Biochimica et Biophysica Acta, 396 (1975) 17–23

BBA 46947

ENERGY-LINKED AND ENERGY-INDEPENDENT TRANSHYDROGENASE ACTIVITIES IN *ESCHERICHIA COLI* VESICLES

RAYMOND L. HOUGHTON, ROBERT J. FISHER and D. RAO SANADI

Boston Biomedical Research Institute, Department of Cell Physiology, 20 Staniford Street, Boston, Mass. 02114 (U.S.A.)

(Received January 24th, 1975)

SUMMARY

Active transport vesicles of *Escherichia coli* were shown to possess low levels of energy-independent and energy-dependent nicotinamide nucleotide transhydrogenase activities. Breakage of such vesicles in a French pressure cell resulted in a fraction which had an 8–10-fold increased respiration- and ATP-driven transhydrogenase activities.

Stimulation of the ATPase activity in vesicles with Triton X-100 was also paralleled by a 2-fold increase in the energy-independent transhydrogenase.

Disruption of the vesicles similarly resulted in increases in the energy-independent transhydrogenase, NADH and succinate oxidase activities but a decrease in succinate supported proline uptake.

In the light of these findings, the 'sidedness' of the vesicle membranes is discussed.

INTRODUCTION

Active transport vesicles derived from *Escherichia coli* have little ability to phosphorylate ADP using the energy derived from respiration, but can catalyse respiration-dependent uptake of amino acids and sugars [1]. Such vesicles, however, possess low levels of ATPase activity which can be stimulated by treating with Triton X-100 [2]. This suggests that most of the ATPase is inaccessible to its substrates since it is intravesicular and hence cannot support uptake of amino acids at the expense of ATP in the medium. The low level of ATPase activity is suggestive of a mixed population of 'rightside in' and 'inside out' vesicles.

Mevel-Ninio and Yamamoto [3] have recently shown that disruption of active transport vesicles in a French pressure cell yields membranes which catalyse oxidative phosphorylation at a rate 10 times faster than in the intact vesicles, but do not carry out active transport. The P/O values, however, were quite low, possibly due to the loss of ATPase during treatment of the vesicles.

It is the purpose of this paper to demonstrate that disruption of active transport vesicles results in a membrane fraction which has high levels of ATP- and respiration-linked nicotinamide nucleotide transhydrogenase activities as well as

increased oxidase, ATPase and energy-independent transhydrogenase activities. Furthermore, the ATPase and ATP-driven transhydrogenase activities in vesicles and disrupted vesicles are sensitive to antiserum to *E. coli* ATPase as is the ATPase activity exposed by Triton X-100.

METHODS

Preparation of cells and membrane vesicles

The proline auxo troph, Strain W6 of *E. coli* was grown in 10-l batches, on a minimal salts medium supplemented with 50 μg/ml L-proline. The carbon source was 30 mM glucose and the O_2 tension was regulated at 30 % dissolved oxygen. Cells were harvested in the late exponential phase of growth and membrane vesicles were prepared by the procedure of Kaback [4].

The vesicles were disrupted in a French pressure cell at 20 000 lb/inch^2 and the broken membrane vesicles prepared by the centrifugation schedule of Fisher and Sanadi [5].

Enzyme assays

The energy-independent, ATP- and respiration-dependent transhydrogenase activities were measured by the procedure of Fisher and Sanadi [5].

NADH and succinate oxidase activities were measured polarographically at 37 °C in a medium containing 0.5 M sucrose, 50 mM Tris · sulphate pH 7.8 and 0.2 % bovine serum albumin. Activities were expressed as ngatoms O/min/mg protein.

ATPase activities were measured at 37 °C at either pH 7.5 or pH 9.0 in 50 mM Tris · HCl containing 5 mM ATP and 2 mM $MgCl_2$. The reaction was initiated by addition of ATP and terminated by adding an equal volume of 10 % trichloroacetic acid. Inorganic phosphorus was measured by the method of Fiske and SubbaRow [6]. Proline transport in vesicles was measured by the procedure of Kaback [4], except that succinate was used as the energy source. The cytochrome contents of the various membrane vesicles were determined with a Cary 15 spectrophotometer. Cytochrome b_1 content of the fractions was estimated using the extinction coefficients given by Jones and Redfearn [7]. Antiserum to *E. coli* ATPase was a gift from Dr A. Tzagoloff and was in the form of the whole antiserum.

