Mutants of *Salmonella typhimurium* and *Escherichia coli* Pleiotropically Defective in Active Transport*

(energy-transfer coupling mutants/D-lactate dehydrogenase mutants/membrane vesicles/active transport)

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ABSTRACT Two classes of mutants isolated from *E. coli* and *Salmonella typhimurium* are altered in respiration-coupled active transport, as studied in whole cells and/or isolated membrane vesicles. Mutant cells defective in D-lactate dehydrogenase (*dld*) transport amino acids and lactose normally. Membrane vesicles prepared from these mutants do not exhibit D-lactate-dependent transport, D-lactate oxidation, or D-lactate:dichlorophenolindophenol reductase activity. However, succinate-dependent transport is markedly enhanced in these mutants, without a corresponding increase in succinic dehydrogenase activity.

The second class of mutants is defective in the coupling of electron transfer to active transport. Whole cells and membrane vesicles prepared from these *etc* mutants exhibit markedly reduced ability to transport amino acids, despite the ability of the vesicles to oxidize D-lactate, succinate, and NADH. Vectorial phosphorylation of α -methylglucoside by these mutants is normal. Electron-transfer coupling mutants are similar phenotypically to mutants uncoupled for oxidative phosphorylation (*uncA*), but have normal ATPase activity. Moreover, *uncA* mutants catalyze active transport as well as does the wild type. These experiments indicate that the ETC component is essential for the coupling of respiratory energy to active transport, and provide further evidence that the generation or utilization of ATP is not involved in these transport mechanisms.

The transport of a wide variety of metabolites by membrane vesicles isolated from *Escherichia coli* is coupled primarily to D-lactate oxidation catalyzed by a flavin-linked D-lactate dehydrogenase (1-9).‡ Other oxidizable substrates, such as succinate, L-lactate, or NADH do not serve nearly as effectively as electron donors for transport, and evidence has been presented that shows that the site of energy coupling of D-lactate dehydrogenase to active transport occurs after 80% of the membrane-bound flavin, but before the cytochrome chain (3, 4, 8, 10). The mechanism involved appears to involve specific sulfhydryl-containing "carrier" proteins that reflect the redox potential of the respiratory chain at the site of energy coupling (4, 8). A simplistic conceptual model has been formulated (4) in which the carrier molecules are depicted as electron-transfer intermediates and a critical disulfide group in these carrier molecules is postulated to undergo

reduction by D-lactate or ascorbate-phenazine methosulfate, resulting in conformational change and translocation of substrate from the outside of the membrane to the inside. Concomitant with the conformational change, the affinity of the carriers for ligand is decreased, and substrate is released on the inside of the membrane. Subsequently, the reduced carriers are reoxidized, and the cycle can then be repeated. By this means, the internal solute concentration will increase until it becomes high enough to saturate the "reduced" (sulfhydryl) form of the carrier, at which time the rate of efflux will be equal the rate of influx, and a steady-state will be achieved.

The results presented in this paper represent initial attempts to study these mechanisms by genetic means. The isolation of two classes of mutants and their properties are described. One class, designated electron-transfer coupling (*etc*) mutants, is pleiotropically defective in active transport, and is apparently altered in a component that is essential for coupling respiration to active solute accumulation.

METHODS

Bacterial Strains. All strains were derived from either *S. typhimurium* LT-2 or *E. coli* ML 308-225 (*i⁻z⁻y⁺a⁺*). *E. coli* K12 strains AN120 and AN180 (11) were generous gifts from Prof. Frank Gibson.

Media. Nutrient broth or minimal salts medium E (12) containing 2% glucose was supplemented with 1.5% agar for plates. Minimal salts medium (13) supplemented with 1.0 g/liter of NH_4Cl was used to prepare D-lactate-, succinate-, or pyruvate-containing media.

Phage. The phage used for mutagenesis and transduction was a nonintegrating mutant of P22, P22-int-4 (14).

Isolation of Mutants. Mutants unable to grow on D-lactate as sole carbon source, but able to grow on glucose, were isolated by mutagenesis with diethylsulfate or nitrosoguanidine (15), penicillin enrichment, and replica plating. Because *E. coli* and *S. typhimurium* grow very slowly on D-lactate, the penicillin enrichment step was usually performed for 4-6 hr.

Preparation of Membrane Vesicles. Membrane vesicles were prepared according to described methods (16). Spheroplasts from *S. typhimurium* were prepared essentially according to the method of Osborne *et al.* (17), and vesicles were prepared as described for *E. coli* (16).

