoprotein was performed as previously described (O'Brien et al., 1976). In some cases, a second DEAE chromatography step was substituted for the TPP affinity chromatography step. Enzyme that was used had a specific activity greater than 250 μmol of ferricyanide min^{-1} mg^{-1} in the standard assay (Blake et al., 1978). This specific activity corresponds to about 5000 decarboxylase units/mg as reported in early publications on this enzyme [e.g., Williams & Hager (1966)].

The preparation of the cytochrome *d* terminal oxidase complex was performed as recently described (Miller & Gennis, 1983). The specific heme *b* content of the terminal oxidase used in these studies was approximately 18 nmol/mg of protein.

Isolation of Quinones. Ubiquinone 8 (UQ-8) and menaquinone 8 (MK-8) were purified from lyophilized stationary phase *E. coli* cells as follows. First, 75 g of cells was extracted for 10 h by being stirred in a mixture of 900 mL of methanol and 900 mL of pentane. Then, 200 mL of aqueous 0.1 M ferric chloride was added to oxidize the quinones. After centifugation, the upper pentane phase was collected and dried in a rotary evaporator. The brown residue was dissolved in a small amount of hexane and applied to a 30-mL silica gel column equilibrated with hexane. The column was then washed with 60 mL of hexane. The quinones were eluted with 2% ethyl acetate in hexane. Menaquinone eluted rapidly as a yellow band followed by a dark orange ubiquinone-containing band. The menaquinone fraction was concentrated and used without further purification. The ubiquinone fraction was dried, redissolved in 3 mL of hexane, and applied to a small alumina column (activity grade I, Sigma). After a hexane wash, ubiquinone was eluted with 2.5% acetone in hexane. The menaquinone and ubiquinone were judged pure by thin-layer chromatography. Mass spectroscopic and ultraviolet spectroscopic analyses indicated that MK-8 and UQ-8 had been purified. Some preparations of MK-8 may have contained small amounts of demethylmenaquinone 8.

Strains and Growth Conditions. Stationary-phase *E. coli* MR43L(F152) cells were grown as previously described (Miller & Gennis, 1983). The cytochrome *d* deficient strain, GR19N, and growth conditions were described previously (Green & Gennis, 1983). The strains AN384, AN385, AN386, and AN387 (Wallace & Young, 1977) were gifts of I. G. Young, John Curtin School of Medical Research, Australian National University, Canberra. They were grown in a basal medium (Cohen & Rickenburg, 1956) supplemented with 1.0 g/L yeast extract, 10 g/L glucose, and 10 mg/L thiamin hydrochloride, with aeration at 37 °C. Approximately 3 g/L of the strains AN385, AN386, and AN387 was harvested. The yield of AN384, the strain lacking both ubiquinone and menaquinone, was 10–fold lower. The phenotypes of the cells were verified by extraction of quinones and ultraviolet spectroscopic analysis (see below). As judged by reduced minus oxidized spectroscopy (see below), the specific heme *b* and heme *d* contents of membranes prepared from these strains were essentially the same for all four strains.

Isolation of Cell Membranes. Cell paste (3 g) was suspended in 15 mL of 0.1 M potassium phosphate at pH 7.0. Cells were disrupted by sonication and unbroken cells removed by centrifugation at 10000*g* for 15 min. Membranes were collected by centrifugation at 140000*g* for 1 h and resuspended in 0.1 M potassium phosphate buffer at pH 7.0 by brief sonication.

Proteolytic Activation of Pyruvate Oxidase Flavoprotein. Activation of the flavoprotein by α-chymotrypsin was performed as previously described (Russell et al., 1977a). Action of the protease was subsequently inhibited by the addition of 0.1 mg/mL phenylmethanesulfonyl fluoride from a 2-propanol stock. An unactivated control sample was prepared in an identical manner except that phenylmethanesulfonyl fluoride was added to the flavoprotein solution prior to the addition of the protease.

Reconstitution Methods. To prepare homogeneous mixtures of quinones and phospholipids, the lipids and quinone solutions in organic solvents were first combined, and the solvent was evaporated under a stream of nitrogen. The compositions of the mixtures used in each experiment are described in the figure legends. The solid lipid–quinone mixtures were twice redissolved in diethyl ether and dried under nitrogen. Vesicles were prepared from the dried lipids by two different means: detergent dialysis and sonication. To form vesicles by detergent dialysis (Racker, 1979; Jackson & Litman, 1982), lipids were dissolved at 20 mg/mL in either 0.1 M potassium phosphate, 0.2 M potassium chloride, and 30 mg/mL octyl β-D-glucoside or 0.1 M potassium phosphate and 2.0% cholate at pH 7.0 and briefly sonicated. In most cases, cholate was used. The solutions were then dialyzed against three changes of detergent-free buffer for 8 h each. When the terminal oxidase complex was to be incorporated in phospholipid ves-

icles, the cholate-solubilized complex was added to the lipid–detergent solution just prior to dialysis.

Vesicles loaded with sodium ferricyanide were prepared by sonication (Futami et al., 1979). Solid lipid mixtures were suspended at 20 mg/mL in 50 mM sodium phosphate, 10 mM magnesium chloride, and 0.2 M sodium ferricyanide at pH 6.0 by sonication. External ferricyanide was removed by passing the vesicle suspension through a Sephadex G-50 (Pharmacia Fine Chemicals) gel-filtration column equilibrated and eluted with 50 mM sodium phosphate, 10 mM magnesium chloride, and 0.3 M potassium chloride at pH 6.0.

Oxidase Activity Assays. Rates of oxygen consumption were measured with a YSI Model 53 oxygen monitor (Yellow Springs Instrument Co.) and a temperature-controlled electrode chamber with 1.8-mL volume (Gilson). The temperature was held constant at 36 °C, and the concentration of O_2 in the air-saturated buffer was assumed to be 220 μM. Experimental details are provided in the figure and table legends.

Protein Determinations. The procedure of Lowry (Lowry et al., 1951) was used in the determination of the protein content of membrane preparations. Bovine serum albumin was used as protein standard. The procedure was modified so the final solution contained 1% SDS.