RESULTS AND DISCUSSION

Table I illustrates the increase in the transhydrogenase and ATPase activities on disruption of active transport vesicles. The data demonstrate that the ATP- and respiration-driven transhydrogenase are increased 7–10-fold. This parallels the increase in P/O ratios from $0.7 \cdot 10^{-3}$ to $6 \cdot 10^{-3}$ observed by Mevel-Ninio and Yamamoto [3]. Increases of 3–4-fold also occur in the energy-independent transhydrogenase and ATPase activities.

Table II shows the effects of breakage on the oxidase activities and cytochrome b_1 content of the vesicles. Since there is no increase in total cytochrome b_1 content, the enhanced activities are probably due to a change in sidedness of the membrane and not to any purification during the preparation of the broken vesicles. The 3–4-fold increase in NADH and succinate oxidase activities is different from the finding of

TABLE I

ATPase AND TRANSHYDROGENASE ACTIVITIES IN INTACT AND BROKEN VESICLES

Energy-dependent transhydrogenase activities were measured using the assay procedure of Fisher and Sanadi [5]. The medium contained 50 μmol Tris–sulphate pH 7.8, 5 μg crystalline yeast alcohol dehydrogenase, 90 μmol ethanol, 0.3–0.5 mg membrane protein and water to 2.95 ml. The reaction was initiated by the addition of 1.5 μmol of NADP. The succinate-stimulated transhydrogenase was measured by inclusion of 60 μmol sodium succinate, and the NADH driven+ATP/Mg-driven transhydrogenase assayed by inclusion of 6 μmol ATP and 10 μmol $MgSO_4$. The energy-independent transhydrogenase was measured in the reverse direction by following the reduction of acetylpyridine NAD by NADPH as described by Fisher and Sanadi [5]. ATPase was measured as described in the Methods section. The NADPH/O value is the ratio of NADH oxidation-driven transhydrogenase to NADH oxidase activities.

Membrane fraction	Energy-dependent transhydrogenase (nmol NADPH/min/mg)			Energy-independent transhydrogenase (nmol/min/mg)	ATPase (μmol/min/mg)		NADPH / O
	NADH	+Succinate	+ATP/Mg driven		pH 7.5	pH 9.0	
Vesicles	9.8	20.5	35.6	430	0.26	0.61	0.07
Broken vesicles	91	172	262	1209	0.96	2.00	0.20

TABLE II

PROLINE UPTAKE, CYTOCHROME b_1 AND OXIDASE ACTIVITIES IN INTACT AND BROKEN VESICLES

Activities were measured as described in the Methods section. In the case of succinate supported proline uptake the activity is given as the initial rate in nmol/min/mg at 25 °C. Cytochrome b_1 was estimated from the dithionite reduced minus air-oxidized spectra using the extinction coefficient published by Jones and Redfearn [7].

Membrane fraction	Oxidases (ngatoms O/min/mg)		Cytochrome b_1 (nmol/mg)	Succinate supported proline uptake (nmol/min/mg)
	NADH	Succinate		
Vesicles	347	222	0.46	0.31
Broken vesicles	861	720	0.37	0.06

Mevel-Ninio and Yamamoto [3] that D-lactate oxidation was not significantly enhanced during the breakage of the vesicles.

In accordance with earlier work we found that breakage of the vesicles also resulted in a decrease in the ability to actively transport proline as measured using the Millipore filtration method. The significance of the low level of proline uptake in inverted vesicles is uncertain. Kaback and Deuel [8] showed that sonicated vesicles when separated from intact vesicles by gel filtration could still accumulate proline at a rate about one tenth that in the intact vesicles. These low amounts of activity could, however, be due to a small population of 'right sided' vesicles remaining after sonication.

Effect of Triton X-100 and antiserum

Van Thienen and Postma [2] showed that the ATPase activity of intact vesicles could be stimulated by Triton X-100. Membrane particles prepared by sonication of whole cells, however, did not exhibit this increase in activity on treatment with the detergent. Thus, by using ATPase as a marker of membrane polarity, these authors established that vesicles prepared by the procedure of Kaback [4] were a heterogeneous population of 'rightside in' and 'inside out' membranes.

We have confirmed that the ATPase activity in the intact vesicles could be stimulated to an activity comparable to that in the broken vesicles by increasing levels of Triton X-100. In a particular experiment, the activity was increased from 0.63 to 2.12 μmol P_i/min/mg protein (at pH 9.0) in the presence of 0.5 % (v/v) Triton X-100. A similar increase, however, could not be produced with the membrane fraction obtained after breakage of these intact vesicles; the activities in the absence and presence of 0.5 % (v/v) Triton X-100 were 2.20 and 2.15 μmol P_i/min/mg protein respectively. These findings would tend to indicate that treatment of the vesicles with detergent or breakage in a French pressure cell results in inverted membranes which have their ATPase exposed to the medium.

