Different Mechanisms of Energy Coupling for the Active Transport of Proline and Glutamine in *Escherichia coli*

(inhibitors/mutants/ATP/energized membrane state/starvation)

EDWARD A. BERGER

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

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ABSTRACT The ability of either glucose or D-lactate to energize active transport of amino acids in *E. coli* was studied in starved cells blocked at specific sites of energy metabolism. Proline uptake could be driven by either oxidative or substrate-level processes. The oxidative pathway was sensitive to cyanide but not to arsenate, and operated normally in a mutant deficient in the Ca, Mg-dependent ATPase. The substrate-level pathway, which was active with glucose but not with D-lactate as the carbon source, was sensitive to arsenate but not to cyanide, and required a functional ATPase. Uncouplers prevented the utilization of energy for proline uptake by either pathway.

Energy coupling for glutamine uptake was quite different. The oxidative pathway was sensitive to cyanide and uncouplers and, in contrast with proline, required an active ATPase. The glycolytic component was resistant to cyanide and uncouplers, and functioned normally in the ATPase mutant: Arsenate abolished glutamine transport energized by either pathway.

The results suggest that proline transport is driven directly by an energy-rich membrane state, which can be generated by either electron transport or ATP hydrolysis. Glutamine uptake, on the other hand, is apparently driven directly by phosphate-bond energy formed by way of oxidative or substrate-level phosphorylations.

Early investigations of bacterial active transport have implicated the high-energy phosphate bond in the energy-coupling process (1, 2). Recent evidence, however, suggests that the role of ATP is probably indirect. The extensive studies of Kaback and his colleagues (3) demonstrated that membrane vesicles incapable of oxidative phosphorylation can still use respiration to drive the uptake of a wide variety of amino acids and sugars. Klein and Boyer recently showed (4) that aerobic proline transport in intact cells of *Escherichia coli* is retained under conditions where intracellular ATP and phosphoenolpyruvate levels are drastically reduced by arsenate. Furthermore, a functional Ca,Mg-dependent ATPase is not required for aerobic accumulation of thiomethyl-galactoside (5) or proline (4, 6), confirming that transport can proceed independently of oxidative phosphorylation.

While it is clear that the respiration-linked uptake of certain substrates does not involve the formation or use of high-energy phosphates, these same transport systems can apparently be energized by an alternate nonoxidative mechanism that uses ATP. *E. coli* is able to accumulate various substrates anaerobically (4, 7), and it has been shown for proline that anaerobic uptake is abolished by arsenate (4). The essential role of the Ca, Mg-ATPase in the use of ATP for transport is inferred from the sensitivity of anaerobic proline accumulation to the ATPase inhibitor *N*,*N*-dicyclohexylcarbodiimide (4), as well as from the loss of cyanide-resistant thiomethyl-galactoside uptake in an ATPase mutant (5).

Energy for active transport can thus be derived independently from either respiration or ATP hydrolysis. The observations (4, 7) that uptake driven by either pathway is sensitive to uncouplers of oxidative phosphorylation has led several workers (4–8) to propose that a high-energy membrane state is the immediate energy donor for bacterial transport, though other models have recently been formulated (3, 9). Since this conclusion is based upon studies with only a few uptake systems, it is necessary to identify the energy donors for the active transport of other metabolites before any generalizations can be applied.

In this study, the ability of different carbon sources to provide energy for active transport was investigated in starved *E. coli* cells blocked at specific sites of energy metabolism. By the judicious choice of energy sources and inhibitors, it is possible to distinguish whether respiration per se, the energized membrane state, or substrate-energy donor is the obligatory energy donor for a particular system. For example, glucose can provide ATP by either oxidative phosphorylation, which requires both electron transport and a functional Ca,Mg-ATPase, or by the substrate-level phosphorylations of glycolysis, which require neither process. Glucose can also give rise to an energy-rich membrane state by two pathways: through hydrolysis of glycolytic ATP by ATPase in the presence or absence of respiration, or through the oxidations of the respiratory chain, which may occur in the absence of ATPase. Alternatively, D-lactate is oxidized directly by a membrane-bound dehydrogenase coupled to the cytochrome chain (8), and can provide energy only in the presence of electron transport. The synthesis of ATP requires an active ATPase, whereas the generation of the energized membrane state does not.

The results presented here confirm the suggestions of others (4, 6) that proline uptake is driven by the energized membrane state. Glutamine transport, on the other hand, is driven directly by phosphate-bond energy formed by either oxidative phosphorylation or glycolysis.

