Mechanisms of Active Transport in Isolated Membrane Vesicles

I. THE SITE OF ENERGY COUPLING BETWEEN D-LACTIC DEHYDROGENASE AND β-GALACTOSIDE TRANSPORT IN ESCHERICHIA COLI MEMBRANE VESICLES*

EUGENE M. BARNES, JR.,† AND H. R. KABACK
From the Roche Institute of Molecular Biology, Nutley, New Jersey 07110

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SUMMARY

Transport of a wide variety of amino acids and sugars by membrane vesicles isolated from Escherichia coli ML 308-225 is coupled primarily to d-lactic dehydrogenase. This membrane-bound, flavin-linked primary dehydrogenase is coupled to oxygen via a cytochrome system also present in the vesicle membrane. Spectrophotometric evidence shows that d-lactic dehydrogenase, succinic dehydrogenase, l-lactic dehydrogenase, and NADH dehydrogenase all utilize the same cytochrome system. There is no relationship between rates of oxidation of electron donors by the respiratory chain (succinate > NADH = d-lactate > l-lactate) and the ability of these compounds to stimulate lactose transport (d-lactate >> succinate > l-lactate > NADH). Furthermore, d-lactate in combination with other electron donors is no more effective than l-lactate alone for support of lactose transport. These findings indicate that the site of energy coupling of d-lactic dehydrogenase to active transport lies between the primary dehydrogenase and cytochrome b.

Supportive evidence for this conclusion is obtained from experiments showing that N-ethylmaleimide and p-chloromercuribenzoate inhibit transport and D-lactate-induced respiration. However, these sulfhydryl reagents do not inhibit d-lactic dehydrogenase with dichlorophenolindophenol as an artificial acceptor, nor do they significantly block NADH-induced respiration.

Previous work from this laboratory showed that concentrative uptake of amino acids (2) and β-galactosides (3) by isolated cytoplasmic membrane vesicles from Escherichia coli is coupled primarily to a membrane-bound d-lactic dehydrogenase. Subsequent studies have shown that transport systems for galactose,1 glucose-6-P,2 mannanase (4), and potassium (in the presence of valinomycin) (5) are also coupled primarily to this dehydrogenase. Although it was apparent from the initial studies that generation of high energy phosphate compounds was not involved in these transport systems, a requirement for electron transport was clearly shown.

This paper reports further studies on the nature of coupling of d-lactic dehydrogenase to active transport in isolated membrane vesicles. For the purposes of this communication, the β-galactoside transport system has been selected as an example; however, it should be emphasized that the studies reported here have been carried out with each d-lactate coupled transport system with qualitatively similar results. The results of these studies will be published at a later date.

METHODS

Membrane Preparations—E. coli ML 308-225 (g-y+a+) was grown on Medium A containing 1% disodium succinate (hexahydrate). Membrane vesicles were prepared from these cells as described previously (6, 8).

Sugar Uptake—The assay for lactose uptake by the vesicles has been reported (3).

D-Lactic Dehydrogenase Assay—Either the assay utilizing d-[14C]lactate described previously (2) or DCI* acceptor was used for d-lactic dehydrogenase. Incubation mixtures for the latter procedure contained (in 1.0 ml): 20 mM potassium phosphate (pH 7.0); 20 mM d-lactate (lithium salt), 100 to 500 μg of membrane protein, and 0.002% dichlorophenolindophenol. Reduction of DCI was followed at 620 μm with a Gilford recording spectrophotometer.

Oxygen Uptake Measurements—Rates of oxygen uptake were measured with the Clark electrode of a YSI model 53 oxygen monitor (Yellow Springs Instrument Company, Yellow Springs, Ohio). The oxygen-monitoring system was calibrated by the method of Chappell (9) and gave a typical tension of 0.47 μg-atom of oxygen per ml of reaction mixture at 25°. Assay mixtures (3.0 ml) contained 50 mM potassium phosphate (pH 6.6), 10 mM MgSO4, membrane protein (0.2 to 1.0 mg), and 20 mM electron donor (D-lactate, succinate, etc.) with the exception of NADH which was assayed at 5 min. All assays were carried out at 25° and rates of oxygen consumption were linear with time for a minimum of 5 min.

D Difference Spectra—An Amino Chance dual wave length-split

* The abbreviations used are: DCI, dichlorophenolindophenol; BQNO, 2-heptyl 4-hydroxyquinoline-N-oxide; NEM, N-ethylmaleimide; PCMB, p-chloromercuribenzoate.

† Postdoctoral fellow of the American Cancer Society (PF 545).

‡ Present address, Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77045.

$ A preliminary report of portions of this work has been submitted for publication (1).

