ATP-driven active transport in right-side-out bacterial membrane vesicles

(*Salmonella typhimurium*/*Escherichia coli*/phosphoglycerate transport/electrochemical proton gradient/cloning)

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ABSTRACT Membrane vesicles from *Salmonella typhimurium* induced for phosphoglycerate transport, were loaded with pyruvate kinase and ADP by lysing spheroplasts under appropriate conditions. Vesicles so prepared catalyze active transport of proline and serine in the presence of phosphoenolpyruvate; this activity is abolished by the protonophore carbonyl cyanide-*m*-chlorophenylhydrazone and by the H⁺-ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide but not by anoxia or cyanide. In contrast, D-lactate-driven active transport is abolished by the hydrazone and by anoxia or cyanide but not by the carbodiimide. Moreover, phosphoenolpyruvate does not drive transport effectively in vesicles that lack the phosphoglycerate transport system. The results are consistent with an overall mechanism in which phosphoenolpyruvate gains access to the interior of the vesicles by means of the phosphoglycerate transporter and is then acted on by pyruvate kinase to phosphorylate ADP. ATP formed inside of the vesicles is then hydrolyzed by the H⁺-ATPase, leading to the generation of a proton electrochemical gradient that drives H⁺/solute symport. By using pBR322 as vector and *Escherichia coli* as host, a fragment of *S. typhimurium* DNA coding for the phosphoglycerate transport system has been cloned. *E. coli* membrane vesicles containing the phosphoglycerate transport system also catalyze transport in the presence of phosphoenolpyruvate when they are loaded with pyruvate kinase and ADP.

According to the chemosmotic hypothesis of Mitchell (1–5), energy derived from respiration, light, or ATP hydrolysis can be transformed into a transmembrane electrochemical gradient of protons ($\Delta\mu_{H^+}$) that represents the immediate driving force for active transport and various other energy-dependent processes. Cytoplasmic membrane vesicles from *Escherichia coli* that have the same (right-side-out; refs. 6–11) or the opposite (inverted; refs. 12–15) orientation as the membrane in the intact cell retain the capacity to convert respiratory energy into a $\Delta\mu_{H^+}$, and studies with these preparations have provided virtually unequivocal support for the central obligatory role of chemosmotic phenomena in active transport (16–29). Similarly, plasma membrane vesicles from *Halobacterium halobium* (30) and *Rhodospseudomonas spheroides* (31) generate a $\Delta\mu_{H^+}$ and catalyze active transport when exposed to light. Nonetheless, one important aspect of the general chemosmotic hypothesis remains unresolved in the vesicle system. Although it is clear that ATP hydrolysis leads to the generation of a $\Delta\mu_{H^+}$ in inverted vesicles (21–29), this phenomenon has not been elucidated in right-side-out vesicles despite numerous and varied attempts to make ATP accessible to the inner surface of the vesicle membrane (7).

In 1975, Saier *et al.* (32) described and characterized an inducible phosphoglycerate transport system in *Salmonella ty-*

phimurium LT-2 that catalyzes the uptake of 2-phosphoglycerate, 3-phosphoglycerate, and phosphoenolpyruvate, and, subsequently, they demonstrated that the transport system allows energy-depleted cells to use external phosphoenolpyruvate more efficiently for vectorial phosphorylation of methyl α -D-glucopyranoside (33). These workers also suggested the possibility of using the phosphoglycerate transport system to affect the intravesicular generation of ATP from phosphoenolpyruvate added to the medium (32).

The experiments presented here demonstrate that external phosphoenolpyruvate drives active transport in right-side-out vesicles containing the phosphoglycerate transport system and an ATP-generating system consisting of pyruvate kinase and ADP. They also provide evidence suggesting that transport activity under these conditions is due to the intravesicular formation of ATP that is subsequently hydrolyzed by the H⁺-ATPase with generation of a $\Delta\mu_{H^+}$. In addition, the *S. typhimurium* gene encoding for phosphoglycerate transport activity has been cloned by transformation into *E. coli*, in which it is expressed functionally.

MATERIALS AND METHODS

Growth of Cells and Preparation of Membrane Vesicles. *S. typhimurium* LT-2 was grown on medium A (34) with either 0.5% sodium DL-lactate (uninduced) or 0.5% sodium 3-phosphoglycerate (induced) as indicated. Cells were harvested in the middle of the logarithmic growth phase (≈ 180 Klett units). *E. coli*/pBR322-pgt2 (see below) was grown on medium A containing 0.5% sodium 3-phosphoglycerate, methionine at 200 μ g/ml, thiamine·HCl at 20 μ g/ml, and ampicillin at 20 μ g/ml; cells were harvested at the end of logarithmic growth (≈ 200 Klett units). Spheroplasts and membrane vesicles were prepared as described from *S. typhimurium* (19) and *E. coli* K-12 (18). Where indicated, ADP and pyruvate kinase were included in the lysis buffer at final concentrations of 5 mM and 50 μ g/ml, respectively.

