Reconstitution of Ubiquinone-Linked Functions in Membrane Vesicles from a Double Quinone Mutant of *Escherichia coli*†

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ABSTRACT: Membrane vesicles prepared from a mutant of *Escherichia coli* that is deficient in ubiquinone and menaquinone are markedly defective in their ability to oxidize D-lactate, NADH, and other substrates, and these electron donors do not drive proline transport effectively. Reconstitution of mutant vesicles with each of eight ubiquinone side-chain homologues (ubiquinone-1 through ubiquinone-8) under appropriate conditions restores certain activities to levels that are comparable to those of vesicles prepared from the parent strain, while menaquinone homologues are ineffective. However, there are dramatic differences in the efficiency of reconstitution with various ubiquinone homologues. High levels of D-lactant oxidation and D-lactate-driven proline transport are restored with both long- and short-chain homologues, but ubiquinone-1 functions at much lower membrane-bound concentrations than ubiquinone-8 NADH is oxidized at a high rate by vesicles from the parent, but does not drive transport effectively, and addition of ubiquinone-1 stimulates proline transport over tenfold with no increase in NADH oxidation. In contrast, addition of ubiquinone-8 causes very little stimulation of proline transport. Moreover, in mutant vesicles, reconstitution with ubiquinone-1 results in both NADH oxidation and NADH-driven proline transport, while ubiquinone-8, on the other hand, reconstitutes NADH oxidation but has almost no effect on proline transport. The results indicate that functional reconstitution with ubiquinone side-chain homologues does not result simply from binding of the quinones to the membrane, but requires interaction of the bound quinone with specific sites. Moreover, the data suggest that ubiquinone-8 is not freely mobile within the membrane and imply that the enhanced mobility of ubiquinone-1 allows it to shunt reducing equivalents from NADH dehydrogenase to a segment of the respiratory chain containing the site at which the electrochemical proton gradient is generated. In addition, experiments with dithiothreitol as a reducing agent suggest that ubiquinone-8 is not accessible from the surface of the membrane.

The chemiosmotic hypothesis (Mitchell, 1961, 1966a,b, 1968, 1970, 1973; Greville, 1969; Harold, 1972) has focused widespread attention on the fundamental role of the "protonmotive force" in bioenergetics and has stimulated intensive research into the nature of the electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$),[1] the mechanism(s) by which it is generated, and the means by which its energy is coupled to endergonic processes. Bacterial membrane vesicles (Kaback, 1974b) have contributed increasingly to an understanding of chemiosmotic phenonema, particularly with respect to active transport, and recent experiments with this system have led to considerable clarification of the relationship between the chemical and electrical components of $\Delta\bar{\mu}_{H^+}$ and the accumulation of specific transport substrates (Ramos et al., 1976; Kaback, 1976; Ramos & Kaback, 1977a–c; Tokuda & Kaback, 1977).

Although it is clear that $\Delta\bar{\mu}_{H^+}$ plays a central, obligatory role in active transport, the mechanism by which respiration leads to $\Delta\bar{\mu}_{H^+}$ remains obscure, especially in bacterial systems. The prevailing hypothesis involves the concept of "loops" in the respiratory chain (Mitchell, 1966b, 1968). According to this notion, the proton and/or electron carriers comprising an "energy-coupling site" are disposed across the membrane in such a fashion that, in the first arm of the loop, 2 protons and 2 electrons pass vectorially from one carrier on the inner surface of the membrane to the next carrier on the external surface. The second arm of the loop involves the vectorial transfer of electrons (but not protons) from the carrier on the outer surface of the membrane to an electron acceptor on the inner surface, resulting in the release of 2 protons in the external medium. The sum of the two processes (i.e., extrusion of protons into the external medium and vectorial flow of electrons from the outer to the inner surface of the membrane) results in the generation of $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline). In addition, Mitchell (1975a,b, 1976) has proposed more recently the "protonmotive ubiquinone cycle" primarily to explain site II in the mitochondrial membrane. This mechanism envisages ubiquinone acting as a respiratory intermediate both before and after cytochrome *b* and stipulates a direct role for ubiquinone in the generation of $\Delta\bar{\mu}_{H^+}$.

An evaluation of these possibilities in *Escherichia coli* is difficult since little is known concerning the molecular structure of the respiratory chain in the cytoplasmic membrane. One distinct advantage of this organism, however, is the applicability of biochemical genetics. In this context, observations regarding quinone function have been presented leading to the important conclusions that ubiquinone functions before as well as after cytochrome b_{556} (Cox et al., 1970; Downie & Cox, 1978) and that individual dehydrogenases have specific requirements for ubiquinone or menaquinone (Wallace & Young, 1977a,b).