MATERIALS AND METHODS

Bacterial Strains. *E. coli* ML 308-225 and its derivative DL-54 were the generous gifts of Dr. Robert D. Simoni. DL-54 is missing more than 98% of the Ca,Mg-ATPase activity and is unable to grow on carbon sources that require oxidative phosphorylation for ATP formation (6).

Chemicals. L-[U-14C]proline and L-[U-14C]glutamine were purchased from New England Nuclear Corp. The isotopes

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were diluted with nonradioactive amino acids to final specific activities of 20–25 Ci/mol. 2,4-Dinitrophenol was purchased from Sigma. Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was a gift from Dr. Efrem Racker. All other compounds were analytical grade.

**Growth of Cells.** Bacteria were grown in a synthetic, phosphate-buffered minimal medium (10) supplemented with 11 mM glucose as a carbon source. Optical density at 600 nm was monitored with a Gilford spectrophotometer model 240 (OD

**Starvation of Cells.** Washed cells were suspended in minimal medium containing 5 mM dinitrophenol at a density of 1 g (wet weight) of cells per 200 ml. The suspension was incubated at 37° for 5 min. 11 mM Glucose or 10 mM D-lactate was added and the incubation was continued for 10 min. The flask was brought to room temperature and the transport reaction was initiated by the addition of labeled amino acid to a concentration of 10 μM. The final volume of the mixture was 0.5 ml. At various times, 0.2-ml aliquots were withdrawn, filtered on 25-mm nitrocellulose filters (0.45 μm, Matheson-Higgins), and washed with 10 ml of a solution containing 0.01 M Tris·HCl (pH 7.3)–0.15 M NaCl–0.5 mM MgCl\(_2\) (11) at 23°. The filters were dried and counted in 7.5 ml of a solution of 15 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis-[4-methyl-5-phenyloxazolyl]benzene dissolved in 3.8 liters of toluene. To ensure linearity of initial rates of uptake, 15-sec and 30-sec time periods were taken, and the 15-sec points were used for the calculations. Uptake values are expressed as nmol/min per mg of cellular protein.

For studies on the effects of arsenate, starved cells were washed three times with 25 mM Tris·HCl (pH 7.3) at 4°, and suspended at 23° in a minimal medium (12), modified by replacing the potassium phosphate with 0.1 M Tris·HCl (pH 7.0) (referred to as phosphateless medium). Transport assays were performed as described above, except that the reaction flask contained phosphateless medium instead of the normal minimal medium, and the cells were incubated at 37° with arsenate for 15 min before addition of the carbon source.

**Protein Assay.** The protein content of cell suspensions was determined by a micromodification of the method of Lowry et al. (13), with bovine-serum albumin as a standard.

**RESULTS**

**Starvation of the Cells.** To study transport driven by a particular carbon source, endogenous energy stores must be sufficiently low so that the bulk of the measured uptake is supported by the added compound. In Table 1A, I show that the endogenous rates of proline uptake in unstarved cells were so high as to partially or completely mask the effects of glucose or D-lactate. Glutamine uptake in the absence of added carbon source was not as high, suggesting that the two transport systems might be using different energy stores.

Several starvation methods were examined for their ability to deplete endogenous energy reserves. Vigorous overnight aeration of the cells at 37° in the presence or absence of thiomethylgalactoside failed to reduce endogenous rates of proline transport significantly. Incubation with α-methyl-glucoside in the presence of sodium azide (14) was more effective, though the inhibitory effects of azide on transport could not be completely reversed by washing. The most satisfactory results were obtained with the uncoupler dinitrophenol. Table 1B shows that incubation of ML 308-225 with 5 mM dinitrophenol for 10 hr at 37°, followed by extensive washing, reduced the endogenous rates of proline uptake sufficiently to permit large stimulations by added energy sources. Furthermore, the transport rates in the presence of glucose and D-lactate were very similar to those observed with unstarved cells (Table 1A).

DL-54 was much more sensitive to this starvation procedure than the wild type. Incubations as short as 30 min greatly reduced endogenous rates of proline uptake in this strain, while they had little effect on ML 308-225. If the dinitrophenol treatment was continued beyond 2 hr with the mutant, endogenous uptake rates were reduced below detectable limits, but the rates with added energy sources also began to decline. A 1-hr incubation was therefore chosen for DL-54, since as shown in Table 1B, endogenous proline transport was adequately depleted and the rates of glucose- and D-lactate-supported uptake of both proline and glutamine were similar to those in unstarved cells.

