Mutant of Escherichia coli K-12 with Defective Phosphorylation of Two Periplasmic Transport Proteins*

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Bacterial periplasmic transport systems require the function of a specific substrate-binding protein, located in the periplasm, and several cytoplasmic membrane transport components. In Escherichia coli K-12, the arginine-ornithine transport system requires an arginine-ornithine-binding protein and the lysine-arginine-ornithine (LAO) transport system includes a LAO-binding protein. Both periplasmic proteins can be phosphorylated by a single kinase. The enzyme exhibits a kinase activity and an ATPase activity. A mutant, defective in the phosphorylation of the arginine-ornithine and the LAO periplasmic proteins, was isolated and characterized. The defective enzymatic activity was reflected in substantially reduced levels of transport activity of the periplasmic transport systems that include each of the binding proteins. The binding proteins, extracted from the mutant, showed no detectable alterations in terms of quantity, electrophoretic mobility, or affinity constants. An apparent $K_m$ value of 1.0 mM was calculated for the ATPase activity of the defective enzyme. The ATPase activity of the wild-type enzyme yielded an apparent $K_m$ value of 50 $\mu$M. The amount of inorganic phosphate incorporated in vivo and in vitro into the binding proteins by the activity of the defective kinase was reduced to very low levels. A structural gene for the phosphorylating enzyme was located near the serA marker on the linkage map of E. coli. These results indicate that phosphorylation of the periplasmic transport protein is obligatorily linked to the normal function of the periplasmic transport system.

The transport of L-arginine in Escherichia coli K-12 is mediated by two kinetically distinct transport systems (1). There is one high affinity system which is common to L-lysine, L-arginine, and L-ornithine (LAO system) (1, 2) and a low affinity system for L-arginine and L-ornithine (arginine-ornithine system) (3). The high affinity system is repressible only by lysine, whereas the low affinity system is sensitive to multiple amino acids (arginine, lysine, L-arginine, and L-ornithine (LAO) system) (3). The binding proteins, extracted from the mutant, showed no detectable alterations in terms of quantity, electrophoretic mobility, or affinity constants. An apparent $K_m$ value of 1.0 mM was calculated for the ATPase activity of the defective enzyme. The ATPase activity of the wild-type enzyme yielded an apparent $K_m$ value of 50 $\mu$M. The amount of inorganic phosphate incorporated in vivo and in vitro into the binding proteins by the activity of the defective kinase was reduced to very low levels. A structural gene for the phosphorylating enzyme was located near the serA marker on the linkage map of E. coli. These results indicate that phosphorylation of the periplasmic transport protein is obligatorily linked to the normal function of the periplasmic transport system.

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incubation at 37 °C (17) were purified by restreaking on the same agar plate and then grown in liquid medium A in preparation for transport studies.

**TABLE I**

| Relevant genotype | Source or reference |
|-------------------|---------------------|
| Strains           |                     |
| MA176             | thr leu serA lysA thi rpsL |
| MA177             | pro trp argE serA speB thi |
| JC-182            | ade thi |
| JC-182-5          | ade thi argP |
| JM103             | Δ(lac pro) thi strA end AsbB15 hetR4 supE F' traD36 proA B lacI |
| Plasmids          |                     |
| pTZ218            | Ap' Ori IacZ' f1IC |
| pRC96             | Ap, Ori f1IG argK* |
|                | Derivative of pTZR18 carrying argK* |

* Symbols and map positions are from Bachman (9). argK, arginine kinase/ATPase. Ap* = resistance to ampicillin.

**RESULTS**

Transport Studies in a Mutant Carrying a Defective Kinase. L-Canavanine, a naturally occurring structural analogue of arginine, inhibits the growth of *E. coli* (14) and is incorporated into the cell by the arginine-ornithine and the LAO transport systems (2, 7). Both transport systems include a specific periplasmic transport protein that can be phosphorylated by a single protein kinase (6). Therefore, to isolate mutants carrying a defective protein kinase I selected for canavanine-resistant phenotypes with low transport activities of the arginine-ornithine and the LAO transport systems, but carrying normal periplasmic transport proteins. Fig. 1 shows the activity of the different transport systems tested in the parent strain and its canavanine-resistant derivative used in these studies. At the initial external concentration used for labeled arginine (1.0 μM) most of the substrate is taken up by the arginine-ornithine system (1). When ornithine was used at a concentration of 0.1 μM, the amino acid is incorporated into the cell through the LAO system (1). The activities of these two transport systems were present in the mutant at greatly reduced amounts. The lysine-specific system, an inner membrane transport system (1), were also reduced in the mutant (data not shown). Transport assays were performed as previously reported (3). ○, strain RC04; ●, strain RC101.
membrane-bound system that requires no periplasmic binding protein (23) and is related to the transport of arginine and ornithine in E. coli K-12 (1), remained unchanged in the mutant. Similarly, studies with two unrelated transport systems, the glutamine and the proline (not shown) systems indicated that they had not been affected by the mutation.