Quinone Determinations. The simultaneous extraction of ubiquinone and menaquinone from membrane preparations was performed as previously described (Kroger, 1978). The ultraviolet reduced minus oxidized spectrum of the extracted quinones and reference spectra of purified UQ-8 and MK-8 were recorded and stored on magnetic discs. A second derivative of the spectrum was obtained by the method of Savitzky & Golay (1964) and was fit by a least-squares procedure to a weighted sum (Sternberg et al., 1960) of the second derivatives of the reference spectra. The weights obtained were estimates of the concentrations of ubiquinone and menaquinone in the sample analyzed. Use of second derivatives in this procedure minimized the effect of light-scattering artifacts.

Dual-Wavelength Spectroscopic Measurements. Measurements of the difference in optical density at two wavelengths were performed with an Aminco DW-2 spectrophotometer in the dual-wavelength mode. Wavelength pairs are specified in the figure legends. Dual-wavelength measurements allowed determination of the rates of substrate reduction in light-scattering suspensions.

Cytochrome Measurements. Cytochrome *b* was quantitated from reduced minus oxidized spectra measured at 25 °C with an Aminco DW-2 spectrophotometer. For membranes, the wavelength pair and extinction coefficient used were 560/580 nm and 17.5 mM^{-1} cm^{-1} (Deeb & Hager, 1964). For the pure cytochrome *d* terminal oxidase, the extinction coefficient used was 10.8 mM^{-1} cm^{-1} (562/580 nm) as previously determined for the cytochrome b_{558} component of this complex (Miller & Gennis, 1983).

Transmembrane Potential Measurement. The fluorescent dye 3,3′-bis(propylthio)dicarbocyanine at a final concentration of 1 μM was used to measure the transmembrane potential in the reconstituted vesicle system. The use of similar techniques in proteoliposome systems has been recently reported by others (Kita et al., 1982; Hurt et al., 1982). Fluorescence was recorded at 25 °C on a Perkin-Elmer MPF44. Excitation was at 620 nm and emission recorded at 670 nm. The dyes 3,3′-dipentyloxacarbocyanine and oxonol VI were also used as described previously (Hurt et al., 1982; Bashford & Smith, 1978).

The proteoliposomes were prepared from equal amounts of phosphatidylethanolamine and phosphatidylglycerol and contained about 12 nmol of UQ-8 and 0.05 nmol of heme *b*/mg of lipid. The stock solution of proteoliposomes (20 mg/mL) was prepared by cholate dialysis as described above in 50 mM potassium phosphate buffer, pH 7.5, and was diluted 1000-fold into the fluorescence cuvette for measurements. When the pyruvate oxidase activity was measured, the proteoliposomes were diluted into 100 mM potassium phosphate, 200 mM pyruvate, 100 mM thiamin pyrophosphate, and 10 mM $MgCl_2$ at pH 6.5. The reaction was initiated by the addition of 0.36 μg of the flavoprotein to the 3-mL cuvette. The final solutions contained 20 μg/mL phospholipid, 240 nM UQ-8, 1 nM heme *b*, and 2 nM flavoprotein subunit (0.5 nM tetramer). For measuring UQ-1 oxidase activity, no UQ-8 was incorporated in the liposomes, and the reaction was carried out in 50 mM potassium phosphate, pH 7.5, containing 25 μM UQ-1 and 2 mM dithioerythritol. Higher concentrations of UQ-1 made the vesicles leaky as judged by the rapid return of the fluorescence to the initial value. CCCP, HQNO, valinomycin, and nigericin were added when indicated from ethanol solutions. In no case were the final concentrations of ethanol greater than 0.1%.

For the calibration, the liposomes were prepared in a 50 mM potassium phosphate buffer, pH 7.5, and diluted into mixtures of 50 mM potassium phosphate and 50 mM sodium phosphate buffers to attain various K^+ ratios of inside to outside. The presence of the buffer components required for the pyruvate oxidase assay had no effect on the calibration curve relating fluorescence quenching to the diffusion potential. The diffusion potential was induced by the addition of valinomycin to a final concentration of 10^{-8} M.

Results

Reconstitution of Pyruvate Oxidase Flavoprotein with E. coli Membrane Vesicles. Previous studies have demonstrated that pyruvate oxidase activity (oxygen utilization) is reconstituted upon combining the purified flavoprotein with *E. coli* membranes [e.g., Cunningham & Hager (1975)]. These experiments were repeated in order to obtain kinetic data that could be compared with data obtained when the enzyme was reconstituted with purified membrane components. The reconstitution was performed at pH 6.0 at 37 °C, with the concentrations of flavoprotein and membrane varied from 0 to 0.18 μM (flavin) and 0 to 0.95 μM (*b* cytochrome), respectively. Membranes from two *E. coli* strains were used. Strain MR43L(F152) grown to stationary phase had little cytochrome *o* but contained cytrochrome *d* (R. G. Kranz, R. Lorence, and R. B. Gennis, unpublished results), and strain GR19N lacked cytochrome *d* due to a mutation in the *cyd* locus but had cytochrome *o* (Green & Gennis, 1983). Results were similar with both membranes, indicating that pyruvate-derived reducing equivalents can flow through either branch of the respiratory chain.