1 G. K. Kerwar and H. R. Kaback, manuscripts in preparation.

2 P. Bhattacharyya, F. J. Lombardi, and H. R. Kaback, manuscript in preparation.
Substrate Oxidation and Lactose Uptake—The effect of a series of electron donors on lactose uptake is shown in Table I. Addition of n-lactate to membrane vesicles produces a 30-fold increase in the rate of lactose transport and a 20-fold stimulation of the steady-state level of lactose accumulation. Under these conditions intravesicular lactose accumulates to a level over 100 times that of the surrounding medium. As indicated, other substrates such as α-hydroxybutyrate, succinate, and L-lactate are able to stimulate both initial rates and the steady-state level of lactose accumulation, but none are as effective as n-lactate. The second most effective compound, DL-α-hydroxybutyrate, is apparently utilized as substrate for n-lactate dehydrogenase (10). Only very slight stimulation of lactose uptake is observed with NADH.

The rates of oxygen uptake by membrane vesicles in the presence of these substrates are also presented in Table I. In the absence of donor, oxygen utilization by the vesicles is negligible. Addition of oxidizable substrate to membrane preparations produces an immediate and rapid uptake of oxygen from the medium. The most effective electron donors for respiration in these preparations are succinate, n-lactate, and NADH, in that order of effectiveness. The concentrations of substrates used in these studies are saturating both for oxygen uptake and lactose transport. It is clear that no apparent relationship exists between rates of oxidation of these electron donors and their ability to support lactose transport. Although succinate is the best substrate for respiration, it ranks third in stimulation of transport, being less than half as effective as n-lactate. Oxidation of NADH proceeds at nearly the rate of n-lactate, but NADH is only marginally effective in stimulating lactose transport. These findings indicate that the observed specificity of B-galactoside transport for n-lactate dehydrogenase, as opposed to other dehydrogenases, cannot be accounted for solely on the basis of rates of electron flow to oxygen.

Although not shown in Table I, vesicles prepared from cells grown on complex media (i.e. Difco Penassay broth or nutrient broth) have both α-glycerol-P and formate dehydrogenase activities. Moreover, in such membrane preparations, addition of α-glycerol-P or formate stimulates lactose or amino acid uptake about as well as succinate.

Effect of Inhibitors on n-Lactate Respiration—The effect of inhibitors and other agents on n-lactate-dependent oxygen uptake by membrane vesicles is shown in Table II. Amytal, HOQNO, and cyanide all inhibit respiration by the vesicles in the presence of n-lactate. Previous experiments from this laboratory have established that these inhibitors also block lactose uptake by membrane preparations with similar degrees of effectiveness (3). Recent investigations of the respiratory chain of E. coli by Cox et al. (11) have identified the amytal-sensitive site as a flavoprotein between n-lactate dehydrogenase and cytochrome b1. In addition, these workers have indicated that HOQNO acts between cytochrome b1 and cytochrome a2, perhaps as a quinone-containing component, and that cyanide blocks cytochrome a2.

It is also significant that NADH oxidation is also sensitive to HOQNO (76% inhibition at 2 × 10⁻⁴ M) and cyanide (86% inhibition at 10⁻⁴ M) as is succinate oxidation (14% inhibition at 2 × 10⁻⁴ M HOQNO and 93% inhibition at 10⁻³ M cyanide).
Table III

Effect of oxamate on dehydrogenase activities

Rates of DCI reduction and oxygen uptake were determined as described under "Methods." Sodium oxamate (5 mM) was incubated with membrane vesicles in the assay mixture for 2 min prior to the initiation of the reaction with substrate. Results are expressed as per cent inhibition due to oxamate relative to untreated controls.

| Substrate  | Assay method | Inhibition by 5 mM oxamate |
|------------|--------------|---------------------------|
| D-Lactate  | DCI reduction| 91                        |
| D-Lactate  | Oxygen uptake| 91                        |
| NADH       | Oxygen uptake| 3                         |
| Succinate  | Oxygen uptake| <1                        |

indicating that most of the NADH and succinate oxidation observed is cytochrome-linked. These findings show that each dehydrogenase studied is coupled to oxygen via a membrane-bound respiratory chain.

Dinitrophenol, carbonyl cyanide m-chlorophenylhydrazone, and valinomycin do not significantly affect D-lactate oxidation (Table II), despite profound inhibition of lactate transport (3). This finding is not unexpected since most bacterial electron transport systems are not subject to respiratory control. Regarding the failure of antimycin and azide to block respiration, it is noteworthy that many bacterial respiratory systems are resistant to these inhibitors (12). Moreover, antimycin inhibition of δ-galactoside transport by vesicles (8) may be due to induction of passive leakage due to detergent effects of this compound (13).