Studies with the Periplasmic Transport Proteins—Since the mutant RC101 showed reduced uptake of substrates for the arginine-ornithine and the LAO transport systems, and since both transport systems include a specific periplasmic binding protein, it seemed conceivable that the mutation could have affected either the synthesis or the structure of the binding proteins. Therefore, studies were carried out with the binding proteins from the mutant and parent strains isolated as unphosphorylated species (5). The data from binding activities of labeled arginine, ornithine, and lysine with crude osmotic shock fluid released from parent and mutant strains are presented in Table II. As shown, the same amounts of binding activities were detected by equilibrium dialysis with preparations of the two strains. In addition, the binding proteins were purified, subjected to electrophoresis analysis on sodium dodecyl sulfate-acrylamide on slab gels and the affinity constants for their respective substrates were determined. The two binding proteins, from the mutant and parent strain, were released by osmotic shock in similar amounts and each of them from both strains exhibited protein bands with identical electrophoretic mobilities. The dissociation constants of protein-amino acid complexes yielded similar Kd values from the analysis of Scatchard plots of data with preparations of both strains (Table III). Thus, the reduced transport activity in the mutant cannot be ascribed to changes in the structure or the formation of the periplasmic binding proteins.

Metabolism of Arginine in Strain RC101 In addition to arginine transport-defective mutants, three different classes of canavanine-resistant mutants have been isolated from E. coli K-12 (7). In nonrepressible (argR) strains, the resistance to canavanine is a consequence of an increase in the internal pool of arginine resulting from derepression of enzymes of the arginine biosynthetic enzymes (24). A second type of mutants, argS, carry a defective arginyl-transfer RNA synthetase and the partial block in the incorporation of arginine into tRNA leads to an increase in the pool of arginine (25). Finally, a block in the conversion of arginine into putrescine also leads to a canavanine-resistant phenotype with a larger pool of endogenous arginine (19). It was, therefore, necessary to measure the levels of the enzymes involved in the biosynthesis and conversion of arginine, to rule out a possible influence of changes in the internal pool of the amino acid on the activity of the transport systems. Table IV shows that the repression of the arginine biosynthetic pathway by arginine, as measured by the specific activity of ornithine carbamoyltransferase remained unaffected in the mutant and extracts prepared from cells of mutant and parent strain showed similar levels of arginyl-tRNA synthetase activity. The levels of the enzymes involved in the conversion of arginine into agmatine and putrescine are also presented in Table IV. Here again, it can be seen that the canavanine-resistant phenotype of strain RC101 cannot be attributed to differences in the conversion of arginine into polyamines.

Enzymatic Activities of the Phosphorylating Enzyme—The enzyme was extracted and purified from the canavanine-resistant mutant and the parent strain. Through the different steps of purification the mutant enzyme was indistinguishable from the wild-type enzyme, except in its catalytic activities. The purified preparations produced a single band on sodium dodecyl sulfate-polyacrylamide electrophoresis in slab gels with identical electrophoretic mobilities.

The kinase activity of the enzyme was studied by the extent of in vivo and in vitro phosphorylation of the two periplasmic transport proteins: the arginine-ornithine and the LAO-binding proteins. ATPase activity was measured by hydrolysis of [γ-32P]ATP in the absence of a phosphoryl acceptor.

The amount of 32P, incorporated in vivo into the two periplasmic proteins is shown in Fig. 2. Binding activity and specific 32P radioactivity, measured in the DEAE-Sephacel fractions, showed that phosphorylation of the binding proteins, at the time of arginine incorporation into the cell, was markedly reduced in strain RC101. The phosphorylation activity of the enzyme, tested in vitro in the presence of purified preparations of the arginine-ornithine and the LAO-binding proteins, is presented in Table V. The mutant is clearly defective in the kinase activity of the enzyme. Similarly, a substantially reduced ATPase activity was found in the en-

### TABLE II

| Strain | Arginine | Lysine | Ornithine |
|--------|----------|--------|-----------|
| RC04 (parent) | 14.5 | 7.8 | 6.6 |
| RC101 (mutant) | 15.0 | 7.5 | 7.0 |