The addition of a sufficient amount of membrane to a fixed concentration of flavoprotein saturated the enzyme so the maximum turnover of the flavoprotein could be measured. With the highest quantity of membranes used, the specific activity observed was 105 μequiv min^{-1} (mg of flavoprotein)$^{-1}$. This value assumes four reducing equivalents required to reduce oxygen. Although the maximal turnover of the flavoprotein was similar with different membrane preparations, the amount of membrane required for the maximal activity with a given amount of flavorprotein was variable and depended on the manner in which the membranes were prepared. This was not systematically investigated. In the following sections it is demonstrated that the pyruvate oxidase activity observed in these reconstitution experiments requires (1) that the fla-

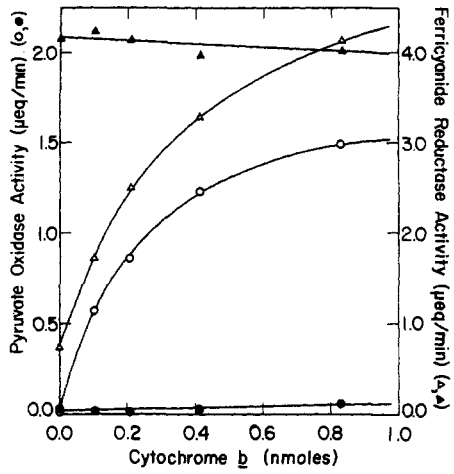

FIGURE 1: Reconstitution of *E. coli* membranes with the protease-activated form of pyruvate oxidase. The ferricyanide reductase (▲) and pyruvate oxidase (●) activities of the protease-activated enzyme (25 μg) were determined with varying amounts of the membrane preparation used in the reconstitution. The corresponding ferricyanide reductase (△) and pyruvate oxidase (○) activities of the unactivated control sample (25 μg) were also determined. Note that the ordinate scales of the two activities are different.

voprotein have the capacity to bind to the membrane and (2) that ubiquinone 8 be present in the membrane.

The protease-activated form of the flavoprotein (Recny & Hager, 1983; Russell et al., 1977a,b) has been shown to exhibit ferricyanide reductase activity approximately equal to that of the lipid-activated native enzyme (Russell et al., 1977a) but has a reduced affinity for detergents and phospholipids (Russell et al., 1977b). When protease-activated flavoprotein was added to *E. coli* membrane preparations, pyruvate oxidase activity was not observed (Figure 1), although the ferricyanide reductase activity was high. This result indicates that binding of the flavoprotein to the membrane surface is a prerequisite for functional reconstitution of the pyruvate oxidase system.

Reconstitution of Pyruvate Oxidase Flavoprotein with Quinone-Deficient Membrane Vesicles. The role of quinones was investigated with quinone-deficient strains of *E. coli* (Wallace & Young, 1977). The flavoprotein was added to membrane vesicle suspensions prepared from either *ubi*$^-$, *men*$^-$, *ubi*$^-$ *men*$^-$, or wild-type cells. The pyruvate oxidase activity of each reconstituted system was compared with the endogenous succinate, lactate, and NADH oxidase activities of the membranes (Table I). The results show conclusively that ubiquinone is required for efficient electron transport from pyruvate to oxygen. Menaquinone can apparently function to a very limited extent in this capacity, as it can in the succinate, lactate, and NADH oxidase systems [see Wallace & Young (1977) and Table I].

Quinone Reductase Activity of Pyruvate Oxidase Flavoprotein. The ability of the flavoprotein to reduce ubiquinone was tested in several systems. In one assay method (Figure 2A), the reduction of water-soluble UQ-1 by sodium dodecyl sulfate activated flavoprotein was monitored by dual-wavelength spectroscopy. The maximal velocity (V_{max}) for UQ-1 reduction was estimated to be 140 μequiv min^{-1} (mg of flavoprotein)$^{-1}$, and the K_m for UQ-1 was 200 μM. These values can be compared with the V_{max} of approximately 350 μequiv min^{-1} (mg of flavoprotein)$^{-1}$ and K_m of 3.0 mM observed in the ferricyanide reductase assay with sodium dodecyl sulfate activated enzyme (Blake et al., 1978).

Whereas it is apparent that the flavoprotein readily reduces water-soluble UQ-1, this system is not an adequate model of an in vivo quinone reductase reaction, which would involve a

Table I: Reconstituted Pyruvate Oxidase and Endogenous Oxidase Activities of Quinone-Deficient *E. coli* Membrane Preparations[a]

strain	oxidase activity[b]			
	succinate	DL-lactate	NADH	pyruvate
AN384 (*ubi*$^-$ *men*$^-$)	0	4	10	27
AN385 (*ubi*$^-$)	11	33	75	71
AN386 (*men*$^-$)	23	62	420	440
AN387 (wild type)	19	64	340	410

[a] Membranes of quinone-deficient strains of *E. coli* were prepared as described in the text. Membrane suspensions (0.3 nmol of heme *b*, 0.6 mg of membrane protein) were diluted into 0.1 M potassium phosphate, pH 7.0, in the electrode chamber. Assays were initiated by addition of either succinate (to 11 mM), DL-lactate (to 11 mM), or NADH (to 8 mM). In pyruvate oxidase assays, the phosphate buffer also contained 200 mM pyruvate, 10 mM magnesium chloride, and 100 μM TPP. Pyruvate oxidase assays were initiated by the addition of 50 μg (0.83 nmol) of purified flavoprotein to the oxygen electrode chamber. [b] All activities are expressed as nmol of O_2 min^{-1} (mg of membrane protein)$^{-1}$.

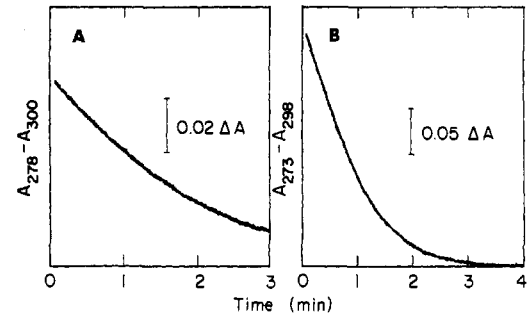

FIGURE 2: Reduction of UQ-1 and UQ-10 by pyruvate/pyruvate oxidase as monitored by dual-wavelength spectroscopy. (A) The initial velocity for UQ-1 reduction was determined with $\Delta\epsilon_{278-300}$ = 10.6 mM^{-1} cm^{-1}. Pyruvate oxidase (2.5 μg) was added to 0.80 mL of 0.125 M sodium phosphate, 12.5 mM magnesium chloride, 125 mM pyruvate, and 125 μM TPP at pH 6.0. A 0.20-mL aliquot of 50 μM sodium dodecyl sulfate was added as a detergent activator. After a 10-min incubation, the assay was initiated by the addition of UQ-1 from an 80 mM ethanol stock. The total volume was 1.0 mL. (B) The reduction of octyl β-D-glucoside solubilized UQ-10 was determined with $\Delta\epsilon_{273-298}$ = 13.0 mM^{-1} cm^{-1}. Pyruvate oxidase (10 μg) was incubated in diluted assay buffer (detergent free) and the assay initiated by addition of 0.10 mL of a 200 μM UQ-10 solution. A 1-cm path-length quartz cuvette was employed. Pyruvate oxidase (10 μg) was incubated in diluted assay buffer (detergent free) and the assay initiated by addition of 0.10 mL of a 200 μM UQ-10 solution in 200 mg/mL octyl β-D-glucoside. The final volume was 1.0 mL, with 0.1 M sodium phosphate, 20 mM pyruvate, 1.0 mM magnesium chloride, and 10 μM TPP present at pH 6.0.

