JOURNAL OF BACTERIOLOGY, Nov. 1974, p. 861-865

Vol. 120, No. 2
Printed in U.S.A.

Stimulation of Transport into *Escherichia coli* Membrane Vesicles by Internally Generated Reduced Nicotinamide Adenine Dinucleotide

MASAMITSU FUTAI[1]

Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14850

Received for publication 24 June 1974

Membrane vesicles containing internal alcohol dehydrogenase and nicotinamide adenine dinucleotide (NAD) were prepared from *Escherichia coli* ML308-225. Upon the addition of ethanol, these vesicles respired and transported several amino acids. Transport rates driven by internally generated reduced NAD (NADH) were about 60 to 80% of that stimulated by D-lactate. This transport was inhibited by cyanide and anaerobiosis. Ferricyanide, a nonpermeable electron acceptor, inhibited transport stimulated by external NADH, but not that stimulated by internally generated NADH.

The oxidation of D-lactate by the membrane-bound D-lactate dehydrogenase greatly stimulates the active transport of various amino acids and sugars into membrane vesicles (8). Succinate and nicotinamide adenine dinucleotide (NAD), reduced form (NADH), support transport less efficiently than D-lactate, although membrane vesicles oxidize both substrates at rates comparable to that for D-lactate. From this evidence, Kaback and his colleagues have suggested that D-lactate dehydrogenase plays a special role in the coupling of energy to active transport (8). The available evidence does indicate that D-lactate is particularly effective as an energy source for transport. However, comparison with NADH may not be justified since, as pointed out by Harold (5), this is probably an impermeant coenzyme which could not be oxidized by correctly oriented vesicles. In a recent study, the apparent inefficiency of NADH in driving transport has been attributed to the presence of open or inverted vesicles in membrane preparations, which are thought to be responsible for the bulk of the NADH oxidation (4). I have independently suggested that membrane vesicles prepared as described (8) are not a simple, homogeneous population (M. Futai, Biochemistry, in press; 3).

Since no uptake system for NADH is known, the most logical way to test the ability of NADH to stimulate transport of other substances is to generate it inside membrane vesicles. A method to accomplish this became possible as a result of recent work by Konings and Kaback (9), who modified their method of making membrane vesicles by lysing spheroplasts in a much smaller volume of buffer than was used in the original procedure. In the present investigation, the modified procedure made it practical to carry out the lysis step in the presence of NAD and alcohol dehydrogenase, thereby incorporating both materials inside the vesicles. When ethanol was added to a suspension of these vesicles, amino acid transport was stimulated to 60 to 70% of the rate observed when D-lactate was added to the same vesicles. This stimulation must be caused by internally generated NAD, since when NAD and alcohol dehydrogenase were absent from the vesicles, addition of ethanol gave no stimulation. The transport stimulated by internally generated NADH, if it is expressed as nanomoles transported per unit of oxygen consumption, was much greater than for D-lactate.

MATERIALS AND METHODS

Bacteria and growth conditions. *E. coli* ML308-225 (i^- z^- y^+ a^+) was grown aerobically in a synthetic medium (14) supplemented with 0.5% DL-lactate. Physiologically young cells (2) were used for the preparation of ethylenediaminetetraacetate-lysozyme spheroplasts (3).

Preparation of membrane vesicles containing internal NAD and alcohol dehydrogenase. Membrane vesicle preparation was done essentially by a modification of a recently published method (9). Spheroplasts (1 g, wet weight) were suspended in 5 ml of 0.01 M potassium phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$, 200 μg of alcohol dehydrogenase, 2 mM NAD, and 10 μg of ribonuclease and 10 μg of deoxyribonuclease per ml. The spheroplasts were dispersed by means of a hypodermic syringe

[1] Present Address: Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan.

fitted with an 18-gauge needle. After apparently complete dispersion, the suspension was homogenized by forcing it through the syringe needle five more times. The mixture was incubated at 37 C for 30 min in a rotatory shaking bath and centrifuged at 2,500 × *g* for 20 min to remove whole cells and unlysed spheroplasts. The supernatant was centrifuged at 100,000 × *g* for 20 min. The pellet was suspended in 10 ml of 0.05 M potassium phosphate buffer (pH 7.0) and centrifuged at approximately 100,000 × *g* for 20 min. The supernatant material was discarded, and the pellet was suspended in 0.05 M potassium phosphate (pH 7.0) at a protein concentration of about 10 mg/ml. Experiments using these vesicles were performed on the day of preparation because vesicles stored overnight in an ice bath or at −80 C lost about half of their uptake activity for proline. Membrane vesicles were prepared by lysing spheroplasts in the same buffer but without NAD and alcohol dehydrogenase. Control vesicles also lost activity on storage.