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Transport Assays. Amino-acid and lactose transport were assayed (16) in whole cells and membrane vesicles. Unless otherwise indicated, the specific activities and final concentrations of the radioactive compounds used were as follows: L-[U-¹⁴C]proline (209 Ci/mol), 14.4 μ M; L-[U-¹⁴C]lysine (257 Ci/mol), 11.8 μ M; L-[U-¹⁴C]serine (118 Ci/mol), 17.0 μ M; L-[U-¹⁴C]glutamic acid (205 Ci/mol), 23.4 μ M; L-[U-¹⁴C]phenylalanine (385 Ci/mol), 9.6 μ M; L-[U-¹⁴C]tyrosine (410 Ci/mol), 5.7 μ M; L-[U-¹⁴C]histidine (256 Ci/mol), 9.5 μ M; L-[U-¹⁴C]valine (72.8 Ci/mol), 50 μ M; [U-¹⁴C]lactose (14.9 Ci/mol), 200 μ M; methyl[¹⁴C] α ,D-glucopyranoside (52.2 Ci/mol), 36 μ M.

D-Lactate Dehydrogenase (EC 1.1.2.3). Activity was measured spectrophotometrically with dichlorophenolindophenol as electron acceptor (3).

Oxygen Uptake. Rates of oxygen uptake were measured with a Clark electrode as described (3).

Protein Determinations. Protein was determined as described by Lowry *et al.* (18) with bovine-serum albumin as standard.

RESULTS

D-Lactate dehydrogenase (*dld*) mutants

dld Mutants do not grow on D-lactate as a sole carbon source, but grow normally on glucose, D-ribose, glycerol, succinate, fumarate, and malate. They lack D-lactate dehydrogenase activity, as measured by dichlorophenol indophenol reduction (data not shown) and on the basis of oxygen uptake measurements (Table 1). Mutants *dld-3* and *dld-5* were derived from diethylsulfate-treated *E. coli* ML 308-225, and *dld-16* from nitrosoguanidine-treated *S. typhimurium* LT-2. Preliminary mapping studies with the *Salmonella dld* mutants place the defect at about 74 min on the *Salmonella* chromosome map (19).

The data presented in Fig. 1 show the uptake of proline, glutamic acid, and lactose by washed cells of *E. coli* ML 308-225 wild-type and mutant *dld-3*, and of proline, glutamic acid, and tyrosine by isogenic strains of *S. typhimurium*, *dld-27* and a Dld⁺ transductant obtained by transduction of mutant *dld-27* with phage grown on wild-type LT-2. There is no significant difference in the initial rates of uptake of any of the solutes tested between wild-type and mutant strains.

TABLE 1. Oxidation of D-lactate, succinate, and NADH by *dld* and *etc* membrane vesicles

Membrane vesicles	Rate of oxygen uptake in the presence of:		
	D-lactate	Succinate	NADH
	ng-atoms/min per mg		
<i>E. coli</i> ML 308-225 wild-type	139	47	374
<i>E. coli</i> ML 308-225 <i>dld-3</i>	<4	48	472
<i>E. coli</i> ML 308-225 <i>dld-5</i>	0	37	333
<i>E. coli</i> ML 308-225 <i>etc-7</i>	108	33	523
<i>S. typhimurium</i> wild-type	109	102	278
<i>S. typhimurium dld-16</i>	0	54	225
<i>S. typhimurium dld-27</i>	<3	78	255
<i>S. typhimurium</i> TH31 (<i>etc-10</i>)	168	71	196

Membrane vesicles were prepared from glucose-grown cultures.

FIG. 1. Uptake of amino acids and lactose by intact cells. Overnight cultures (0.5 ml) grown on nutrient broth were transferred to 250-ml Erlenmeyer flasks containing 70 ml of minimal glucose (0.5%) medium, and the cultures were grown at 37° with vigorous shaking. Cells were harvested at mid-log phase by centrifugation, washed twice, and resuspended in carbon-free salt medium (13) to about 300 Klett units (No. 66 filter). The cells were then starved for 2 hr by shaking at 37°. Finally, they were incubated with 50 μ g/ml of chloramphenicol for 15 min at 25°. Transport was assayed (16) at 25°. Top: *E. coli* wild-type, $\circ$ — $\circ$; *dld-3*, $\bullet$ — $\bullet$; Bottom, *S. typhimurium*, *dld-27*, $\bullet$ — $\bullet$; Dld⁺ transductant, $\circ$ — $\circ$.