long-chain quinone (UQ-8) situated in a hydrophobic lipid bilayer. Therefore, the quinone reductase activity of the pyruvate oxidase flavoprotein was measured with both UQ-8 and UQ-10 in hydrophobic environments. Both detergent-solubilized ubiquinone and mixed ubiquinone/phospholipid vesicles were tested as electron acceptors for the flavoprotein.

Pyruvate oxidase flavoprotein was found to reduce UQ-10 with an initial velocity of 5.8 μequiv min^{-1} (mg of flavoprotein)$^{-1}$ (Figure 2B) when solubilized in octyl β-D-glucoside. The reduction of detergent-solubilized UQ-10 was, therefore, much slower than the reduction of water-soluble electron acceptors such as ferricyanide and UQ-1. However, because both pyruvate oxidase and UQ-10 probably bind to detergent micelles under these conditions, intermicelle exchange of enzyme or quinone may have been rate limiting in this assay.

Mixed vesicles of UQ-8 and phospholipid were prepared by detergent dialysis. The ability of the flavoprotein to reduce

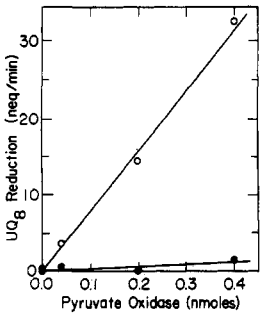

FIGURE 3: Reduction of UQ-8 in mixed UQ-8/phospholipid vesicles by pyruvate/pyruvate oxidase. Mixed UQ-8/phospholipid vesicles (25 nmol of UQ-8/mg of phospholipid, 20 mg/mL phospholipid) were prepared by the cholate dialysis procedure. The indicated amounts of pyruvate oxidase were added to 40 mL of the vesicle preparation, and the mixture was diluted to 0.80 mL with 0.1 M sodium phosphate and 0.2 M potassium chloride, pH 7.0. The assay was initiated by addition of 0.2 mL of a substrate solution, so that 67 mM pyruvate, 3.3 mM magnesium chloride, and 33 μM TPP were present in the final 1.00-mL volume. Reduction of UQ-8 was monitored by dual-wavelength spectroscopy as in Figure 2. Vesicles were prepared from either phosphatidylcholine (●) or phosphatidylcholine/phosphatidylglycerol (1/1 by weight) (○).

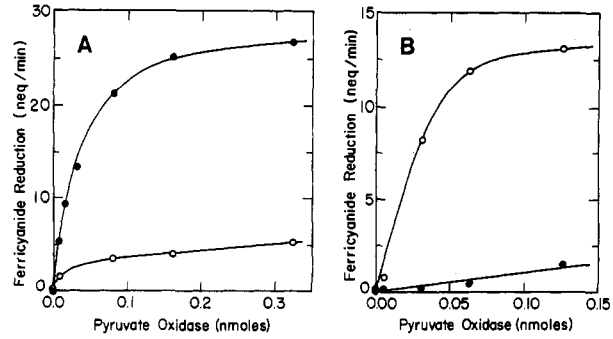

FIGURE 4: Reduction by pyruvate/pyruvate oxidase of ferricyanide trapped in mixed UQ-10/phospholipid vesicles. Mixed vesicles of UQ-10 and crude soybean phosphatidylcholine (5.0 nmol of UQ-10/mg of phospholipid, 10 mg/mL phospholipid) were prepared as described under Experimental Procedures. The indicated amounts of pyruvate oxidase were added to 0.10 mL of the vesicle preparation. The assay was initiated by addition of 0.90 mL of 0.05 M sodium phosphate, 10 mM magnesium chloride, 100 μM TPP, 200 mM pyruvate, and 100 mM potassium chloride, pH 6.0. Reduction of the internally trapped ferricyanide was monitored by dual-wavelength spectroscopy with $\Delta\epsilon_{420-450} = 0.78\ mM^{-1}\ cm^{-1}$. (A) Reduction of internally trapped ferricyanide by native pyruvate oxidase with 5.0 nmol of UQ-10 incorporated/mg of phospholipid (●) or with UQ-10 omitted (○). (B) Reduction of ferricyanide trapped in UQ-10/phospholipid vesicles by protease-activated pyruvate oxidase (●) or native enzyme (○).