Numerous experiments including those shown in Table II have established that lactose is totally without effect on δ-lactate oxidation. These findings are in opposition to the experiments of Kepes (14) who found that thiocetylglactoside stimulated oxygen uptake by whole cells.

Oxamic acid, an inhibitor of the cytoplasmic NAD-dependent δ-lactic dehydrogenase (15), also blocks oxidation of δ-lactate by the membrane-bound, NAD-independent δ-lactic dehydrogenase (cf. Table II). A similar degree of oxamate inhibition is observed for either oxygen uptake or DCI reduction in the presence of δ-lactate (cf. Table III). Furthermore, oxamate inhibition is specific for δ-lactic dehydrogenase, since neither succinate nor NADH oxidation is affected. Thus oxamate inhibits at the level of the primary dehydrogenase for δ-lactate.

Utilization of Electron Donors by Cytochrome System—Reduction of the membrane-bound cytochromes by δ-lactate, succinate, NADH, and dithionite is illustrated in Fig. 1. This figure shows difference spectra of substrate-reduced membrane vesicles with respect to control preparations in the oxidized state. Line 1 is a plot of δ-lactate reduced against oxidized spectrum of E. coli ML 308-225 membrane vesicles. The absorption bands are identified (12) as Soret (432 nm), due mainly to the δ band of cytochrome b, flavoprotein trough (465 nm), δ band of cytochrome b, (533 nm), a band of cytochrome b, (590 nm), a band of cytochrome a, (595 nm), and α band of cytochrome a, (630 nm). These peaks account for all known classes of cytochromes of E. coli (12) except for cytochrome o which is not detectable by these methods. This observation is also supported by comparison of the δ-lactate (Line 1), succinate (Line 3), and NADH (Line 4) reduced-oxidized spectra with the dithionite reduced-oxidized spectrum (Line 2). Reduction of the cytochrome system by each substrate studied reveals nearly complete reduction of all available chromophores. Moreover, essentially the same absorption spectra are obtained with vesicles whose electron transport chain is reduced with δ-lactate (Line 1), succinate (Line 3), and NADH (Line 4). Indeed, the difference spectrum between NADH reduced and δ-lactate reduced vesicles (Line 5) shows no significant bands.

Quantitative determination of the relative amounts of each chromophore reduced by various electron donors is shown in Table IV. No significant differences are observed for reduction of Soret, cytochrome b, or cytochrome a, (cytochrome oxidase) by any of the three enzymatic electron donors. There was insufficient cytochrome a, to permit quantitative comparison.

These findings indicate that δ-lactic, succinic, and NADH dehydrogenases utilize the same cytochrome system for electron flow in membrane vesicles. Thus the specificity of the coupling between lactose transport and δ-lactic dehydrogenase (as opposed...
Table IV  
Anaerobic reduction of respiratory components by dehydrogenase substrates

Values for reduction are expressed as percentages of controls reduced with dithionite and were based on the spectra shown in Fig. 1. Wave length pairs were those suggested by Jones and Redfearn (16) and values for the absorption of each component were based on the absorption difference between these wave lengths. Flavoprotein measured by this method may be subject to interference by non-heme iron (11).

| Component         | Wave length pair | Reduction of component  |
|-------------------|------------------|-------------------------|
|                   |                  | $\Delta\%$ Succinate     |
| Soret             | 432-419          | 105                     |
| Cytochrome $b_1$  | 560-575          | 97                      |
| Cytochrome $a_2$  | 630-615          | 99                      |
| Flavoprotein      | 465-310          | 63                      |

Table V  
Effect of thiol reagents on $\alpha$-lactate and NADH oxidation

Rates of oxygen uptake by ML 308-225 membrane vesicles were measured as described under "Methods." N-Ethylmaleimide (0.5 mM) and p-chloromercuribenzoate (0.05 mM) were incubated with membrane protein (105 $\mu$g per ml) for 15 min prior to initiation of the oxidase reaction with substrate. Dithiothreitol (1.0 mM) was added to reaction mixtures (where indicated) after initiation of the oxidase reaction and the rate of oxygen uptake was measured immediately.

| Reagent            | Rate of oxygen uptake |
|--------------------|-----------------------|
|                    | $\Delta\%$ Succinate   |
| NEM                | 27                    |
| PCMB               | 18                    |
| PCMB + dithiothreitol | 92                    |

Discussion

A diagram of some features of the E. coli respiratory chain is shown in Fig. 2. This figure illustrates the order of the components involved in electron flow as suggested by Cox et al. (11). Electrons from $\alpha$-lactic dehydrogenase flow to cytochrome $b_1$ via flavoproteins (in perhaps several stages). Electrons are then transferred to a ubiquinone component perhaps involving non-heme iron (11), which in turn transfers electrons to the cytochrome oxidases, $a_2$ and $a$. Succinic and NADH dehydrogenases also feed electrons via flavins to cytochrome $b_1$ and in the latter case a second quinone site appears to be involved (11). This scheme is not intended to represent complete pathways for electron transport but merely the order of events thought to occur. With reference to the scheme, the findings presented here indicate that the site of energy coupling for lactose transport is localized primarily between $\alpha$-lactic dehydrogenase and cytochrome $b_1$. It is re-emphasized that the same observations and thus the same conclusions can be extended to the coupling of $\alpha$-lactic dehydrogenase to the other transport systems mentioned previously.