Transport Assays. Respiration-driven transport of phosphoenolpyruvate, proline, or serine was measured in the presence of ascorbate and phenazine methosulfate (PMS) or lithium D-lactate as described (35). In the case of phosphoenolpyruvate transport, the samples were washed twice after filtration to decrease background activity further. Phosphoenolpyruvate-driven proline or serine transport was measured under identical conditions except that ascorbate, PMS, and D-lactate were omitted, sodium phosphoenolpyruvate (final concentration, 10 mM) was added to the reaction mixtures, and the samples were incubated at 30°C for 20 min before addition of radioactive transport sub-

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Abbreviations: $\Delta\mu_{H^+}$, the proton electrochemical gradient; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; PMS, phenazine methosulfate.

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strate. When carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) or *N,N'*-dicyclohexylcarbodiimide (DCCD) were used, small aliquots from concentrated ethanolic stock solutions were added such that the final concentration of ethanol in the reaction mixtures did not exceed 1% (vol/vol).

Cloning of the Phosphoglycerate Transport System. The phosphoglycerate transporter of *S. typhimurium* LT-2 was cloned by using the plasmid pBR322 and phenotypic complementation. Chromosomal DNA from *S. typhimurium* LT-2 was prepared by the method of Saito and Miura (36) and plasmid DNA was prepared by the method of Clewell and Helinski (37). pBR322 DNA (10 μ g) was linearized in 300 μ l of 20 mM Tris-HCl, pH 7.0/100 mM NaCl/7 mM MgCl₂/2 mM 2-mercaptoethanol containing 10 units of *Bam*HI at 37°C for 15 hr. Partially digested chromosomal DNA was prepared by incubating 15 μ g of DNA in 150 μ l (final vol) of 6 mM Tris-HCl, pH 7.5/50 mM NaCl/6 mM MgCl₂ containing 1 unit of *Sau*3a for 4 min at 37°C. Ligation of *Bam*HI-treated plasmid DNA with *Sau*3a partially digested DNA was carried out in 0.5 ml (final vol) of 66 mM Tris-HCl, pH 7.6/10 mM MgCl₂/1 mM ATP/30 mM 2-mercaptoethanol containing 1 unit of T4 ligase for 15 hr at 4°C. Transformation was carried out according to Mandel and Higa (38) using the *E. coli* K-12 strain MS401 (*thi*, *R*⁻*M*⁻, *endB*, *metC*). Transformants able to use 3-phosphoglycerate as the sole carbon source for growth were selected on minimal medium (300 μ M methionine/10 μ M thiamine-HCl/0.5% sodium 3-phosphoglycerate/1.5% agar; ref. 39). Six clones were obtained from $\approx 2 \times 10^5$ ampicillin-resistant transformants. One of these clones was designated *E. coli*/pBR322-pgt2 and used for the experiments reported here. The hybrid plasmid pBR322-pgt2 was shown to contain a 14.4-kilobase pair insert.

Protein Determinations. Protein was measured as described by Lowry *et al.* (40) using bovine serum albumin as standard.

Materials. L-[U-¹⁴C]Proline and L-[U-¹⁴C]serine were purchased from New England Nuclear and phosphoenol[1-¹⁴C]pyruvate was from Amersham/Searle. Pyruvate kinase (type II, rabbit muscle) and ADP (grade III, yeast) were obtained from Sigma. All other materials were reagent grade and obtained from commercial sources.

RESULTS

Respiration-Driven Phosphoenolpyruvate Transport. In the absence of exogenous electron donors, phosphoenolpyruvate transport is minimal in vesicles prepared from *S. typhimurium* LT-2 or *E. coli*/pBR322-pgt2 grown on 3-phosphoglycerate (Fig. 1). On addition of ascorbate/PMS, dramatic stimulation of the initial rates and the steady-state levels of accumulation are observed; by 2–3 min, both preparations take up 12–14 nmol of phosphoenolpyruvate per mg of membrane protein. Also, addition of the protonophore CCCP (final concentration, 10 μ M) completely blocks the stimulatory effect of ascorbate/PMS (data not shown). Furthermore, uptake is negligible in vesicles prepared from lactate-grown *S. typhimurium* LT-2 and *E. coli* K-12 or ML 308-225 in the presence or absence of ascorbate/PMS. It is apparent therefore that the phosphoglycerate transport system is retained in membrane vesicles, that it is driven by a $\Delta\mu_{H^+}$ or one of its components, and that it is expressed in *E. coli* after transformation with the hybrid plasmid pBR322-pgt2.