The dinitrophenol starvation procedure thus permitted assessment of the ability of different energy sources to stimulate transport, and starved cells were used in the remainder of the experiments.

**Transport in DL-54.** The membrane-bound Ca, Mg-ATPase is believed to catalyze the reversible interconversion of ATP and the energized membrane state (15). Table 1B shows that loss of this activity by mutation had marked effects on transport, but only under certain conditions. In wild-type cells, D-lactate supported a relatively high rate of glutamine uptake, while in DL-54 this uptake was greatly impaired. D-

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**TABLE 1.** Aminoacid transport in unstarved and starved cells of ML 308-225 and DL-54.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Addition</th>
<th>Initial rate of proline uptake (nmol/min per mg)</th>
<th>Initial rate of glutamine uptake (nmol/min per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ML 308-225</td>
<td>DL-54</td>
</tr>
<tr>
<td>Proline</td>
<td>None</td>
<td>2.26</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.37</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td>5.78</td>
<td>5.52</td>
</tr>
<tr>
<td>D-Lactate</td>
<td></td>
<td>5.37</td>
<td>2.62</td>
</tr>
</tbody>
</table>

**A. Unstarved cells**

**B. Starved cells**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Addition</th>
<th>Initial rate of proline uptake (nmol/min per mg)</th>
<th>Initial rate of glutamine uptake (nmol/min per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proline</td>
<td>None</td>
<td>0.11</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td>4.05</td>
<td>5.78</td>
</tr>
<tr>
<td>D-Lactate</td>
<td></td>
<td>3.29</td>
<td>4.21</td>
</tr>
</tbody>
</table>
Lactate-driven proline transport, however, was nearly identical in the two strains. Phosphorylation is thus necessary for glutamine, but not for proline, uptake. With glucose as the carbon source, glutamine transport in DL-54 was nearly identical to that in the wild type (Table 1B). Energy derived from glycolysis can thus drive glutamine uptake in the absence of the Ca, Mg-ATPase. Glucose-driven proline transport was only partially reduced in DL-54 (Table 1B).

In the remaining experiments, the effects of various metabolic inhibitors were examined to see whether they support the interpretation that phosphate-bond energy can directly drive glutamine transport, and to distinguish whether respiration per se or the energized membrane state derived from it (or from ATP hydrolysis) is the primary energy donor for proline uptake.

The Effects of Cyanide. Inhibitors of respiration would be expected to block uptake processes under conditions where energy is derived from electron transport, but should have relatively little effect on transport supported by substrate-level phosphorylations. Fig. 1 shows that this was indeed the case. Cyanide abolished D-lactate-driven proline uptake in both the wild type and the ATPase mutant, while glucose-driven proline uptake in ML 308-225 was only partially sensitive. Furthermore, cyanide completely inhibited glucose-supported proline transport in DL-54. Thus, in contrast with findings in membrane vesicles (3), proline uptake in whole cells can occur in the presence of high levels of cyanide. This process requires a nonoxidative pathway for ATP formation (glycolysis), as well as a functional Ca, Mg-ATPase for generation of the energized membrane state from ATP. The need for the ATPase for energy coupling in the absence of electron transport has been noted for the uptake of proline (4) and β-galactosides (5).

As shown in Fig. 1, D-lactate-driven glutamine uptake was completely inhibited by cyanide. Glucose, on the other hand, supported a substantial rate of cyanide-resistant glutamine transport. Utilization of this energy did not require the Ca, Mg-ATPase, since DL-54 displayed a comparable level of glucose-driven glutamine uptake in the presence of cyanide. This result supports the view that phosphate-bond energy can directly drive glutamine transport.

The Effects of Uncouplers. Uncouplers of oxidative phosphorylation dissipate the energized membrane state and would thus be expected to inhibit those transport systems that use this state directly as a source of energy. Systems driven directly by phosphate-bond energy should be strongly inhibited under conditions where oxidative phosphorylation is the primary source of ATP, but should be relatively resistant where substrate-level phosphorylation is the major source. As shown in Fig. 2, proline uptake driven by glucose or D-lactate was abolished by the uncoupler FCCP in both ML 308-225 and DL-54. Glutamine uptake with D-lactate as the energy source was also quite sensitive. As expected, however, glucose was able to support a substantial rate of glutamine transport in DL-54 in the presence of high levels of FCCP, again implicating a direct role of phosphate-bond energy in this uptake system. FCCP had more drastic effects on glucose-drive glutamine uptake in ML 308-225 than in DL-54. This finding likely reflects the ability of uncouplers to enhance the hydrolytic activity of the ATPase, a phenomenon known to occur in both mitochondria (17) and chloroplasts (18).