Assays. Transport assays were carried out as described (7) except that the pH of the buffer was 7.0. Alcohol dehydrogenase was assayed by a published procedure (15). The NAD concentration in vesicles was determined by a neutralized perchloric acid extract of membranes, equivalent to 4 mg of protein. Oxygen consumption (Futai, in press) and protein (12) were determined by published procedures.

Materials. Chemicals were obtained as follows: lysozyme, NAD, NADH, alcohol dehydrogenase, and ribonuclease, Sigma Chemical Co.; deoxyribonuclease, Worthington Biochemical Corp.; ^{14}C-labeled amino acids, New England Nuclear; and D-lactate, Calbiochem.

RESULTS

Properties of membrane vesicles containing internal alcohol dehydrogenase and NAD. Electron micrographs of negatively stained preparations revealed vesicles of approximately the same size as those obtained by the original procedure (8). A minor degree of contamination by membranous material other than vesicles was also observed. Almost all of the alcohol dehydrogenase activity in vesicles was cryptic and could not be measured unless the vesicles were treated with toluene or detergents; the specific activities of alcohol dehydrogenase before and after treatment with 1% cholate were 0.02 and 0.17 U per mg of membrane protein. Approximately the same specific activity was observed after treating the vesicles with 1% toluene or 1% Triton X-100. These results suggest that most of the alcohol dehydrogenase is inside the membrane vesicles. The concentration of NAD in vesicles, determined by alcohol dehydrogenase, was 0.01 μmol per mg of membrane protein. Alcohol dehydrogenase and NAD could not be detected in control vesicles prepared without addition of these compounds during lysis.

Oxygen uptake of membrane vesicles with different substrates. Table 1 shows data on the uptake of oxygen stimulated by D-lactate, by NADH added to the outside medium, and by NADH generated internally upon the addition of ethanol. The extent of respiratory stimulation by ethanol was about 10% of that resulting from the addition of NADH or D-lactate. Oxygen consumption caused by addition of ethanol showed no lag period. A greater extent of respiratory stimulation by ethanol had been expected from the amount of alcohol dehydrogenase incorporated into vesicles. However, it should be pointed out that alcohol dehydrogenase was assayed (15) at pH 8.6 in pyrophosphate buffer, and the experiments with vesicles were carried out with neutral phosphate buffer. Also, the rate of entry of ethanol is unknown. Vesicles prepared in the absence of NAD and alcohol dehydrogenase showed no uptake of oxygen upon the addition of ethanol.

Transport of amino acids into membrane vesicles. The transport of proline into vesicles stimulated by the addition of NADH was much lower than was observed for D-lactate, confirming the observation of Lombardi and Kaback (10). On the other hand, the initial rate of transport activity stimulated by NADH generated internally was about 70% of that stimulated by D-lactate (Table 2). The efficiency of

TABLE 1. *Uptake of oxygen by membrane vesicles loaded with NAD and alcohol dehydrogenase*[a]

Membrane vesicles	Substrate	O_2 uptake (ng of atoms/min per mg)
Control	D-Lactate	258
	NADH (external)	168
	NADH generated by ethanol[b]	1.3
Vesicles with internal NAD and alcohol dehydrogenase	D-Lactate	236
	NADH (external)	238
	NADH generated by ethanol	25.5

[a] Membrane vesicles (about 100 μg of membrane protein) were placed in the sample chamber of the oxygen electrode containing 1.2 ml of 0.05 M potassium phosphate (pH 7.0) and 10 mM $MgCl_2$. Ten millimolar D-lactate, 2.5 mM NADH, or 0.1 M ethanol was added as energy source. Oxygen consumption was monitored for at least 10 min. Endogenous oxygen consumption was less than 1 ng of atom/min per mg of protein.