ATP-Driven Active Transport. When vesicles containing the phosphoglycerate transport system and loaded with pyruvate kinase and ADP are incubated with phosphoenolpyruvate for 20 min and then exposed to radioactive proline, marked stimulation of uptake is observed relative to control samples incubated in the absence of an energy source (Fig. 2). Both the rate

FIG. 1. Transport of phosphoenolpyruvate by isolated membrane vesicles. Vesicles were prepared from *S. typhimurium* LT-2 (○ and ●) and *E. coli*/pBR322-pgt2 (□ and ■) grown on 3-phosphoglycerate. Aliquots of membrane vesicles containing 0.08 mg of membrane protein were diluted to 0.05 ml (final volume) containing (in final concentrations) 50 mM potassium phosphate (pH 6.6) and 10 mM magnesium sulfate. The samples were incubated at 30°C and gassed with oxygen for 2–3 min before further additions and throughout the incubation as described (35). Where indicated (open symbols), ascorbate/PMS was added to the reaction mixtures to final concentrations of 20 mM and 0.1 mM, respectively, and 30 sec later, phosphoenol[1-¹⁴C]pyruvate (12.4 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels) was added to a final concentration of 0.15 mM. Closed symbols signify identical incubations carried out in the absence of ascorbate/PMS. After incubation for given times, the reactions were terminated and the samples were assayed as described (35).

and steady-state level of accumulation are approximately half those observed in the presence of D-lactate, the most effective physiological electron donor for active transport in these vesicles and, in either case, transport activity is abolished by CCCP. Furthermore, when vesicles containing the ATP-generating system but lacking the ability to transport phosphoenolpyruvate are used, external phosphoenolpyruvate has little, if any, significant effect on proline uptake. Finally, a number of additional points are noteworthy: (i) although the experiments shown were carried out with vesicles from *S. typhimurium* LT-2, essentially identical results were obtained with *E. coli*/pBR322-pgt2 vesicles; (ii) similar observations were made with serine as transport substrate; (iii) vesicles containing the phosphoglycerate transport system and loaded with either pyruvate kinase or ADP do not exhibit active transport when phosphoenolpyruvate and the other component of the ATP-generating system are added to the medium; and (iv) increasing the external phosphoenolpyruvate concentration to 50 mM does not obviate the need for a 20-min preincubation period to achieve maximum transport activity (data not shown).

The studies presented in Figs. 3 and 4 provide additional support for the argument that active transport in the presence of phosphoenolpyruvate involves the intravesicular formation of ATP and its hydrolysis by the H^+ -ATPase with concomitant generation of a $\Delta\mu_{H^+}$. As expected (41, 42), DCCD, a potent and relatively specific inactivator of the H^+ -ATPase, has essen-

FIG. 2. Phosphoenolpyruvate-driven proline transport by *S. typhimurium* LT-2 vesicles. Cells grown on 3-phosphoglycerate (unless indicated otherwise) were converted into spheroplasts and lysed in the presence of pyruvate kinase and ADP. Reaction mixtures (total vol, 0.05 ml) contained (final concentrations) 50 mM potassium phosphate, pH 6.6/10 mM magnesium sulfate/0.08 mg of membrane protein/3.5 μ M L-[U- 14 C]proline (287 mCi/mmol) and either 20 mM lithium D-lactate or 10 mM sodium phosphoenolpyruvate, as indicated. With phosphoenolpyruvate as energy source, the samples were incubated for 20 min before addition of proline; with D-lactate as energy source, preincubation was carried out for 3 min. ○, Uptake in the presence of D-lactate; □, uptake in the presence of phosphoenolpyruvate; △, uptake by vesicles lacking the phosphoglycerate transport system in the presence of phosphoenolpyruvate (i.e., vesicles were prepared from lactate-grown cells); ●, uptake in the absence of exogenous energy sources; ■, uptake in the presence of D-lactate or phosphoenolpyruvate and 10 μ M CCCP.

tially no effect on D-lactate-driven proline transport (Fig. 3A). In contrast, phosphoenolpyruvate-driven proline accumulation is completely abolished when the vesicles are treated with the

FIG. 3. Effect of DCCD on proline uptake in the presence of D-lactate (A) or phosphoenolpyruvate (B). *S. typhimurium* LT-2 vesicles containing the phosphoglycerate transport system and loaded with pyruvate kinase and ADP were prepared, and transport with D-lactate or phosphoenolpyruvate as energy source was assayed. Where indicated, samples were incubated with 50 μ M DCCD for 15 min at 30°C before assaying transport activity. ●, Uptake in the presence of D-lactate; ○, uptake in the presence of D-lactate after treatment with DCCD; □, uptake in the presence of phosphoenolpyruvate; ■, uptake in the presence of phosphoenolpyruvate after treatment with DCCD; △, uptake in the absence of exogenous energy sources and DCCD.