As shown in Fig. 3, dinitrophenol produced results similar to those with FCCP. Furthermore, cyanide had relatively little effect on the dinitrophenol-resistant glutamine uptake in DL-54 (data not shown).

The Effects of Arsenate. Incubation of E. coli cells with arsenate causes a drastic reduction of the intracellular ATP (and phosphoenolpyruvate) levels (4). Transport systems that use high-energy phosphates directly as a source of energy should be severely inhibited by arsenate, whereas those driven by the energy-rich membrane state should be relatively resistant, unless this state is derived from ATP hydrolysis. As shown in Fig. 4, glutamine uptake was nearly completely in-

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*Since growth on glucose is known to repress oxidative phosphorylation in certain strains of E. coli (16), the experiments reported here were repeated with glycerol-grown cells with essentially identical results.
hibited by arsenate in both the wild type and the ATPase mutant, with either glucose or D-lactate as the energy source.

The effects on proline transport were much less severe (Fig. 4). With glucose as the energy source, a significant portion of proline uptake in ML 308-225 was inhibited by arsenate, whereas in DL-54, which presumably cannot form the energized membrane state from ATP, arsenate had no effect. Furthermore, glucose-driven proline uptake in the wild type in the presence of arsenate was nearly completely abolished by cyanide (Fig. 5). This result confirms the suggestion that the cyanide-insensitive component of proline transport in ML 308-225 (see Fig. 1) is energized by the hydrolysis of glycolytic ATP. Klein and Boyer reached similar conclusions of the basis of the oxygen requirement for arsenate-resistant proline accumulation (4). As expected, arsenate had only minor effects on D-lactate-driven proline uptake (Fig. 4).

DISCUSSION

The data presented here are consistent with the scheme shown in Fig. 6. Energy for proline transport can be derived from either electron transport or glycolysis. The oxidative pathway is sensitive to cyanide and uncouplers, does not require the Ca, Mg-ATPase, and is resistant to arsenate. The cyanide-resistant component is abolished by both uncouplers and arsenate, and requires an active ATPase. The two pathways converge in the presence of the Ca, Mg-ATPase to generate the energized membrane state that can drive proline uptake.

In contrast, glutamine transport is apparently driven directly by phosphate-bond energy, which can be formed by either oxidative or substrate-level processes. The oxidative pathway is sensitive to cyanide and uncouplers, but in contrast to proline, requires the Ca, Mg-ATPase. The glycolytic portion is resistant to cyanide and uncouplers, and does not require the ATPase. Arsenate prevents the utilization of energy for glutamine uptake by either pathway, presumably by preventing the synthesis of ATP.

A major portion of glucose-supported glutamine uptake in DL-54 is sensitive to both cyanide (Fig. 1) and uncouplers (Figs. 2 and 3). It is unlikely that this sensitivity represents side effects of these reagents on the formation of glycolytic ATP, since the inhibitions plateau with increasing inhibitor concentrations, and the effects are observed with the chemically dissimilar reagents, cyanide, FCCP, and dinitrophenol. Nor does this resistant uptake seem to reflect a component of transport driven directly by the energy-rich membrane state, since D-lactate-driven glutamine uptake in DL-54 is extremely low under conditions where this carbon source can support a full level of proline transport (Table 1B). A more reasonable explanation is that the cyanide- and uncoupler-sensitive component of glucose-driven glutamine uptake in DL-54 arises from a quantity of ATP formed by oxidative phosphorylation via residual ATPase activity in the mutant. Indeed, D-lactate is able to stimulate glutamine transport in DL-54 slightly (Table 1). If the ATP requirements for maximum uptake are small, it is possible that a low rate of oxidative phosphorylation could provide enough ATP to support a substantial level of glutamine transport with glucose, but not with D-lactate, as the carbon source.

Simoni and Shallenberger (6) have reported that in membrane vesicles, the transport of proline and alanine driven by D-lactate is markedly reduced in DL-54. These findings led Hong and Kaback (9) to suggest that this strain might possess a secondary or polar mutation affecting an energy-transfer coupling locus, an interpretation that seems unlikely in view of my finding that intact cells of DL-54 show normal lactate-supported proline transport. An alternative explanation for the defect in vesicles is provided by the recent

![Fig. 3](image-url) The effects of dinitrophenol on aminoacid uptake in ML 308-225 and DL-54. Additions: \(\bullet \), glucose; \(\square \), D-lactate.