Fig. 1 shows that the energy-independent transhydrogenase of vesicles could be similarly stimulated roughly 2-fold by Triton X-100, and again similar enhancement was not produced in the broken vesicles; in fact, higher levels of the detergent inhibited the activity. By comparison with the situation with ATPase, Triton X-100 seems to expose the energy-independent transhydrogenase located on the inner surface of the membrane. The same effect can also be achieved by disruption of the vesicles in which case the activity increases from 430 to 1209 nmol acetyl pyridine NADH/min/mg protein.

Hanson and Kennedy [9] demonstrated that at sufficiently high levels, the membrane bound ATPase and ATP dependent transhydrogenase activities could be inhibited by antiserum to *E. coli* ATPase. The ATPase activity of the vesicles could be inhibited 75–80 % by the antiserum (Fig. 2). Treatment with Triton X-100 to enhance the ATPase activity resulted in nearly 100 % sensitivity to the antiserum. The activity in the broken vesicles was also inhibited but at higher levels of antiserum,

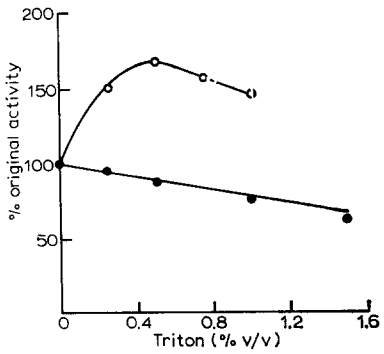

Fig. 1. Effect of Triton X-100 on the energy-independent transhydrogenase. Vesicles (4 mg/ml) or broken vesicles (6.4 mg/ml) were preincubated on ice for 5 min with the relevant concentrations of Triton X-100. Aliquots (20 μl) were then removed and assayed as described in the Methods section. The 100 % values for the vesicles and broken vesicles were 379 and 1101 nmol acetyl pyridine NADH/min/mg protein, respectively. ○–○, vesicles; ●–●, broken vesicles.

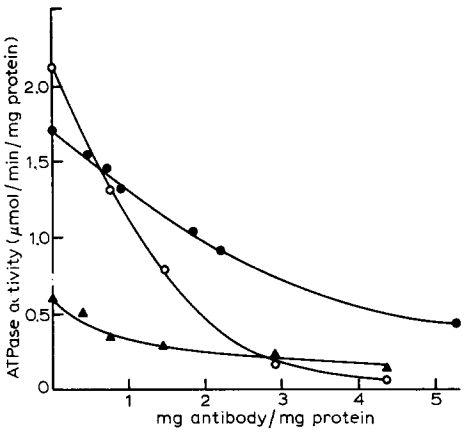

Fig. 2. Inhibition of ATPase by antiserum. Intact (1.65 mg/ml) or broken (1.92 mg/ml) vesicles were incubated on ice with varying concentrations of antibody. Incubations were performed in 50 mM Tris–sulphate pH 7.8+10 mM $MgSO_4$. Activated vesicles were obtained by prior treatment of the vesicles with 1 % Triton X-100. Aliquots (10 μl) were removed to determine the ATPase activity at pH 9.0. ▲–▲, vesicles; ○–○, vesicles in 1 % Triton; ●–●, broken vesicles.

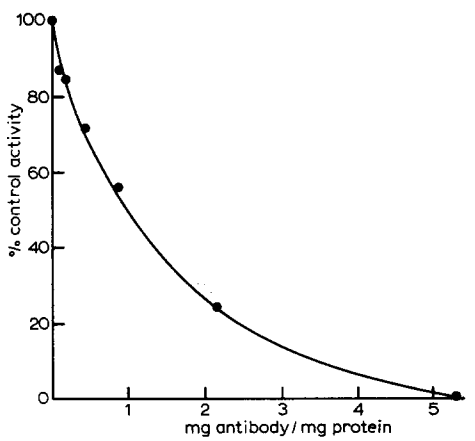

Fig. 3. Inhibition of ATP-driven transhydrogenase by antiserum. Broken vesicles at a final concentration of 3.2 mg/ml in 50 mM Tris · sulphate pH 7.8+10 mM $MgSO_4$ were incubated on ice with varying levels of antiserum. Aliquots (20 μl) were then removed and assayed for ATP dependent transhydrogenase as described in the Methods section. The control activity was 159 nmol/min/mg after correcting for the NADH driven transhydrogenase activity.