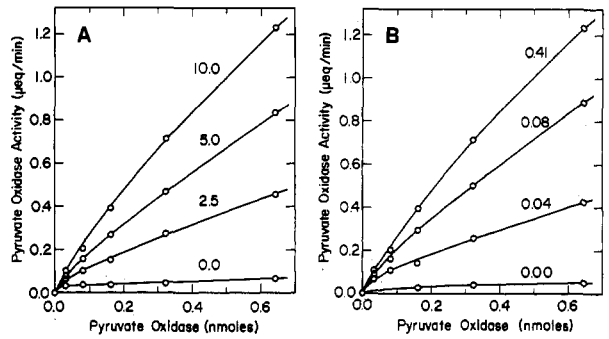

FIGURE 5: Activity of pyruvate oxidase system reconstituted from purified components. The purified cytochrome *d* terminal oxidase complex and UQ-8 were incorporated together in mixed phosphatidylethanolamine/phosphatidylglycerol (2/1 by weight) vesicles by cholate dialysis. A 40-μL aliquot of the vesicle suspension (20 mg of phospholipid/mL) was diluted into 1.80 mL of assay buffer air-saturated at 37 °C in the oxygen electrode chamber. The assay buffer was 0.1 M potassium phosphate, 10 mM magnesium chloride, 200 mM pyruvate, and 100 μM TPP, pH 6.0. The assay was initiated by the addition of varying amounts of pyruvate oxidase. (A) The indicated quantity of UQ-8 was incorporated in the vesicles (nmol/mg of phospholipid), with the amount of terminal oxidase fixed (0.41 nmol of heme *b*/mg of phospholipid, 0.33 nmol of heme *b*/assay). (B) The indicated quantity of terminal oxidase was incorporated in the vesicles (nmol of heme *b*/mg of phospholipid), with the amount of UQ-8 fixed (10 nmol/mg of phospholipid, 8.0 nmol/assay).

the quinone was strongly dependent on the phospholipid composition of the vesicles (Figure 3). When UQ-8 was incorporated in crude soybean phosphatidylcholine vesicles (25 nmol of UQ-8/mg of phospholipid or 2 mol % UQ-8), it was reduced only very slowly [0.06 μequiv min^{-1} (mg of flavoprotein)$^{-1}$]. In phosphatidylcholine/phosphatidylglycerol (1/1 by weight) vesicles, UQ-8 was reduced much more rapidly [1.2 μequiv min^{-1} (mg of flavoprotein)$^{-1}$]. Even this rate is small relative to the rate of reduction observed with water-soluble electron acceptors. However, it was demonstrated by ferricyanide reductase assays that the flavoprotein was not fully activated by the phospholipid vesicles (data not shown). Since lipid binding and activation is a slow process that does not begin until the flavoprotein is reduced (Russell et al., 1977a; Schrock & Gennis, 1980), reduction of all the membrane-incorporated UQ-8 might be accomplished before the entire enzyme population became bound to the lipid and activated.

In order to extend the time of the ubiquinone reductase assay and to facilitate more complete activation of the flavoprotein, experiments were performed with vesicles loaded with sodium ferricyanide. In this system, ubiquinone reduced on the outer surface of the vesicles can traverse the lipid bilayer and become reoxidized by the internally trapped ferricyanide (Hinkle, 1970; Deamer et al., 1972; Futami et al., 1979). Thus, repeated turnover of the incorporated ubiquinone can be observed and the time course of the reaction extended. The rate of reduction of the trapped ferricyanide can be monitored spectrophotometrically and indicates the rate of quinone turnover.

Figure 4A shows that reduction of ferricyanide trapped in soybean phosphatidylcholine vesicles was catalyzed by the incorporated UQ-10 (5.0 nmol of UQ-10/mg of phospholipid). Apparently, when the enzyme can turnover for an extended period of time, a larger fraction of the flavoprotein will bind to crude soybean phosphatidylcholine vesicles than when turnover is limited by the amount of quinone. The rate of ferricyanide reduction was enhanced when the ratio of quinone to phospholipid was increased (data not shown). When the flavoprotein was rate limiting, the rate of ferricyanide reduction was as high as 35 μequiv min^{-1} (mg of flavoprotein)$^{-1}$ with 15 nmol of UQ-10/mg of phospholipid. Only very slow reduction of ferricyanide was observed when ubiquinone was absent from the vesicles (Figure 4A). Results obtained with UQ-8 incorporated in vesicles of phosphatidylcholine/phosphatidylglycerol (1/1 by weight) were essentially the same (data not shown).

The protease-activated form of the flavoprotein did not reduce the liposome-entrapped ferricyanide (Figure 4B). Apparently, as observed in the membrane reconstitution experiments, the binding of the flavoprotein to the lipid bilayer is a prerequisite for effective transfer of reducing equivalents to the membrane-bound acceptor.

Reconstitution of Pyruvate Oxidase Flavoprotein, Ubiquinone, and the Cytochrome d Terminal Oxidase in Phospholipid Vesicles. The cytochrome *d* terminal oxidase complex and UQ-8 were incorporated together into the bilayer membrane of phospholipid vesicles by cholate dialysis. When the

Table II: Summary of Maximal Observed Turnover Number of the Pyruvate Oxidase Flavoprotein in Different Systems

assay system	turnover number (electrons FAD^{-1} s^{-1})
(1) flavoprotein plus *E. coli* membrane, measuring oxygen utilization	105
(2) same as (1), but measuring ferricyanide reductase activity	290
(3) flavoprotein activated by SDS, measuring ferricyanide reductase activity	350
(4) flavoprotein activated by SDS, measuring UQ-1 reductase activity	140
(5) flavoprotein in octyl β-D-glucoside, measuring UQ-10 reductase activity	5.8
(6) flavoprotein with phospholipid vesicles containing 25 nmol of UQ-8/mg of lipid, measuring UQ-8 reductase activity	1.2
(7) flavoprotein with phospholipid vesicles containing 15 nmol of UQ-10/mg of lipid and with ferricyanide trapped within, measuring ferricyanide reductase activity	35
(8) flavoprotein with phospholipid vesicles containing 10 nmol of UQ-8 and 0.41 nmol of heme *b* (from terminal oxidase complex) per mg of lipid, measuring oxygen utilization	30
(9) same as (8), but measuring ferricyanide reductase activity	395

flavoprotein was added to suspensions of these vesicles, a functional pyruvate oxidase system was created. Figure 5 shows that the pyruvate oxidase activity of this system required the presence of each of the catalytic components: flavoprotein, UQ-8, and the terminal oxidase complex. The flavoprotein was fully activated in this reconstituted system. A steady-state ferricyanide reductase activity of 395 μequiv min^{-1} (mg of flavoprotein)$^{-1}$ was measured.