Membrane vesicles prepared from *dld* mutants are markedly defective in D-lactate-dependent transport (Figs. 2 and 3). Membrane vesicles prepared from *E. coli* ML 308-225 wild-type and a representative *dld* mutant grown on succinate are unable to transport lactose (Fig. 2A) or proline (Fig. 2B) with D-lactate as electron donor. However, succinate-dependent transport in this mutant, as in other *dld* mutants, is dramatically enhanced. As shown previously (1–3, 6) and in the left-hand panels of Fig. 2A and B, succinate is a poor electron donor for transport in wild-type vesicles. In *dld* mutants, however, succinate is almost as effective as D-lactate with wild-type vesicles (right-hand panels in Fig. 2A and B). This phenomenon is also observed with vesicles prepared from glucose-grown cells (Fig. 3A), in which succinate oxidation is markedly diminished. As shown in Fig. 3B, *dld* mutants of *S. typhimurium* also exhibit similar properties. Despite marked enhancement of succinate-stimulated transport in these *dld* mutants, no increase in succinate oxidation is observed (Table 1). Although not shown, it should be noted that transport stimulated by ascorbate-phenazine methosulfate is similar in wild type and *dld* vesicles.

Electron transfer coupling (*etc*) mutants

A few strains that do not grow on D-lactate have normal D-lactate:dichlorophenol indophenol reductase activity and,

FIG. 2. Uptake of lactose (A) and proline (B) by membrane vesicles prepared from *E. coli* ML 308-225 and a D-lactate dehydrogenase mutant, *dld-3*, grown on succinate (*succ*). *Lac*, lactate; *Q-HB*, α -hydroxybutyrate.

unlike *dld* mutants, cannot grow on succinate, fumarate, malate, or D-ribose in addition to D-lactate. Preliminary mapping data indicate that this mutation, as opposed to *dld*, is located at 120 min on the *S. typhimurium* chromosome map (19), and is 40% cotransducible with *asn-1*, an asparagine auxotroph derived from diethylsulfate-treated wild-type LT-2 in this laboratory. Several *etc* mutants were also isolated by localized mutagenesis (20) with *asn-1* as the recipient for transduction. One of these mutations, *etc-10*, was transduced into *asn-1* yielding TH31 (*etc-10*), and one of the *Etc*⁺ transductants, TH32, was picked as an isogenic wild-type strain of TH31. Electron-transfer coupling mutants grow on glucose and glycerol at about half the rate of the wild type, have a lower growth yield on these sugars, and are phenotypically similar to *uncA* mutants of *E. coli* that are uncoupled for oxidative phosphorylation (11). However, unlike *uncA* mutants that lack Mg^{++} , Ca^{++} -activated ATPase activity, *etc* mutants have normal ATPase activity, as shown in Table 2. ATPase assays with AN120 (*uncA*) and AN180, an isogenic *UncA*⁺ derived from AN120, are also shown for comparison.

In contrast to *dld* mutants, *etc* mutant cells exhibit markedly reduced ability to transport various amino acids (Fig. 4). Clearly, the *etc* mutant TH31 (*etc-10*) does not take up any of the amino acids tested as well as the isogenic *Etc*⁺ strain TH32. In order to insure that the defect in amino-acid transport is not due to a nonspecific membrane lesion, the trans-

TABLE 2. Mg^{++} -Activated ATPase activities

Strain	P _i released μmol/mg protein per 10 min	
	- Mg^{++}	+ Mg^{++}
<i>S. typhimurium</i>		
TH32 (<i>Etc</i> ⁺)	0.23	0.57
TH31 (<i>etc-1</i>)	0.50	1.27
<i>E. coli</i> ML 308-225		
wild-type	0.29	0.78
<i>etc-15</i>	0.03	0.85
<i>E. coli</i> K12		
AN180 (<i>UncA</i> ⁺)	0.31	0.91
AN120 (<i>uncA</i>)	0.25	0.37

Mg^{++} -Activated ATPase activity was assayed at 37° in a reaction mixture (200 μl) containing 0.1 M Tris-HCl (pH 8.0), 20 mM $MgSO_4$ (when present), membrane vesicles (30–60 μg of protein), and 0.05 M [γ -³²P]ATP (1.5×10^6 cpm/μmol). ³²P_i Released was extracted by the method of Martin and Doty (21) and counted in a liquid scintillation spectrometer.

port of α -methylglucoside was also studied (Fig. 4H). This glucose analogue is transported via the P-enolpyruvate-P-transferase system (22) by a mechanism that has no direct relationship to respiration-linked transport (1, 2, 5, 6, 8). As shown, the mutant takes up α -methylglucoside at the same rate as does the wild type.