The use of membrane vesicles prepared from quinone-deficient mutants offers certain clear advantages in studying quinone function, particularly in view of the vectorial nature of both $\Delta\bar{\mu}_{H^+}$ and the transport processes driven by it. Menaquinone-deficient membrane vesicles from *Bacillus subtilis* have been reconstituted with the menaquinone analogue menadione (Bisschop & Konings, 1976), and light-inactivated membrane vesicles from *Bacillus licheniformis* have been reconstituted with short-chain menaquinone homologues (Macleod et al., 1973). However, reconstitution with the native quinone homologues has proven difficult, although such

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[1] Abbreviations used: $\Delta\mu_{H^+}$, the electrochemical gradient of protons; Q_n, the ubiquinone side chain homologue with n isoprene units in the polyprenyl side chain.

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an achievement is important for investigating the function of physiological quinones.

This paper describes the functional reconstitution of membrane vesicles from a double quinone mutant of *E. coli* with a series of ubiquinone homologues. The results demonstrate that there are important differences in the properties of reconstituted mutant vesicles that are related to the length of the ubiquinone side chain and provide information concerning the site of action and accessibility of functional ubiquinone in the membrane.[2]

Experimental Section

Methods

Bacterial Strains. The double quinone mutant AN384 (ubiA420, menA401) and its isogenic parent AN387 (ubi$^+$, men$^+$), both *E. coli* K 12 derivatives, were generously provided by Dr. I. G. Young of the John Curtin School of Medical Research, Australian National University, Canberra, Australia.

Growth of Cells and Preparation of Membrane Vesicles. After purifying the strains and preparing inocula as described (Wallace & Young, 1977b), AN 387 and AN 384 were grown on minimal medium A (Davis & Mingioli, 1950) containing 30 mM lactose and 1 μM thiamine hydrochloride. Cultures were grown at 37 °C in 10-L quantities in glass fermentor jars (New Brunswick Scientific Co., Inc.) with stirring (500 rpm) and aeration (10-L per min), and cells were harvested during late logarithmic phase. Membrane vesicles were prepared as described (Kaback, 1971; Short et al., 1975), except that lysozyme and sucrose were used at 40 μg per mL and 30%, respectively, for spheroplast formation.

Transport. Transport was assayed by filtration as described (Kaback, 1974a).

Oxygen Consumption. Rates of oxygen uptake were measured with a Clark electrode (YSI Model 53 oxygen monitor) as described (Barnes & Kaback, 1971).

Reconstitution with Quinone Homologues. Aliquots (500 μL) of membrane vesicles suspended in 0.1 M potassium phosphate (pH 6.6) at a concentration of 2 mg of protein per mL were mixed rapidly with 125 μL of dimethyl sulfoxide containing given concentrations of appropriate quinone homologues at room temperature. The mixtures were incubated at 25 °C for 20 min and then centrifuged at 40 000*g* for 20 min. The supernatants were discarded, and the pellets were washed once in 0.1 M potassium phosphate (pH 6.6) and resuspended to 500 μL in the same buffer. Treatment with dimethyl sulfoxide under these conditions inhibits the initial rate of D-lactate-dependent proline transport in AN387 membrane vesicles by about 20%, but has no effect on D-lactate-dependent oxygen uptake. Where indicated, ubiquinone-1 (Q_1) was added to the vesicles in an aliquot of ethanolic solution to give a final ethanol concentration of 0.1% (Stroobant & Kaback, 1975). At this concentration, ethanol has no effect on either transport or oxygen consumption in the presence of D-lactate. Alternatively, Q_1 was added in dimethyl sulfoxide as described above. In reconstitution experiments where D-lactate oxidation and D-lactate-driven transport activity were compared with levels of membrane-bound quinones (cf. Figures 2 and 3), all assays were carried out with vesicles reconstituted under identical conditions.

Quinone Extraction and Determination. Membrane vesicles were extracted using the method described for determination of the redox state of ubiquinone (Crane & Barr, 1971) with the exception that pyrogallol was omitted. Subsequently, ubiquinone and menaquinone were estimated by the methods of Crane & Barr (1971) and Dunphy & Brodie (1971), respectively.

Protein Determinations. Protein was assayed as described by Lowry et al. (1951) using bovine serum albumin as a standard.