The observed pyruvate-driven O_2 consumption catalyzed by this system depended strongly upon the content of UQ-8 (Figure 5A) and terminal oxidase (Figure 5B) in the vesicles. At the highest levels of UQ-8 (10 nmol/mg of lipid) and terminal oxidase (0.4 nmol of heme *b*/mg), the activity was approximately 30 μequiv min^{-1} (mg of flavoprotein)$^{-1}$. Thus, the pyruvate oxidase activity of this system was less then 10% of the observed ferricyanide reductase activity (395 μequiv min^{-1} mg^{-1}) but was nearly 30% of the oxidase activity observed when the enzyme was coupled to the *E. coli* membranes (105 μequiv min^{-1} mg^{-1}). The steady-state kinetics data are summarized in Table II.

In these reconstitution experiments, a mixture of phosphatidylethanolamine and phosphatidylglycerol was used in the preparation of vesicles. When phosphatidylglycerol was omitted, the pyruvate oxidase activity of the reconstituted system was greatly decreased (data not shown). When MK-8 was substituted for UQ-8 in the reconstitution, the resulting pyruvate oxidase activity was roughly 10% of that observed with vesicles with an equivalent content of UQ-8 (Figure 6). This latter finding is in accord with the results of the studies of the pyruvate oxidase activity with quinone-deficient *E. coli* membranes (see Table I).

When the protease-activated form of the flavoprotein was used in the reconstitution, very little pyruvate oxidase activity was observed (not shown). This is analogous to the results obtained in the *E. coli* membrane vesicle reconstitution experiments (Figure 1) and in the ubiquinone reductase assay of pyruvate oxidase (Figure 4).

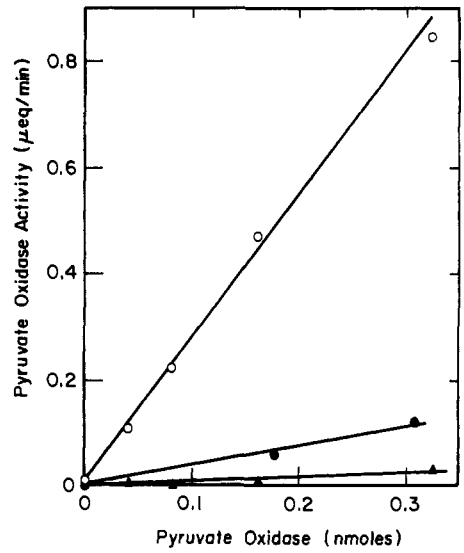

FIGURE 6: Reconstitution of pyruvate oxidase flavoprotein with UQ-8 and MK-8. The pyruvate oxidase system was reconstituted as described in the legend to Figure 5, with either 10 nmol/mg of phospholipid ubiquinone 8 (○), menaquinone 8 (●), or no quinone (▲). The vesicle preparation contained the cytochrome *d* complex (0.41 nmol of heme *b*/mg of phospholipid, or 0.33 nmol of heme *b*/assay). Oxygen utilization was measured as a function of the flavoprotein added (nmol of flavin).

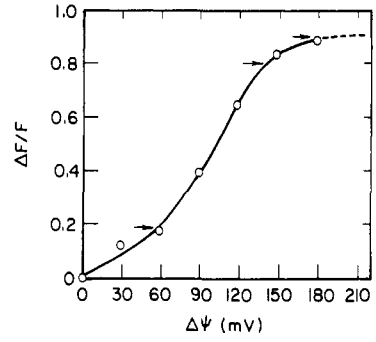

FIGURE 7: Calibration curve relating extent of fluorescence quenching of the fluorophore 3,3′-bis(propylthio)dicarbocyanine to a diffusion potential across the membrane of phospholipid vesicles. The proteoliposomes were prepared as described under Experimental Procedures, and the diffusion potential was induced by valinomycin with vesicles with various inside to outside K^+ concentration gradients. Details are given in the text. The three arrows indicate the extent of quenching observed due to (1) UQ-1 oxidase activity (20%, 60 mV), (2) UQ-1 oxidase activity in the presence of 0.3 μM nigericin (80%, 140 mV), and (3) pyruvate oxidase activity (90%, 180 mV).

The voltage-sensitive fluorescent dye 3,3′-bis(propylthio)-dicarbocyanine was used to determine if a transmembrane potential developed across the liposome bilayer concomitant with pyruvate oxidase activity. Figure 7 is a calibration showing the fluorescence quenching in response to an increase in a valinomycin-induced potassium diffusion potential in the reconstituted liposomes. When proteoliposomes containing the cytochrome *d* complex were reacted with UQ-1, a transmembrane potential of 60 mV (negative inside) was created, as judged by the quenching of the fluorescence of the dye. The rate of oxygen reduction was one-third of the rate observed when the pyruvate oxidase system was examined under the same conditions. When nigericin was present, the value of $\Delta\psi$ increased to 140 mV, as expected if the electron-transport reaction was accompanied by a net proton translocation from inside to outside the vesicles. The formation of $\Delta\psi$ by UQ-1 oxidation was blocked by the presence of the repiratory inhibitors HQNO and cyanide, and $\Delta\psi$ was collapsed by the addition of CCCP or valinomycin (data not shown). Since