**TABLE III**

**Affinity constants of periplasmic binding proteins**

Disassociation constants were determined by equilibrium dialysis as previously reported (3).

| Binding protein | Substrate | Kd (μM) | Strain |
|----------------|-----------|---------|--------|
| Arginine-ornithine | Arginine | 0.2 | RC04 |
| | Ornithine | 10.0 | RC101 |
| Lysine-arginine-ornithine | Arginine | 0.05 | RC04 |
| | Ornithine | 1.0 | RC101 |

### TABLE IV

**Levels of enzymes involved in the biosynthesis and conversion of arginine in parent and mutant strains**

| Enzyme | Product measured | Enzyme specific activity |
|--------|-----------------|-------------------------|
| | Parent (RC04) | Mutant (RC101) |
| Ornithine carbamoyltransferase | Citrulline | 2.09 | 2.20 |
| Arginyl-tRNA synthetase | Arg-tRNA | 14.10 | 12.80 |
| Arginine biosynthetic decarboxylase (constitutive) | CO2 | 0.01 | 0.01 |
| Arginine degradative decarboxylase (inducible) | CO2 | 0.16 | 0.18 |
| Agmatine urea hydratase | Urea | 0.06 | 0.00 |

*Ornithine carbamoyltransferase units are micromoles of citrulline produced in 1 h. Arginyl-tRNA synthetase units are micromoles of arginyl-tRNA produced in 1 min. References for enzyme assays are included under "Experimental Procedures."
TABLE V

| Activity measure                                    | Specific activity* |
|-----------------------------------------------------|--------------------|
| Phosphorylation of arginine-ornithine-binding protein| Parent (RC04) 83.2  Mutant (RC101) 0.08 |
| Phosphorylation of lysine-arginine-ornithine-binding protein | Parent (RC04) 30.4  Mutant (RC101) 0.32 |
| ATPase                                              | Parent (RC04) 40.2  Mutant (RC101) 8.10 |

* Procedures used for enzyme assays are described in the accompanying paper (6).

Enzyme purified from the mutant (Table V). The ATPase activity of the wild-type enzyme yielded an apparent \( K_m \) value for ATP of 50 \( \mu \)M. An apparent \( K_m \) value of 1.0 mM was calculated for the ATPase activity of the defective enzyme with variations in the concentration of ATP (Fig. 3). Due to the low values of phosphorylation of the plasmidic proteins with the defective enzyme, it was impossible to calculate accurate kinetic constants for the kinase activity of the enzyme.

Transport studies carried out with strain RC112 (Table I) showed values of activity of the arginine-ornithine and the LAO transport systems similar to those present in strain RC101. Similarly, the kinase and ATPase enzymatic values of the enzyme purified from strain RC112 were identical to those present in the enzyme from the mutant strain RC101.

Mapping of the Mutation on Strain RC101—Strains of E. coli K-12 with normal uptake and metabolism of arginine are inhibited in their growth by canavanine during the first 24 h of incubation, at 37 °C, on solid media (14), i.e. they are canavanine-sensitive (CanS). They can grow, however, after 36 h of incubation on arginine-free plates supplemented with 100 \( \mu \)g/ml canavanine (17). Hfr × F− mating experiments were carried out by using different Hfr strains (20) as donors and strain RC102 as recipient. Streptomycin was used to counterselect the Hfr strains (20). The selection plates contained \( 10^{-5} \)M medium supplemented with canavanine and a trace of L-arginine (0.1 \( \mu \)g/ml). Arginine was included in the medium to allow for the phenotypic lag in the expression of canavanine sensitivity (26). Recombinants that failed to grow in the first 24 h but grew after 36 h appeared with the Hfr donor strains PK191 (point of origin near his) and KL228 (point of origin near ilvE) (Fig. 4). Since no recombinants were detected with either Hfr KL16 or Hfr KL14, the area between lysA and his and the area between metC and ilvE were ruled out, and the region between lysA and metC were explored in detail by transduction experiments.

Linkage between the sap locus and the mutations conferring canavanine resistance (CanR) to strain RC101 was established when a lysate of bacteriophage 363 grown in E. coli K-12 wild-type cells was used to transduce the sap locus marker into strain RC106. Ninety-two percent of sapA transductants became canavanine sensitive and only 8% remained canavanine-resistant. The mutation was further localized by 363 transduction experiments, crossing strain RC101 (sapA, CanR) as donor and strain MA176 (sapA, lysA, CanS) as recipient. Among 460 lysA+ recombinants tested, 12 were sapA+ in this experiment, were 90% for canavanine resistance and 42% for speB+. All speB+ derivatives were canavanine-resistant. Therefore, the mutation was located in a gene closely linked to sapA. The genotypic
symbol argK has been assigned to the locus containing the mutation.