probably due to the higher level of ATPase exposed to the medium. The ATP-dependent transhydrogenase in the broken vesicles was also shown to be inhibited (Fig. 3) (but at slightly lower levels of antiserum than for ATPase). The inability to inhibit the ATPase activity 100 % in the vesicles is probably due to the 'leakiness' of the intact vesicles to ATP, in which case a small amount of the vesicle ATPase activity would be due to some of the enzyme located on the inner membrane. Treatment of the vesicles with Triton X-100 exposes the remaining ATPase to the medium and hence to the antiserum. The lower titer of ATPase antiserum required to inhibit

the ATP driven transhydrogenase in the broken vesicles may indicate that only some of the ATPase activity is associated with the transhydrogenase.

The data presented here provide further evidence that active transport vesicles do possess a full complement of enzymes for energy-dependent reactions. Their inability to do so in the intact vesicles is probably related to the differing abilities of substrates to reach their site of action due to the orientation of the membranes. The increase in NADH and succinate oxidation rates on breakage of the vesicles indicates these two dehydrogenases are located on the inner surface of the intact vesicle membrane. The same is true for the ATPase and energy-independent transhydrogenase enzymes, as evidenced by the increase in activities of these enzymes either by vesicle breakage or treatment with Triton X-100. Recently Futai [10] demonstrated that vesicles isolated with an internally generated NADH pool could actively transport proline at 60–80 % of the rate supported by D-lactate, further indicating the location of NADH dehydrogenase on the inner membrane. In experiments with *Mycobacterium phlei* [11] it was also concluded that succinate dehydrogenase had to be located on the inside of the membrane to be able to accumulate proline. In explaining why D-lactate supports proline uptake at a rate faster than succinate, one has to take into account the fact that the respiration dependent uptake of D-lactate is faster than the uptake of succinate [12]. The rate limiting step controlling proline uptake may not be the rate of oxidation of the substrates, such as D-lactate or succinate by the internally located dehydrogenases, but the rate of translocation of these substrates across the membrane. D-lactate is translocated at five times the rate of succinate [12]. Thus, one may expect succinate-driven proline uptake to be slower than D-lactate-driven uptake, which is the case by a factor of approximately five.

Since the ATPase present on the inner face of the cytoplasmic membrane can be exposed to the medium by mechanical breakage or by treatment with Triton X-100; then, by analogy, increases of other activities by either of these agents is also indicative of their location on the cytoplasmic membrane. Thus, the enzymes required for the oxidation of succinate and NADH, and for transhydrogenation, must be located on the inner face of the cytoplasmic membrane. The lack of increase in D-lactate oxidation rate on disruption of vesicles, as reported by Mevel-Ninio and Yamamoto [3], may indicate that D-lactate dehydrogenase is a transmembrane enzyme. This may also account for the high rate of D-lactate-driven proline uptake.

ACKNOWLEDGMENTS

This work was supported by grants from the National Science Foundation (GB-33919) and the National Institutes of Health, USPHS (GM 13641).

REFERENCES

1 Konings, W. N. and Freese, E. (1972) J. Biol. Chem. 247, 2408–2418
2 Van Thienen, G. and Postma, P. W. (1973) Biochim. Biophys. Acta 323, 429–440
3 Mevel-Ninio, M. and Yamamoto, T. (1974) Biochim. Biophys. Acta 357, 63–66
4 Kaback, H. R. (1971) Methods in Enzymology (Jacoby, W. B., ed.), Vol. 22, pp. 99–120, Academic Press, New York
5 Fisher, R. J. and Rao Sanadi, D. (1971) Biochim. Biophys. Acta 245, 34–41

6 Fiske, C. H. and SubbaRow, J. (1925) J. Biol. Chem. 66, 375–400
7 Jones, C. W. and Redfearn, E. R. (1966) Biochim. Biophys. Acta 113, 467–481
8 Kaback, H. R. and Deuel, T. E. (1969) Arch. Biochem. Biophys. 132, 118–129
9 Hanson, R. L. and Kennedy, E. P. (1973) J. Bacteriol. 114, 772–781
10 Futai, M. (1974) J. Bacteriol. 120, 861–865
11 Asano, A., Cohen, N. S., Baker, R. F. and Brodie, A. F. (1973) J. Biol. Chem. 248, 3386–3397
12 Matin, A. and Konings, W. N. (1973) Eur. J. Biochem. 34, 58–67