Although detailed data will not be presented in this paper, membrane vesicles prepared from *etc* mutants, as expected, are markedly defective in amino-acid transport with D-lactate, ascorbate-phenazine methosulfate, succinate, L-lactate, α -hydroxybutyrate, or NADH as electron donors. On the other hand, vesicles prepared from *uncA* mutants transport normally in the presence of these electron donors. It is of considerable importance, furthermore, that *etc* vesicles oxidize D-lactate, succinate, and NADH at the same rate as vesicles prepared from the wild type (Table 1), indicating that the defect in *etc* mutants cannot be ascribed to an obligatory component of the respiratory chain.

DISCUSSION

Since D-lactate is the most effective physiological electron donor for the transport of various metabolites by isolated membrane vesicles from *E. coli* and *S. typhimurium*, *dld* mutants were expected to exhibit defective transport properties. As demonstrated here, this is not the case, and such mutants catalyze transport as well as the wild type. In view of the finding that membrane vesicles isolated from *dld* mutants exhibit a dramatic enhancement of succinate-dependent transport in the absence of a corresponding increase in succinate oxidation, it is likely that the absence of a defect in the whole cells is due to a compensatory increase in the coupling of succinate dehydrogenase to transport. The underlying mechanism is obviously of considerable importance, and attempts to study this phenomenon are currently in progress with temperature-sensitive *dld* mutants.

One of the most intriguing problems in this area is the mechanism of coupling of respiration to active transport. Previous experiments from this laboratory (1–8) have led to

the postulate that redox-driven conformational changes are the primary driving force for active transport, and this hypothesis is supported by recent fluorescence studies with 1-anilino-8-naphthalenesulfonate (23). In addition to studies with *uncA* mutants mentioned here, various other observations (8)§ indicate that neither the generation nor utilization of high-energy phosphate compounds is involved in this phenomenon. Chemiosmotic coupling (24-26) has also been considered as a mechanism, but appears to be inconsistent with the available data (6, 8). Although the carriers reflect the redox potential of a specific portion of the respiratory chain between the site of ascorbate-phenazine methosulfate reduction and the cytochromes (4, 8, 10), the evidence presented in this paper demonstrates conclusively that they cannot be direct electron-transfer intermediates within the main portion of the respiratory chain. Membrane vesicles prepared from *etc* mutants oxidize D-lactate and other electron donors normally, but cannot catalyze respiration-linked transport. Thus, the simplistic conceptual model presented earlier must be refined. Possibly, the carriers are components of shunts from the main portion of the electron-transfer chain. Electron transfer could then occur independently of transport, as is the case in *etc* mutants or in the presence of uncoupling agents. In this context, the *ETC* component(s)

FIG. 3. Uptake of proline by membrane vesicles prepared from glucose-grown cultures. (A) *E. coli* ML 308-225 wild-type and mutant *dld-3*. (B) *S. typhimurium* LT-2 wild-type and mutant *dld-27*. Abbreviations: see Fig. 2.

FIG. 4. Uptake of amino acids and α -methylglucoside by intact cells of *etc* mutant TH31 (●—●) and wild-type TH32 (○—○). Cells were prepared as described in Fig. 1, except without starvation. α -MG, α -methylglucoside.

may be involved in "shunting" electrons to and from the main trunk of the respiratory chain to the carriers.

Another point of considerable interest is that the *etc* mutation co-transduces with asparagine synthetase (*asn-1*), and *uric A* maps very close to the same phenotypic marker (11). These preliminary findings suggest that there may be a linkage group coding for at least two independent energy-transducing components, *ETC* and ATPase. Although active transport and oxidative phosphorylation are apparently independent phenomena, it is possible that the *ETC* component is common to both, since *etc* mutants exhibit extremely low aerobic growth yields on glucose or glycerol.

When vesicles prepared from *dld* and *etc* mutants are mixed, D-lactate dehydrogenase-coupled transport is partially reconstituted. Moreover, the increase in D-lactate-dependent transport is correlated with a decrease in the ability of succinate to stimulate transport. Mixing experiments with centrifugal supernatants derived from these vesicles are completely negative. A possible explanation for these results is inter-vesicular transfer of the *ETC* component and/or D-lactate dehydrogenase, as a result of collision between membrane vesicles. Experiments with density-labeled vesicles are currently in progress to investigate this possibility.

NOTE ADDED IN PROOF

In view of the findings presented in this paper and the considerable body of evidence indicating that oxidative phosphorylation or the use of nucleoside triphosphates is not involved in respiration-linked transport (8)§, it seems likely that the ATPase mutant recently reported by Simoni and

Shallenberger (27), which exhibits a defect in amino-acid transport, is a multiple mutant or possibly an *uncA* mutant with a secondary polarity effect on the *etc* locus. Resolution of this inconsistency should be forthcoming in the near future.

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