Materials

Ubiquinone and menaquinone side-chain homologues were generously provided by Hoffmann-La Roche, Inc., Basle, Switzerland. L-[U-^{14}C]Proline was purchased from New England Nuclear. All other materials were reagent grade obtained from commercial sources.

Results

Properties of a Double Quinone Mutant of E. coli K-12. The double quinone mutant, AN384, carries mutations in the biosynthetic pathways for ubiquinone and menaquinone such that addition of the octaprenyl side chain to the appropriate intermediate is blocked (Wallace & Young, 1977b). Consequently, this strain forms very low levels of ubiquinone and menaquinone, and, in addition, contains no octaprenylquinone intermediates. Moreover, a comparison of the concentrations of respiratory components in membranes from aerobically grown AN384 with those of the isogenic parent, AN387, demonstrates that the levels of flavine adenine dinucleotide, flavine mononucleotide, and cytochromes *b*, *o*, and *d* are within the normal range (Wallace & Young, 1977b). Hereafter, AN387 and AN384 will be referred to as parent and mutant, respectively.

Membrane vesicles prepared from the mutant grown aerobically as described in Methods contain no detectable menaquinone and about 3% of the level of ubiquinone found in the parent (parent level, 5.9 nmol of ubiquinone per mg of membrane protein). Furthermore, the ability of the mutant vesicles to oxidize D-lactate and NADH is markedly reduced relative to vesicles prepared from the parent (Table I). In addition, L-lactate and succinate oxidation are reduced in the mutant vesicles, although it should be noted that both of these dehydrogenases are inducible (i.e., the activity of the parent vesicles is not maximal).

Active transport of proline by parent and mutant vesicles in the presence of various electron donors is shown in Figure 1. As demonstrated previously (Kaback & Milner, 1970; Barnes & Kaback, 1970, 1971; Stroobant & Kaback, 1975), D-lactate is clearly the best physiological electron donor for transport in the parent vesicles, while NADH, L-lactate, and succinate stimulate initial rates and steady-state levels of proline accumulation to less than 20% of those observed with

Table I: Oxidase Activities of Parent and Mutant Membrane Vesicles

substrate[a]	oxygen uptake[b] (ng of O atoms/(min·mg of protein))	
	parent	mutant
D-lactate	83	18
NADH	230	31
L-lactate	31	18
succinate	37	14

[a] Substrates were used at the following final concentrations: lithium D-lactate, 20 mM; NADH (sodium salt), 5 mM; lithium L-lactate, 20 mM; disodium succinate, 20 mM. [b] Rates of oxygen uptake were measured at a membrane protein concentration of 0.6 mg per mL as described in Methods.

[2] A preliminary report on some of this work has been presented (Stroobant & Kaback, 1976).