FIG. 4. Effect of anoxia on proline uptake in the presence of D-lactate (A) or phosphoenolpyruvate (B). *S. typhimurium* LT-2 vesicles containing the phosphoglycerate transport system and loaded with pyruvate kinase and ADP were prepared. Proline transport was assayed under aerobic conditions with D-lactate (○) or phosphoenolpyruvate (□) as energy sources. Transport was also assayed under argon (35) with D-lactate (●) or phosphoenolpyruvate (■) as energy source. △, Uptake in the absence of exogenous energy sources under aerobic or anaerobic conditions.

carbodiimide (Fig. 3B). Conversely, anoxia (Fig. 4A) or 10 mM potassium cyanide (data not shown) completely inhibits D-lactate-dependent proline transport but has no effect on proline transport in the presence of phosphoenolpyruvate (Fig. 4B).

DISCUSSION

These results are fully consistent with the model presented in Fig. 5. As shown, phosphoenolpyruvate added to the medium enters the intravesicular space via the phosphoglycerate transporter and is then used to phosphorylate ADP in a Mg^{2+} -dependent reaction catalyzed by pyruvate kinase. Subsequently, the ATP formed is hydrolyzed by the membranous H^+ -ATPase, generating a proton electrochemical gradient that drives active transport of proline, serine, and presumably many other substrates by means of coupled movements with protons.

One interesting aspect of the experiments is the need for a 20-min preincubation period with phosphoenolpyruvate to achieve maximum stimulation of transport. As the lag is not alleviated by increasing the concentration of phosphoenolpyruvate to >10 mM, it is apparently not due to a limitation in

FIG. 5. Model for phosphoenolpyruvate-driven active transport in vesicles containing the phosphoglycerate transport system, pyruvate kinase, and ADP. PKase, pyruvate kinase; S, transport substrate; P-ePrv, phosphoenolpyruvate.

the accessibility of phosphoenolpyruvate to the intravesicular space (in vesicles containing the phosphoglycerate transporter). Moreover, preliminary experiments suggest that intravesicular ATP concentrations of ≈ 1 mM are obtained 2–3 min after addition of phosphoenolpyruvate to the medium (the apparent K_m of the H^+ -ATPase is ≈ 0.2 mM; ref. 43). Thus, it seems unlikely that the lag can be attributed to a slow rate of intravesicular ATP formation. By exclusion, therefore, it seems reasonable to suggest that the lag is related to a limitation in the generation of a $\Delta\mu_{H^+}$ by H^+ -ATPase. This suggestion is also consistent with previous findings (8) indicating that significant amounts of H^+ -ATPase activity may be lost from the vesicles during preparation. It is also relevant that the lag is reduced considerably by carrying out the reactions at pH 7.5, the approximate pH optimum for the H^+ -ATPase or by decreasing the internal ADP concentration. In any event, this aspect of the system should be amenable to study through the use of strains having amplified levels of the H^+ -ATPase (44).

In addition to providing further evidence for the efficacy of bacterial membrane vesicles as a model system for the study of transport and bioenergetics, the results are encouraging with respect to related problems that have been refractory to an *in vitro* approach. For example, there is abundant evidence (45) that the so-called "shock-sensitive transport systems" in Gram-negative bacteria are driven by phosphate-bond energy. Thus, addition of binding proteins to isolated membrane vesicles *per se* does not lead to reconstitution of these transport systems. Similarly, transport systems have been described (46–48) that do not involve periplasmic binding proteins or a $\Delta\mu_{H^+}$. Clearly, the use of vesicles in which ATP or other high-energy phosphate intermediates can be generated internally might be useful in this context. Finally, the availability of hybrid plasmids encoding for the phosphoglycerate transport system may allow amplification of the protein(s) involved in this transport system, thus facilitating studies on the structure and mechanism of action of the transporter as well as of its insertion into the membrane.

Note Added in Proof. By subcloning, the *pgt* gene has been localized to a 2.2-kilobase pair fragment that is expressed constitutively. Furthermore, the product of the *pgt* gene has been tentatively identified as a 46,000-dalton protein.

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