![Fig. 4](image-url) The effects of sodium arsenate on aminoacid uptake in ML 308-225 and DL-54. Assays were performed in phosphateless medium. Open bars, no arsenate; solid bars, 0.5 mM arsenate.

![Fig. 5](image-url) The effects of cyanide on the arsenate-resistant portion of glucose-driven proline uptake in ML 308-225. Assays were performed in phosphateless medium. After the cells were incubated at 37° with 0.5 mM sodium arsenate for 10 min, the designated level of KCN was added and the incubation was continued for 5 min. Glucose was then introduced and the incubation was allowed to proceed for an additional 10 min before initiation of the transport reaction with labeled proline.
experiments of Bragg and Hou (19). From studies of aerobic and ATP-driven transhydrogenase reactions, these workers concluded that in addition to its catalytic functions, the Ca, Mg-ATPase of E. coli also performs a structural role in stabilizing the high-energy intermediate. Furthermore, the modified ATPase of DL-54 appeared to be more readily lost from the membrane than is the wild-type enzyme. The loss of this structural molecule during the preparation of membrane vesicles could readily account for the differences in β-lactate-driven proline uptake between vesicles and whole cells. I have repeated many of the transport experiments described here with another ATPase mutant, AN 120, and its parent, AN 180 (20), and obtained very similar results.

The difference in the mode of energy transduction for glutamine and proline uptake raises the possibility that these two uptake processes proceed by entirely different molecular mechanisms. Mitchell has suggested (21) that an electrochemical potential created by the extrusion of protons during either respiration or ATP hydrolysis can serve as the driving force for the transport of nonelectrolytes in bacteria. The finding that entry of lactose into E. coli is accompanied by the influx of protons (22, 23) lends support to this concept. A potential gradient model could equally apply to proline transport, though alternative hypotheses are certainly possible. Similarly, several models could explain how phosphate-bond energy drives glutamine uptake. Direct phosphorylation of the carrier molecule, a mechanism that has been elaborated for sodium uptake in erythrocytes (24), is an attractive hypothesis that is currently being explored. E. coli MOX19, which is unable to transport substrates of the phosphoenolpyruvate–phosphotransferase system (25), has normal glutamine uptake.

Another question that obviously arises is the identity of the energy donors for the myriad of different permeases in bacteria. In general, it has been observed (26) that aminoacid uptake systems in E. coli fall into at least two broad categories, those whose activities are sharply reduced by osmotic shock and those that are more tightly associated with the plasma membrane. The glutamine permease is a well-characterized shock-releasable system whose activity depends upon a periplasmic binding protein (27, 28). By contrast, proline uptake is resistant to shock (26), and the corresponding binding protein is extracted from the membrane only with difficulty (29). Membrane vesicles display very active uptake of proline, but not of glutamine (30). Preliminary evidence from this laboratory indicates that other aminoacid transport systems associated with shock-releasable binding proteins derive energy directly from the high-energy phosphate bond, whereas other tightly-bound systems are coupled to the energized membrane state. In addition, I have observed (unpublished information) that the tightly-bound aminoacid permeases are irreversibly inactivated by the sulfhydryl reagent N-ethylmaleimide, consistent with findings in membrane vesicles (3). Shock-releasable systems are much less sensitive to this inhibitor. The difference in behavior of various systems towards osmotic shock, which has long been a source of confusion and controversy for workers in this field (26), may thus reflect a fundamental mechanistic difference between these two classes of uptake systems.

Note Added in Proof. B. P. Rosen has found (personal communication) that proline uptake in membrane vesicles from strain NR 70, another ATPase mutant, is markedly stimulated by N,N-dicyclohexylcarbodiimide. I have made similar observations in DL-54 (unpublished information), consistent with the hypothesis that the transport defects in vesicles from this strain are due to loss of the Ca,Mg-ATPase (and its structural function) during vesicle preparation.

These experiments were performed in the laboratory of Dr. L. A. Heppel, who was a constant source of encouragement and advice. Dr. R. D. Simoni kindly donated the bacterial strains used in this study. I thank Dr. D. B. Wilson, Dr. G. Schatz, Dr. E. Racker, and Mr. E. Hertzberg for their valuable discussions and critical appraisals of this manuscript. The expert technical assistance of Miss Amy Shandell and Mrs. Anat Bromberg is gratefully acknowledged. This research was supported by Grant GB-27396X from the National Science Foundation and Grant AM 11789-05 from the National Institutes of Health.