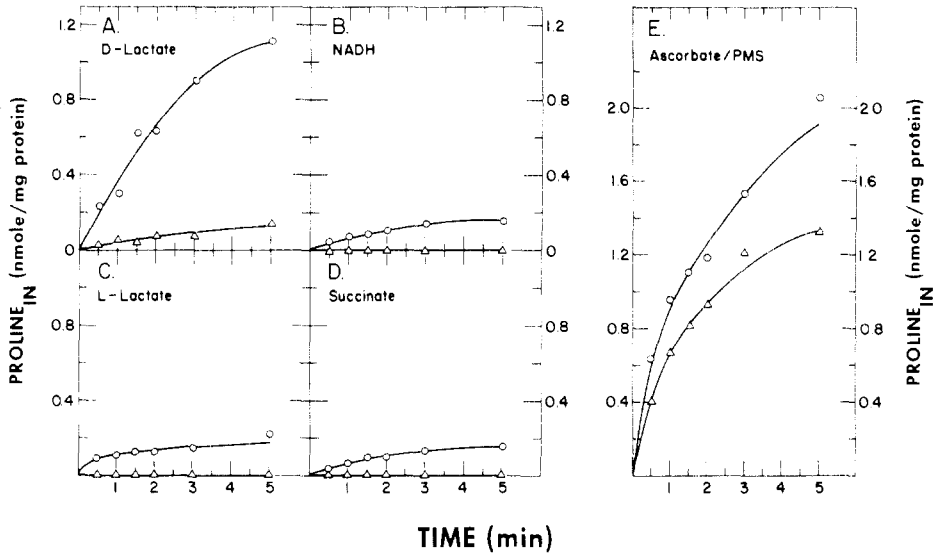

FIGURE 1: Proline transport by parent and mutant membrane vesicles in the presence of various electron donors. Aliquots (25 μL) of membrane vesicles containing about 0.1 mg of membrane protein were diluted to a final volume of 50 μL containing (in final concentrations) 50 mM potassium phosphate (pH 6.6) and 10 mM magnesium sulfate. After 30-s incubation at 25 °C, the indicated dehydrogenase substrates or ascorbate and phenazine methosulfate (PMS) were added, and, immediately thereafter, L-[U-^{14}C]proline (232 mCi per mmol) was added to a final concentration of 16 μM. Final concentrations of dehydrogenase substrates, ascorbate, and PMS were as follows: (A) lithium D-lactate, 20 mM; (B) NADH (sodium salt), 5 mM; (C) lithium L-lactate, 20 mM; (D) disodium succinate, 20 mM; and (E) sodium ascorbate, 20 mM, and PMS, 0.1 mM. Incubations were continued at 25 °C for the times shown, and the reactions were terminated and assayed as described (Kaback, 1974b). (○) Parent; (△) mutant.

D-lactate. In mutant vesicles, the initial rate of transport with D-lactate is only about 10% of that of the parent, and no proline uptake is observed with NADH, L-lactate, or succinate. Strikingly, however, the rate and extent of proline uptake in the mutant vesicles approach parental levels in the presence of ascorbate and phenazine methosulfate.

Reconstitution of D-*Lactate Oxidation and* D-*Lactate-Dependent Proline Transport with Quinone Side-Chain Homologues.* In order to investigate ubiquinone-mediated functions, a procedure was developed to reconstitute mutant vesicles with the hydrophobic physiological ubiquinone homologue, Q_8, under conditions that do not substantially diminish the ability of the parent vesicles to catalyze oxidation or transport. The procedure is based on the unique properties of the aprotic solvent dimethyl sulfoxide.

Treatment of mutant vesicles with each of eight ubiquinone homologues (Q_1 through Q_8) in the presence of dimethyl sulfoxide as described in Methods restores quinone-linked functions, but reveals striking differences among the various quinones. These differences are illustrated by comparing reconstitution of D-lactate oxidation and D-lactate-dependent proline transport in mutant vesicles after treatment with either Q_8 or Q_1. As the concentration of added Q_8 is increased from 0 to 260 μM during reconstitution, the concentration of membrane-bound Q_8 increases in an apparently exponential manner up to about 200 nmol of Q_8 per mg of membrane protein (Figure 2). Over a range of membrane-bound Q_8 concentrations from 0 to about 15 nmol per mg of membrane protein (i.e., 50–60 μM added Q_8), D-lactate oxidation and the initial rate of proline transport increase markedly to approximately 150% and 60%, respectively, of the values observed in parental vesicles. Moreover, there is a linear relationship between D-lactate oxidation and D-lactate-driven proline transport over this range of membrane-bound Q_8 concentrations (Figure 2, inset). At concentrations of membrane-bound Q_8 in excess of about 20 nmol per mg of membrane protein (i.e., over 70 μM added Q_8), there is no further increase in D-lactate oxidation, while the initial rate of transport increases slowly to about 80% of the parental level.

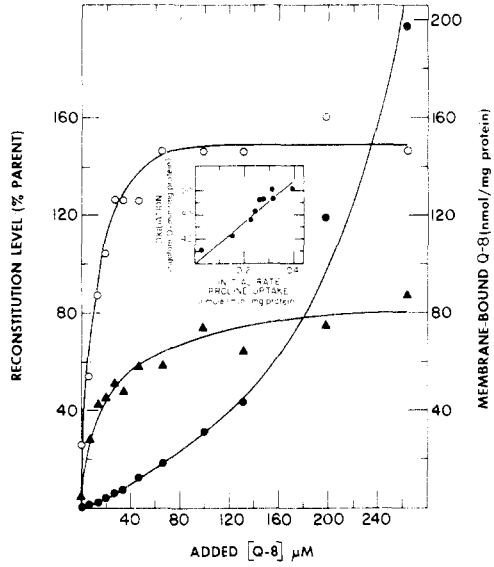

FIGURE 2: Effect of increasing concentrations of Q_8 on D-lactate oxidation, D-lactate-dependent proline transport, and membrane-bound quinone levels in mutant membrane vesicles. Membrane vesicles prepared from the mutant were diluted into dimethyl sulfoxide solutions of Q_8 to give final Q_8 concentrations indicated and treated as described in Methods. Rates of D-lactate oxidation were measured as described in Table I. D-Lactate-dependent proline transport was measured at 0.25, 0.5, and 1 min as described in Figure 1, and initial rates were determined from these data. Membrane-bound quinone levels were measured as described (Crane & Barr, 1971) on samples containing 3 mg of membrane protein. D-Lactate-dependent parameters are expressed as a percentage of the appropriate value obtained with parent vesicles treated with dimethyl sulfoxide in the absence of Q_8. The parent values for D-lactate oxidation and D-lactate-dependent proline transport were 84 ng-atoms/(min·mg of protein) and 0.53 nmol/(min·mg of protein), respectively. (○) D-Lactate oxidation; (▲) D-lactate-dependent proline transport; (●) membrane-bound Q_8. Data presented in the inset show the initial rate of proline uptake plotted as a function of D-lactate oxidation from 0 to 100 μM added Q_8.