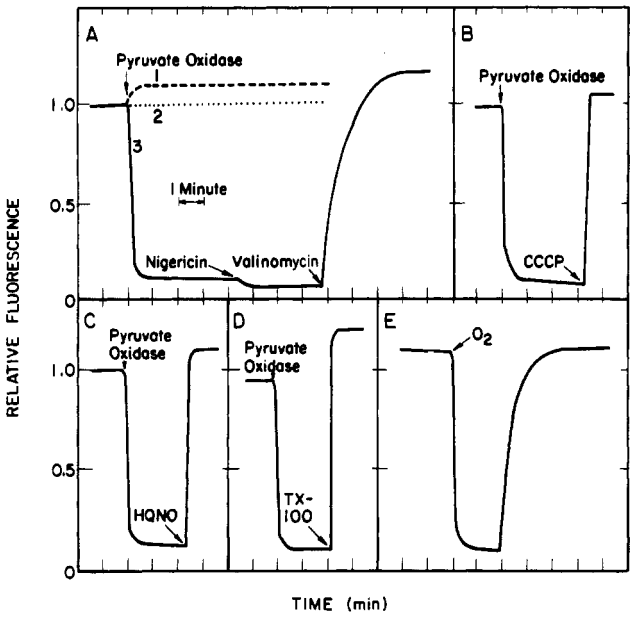

FIGURE 8: Fluorescence quenching of the voltage-sensitive dye 3,3′-bis(propylthio)dicarbocyanine due to reconstituted pyruvate oxidase activity in phospholipid vesicles. The proteoliposomes were prepared as described under Experimental Procedures. The reaction was initiated by the addition of pyruvate oxidase to the cuvette (final concentration, 2 nM flavin). The following were added when indicated: nigericin (0.3 μM), valinomycin (0.3 μM), CCCP (0.3 μM), HQNO (40 μM), and Triton X-100 (0.05%). Without these additions, approximately 15 min elapsed before oxygen was depleted. (Part A) Curve 1 was obtained when cytochrome *d* was omitted from the vesicles; curve 2 was obtained with either no pyruvate or no pyruvate and no pyruvate oxidase present; curve 3 was obtained with all components present. The effects of nigericin and valinomycin are indicated. The presence of the flavoprotein always caused a slight increase in the fluorescence intensity that is apparent in curve 1. (Part B) Effect of CCCP is indicated. (Part C) Effect of HQNO is indicated. (Part D) Effect of Triton X-100 is indicated. (Part E) All the components were present, and the reaction was allowed to run until oxygen was depleted. The fluorescence regained the level shown. At the indicated time, the contents of the cuvette were mixed, introducing oxygen into the solution. The resulting reinitiation of the pyruvate oxidase activity caused the fluorescence quenching, which lasted only until oxygen was depleted.

UQ-1 should be able to reduce the cytochrome on either side of the membrane, these data suggest that the cytochrome *d* complex is preferentially oriented in the liposomes following the cholate dialysis procedure. The data also suggest that the cytochrome *d* complex is a "coupling site" directly involved in generating a protonmotive force.

Figure 8 shows that pyruvate oxidase activity resulted in 90% quenching of the fluorescence, corresponding to a potential of at least 180 mV (negative inside). CCCP and valinomycin both collapsed the gradient, whereas nigericin caused an increase in the electric potential difference (Figure 8). The transmembrane potential was dependent on the intact electron-transport chain and did not develop in the absence of either pyruvate or oxygen or in the presence of the respiratory inhibitor HQNO. At the high concentration of HQNO used, UQ-1 oxidase and pyruvate oxidase activities were inhibited by 70 and 80%, respectively. It is likely that the effect of HQNO at this concentration on the formation of $\Delta\psi$ may be due to an uncoupling action by this inhibitor (Krab & Wikstom, 1980). Cyanide could not be used as an inhibitor due to an apparent interaction with the pyruvate. For example, the presence of 200 mM pyruvate prevented cyanide from inhibiting the UQ-1 oxidase activity of the terminal oxidase. Triton X-100, which disrupts sealed vesicles, also collapsed the potential difference, although the effect due to Triton X-100 may be partly due to a direct interaction with the dye. Similar results were obtained with another dye, 3,3′-dipentyloxacarbocyanine. Neither oxonol VI, which is an indicator of positive membrane potentials inside the vesicles, nor 9-aminoacridine, which detects internal acidification of vesicles, gave any response with either pyruvate oxidase or UQ-1 oxidase activities. All the data are consistent with there being a net proton translocation across the bilayer from inside to outside, which is concomitant with pyruvate oxidase activity. It should be noted that both pyrvuate oxidase activity and UQ-1 oxidase activity in these systems were stimulated by at most a factor of 2 in the presence of CCCP and/or valinomycin (data not shown). The fluorescence quenching of the indicator dye that accompanied the oxidase activities was also observed when the concentration of proteoliposomes was increased from 20 to 330 μg of phospholipid/mL. However, attempts to perform the calibration at the high vesicle concentration were not successful. The cause of this is not understood.

Discussion

There are several important conclusions to be drawn from this work. It is clear the ubiquinone 8 is a critical component in the electron-transport chain from pyruvate to oxygen. It has been demonstrated that the pyruvate oxidase flavoprotein is capable of rapidly reducing ubiquinone 8, which is incorporated in a phospholipid bilayer, and that no intermediate electron carrier is required for this reaction. The product of the reduction of ubiquinone has not been investigated, but it is assumed to be the fully reduced ubiquinol. The ubiquinol is capable of carrying reducing equivalents across the vesicle bilayer and reducing ferricyanide trapped inside.

The successful reconstitution of pyruvate oxidase activity with the cytochrome *d* terminal oxidase complex demonstrates that this enzyme functions as a ubiquinol 8 oxidase. The maximal pyruvate oxidase activity observed in the reconstituted system was about 30 μequiv min^{-1} (mg of flavoprotein)$^{-1}$. This is about the same as the maximal rate observed when ferricyanide trapped inside the vesicles was used as the oxidant. Most likely the rate of reduction of the ubiquinone by the flavoprotein is rate limiting in these experiments. This rate is about one-third of the rate observed when the flavoprotein was added to *E. coli* membranes from cells grown to stationary phase. The cytochrome *d* and ubiquinone levels (per milligram of phospholipid) in such *E. coli* membranes are similar to those used in the liposome reconstitution experiments [e.g. see Green & Gennis (1983)]. Considering artifacts due to possible inhomogeneous protein distribution amongst the vesicles and the existence of multilamellar vesicles (see below), the pyruvate oxidase activity observed in the reconstituted liposome system compares very favorably with that seen with *E. coli* membranes [105 μequiv min^{-1} (mg of flavoprotein)$^{-1}$ or a turnover number of 105 s^{-1}].

