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Energy Coupling in Bacterial Periplasmic Permeases

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INTRODUCTION

Bacterial active transport systems (permeases) can be broadly divided into two classes with regard to their mechanism of energy coupling: those energized by electrochemical ion gradients and those energized by substrate-level phosphorylation. The distribution of the permeases according to this classification is matched by their fundamentally different characteristics. Electrochemical ion gradient-energized permeases, as typified by the β-galactoside (lac) permease (25), are shock-resistant systems usually composed of a single, very hydrophobic membrane protein that acts as a symporter or an antipporter, utilizing an ion or a proton gradient. In contrast, substrate-level phosphorylation-energized permeases constitute a class including numerous shock-sensitive permeases that have a complex composition. The shock-sensitive denotation refers to the inactivation of transport through these systems upon osmotic shock treatment of the intact cell. The inactivation is due to the loss from the periplasm (the space between the outer and inner membranes of gram-negative bacteria) of one of the components of the transport machinery, the periplasmic substrate-binding protein. Thus, shock-sensitive permeases are also called periplasmic permeases. They are composed of a membrane-bound complex, usually comprising between two and four membrane-bound proteins, and a soluble periplasmic protein. They typically transport with high affinity, achieving very large concentration gradients. Numerous such permeases, acting on extremely disparate substrates (sugars, amino acids, peptides, ions, and vitamins) have been characterized, and their properties have been reviewed recently (3, 4). Their overall composition is invariably the same, irrespective of the nature of the substrate being transported. Clearly, the uniformly similar structural design of these permeases can serve to transport vastly different substrates. An understanding of their mechanism of action requires that the energy-coupling process be unravelled first.

The action of periplasmic permeases involves the initial liganding of the substrate to the substrate-binding protein in the periplasm, thus resulting in the formation of the actual transport substrate, the liganded binding protein (33). The practical result of this first step is that the solute to be transported is presented in a concentrated form to the membrane-bound complex, as a consequence of the high binding affinity and very high concentration (of the order of millimolar) of the binding protein in the periplasm. The membrane-bound complex usually contains two very hydrophobic membrane-spanning proteins, which might form a heterodimer (2) or, if only one hydrophobic component is present, a homodimer. The third membrane-bound compo-

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respectively) (9). A troublesome ambiguity also was evident in the correlation between levels of ATP and extent of transport. Upon addition of cyanide to wild-type cells, the ATP level was either unchanged or increased; however, glutamine transport was drastically decreased. The better awareness we have today of the complicated interrelationships between proton motive force and a variety of cell functions suggests that it is unlikely that a simple relationship exists between addition of an inhibitor and its unique effect on proton motive force or ATP level; this more recent knowledge thus explains the conflicting results obtained in the earlier work.

More confusion was generated by results obtained later by two laboratories that directly conflicted with the hypothesis that ATP is the energy source and that implicated the proton motive force as the energy-coupling mechanism. One set of experiments demonstrated that under conditions in which the ATP level of cells is unchanged but the proton motive force is decreased by the addition of valinomycin plus K⁺, the activity of the glutamine permease is decreased. This was taken to indicate that ATP is not sufficient to power transport and that the proton motive force plays a role (directly or indirectly) in periplasmic transport (31). In agreement with Plate's results (31), Singh and Bragg showed that periplasmic permeases are functional only under conditions in which a proton motive force is expected to be generated (37). However, some of these experiments should be reinterpreted today by taking into consideration the fact that a proton motive force could have been built up by way of the proton-conducting ATPase activity (36). Against involvement of the proton motive force it was shown that proton translocation does not occur during transport by several periplasmic systems (11).