Half-maximal reconstitution of both parameters occurs at a membrane-bound Q_8 concentration that is about 50% of the normal Q_8 level in parent vesicles (i.e., 2–3 nmol of Q_8 per mg

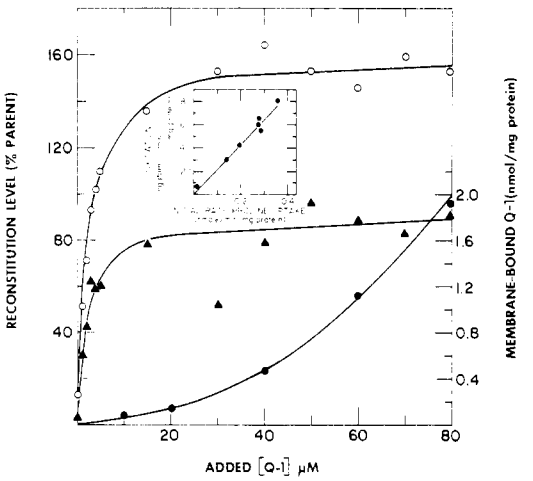

FIGURE 3: Effect of increasing concentrations of Q_1 on D-lactate oxidation, D-lactate-dependent proline transport, and membrane-bound quinone levels in mutant membrane vesicles. Aliquots (200 μL) of mutant membrane vesicles containing 600 μg of membrane protein were diluted to a final volume of 1.0 mL containing (in final concentrations) 50 mM potassium phosphate (pH 6.6), 10 mM magnesium sulfate, 0.1% ethanol, and Q_1 as indicated. After 30-s incubation at 25 °C, rates of D-lactate oxidation were measured as described in Table I. D-Lactate-dependent proline transport was measured at 0.25, 0.5, and 1.0 min as described in Figure 1, and initial rates were determined from these data. Membrane-bound quinone levels were measured as described (Crane & Barr, 1971) on samples containing 3 mg of membrane protein. D-Lactate-dependent parameters are expressed as a percentage of the appropriate value obtained with parent vesicles treated with ethanol in the absence of Q_1. The parent values for D-lactate oxidation and D-lactate-dependent proline transport were 59 ng-atoms of O/(min·mg of protein) and 0.46 nmol/(min·mg of protein), respectively. (○) D-Lactate oxidation; (▲) D-lactate-dependent proline transport; (●) membrane-bound Q_1. Data presented in the inset show the initial rate of proline uptake plotted as a function of D-lactate oxidation from 0 to 18 μM added Q_1.

of membrane protein in the reconstituted mutant vs. 5.9 nmol per mg of protein in the parent), while maximum reconstitution does not occur until the membrane-bound Q_8 concentration is about five times the normal level (i.e., 20–30 nmol of Q_8 per mg of protein). Although not shown, it is also noteworthy that reconstitution of parent vesicles with Q_8 over the concentration range shown in Figure 2 does not result in significant stimulation of either D-lactate oxidation or D-lactate-driven proline transport.

With increasing concentrations of Q_1 added during reconstitution, the concentration of membrane-bound Q_1 also increases in an apparently exponential fashion (Figure 3). Over a range of membrane-bound Q_1 concentrations from 0 to about 0.3 nmol per mg of membrane protein (i.e., about 30 μM added Q_1), D-lactate oxidation and proline transport are restored dramatically to 150% and 80%, respectively, of normal, and, again, there is a linear relationship between D-lactate oxidation and proline transport (Figure 3, inset). Clearly, however, reconstitution in the presence of Q_1 is radically different from that observed in the presence of Q_8. Half-maximal reconstitution with Q_1 occurs at a concentration of membrane-bound Q_1 that is only about 0.3% of the parental level of Q_8 (i.e., about 0.02 nmol of Q_1 per mg of membrane protein in the reconstituted mutant vs. 5.9 nmol of Q_8 per mg of protein in the parent) and maximal reconstitution occurs at about 5% of the parental level of Q_8 (i.e., about 0.3 nmol of Q_1 per mg of membrane protein). In confirmation of earlier observations (Stroobant & Kaback, 1975), reconstitution of parent vesicles with Q_1 over the range of concentrations shown in Figure 3 causes little or no stimulation of D-lactate oxidation or D-lactate-driven proline transport (data not shown).