[b] In this experiment ethanol was added to test for the presence of traces of NAD and alcohol dehydrogenase in vesicles made without adding these materials.

generated NADH in stimulating transport was much higher than for external NADH or D-lactate (expressed as nanomoles of proline transported per nanograms of O_2 atoms consumed); transport of proline stimulated by D-lactate, external NADH, and internally generated NADH were 0.012, 0.0023 and 0.066 nmol per ng of O_2 atom consumed per min, respectively.

In these experiments there was no detectable lag period in the stimulation of proline uptake by generated NADH when uptake was measured at 15-s intervals. Furthermore, preincubation of membrane vesicles with ethanol for 1 min did not increase the rate of transport more than 20%. These results suggest that NADH generated upon the addition of ethanol stimulated transport as well as oxygen consumption. Transport of other amino acids stimulated by generated NADH was 60 to 80% of that driven by D-lactate. In all cases, ethanol had no effect on the transport of vesicles made without NAD and alcohol dehydrogenase.

Effect of various inhibitors on transport by membrane vesicles. To confirm the notion that the transport stimulated by generated NADH is driven by energy derived from the respiratory chain, the effects of inhibitors were tested (Table 3). Cyanide, anaerobiosis, *N*-ethylmaleimide, and dinitrophenol inhibited this transport substantially (Table 3), suggesting that the uptake was energized by the same mechanism as that operating in vesicles prepared by Kaback's usual method (8). Malonate and oxamate, inhibitors of succinate and D-lactate dehydrogenase, respectively, had no effect on this transport activity.

Ferricyanide, a nonpermeant electron acceptor, has been shown to inhibit the glycerol-3-phosphate-driven transport of reconstituted vesicles completely, and of natural vesicles partially (Futai, in press). In those experiments

TABLE 3. *Effect of various compounds on transport of proline stimulated by D-lactate and NADH generated inside vesicles*[a]

Addition	Concentration (mM)	Inhibition of:	
		D-Lactate-driven transport (%)	Transport driven by internally generated NADH (%)
NaCN	2	50	25
NaCN	10	88	87
Anaerobiosis		82	68
N-ethylmaleimide	2	95	90
Dinitrophenol	5	94	95
Oxamate	10	98	0
Malonate	10	0	0

[a] The rate of transport of proline was measured as described in the legend to Table 2. Inhibitors were incubated with membranes for 15 min at 23 C in the absence of an energy source. For the anaerobiosis experiment, the incubation mixture was flushed with nitrogen for 10 min. Reactions were then initiated with ^{14}C-labeled proline after adding an energy source. Transport rates of proline without inhibitor and with ethanol and D-lactate were 1.60 and 2.22 nmol/mg of protein per min, respectively.

TABLE 2. *Effect of various energy sources on uptake of amino acids by E. coli ML308-225 membrane vesicles loaded with NAD and alcohol dehydrogenase*[a]

Membrane vesicle	Amino acid	Transport[b] stimulated by the addition of:			
		Nothing	Ethanol	D-Lactate	NADH
Control	Proline	0.080	0.11	2.78	0.62
	Serine	0.22	0.21	2.17	
	Glycine	0.26	0.20	0.95	
	Phenylalanine	0.11	0.15	0.42	
Vesicles with internal NAD and alcohol dehydrogenase	Proline	0.062	1.68[c]	2.41	0.54
	Serine	0.33	1.28	2.05	
	Glycine	0.13	0.68	0.81	
	Phenylalanine	0.07	0.32	0.44	

[a] A portion (20 μliters) of *E. coli* ML308-225 membranes containing about 0.2 mg of membrane protein was added to the assay mixture (0.5 ml) containing 0.05 M potassium phosphate, 10 mM $MgSO_4$, and energy source (ethanol, 5 μliters; D-lactate, 10 mM; NADH, 2.5 mM) where indicated. After incubation at 23 C for 30 and 60 s, samples (200 μliters) were withdrawn and filtered through membrane filters (Millipore Corp.) and counted.