An interesting set of data implicated acetylphosphate (21) or a compound derived from it (20) as an energy source for periplasmic permeases. These data suffered from the same sources of ambiguity as did the experiments described above, since measurements of acetylphosphate were rarely performed and the correlation between transport and acetylphosphate was therefore indirect. Moreover, the mutants used were still capable of partial acetylphosphate synthesis (and therefore capable of producing ATP). An additional problem was the fact that glutamine transport, the permease under investigation, occurs through multiple systems (30), thus complicating further interpretation of those data. Later preliminary data indicated that under conditions in which arsenate inhibited glutamine transport very strongly, the levels of both ATP and acetylphosphate were normal or only slightly decreased. Therefore, it was suggested that not acetylphosphate, but a compound derived from it, was the true energy-coupling factor (20). Conflicting data obtained with membrane vesicles reconstituted with glutamine-binding protein showed that transport could be energized by the presence of pyruvate (or compounds yielding pyruvate) if NAD was also present and if the enzymes acetylkinase and phosphotransacetylase were active; alternatively, succinate alone could energize transport. It was therefore suggested that the proton motive force was required, in addition to another factor that was synthesized only in the presence of the enzymes involved in the biosynthesis of acetylphosphate, acetylkinase and/or phosphotransacetylase (23).

Additional critical discussions concerning energy coupling in shock-sensitive systems are available (3, 4a, 12, 16, 22). Here it suffices to say that while shock-resistant permeases (e.g., the lacY permease) had been clearly shown to be powered by the proton motive force, the question of energy coupling in periplasmic permeases remained controversial.

**ENERGIZATION IS BY ATP HYDROLYSIS**

Indirect support for the notion that ATP is responsible for energy coupling was the evidence that several members of the conserved family of hydrophilic membrane components did indeed bind ATP and its analogs, as shown by affinity labeling of the histidine and maltose permease components with 8-azido-ATP and ATP (19) and of a chimeric lac fusion derivative of the oligopeptide permease component with fluorosulfonylbenzoyl adenosine (18). These data, while not proving the involvement of ATP in energy coupling, gave a strong impetus to this aspect of the research. Four different experimental systems, one in vivo and three in vitro, were used with the histidine periplasmic permease to prove this point.

(i) **System 1.** The first system used intact cells defective in the proton-conducting ATPase and thus unable to interconvert the two important pools of energy. ATP and the proton motive force (24). This system allowed the separate manipulation of the levels of ATP and proton motive force without the use of metabolic poisons. Using both the histidine and maltose permeases, it was shown that upon dissipation of the proton motive force, while a high ATP pool was maintained, transport was unaffected. This result clearly demonstrates that the proton motive force is unnecessary. Cells which had been depleted of a carbon and energy source had low ATP and high proton motive force and were unable to transport, thus showing that the proton motive force, besides being unnecessary, is also not sufficient. Importantly, the intact cell system was used to demonstrate that the simultaneous inhibition of the proton motive force and histidine transport by valinomycin plus K⁺ (i.e., the result obtained by Plate [31]) was artifactual, since there was no such effect on maltose transport. The artifact was probably due to specific inhibitory effects on the histidine and glutamine permeases resulting from an increase in the internal pH following the treatment. Indeed, agents such as valinomycin-K⁺ and TPP⁺ (tetrphenyl phosphonium ion) are known to cause side effects (32) due to the cell's regulatory efforts at compensating for the loss of proton motive force, such as increasing the internal pH and/or K⁺ concentration (movement of other, unknown, ions might also occur). Thus, dissipation of the membrane potential by this particular method cannot be used for studying the mechanism of energetics of periplasmic permeases.

The whole-cell system is inappropriate for defining the energy source any more accurately, since lowering the ATP pool unavoidably also lowers the pool of several energy-rich molecules. The routine method for lowering the ATP pool makes use of arsenate (26). It was shown that arsenate-treated cells indeed lose the ability to transport histidine and maltose in direct proportion to the drop in ATP levels, while the proton motive force remains unaltered (thus supplying additional evidence for the noninvolvement of the proton motive force) (24). However, while the simultaneous loss of ATP and periplasmic transport could be taken as supportive evidence for the dependence of periplasmic permeases on ATP for energization, such data were considered unsatisfactory to draw a final conclusion, since the arsenate treatment is likely to lower also other energy-rich pools and to have additional effects, such as a direct inhibition of permeases. This was also indicated by the fact that when the ATP pool was depleted drastically by a different, very mild treatment,
involving no addition of poisons (15), no proportionality could be seen between ATP levels and transport: there was essentially no effect on transport despite a decrease in the ATP level to less than 10% of normal. The latter result suggested that a very low level of ATP is sufficient to energize transport and that arsenate has additional effects.