Relationship of the argK Locus to the argP Locus—It has been previously shown that a mutation in the argP locus of E. coli K-12 results in reduced levels of transport activities of the arginine-ornithine and the LAO transport systems (1). In addition, argP mutants the activity of the lysine-specific transport system is also reduced (1). It was, therefore, of interest to investigate what effect, if any, a mutated argP locus would exert on the activities of the phosphorylating enzyme. The enzyme was extracted and purified from strain JC-182-5 (argP) and compared with a purified preparation from the parental strain JC-182. (argP+). The ATPase activity of the enzyme from strain JC-182-5 was found to be reduced to a low value, no higher than 20% of the activity present in the protein from the wild-type strain. Similarly, the level of in vitro phosphorylation of both of the arginine-ornithine and the LAO-binding protein with the mutant enzyme, were significantly reduced. Both enzymes, however, from argP and argP+ strains, yielded the same apparent K<sub>m</sub> value, 100 μM, for their ATPase activities with different concentrations of ATP. This data are consistent with a previous suggestion (27) that the argP locus controls the synthesis of transport elements required for the incorporation of arginine in E. coli K-12.

Complementation studies between the argK and the argP loci were not possible because in argP/argP+ merodiploids the argP allele is dominant. 2

Complementation of the Transport Mutation in Strain RC101 with a Cloned argK Gene—Two bacteriophage λ clones from the Kohara's ordered bank, λ clone 10BA and λ clone 1A2, 3 were identified as capable of complementing the serA mutation on strain RC106.

Among the serA+ derivatives, selected on SF plates, canavanine-sensitive colonies were found in lysogens carrying the λ clone 1A2. The arginine-ornithine and the LAO transport systems, tested on these canavanine-sensitive strains, showed the same values of activity as those present in the parent strain RC04. Purified DNA from λ clone 1A2 was digested with EcoRI and the resultant fragments were ligated into plasmid pTZR18 previously digested with EcoRI. The ligated mixture was used to transform strain JM103. Following overnight incubation, ampicillin-resistant white colonies were isolated. Purified DNA from plasmid pRC96 was then used to transform strain RC101 selecting for ampicillin-resistant transformants. Resistant colonies were purified and tested for their canavanine phenotype and transport activity through the arginine-ornithine and LAO transport systems. Strain RC120, a canavanine-sensitive derivative with normal function of the two transport systems was selected for purification and studies with the phosphorylating enzyme. The values of kinase and ATPase activities obtained with the enzyme from strain RC120 were similar to the values present in the enzyme from strain RC04. The ATPase activity of the enzyme purified from strain RC120, yielded an apparent K<sub>m</sub> value of 50 μM with different concentrations of ATP.

**DISCUSSION**

The data presented in this report indicate that in organisms such as the canavanine-resistant mutant of E. coli K-12 described here, a close parallel exists between the levels of phosphorylation of a periplasmic transport protein and the activity of the transport system that includes the periplasmic protein as an essential component.

The complementation studies with a clone containing the argK + gene and the data from experiments carried out with strain RC112 strongly suggest that the mutation has affected a single gene or closed, linked genes. The analysis of the periplasmic binding proteins clearly showed that the mutation has not affected the synthesis of the structure of the binding proteins but the levels of phosphorylation effects the activity of a defective phosphorylating enzyme. This information supports the interpretation that phosphorylation of the periplasmic protein is an obligatory requirement for the normal operation of the periplasmic transport system.

In bacterial transport systems where a role for periplasmic transport proteins in active transport has been well defined, additional studies have shown that the periplasmic protein operates in concert with two or three additional specific inner membrane proteins (28-30). The amino acid sequences of some of these cytoplasmic membrane proteins have been deduced from the nucleotide sequence of the corresponding genes (28-30). Although there is evidence that suggests an interaction of the periplasmic binding protein with an integral cytoplasmic membrane protein (31), none of these inner membrane transport proteins have been isolated, purified, and characterized.

The requirement of phosphate bond energy by periplasmic transport systems has been previously established (4). There has been, however, some controversy concerning the identity of the energy donor and the mechanism by which energy is coupled to the transport process. Several investigators have claimed a requirement for an electrochemical proton gradient (32-34). It is clear, however, that hydrogen ions are not cotransported with substrates (35, 36), and periplasmic transport systems are capable of attaining concentration gradients that are far in excess of the magnitude that the electrochemical potential of protons could support in a fully energized cell (37). Recent studies with the histidine and the maltose periplasmic transport systems have also shown that these systems do not require a proton potential (38).