[b] Expressed as nanomoles per milligram of membrane protein per minute.

[c] The transport of proline driven by internally generated NADH varied from 0.8 to 1.68 nmol per mg of protein per min in five experiments, with an average value of 1.2. A lower stimulation may be noted in Fig. 1. Uptake driven by D-lactate and NADH varied in corresponding fashion so that the relative rates remained fairly constant.

ferricyanide acted as a competing electron acceptor for glycerol-3-phosphate dehydrogenase that was accessible to the outside. Thus it is of interest to compare the effect of ferricyanide on active transport driven by NADH added externally or generated inside the vesicles by means of ethanol. Ferricyanide had no effect on the transport stimulated by internally generated NADH, although transport stimulated by NADH added to the medium was inhibited about 70% (Fig. 1). Respiration stimulated by externally added NADH was inhibited to about the same extent, but ferricyanide had no effect on respiration induced by internally generated NADH. These effects were observed both when assays were begun with the addition of ferricyanide or when the reaction with NADH followed a preincubation of membranes with ferricyanide.

DISCUSSION

Internally generated NADH has been shown to stimulate transport nearly as much as D-lactate, and with greater efficiency when stimulation of transport is expressed per unit of oxygen consumed. This suggests that NADH dehydrogenase as well as D-lactate dehydrogenase can energize transport. The effectiveness of succinate and L-lactate dehydrogenase has already been documented, using vesicles from a D-lactate dehydrogenaseless mutant (6), and it has been established that L-glycerol-3-phosphate dehydrogenase (G. W. Dietz, Fed. Proc. 30:1062, 1971) also can stimulate transport in vesicles.

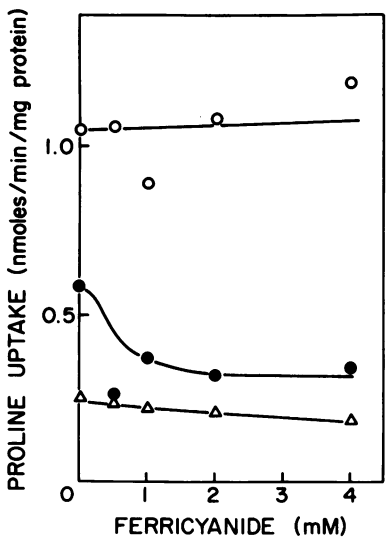

FIG. 1. *Effect of ferricyanide on transport of proline stimulated by NADH added externally and generated inside vesicles. A portion of membranes (about 0.2 mg) was added to the assay mixture containing different concentrations of potassium ferricyanide. Endogenous uptake* (△), *and transport stimulated by NADH* (●) *or ethanol* (○) *were measured.*

In agreement with my results, stimulation of transport in vesicles by externally added NADH has been reported to be very low, although NADH oxidase activity was as great as that for D-lactate (10). In vesicles made from *E. coli* K-12, much greater stimulation of transport by externally added NADH was found (13). Harold (5) has pointed out that NADH oxidase activity measurable in membrane vesicles suggests some damage to the vesicles. In this regard, Lombardi et al. (11) observed that NADH stimulates uptake of oxygen by intact *E. coli* ML308-225, suggesting that NADH is permeable. However, respiration of NADH by *E. coli* ML308-225 grown in synthetic medium was negligible (M. Futai, unpublished observations), confirming results for *Bacillus subtilis* by Hampton and Freese (4). These differences cannot be explained at the present time.

Transport stimulated by external NADH is inhibited by ferricyanide added as a competing electron acceptor, whereas transport stimulated by internally generated NADH is not. Ferricyanide completely inhibited transport driven by L-glycerol-3-phosphate in reconstituted vesicles in which glycerol-3-phosphate dehydrogenase had been mixed with deficient vesicles (Futai, in press). Partial inhibition was obtained with "natural" vesicles, from which it was concluded that glycerol-3-phosphate dehydrogenase becomes partially dislocated to the outer membrane surface during preparation of membrane vesicles. The same explanation could account for the stimulation of transport by external NADH. The lack of inhibition when NADH is generated internally suggests that ferricyanide does not remove electrons from any part of the respiratory chain other than the dehydrogenase. These qualitatively different effects of ferricyanide would also appear to rule out the possibility that external NADH slowly enters the vesicles to stimulate transport. To provide direct proof that a dislocated dehydrogenase is functional in transport, it would be desirable to have a specific antiserum available, but thus far I have not been able to produce potent antisera in rabbits against either D-lactate or glycerol-3-phosphate dehydrogenase.