Several important aspects of these data are immediately apparent. First, it is clear that functional reconstitution with the ubiquinone side-chain homologues does not result simply from binding of the quinones to the membrane, but apparently requires an interaction(s) of the bound quinone with specific sites. Thus, although high levels of D-lactate oxidation and D-lactate-driven proline transport are restored with both long- and short-chain homologues, Q_1 is able to function at much lower membrane-bound concentrations that Q_8. Second, Q_1 functions about 20 times more efficiently than the physiological ubiquinone (i.e., Q_8) present in the parent vesicles. Third, functional reconstitution of the mutant with Q_8 occurs maximally at five times the normal level of membrane-bound Q_8, and it is important in this regard that D-lactate oxidation and proline transport in parental vesicles are not inhibited by treatment with Q_8 at these concentrations (data not shown).

Other reconstitution experiments carried out with each of the homologues Q_2 through Q_7 demonstrate that each homologue at an appropriate concentration restores D-lactate oxidation and D-lactate-dependent proline transport. Interestingly, however, Q_3 and Q_4 exhibit a capacity to stimulate oxidation and inhibit proline transport under certain conditions. For example, treatment of parent and mutant vesicles with 264 μM Q_3 or Q_4 as described in Figure 2 results in 50–100% higher rates of D-lactate and NADH oxidation but 20–30% lower rates of proline transport. The other quinone homologues do not exhibit this property and it is not observed at lower concentrations of Q_3 or Q_4 (i.e., 70 μM). Finally, it should be noted that neither menaquinone-1 nor menaquinone-8 is active to any extent whatsoever in this system (data not shown).

NADH Oxidation and NADH-Dependent Proline Transport with Quinone Side-Chain Homologues. Although wild-type *E. coli* vesicles contain endogenous Q_8 and the quinone is required for NADH oxidation (cf. Table I), oxidation of NADH does not generate $\Delta\bar{\mu}_{H^+}$ or drive active transport unless Q_1 is added to the vesicles (Stroobant & Kaback, 1975; Ramos et al., 1976). Moreover, recent experiments demonstrate definitively that these results cannot be attributed to dislocation of NADH dehydrogenase from the inner to the outer surface of the membrane during vesicle preparation (Owen & Kaback, 1978). In any case, since Q_1 stimulates NADH oxidation only marginally and its effect on transport in the presence of NADH is not due to bulk phase reduction of the quinone (Stroobant & Kaback, 1975), it was suggested that Q_1 shunts reducing equivalents from NADH dehydrogenase to a segment of the respiratory chain containing the site at which $\Delta\bar{\mu}_{H^+}$ is generated. Thus, examination of NADH-linked functions in mutant vesicles reconstituted with Q_1 or Q_8 is of considerable interest.

As shown in Table II, NADH is oxidized at a high rate by wild-type vesicles, but does not drive transport to a notable extent. In confirmation of previous results (Stroobant & Kaback, 1975), when wild-type vesicles are reconstituted with Q_1, proline transport is stimulated over tenfold with essentially no increase in NADH oxidation. On the other hand, when reconstitution is carried out with Q_8, very little stimulation of proline transport is observed and NADH oxidation remains constant. With mutant vesicles, the results are more striking. Reconstitution with Q_1 restores both NADH oxidation and proline transport, while reconstitution with Q_8 restores NADH oxidation with minimal effect on proline transport. In other words, reconstitution of mutant vesicles with the physiological quinone restores the system to its native state where NADH

Table II: Effect of Q_1 and Q_2 on Oxidation and NADH-Dependent Proline Transport in Parent and Mutant Membrane Vesicles

vesicles	addition[a]	NADH oxidation[b] (ng-atoms of O/(min·mg of protein))	initial rate of Pro transport[c] (nmol/(min·mg of protein))
parent	none	316	0.03
	Q_1	320	0.42
	Q_8	299	0.09
mutant	none	51	0.00
	Q_1	154	0.33
	Q_8	299	0.05

[a] Membrane vesicles prepared from the parent and mutant were treated with either Q_1 or Q_8 in dimethyl sulfoxide at final concentrations of 264 μM as described in Methods. For "none", the vesicles were treated with dimethyl sulfoxide in an identical fashion except that quinones were omitted. [b] Rates of NADH oxidation were determined as described in Methods and in Table I. [c] Initial rates of proline uptake were determined as described in Methods and in Figure 1, using final concentrations of NADH (sodium salt) and L-[U-^{14}C] proline (255 mCi per mmol) of 5 mM and 16 μM, respectively.