It is unlikely that direct protein interaction is required between the flavoprotein and the terminal oxidase for the electron-transfer reaction to occur, although this has not been experimentally excluded. Most likely, the ubiquinone 8 is functioning as a mobile pool in carrying reducing equivalents between the two proteins. In principle, such a "pool model" can be kinetically tested (Kroger et al., 1973), but the experimental limitations of this liposome system make this difficult. First, as shown for other systems, the distribution of proteins within the vesicles may be far from random (Madden et al., 1983), with some vesicles containing large quantities of protein and others containing little. In addition, a preliminary examination of the liposomes by electron mi-

croscopy indicated a substantial amount of multilammelar vesicles. Thus, detailed quantitation with this system is unwarranted.

The pyruvate oxidase activity in the reconstituted liposomes generates a transmembrane potential. The substantial fluorescence quenching of the carbocyanine dye suggests a magnitude of at least 180 mV, negative inside. It is interesting that the electron-transfer rate increased only slightly when CCCP or valinomycin was present. This suggests that the electrogenic step in the reaction sequence (e.g., the step resulting in charge separation) may not be fully rate limiting. Presumably, the electrogenic step involves electron transfer within the cytochrome *d* complex. Electron transfer from UQ-1 to oxygen through the cytochrome *d* complex also generates a transmembrane potential. These data show that the cytochrome *d* complex has a preferential orientation in the proteoliposomes and almost certainly functions as a "coupling site" in *E. coli*. A simple bioenergetic mechanism can be postulated, in which quinol is oxidized on the outside of the membrane and oxygen is reduced on the inside following electron transfer through the cytochrome *d* terminal oxidase and across the bilayer. Such a mechanism will be tested.

It is unlikely that the flavoprotein serves as a coupling site in the pyruvate oxidase reaction sequence. The enzyme is not a transmembrane protein. Furthermore, in vivo the enzyme is present on the electrically negative side of the membrane, which is opposite to its position in the reconstituted system. The fact that the value of $\Delta\psi$ is 3 times as large for pyruvate oxidase activity as compared to the UQ-1 oxidase activity probably reflects the greater catalytic velocity and not an additional coupling site due to the presence of the flavoprotein.

The activity of the endogenous pyruvate oxidase flavoprotein has been shown to be coupled to the active transport of amino acids, presumably by passing reducing equivalents through the respiratory chain (Shaw-Goldstein et al., 1978). *E. coli* strains that have been genetically constructed so that pyruvate oxidase provides the only pathway for pyruvate catabolism can, in fact, grow on pyruvate as the only source of energy and carbon (Chang & Cronan, 1982). Growth, however, is very slow, possibly due to the poor ability of cells to utilize the acetate that is formed as a product (Chang & Cronan, 1982). Although the physiological role of the flavoprotein is unclear, it appears that pyruvate oxidase flavoprotein behaves biochemically in a manner similar to other *E. coli* flavoprotein dehydrogenases such as NADH dehydrogenase (Jaworowski et al., 1981), D-lactate dehydrogenase (Short et al., 1975), D-amino acid dehydrogenase (Olsiewski et al., 1981) and glycerol-3-phosphate dehydrogenase (Schryvers et al., 1978). Each of these dehydrogenases has been purified and reconstituted with *E. coli* membrane vesicles (Jaworowski et al., 1981; Short et al., 1975; Olsiewski et al., 1981; Haldar et al., 1982; Schryvers et al., 1978). It is of interest to compare the catalytic velocities of these reconstituted systems. D-Lactate dehydrogenase and D-amino acid dehydrogenase turnover at comparable rates when reconstituted with *E. coli* vesicles, about 20–30 electrons s^{-1} (Haldar et al., 1982; Short et al., 1974). Futai (1974) has reported that the endogenously and exogenously bound D-lactate dehydrogenases function with about the same specific activity with the respiratory chain. The same appears to be true for both glycerol-3-phosphate dehydrogenase (Futai, 1974) and D-amino acid dehydrogenase [see Olsiewski et al. (1980, 1981)]. Endogenous NADH dehydrogenase turnover is about 750 electrons s^{-1}, and the reconstituted enzyme functions at about 38% of the endogenous rate (285 s^{-1}) (Jaworowski et al., 1981). The kinetics of these reconstituted flavoprotein dehydrogenases are comparable to the turnover of the reconstituted pyruvate oxidase flavoprotein (105 s^{-1} with *E. coli* membranes and 30 s^{-1} in the reconstituted liposomes).

Most of the previous kinetic studies with pyruvate oxidase utilized water-soluble electron acceptors, usually ferricyanide. It is now reasonable to conclude that ubiquinone 8 is the natural electron acceptor for the flavoprotein. The present studies indicate that the nonspecific interactions of this enzyme with phospholipids and other amphiphiles that have been studied in vitro [e.g., Blake et al. (1978) and Schrock & Gennis (1980, 1977)] are probably relevant to the membrane binding of the enzyme in vivo. There is no need to postulate nor any evidence to support the existence of a specific membrane protein serving as a binding site for the flavoprotein in the *E. coli* membrane. Similar conclusions have been reached for other *E. coli* flavoprotein dehydrogenases [see Halder et al. (1982)]. The ability of other dehydrogenases to directly reduce long-chain quinones within the bilayer has not been reported as yet. However, it is clear that reconstitution with some other flavoprotein dehydrogenases occurs with both right side out and inverted *E. coli* membrane vesicles [see Haldar et al. (1982)]. The ability to efficiently pass reducing equivalents into the electron-transport chain from either side of the membrane suggests a mobile electron acceptor, such as quinone, although alternative explanations are possible. Certainly, more data are required to determine the natural electron acceptor(s) for each of the flavoprotein dehydrogenases.

The work reported in this paper indicates a relative simplicity for this portion of the *E. coli* respiratory chain. Further work will be necessary to clarify the details of the electron-transfer reactions and the mechanisms of any proton-translocation steps.

Acknowledgments

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Registry No. Pyruvate oxidase, 9001-96-1; cytochrome *d* terminal oxidase, 9035-36-3; UQ-8, 2394-68-5; UQ-10, 606-06-4; ferricyanide, 13408-62-3; ferricyanide reductase, 9047-21-6; UQ-1, 727-81-1; ubiquinol oxidase, 69671-26-7; octyl β-D-glucoside, 65309-84-4.

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