In studies of transport systems that rely for energy coupling on a chemiosmotic proton motive force, the use of bacterial membrane vesicles, introduced by Kaback (39), has been instrumental in establishing the true nature of the energy source. Reconstitution experiments of periplasmic transport systems in bacterial membranes vesicles have been previously reported (40, 41). From published data it has been estimated that the levels of restored transport in the vesicles is very low, no higher than 1% of the activity of transport system in the

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2 R. T. F. Celis, unpublished results.
3 Clone numbers are from Kohara et al. (11).
intact cell (28). Only recently, studies with the histidine transport system in vesicles (42), as well as the use of membrane vesicles obtained from cells producing a tethered maltose-binding protein, for the reconstitution of the transport of maltose (43), seem to indicate that the transport activity of intact cells can be recovered without a drastic inactivation in vesicles.

Binding of ATP analogues with cytoplasmic membrane components of two periplasmic transport systems, the histidine and the oligopeptide permeases, has been detected by using isolated membrane preparations (44, 45). To account for these observations a model has been proposed where conformational changes of inner membrane carriers could be induced by direct hydrolysis of ATP in the coupling of the energy source to the flow of substrate (44, 45). Neither hydrolysis of ATP nor phosphorylation of a transport component was detected in these studies.

The information discussed in the preceding paragraphs shows that substantial progress has been made toward our understanding of the mechanism of accumulation of substrates by periplasmic transport systems, a molecular description of the transport process remains a distant goal. The critical question of how the energy source activates the transport system remains a major unresolved question.

The results presented in this article underscore the importance of hydrolysis of ATP by a membrane enzyme, phosphorylation of the periplasmic binding protein, or a combination of both effects in the accumulation of substrates by two periplasmic transport systems. The arginine-ornithine periplasmic protein, isolated from the cell in a phosphorylated form, showed an affinity for arginine much lower ($K_a = 5.0$ $\mu M$) than the unphosphorylated protein ($K_a = 0.1$ $\mu M$) (5). This observation suggested that phosphorylation of the protein might be required for a change in the ligand-binding protein complex that would ensure an effective release of the substrate for its translocation at the cytoplasmic membrane level. Another interpretation of these results is that phosphorylation of the protein is necessary for an interaction of the substrate-binding protein complex with inner membrane proteins. Finally, since several transport ATPases from E. coli have already been characterized (46–48) a role of the intrinsic ATPase activity of the enzyme, in the operation of the transport systems, should be investigated.

A remaining unresolved problem related to these studies is the localization of the phosphorylating enzyme in the bacterial envelope. The enzyme is released by osmotic shock and can carry out the phosphorylative reaction after having been put in solution; association with membranes is not necessary. Therefore, the enzyme seems to be part of the family of periplasmic proteins. If this proves to be true, it remains to be explained how ATP is translocated from the cytoplasm to the periplasmic space. Although the requirement for ATP in another enzymatic reaction catalyzed by a periplasmic enzyme of E. coli, has been previously reported (49–51), there is no evidence for the existence of a transport system that can give ATP access to the periplasm. If ATP cannot be translocated out, the phosphorylating enzyme is probably located in the cytoplasmic membrane with access, directly or indirectly, through another membrane component, to cytoplasmic ATP. In this situation, the enzyme should contain a substrate-binding protein recognition domain protruding from the periplasmic face of the membrane. Membrane proteins that are sensitive to the effects of osmotic shock are supposed to be located in the periplasmic space. The profiles of proteins released by osmotic shock, however, include proteins from the periplasm as well as enzymes derived from the cytoplasmic membrane (52). It appears that, as a consequence of the abrupt expansion of the inner membrane against the cell wall during the osmotic shock, elements of the cytoplasmic membrane are expelled together with periplasmic proteins. Consistent with a localization of the phosphorylating enzyme in the cytoplasmic membrane is the map location (near the serA locus) of the gene responsible for the structure of the enzyme. Transport-defective mutants previously characterized (1, 7, 53), affected in cytoplasmic membrane proteins required for the incorporation of arginine and ornithine in E. coli K-12, all carry mutations located in the left side of the serA marker. Studies on the amino acid sequence of the enzyme and on the distribution of the protein in the different layers of the bacterial envelope may provide assistance in defining the localization and orientation of the enzyme in the membrane of the bacterium.

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