Table III: Effect of Dithiothreitol on Proline Transport by Parent and Mutant Membrane Vesicles Reconstituted with Q_1 or Q_8

vesicles	addition[a]	initial rate of Pro transport[b] (nmol/(min·mg of protein))
parent	none	0.00
	Q_1	0.85
	Q_8	0.00
	$Q_8 + Q_1$	0.79
mutant	none	0.00
	Q_1	0.44
	Q_8	0.00
	$Q_8 + Q_1$	0.35

[a] As indicated, membrane vesicles prepared from the parent and mutant were treated with Q_8 in dimethyl sulfoxide (final concentration, 264 μM) or with Q_1 in ethanol (final concentration, 80 μM) as described in Figures 2 and 3, respectively, and in Methods. For "none", the vesicles were treated with dimethyl sulfoxide, ethanol, or both in an identical fashion except that quinones were omitted. [b] Initial rates of proline uptake were determined as described in Methods and in Figure 1, using final concentrations of dithiothreitol and L-[U-^{14}C] proline (255 mCi per mmol) of 20 mM and 16 μM, respectively.

is oxidized but does not drive transport. Although data will not be presented, it is noteworthy that these effects are independent of Q_8 concentration (i.e., lower concentrations of Q_8 do not lead to stimulation of proline transport in the presence of NADH) and, of the other ubiquinone homologues tested, only Q_2 behaves like Q_1 with respect to stimulation of proline transport. No NADH-dependent transport is observed with Q_3, and results similar to those obtained with Q_8 are observed with Q_4, Q_5, Q_6, and Q_7.

Accessibility of Q_8 in Native and Reconstituted Vesicles. Although little is known concerning the environment of Q_8 in the cytoplasmic membrane of *E. coli*, on the basis of the location of the ubiquinone biosynthetic enzymes, it has been proposed that the benzoquinone rings of the quinone intermediates are oriented at the inner surface of the membrane (Leppik et al., 1976). Furthermore, dithiothreitol readily reduces Q_1 in solution and Q_8 in aqueous suspension (Hare & Crane, 1971; Stroobant & Kaback, unpublished observations), and this hydrophilic thiol has been used to study quinone accessibility in mitochondrial membranes (Crane, 1977). As shown in Table III, addition of dithiothreitol alone to parent vesicles does not result in proline transport. In contrast, when the thiol is added in the presence of Q_1, dramatic stimulation of proline transport is observed, and the initial rate is about 30% higher than that observed in the presence of D-lactate. However, even when parent vesicles are reconstituted with an excess of Q_8, dithiothreitol-driven proline transport is not observed, and the lack of stimulation cannot be attributed to an uncoupling effect since transport is observed when Q_1 is also added. Similarly, in mutant vesicles reconstituted with Q_8, dithiothreitol-dependent proline uptake is not observed unless Q_1 is added, and reduced Q_1 drives transport in mutant vesicles in the absence of Q_8. The results indicate that Q_8 is inaccessible from the external surface of the membrane, and that even in the Q_8-reconstituted mutant this property is maintained despite a 25-fold excess of the quinone.

Discussion

The results presented in this paper document the functional reconstitution of membrane vesicles prepared from a quinone-deficient mutant of *E. coli* with a range of ubiquinone side-chain homologues from Q_1 to the physiological quinone Q_8. Although the efficiency of reconstitution varies dramatically with respect to the length of the polyprenyl side chain, under appropriate conditions, each quinone restores D-lactate oxidation and the initial rate of D-lactate-dependent proline transport to levels that are comparable to those observed in vesicles prepared from the parent. It is apparent therefore that the length of the polyprenyl side chain is not absolutely critical for quinone function. Furthermore, all of the quinone homologues tested restore NADH oxidation in the mutant vesicles. Surprisingly, however, only those ubiquinones with very short side chains, most notably Q_1, enable NADH oxidation to drive proline transport. These observations provide additional support for the contention that the lack of coupling between NADH oxidation and active transport (i.e., the generation of $\Delta\bar{\mu}_{H^+}$) is not due to an artifact inherent in the vesicle system. Stated otherwise, the data support the postulate (Barnes & Kaback, 1971; Kaback & Barnes, 1971; Schuldiner & Kaback, 1975) that the site at which $\Delta\bar{\mu}_{H^+}$ is generated in *E. coli* membrane vesicles is located in a relatively specific segment of the respiratory chain between D-lactate dehydrogenase and cytochrome *b* (cf. Stroobant & Kaback, 1975; Owen & Kaback, 1978, for a summary of additional evidence).