ACKNOWLEDGMENTS

I am most thankful to Leon A. Heppel, in whose laboratory this work was done, for suggestions and encouragements during this work. I am also grateful to E. Racker, D. B.

Wilson, and J. M. Wood for useful discussions and to S. L. Johnston for technical assistance. I thank John N. Telford for advice and assistance with the electron microscopy.

This work was supported by Public Health Service grant AM 11789 from the National Institute of Arthritis and Metabolism and Digestive Diseases, and by National Science Foundation grant GB27396X.

LITERATURE CITED

1. Barnes, E. M., Jr., and H. R. Kaback. 1971. Mechanism of active transport in isolated membrane vesicles. I. The site of energy coupling between D-lactate dehydrogenase and β-galactoside transport in *Escherichia coli* membrane vesicles. J. Biol. Chem. **246**:5518–5522.
2. Birdsell, D. C., and E. H. Cota-Robles. 1967. Production and ultrastructures of lysozyme and ethylenediaminetetraacetate-lysozyme spheroplasts of *Escherichia coli*. J. Bacteriol. **93**:427–437.
3. Futai, M. 1974. Orientation of membrane vesicles from *Escherichia coli* prepared by different procedures. J. Membrane Biol. **15**:15–28.
4. Hampton, M. L., and E. Freese. 1974. Explanation for the apparent inefficiency of reduced nicotinamide adenine dinucleotide in energizing amino acid transport in membrane vesicles. J. Bacteriol. **118**:497–504.
5. Harold, F. M. 1972. Conservation and transformation of energy by bacterial membranes. Bacteriol. Rev. **36**:172–230.
6. Hong, J.-S., and H. R. Kaback. 1972. Mutants of *Salmonella typhimurium* and *Escherichia coli* pleiotropically defective in active transport. Proc. Nat. Acad. Sci. U.S.A. **69**:3336–3340.
7. Kaback, H. R. 1971. Bacterial membranes, p. 99–120. *In* W. B. Jacoby (ed.), Methods in enzymology, vol. 22. Academic Press Inc., New York.
8. Kaback, H. R. 1972. Transport across isolated bacterial cytoplasmic membranes. Biochim. Biophys. Acta **265**:367–416.
9. Konings, W. N., and H. R. Kaback. 1973. Anaerobic transport in *Escherichia coli* membrane vesicles. Proc. Nat. Acad. Sci. U.S.A. **70**:3376–3381.
10. Lombardi, F. J., and H. R. Kaback. 1972. Mechanism of active transport in isolated bacterial membrane vesicles. VIII. The transport of amino acids by membranes prepared from *Escherichia coli*. J. Biol. Chem. **247**:7844–7857.
11. Lombardi, F. J., J. P. Reeves, S. A. Short, and H. R. Kaback. 1974. Evaluation of the chemiosmotic interpretation of active transport in bacterial membrane vesicles. Ann. N.Y. Acad. Sci. **227**:312–327.
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
13. Matin, A., and W. N. Konings. 1973. Transport of lactate and succinate by membrane vesicles of *Escherichia coli*, *Bacillus subtilis* and a Pseudomonas species. Eur. J. Biochem. **34**:58–67.
14. Tanaka, S., S. A. Lerner, and E. C. C. Lin. 1967. Replacement of a phosphoenolpyruvate-dependent phosphotransferase by a nicotinamide adenine dinucleotide-linked dehydrogenase for the utilization of mannitol. J. Bacteriol. **93**:642–648.
15. Vallee, B. L., and F. L. Hoch. 1955. Zinc, a component of yeast alcohol dehydrogenase. Proc. Nat. Acad. Sci. U.S.A. **41**:327–338.