Although current studies do not provide details of the nature of coupling between $\alpha$-lactic dehydrogenase and active transport, several observations have led to a working hypothesis. Possibly, membrane "carrier" proteins for transport substrates are themselves electron transfer intermediates localized in the membrane to succinie or NADH dehydrogenases) cannot be related either to rates of electron flow to oxygen or to a unique cytochrome system coupled to $\alpha$-lactic dehydrogenase. This conclusion is supported by experiments in which lactose transport was studied in the presence of fixed concentrations of $\alpha$ lactate and increasing concentrations of either succinate or NADH. There was no additional stimulation of the rate of lactose transport over that obtained with $\alpha$-lactate alone under any condition studied. It seems clear therefore that the site of coupling for $\alpha$-lactic dehydrogenase to transport must occur prior to entry of electrons into the cytochrome system.

Effect of Sulfhydryl Reagents on Oxidation  The effect of N-ethylmaleimide and p-chloromercuribenzoate on $\alpha$-lactic oxidations is shown in Table V. Addition of NEM produces a 73% decrease in oxidation of $\alpha$-lactic and PCMB reduces $\alpha$-lactic-dependent oxygen uptake by 82%. These reagents are effective inhibitors of lactose transport by membranes (3). Reversal of PCMB inhibition by dithiothreitol provides further evidence that sulfhydryl groups are necessary for oxidation of $\alpha$-lactate.

Inhibition of oxygen uptake by PCMB and NEM does not appear to be mediated at the level of the primary dehydrogenase for $\alpha$-lactate. Neither $\alpha$-lactate-DC1 reductase activity in intact vesicles (125 nmoles of DC1 reduced per min per mg of protein) nor a solubilized, partially purified preparation of this enzyme4 (300 nmoles of DC1 reduced per min per mg of protein) is sensitive to NEM or PCMB inhibition. It is important to note that NADH oxidation (cf. Table V) is not sensitive to NEM, and that the small amount of inhibition due to PCMB is not reversed by dithiothreitol. Thus, neither the primary $\alpha$-lactic dehydrogenase itself nor the cytochrome system contains a sulfhydryl-sensitive site. Therefore, the site of inhibition of $\alpha$-lactic dehydrogenase by NEM and PCMB must lie between $\alpha$-lactic dehydrogenase and the cytochromes.

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4 In collaboration with Dr. Leonard D. Kohn of the Laboratory of Biochemical Pharmacology, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland, the membrane-bound $\alpha$-lactic dehydrogenase has been solubilized and purified approximately 250- to 300-fold from whole cells of E. coli ML 308-225. At this stage, the preparation is approximately 80% pure as judged by disc gel electrophoresis, and contains cytochrome $b_1$ but no phospholipid or dehydrogenase activity toward succinate or NADH. The purification and properties of this enzyme will be published at a later date.
between primary dehydrogenases (principally d-lactic dehydro-
genase in E. coli) and cytochrome b1. A cycle of d-lactate-de-
gen-}dependent reduction of the carriers followed by oxidation through
cytochrome b1 (perhaps by sulfhydryl-disulfhydryl interconver-
sion) could account for reversible conformation changes. De-
creased affinity of the reduced form of a carrier protein for its
transport substrate at the interior face of the membrane could be
a result of this conformational change.

There are several observations which are consistent with such
a model.

1. Oxidation-reduction coupling of carriers accounts directly
for utilization of electron flow for active transport.

2. Association of carrier molecules with dehydrogenase systems
branched before cytochrome b1 explains electron donor speci-
city.

3. Conformational changes in the carrier model do not require
participation of high energy phosphate compounds. Participation
of such energy sources has been suggested in an earlier model
(17) but such compounds are not required for d-lactic dehydro-
genase-coupled sugar or amino acid transport in vesicles (2, 3).

The model proposed here is intended only to summarize some
of our working hypotheses. Another possible mechanism for
energy coupling of d-lactic dehydrogenase to active transport in-
volving participation of proton or potential gradients has been
suggested recently (18, 19). However, this type of mecha-
nism appears to be ruled out by studies presented in the
following paper (20).

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