It is important to consider the results presented here within the context of a recently published sequence of respiratory intermediates in the electron transfer chain of *E. coli* (Downie & Cox, 1978). This sequence, together with a sulfhydryl-dependent intermediate (Kaback & Patel, 1978) and a schematic representation of the site at which $\Delta\bar{\mu}_{H^+}$ is presumably generated (Kaback et al., 1977), is shown in Figure 4. Given the two sites at which ubiquinone purportedly functions, reconstitution of D-lactate-linked processes must involve the second site in that portion of the respiratory chain common to both D-lactate dehydrogenase and NADH dehydrogenase (i.e., that portion to the right of cytochrome b_{556}). Although all of the ubiquinone homologues tested function at this site, Q_1 is able to do so at a membrane-bound concentration that is only about 5% of the level at which Q_8 is present in the parent. This finding is relevant to previous studies with another ubiquinone-deficient mutant of *E. coli* (Newton et al., 1972) showing that the concentration of ubiquinone normally

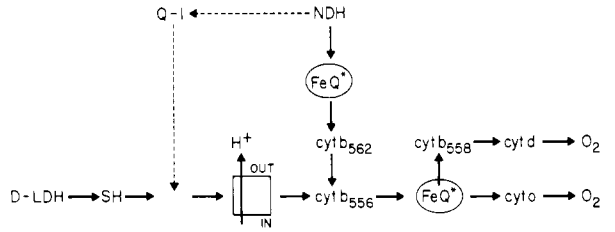

FIGURE 4: Schematic representation of sequence of D-lactate and NADH dehydrogenases, cytochrome and ubiquinone in the electron transfer chain of *E. coli* showing a sulfhydryl-dependent respiratory intermediate, the site at which $\Delta\bar{\mu}_{H^+}$ is generated, and the Q_1-mediated transfer of reducing equivalents from NADH dehydrogenase to a site containing ubiquinone-1 dehydrogenase activity. D-LDH, D-lactate dehydrogenase; NDH, NADH dehydrogenase; SH, sulfhydryl-dependent respiratory intermediate; Q*, ubisemiquinone.

present in wild-type membrane preparations (27 times the concentration of cytochrome b_1) is necessary for maximum oxidase activity. The findings presented here imply therefore that, although the quinone ring of Q_1 can interact with the same functional sites as Q_8, the short side chain of Q_1 allows it an enhanced mobility within the membrane that enables it to function much more efficiently. If this interpretation is correct, Q_8 cannot be freely mobile within the membrane as is commonly assumed, but must move in a restricted manner. Moreover, this restriction in mobility may represent a limiting factor in quinone function.

Previous evidence (Schuldiner & Kaback, 1975; Stroobant & Kaback, 1975) indicates that the site at which $\Delta\bar{\mu}_{H^+}$ is generated in *E. coli* membrane vesicles is located between D-lactate dehydrogenase and cytochrome *b*. In view of the scheme shown in Figure 4, this contention makes it seem unlikely that ubiquinone plays a direct role in the generation of $\Delta\bar{\mu}_{H^+}$ in this system because there is no quinone site between D-lactate dehydrogenase and cytochrome b_{556} and NADH oxidation does not generate $\Delta\bar{\mu}_{H^+}$. Clearly, however, when Q_1 is added to the vesicles, NADH oxidation is productive for active transport. Possibly, the short side chain of Q_1 enables the quinone, after reduction by NADH dehydrogenase or dithiothreitol, to bypass cytochrome b_{562} and reach a ubiquinol-1 dehydrogenase site located prior to the region of the chain at which $\Delta\mu_{H^+}$ is generated. This suggestion is supported by recent studies of the steady-state level of reduction of the *b* cytochromes in a ubiquinone-deficient mutant of *E. coli* (Downie & Cox, 1978). These studies indicate that addition of Q_1 partially inhibits the reduction of cytochrome b_{562} when added in the presence of NADH. Finally, it should be emphasized that, although the scheme presented in Figure 4 can account for many of the observations at hand, it is largely conjectural, and more definitive conclusions must await a detailed analysis of the respiratory chain in this experimental system.

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