Whole cells were recently used to study the stoichiometry between transport and ATP hydrolysis rates (see below).

(ii) System 2. The second system was composed of right-side-out membrane vesicles reconstituted with added binding protein and energized with ascorbate and phenazine methosulfate or with D-lactate. This system was initially developed in the Hong laboratory by using the glutamine permease (22) and later was used by Rotman with the galactose periplasmic permease (35). By using the histidine permease, this system was improved extensively (33). The apparent contradiction that ascorbate-phenazine methosulfate or lactate, both proton motive force-producing energizing systems, allowed periplasmic transport in the reconstituted vesicles was easily understood once it became clear that the vesicles converted the proton motive force into ATP by using residual proton ATPase activity: uncleaved mutants could not transport, and various treatments lowering or raising the ATP pool correspondingly inhibited or increased transport.

The possible involvement of acetylphosphate was excluded by the use of mutants lacking acetylkinase and phosphotransacetylase (33). An interesting modification was introduced in the study of maltose transport, which used a mutant of the maltose permease that could not fully secrete the maltose-binding protein, thus producing vesicles that carried the binding protein "stuck" in the membrane (13). Despite the important information contributed by reconstituted right-side-out vesicles, this system cannot be easily manipulated to control accurately the level of ATP in order to determine its hydrolysis concomitant with transport, and it is still metabolically too complicated to be useful for detailed energetic studies. Thus, the results obtained with the histidine permease (33) and with the maltose permease (13) can be taken as strongly suggestive that ATP is the energy source, but they cannot in any way be considered conclusive.

(iii) System 3. Very strong support that ATP is indeed the direct energy source and that its hydrolysis is necessary was obtained for the histidine permease with a novel inside-out vesicle system that contained the histidine-binding protein trapped internally (6). This system allows the controlled and direct presentation of the normally impermeable energy substrate to the energy-coupling side of the membrane. Thus, addition of ATP caused histidine translocation from inside out; this movement corresponds to inward transport in intact cells. A nonhydrolyzable analog of ATP (adenyl-5′-yl imidodiphosphate) was inactive, suggesting the need for ATP hydrolysis. Pretreatment of the vesicles with 8-azido-ATP and UV light (thus inactivating the nucleotide-binding protein, HisP) (19) eliminated transport, as expected. With this system it was also possible to establish that the affinity of the permease for ATP is about 200 μM. This value explains the ability of whole cells to transport when the ATP level is lowered 10-fold (24).

(iv) System 4. The incontrovertible demonstration of ATP involvement came with the development of a reconstituted proteoliposome system utilizing a solubilized, partially purified histidine permease membrane complex and Escherichia coli phospholipids (10). It should be stressed that the success in developing this system owes a great deal to the prior studies on the reconstitution of the lac permease (29) and of anion-exchange permeases (1). In addition, the understanding of the important parameters of periplasmic function, such as the nature of the liganded binding protein as the true transport substrate (33), the elimination of the proton motive force and acetylphosphate as energy sources (24, 33), and the knowledge of the appropriate concentration of ATP needed (6), resulted in the confidence necessary for our laboratory to attack this challenging problem.

Using reconstituted proteoliposomes, it was shown that internally trapped ATP allowed active uptake upon addition of liganded binding protein. The reconstituted system was entirely dependent on all four permease proteins and on the presence of ATP. Dissipators of the membrane potential had absolutely no effect on transport. The demonstration that ATP was hydrolyzed only concomitantly with histidine transport finally confirmed incontrovertibly that ATP drives solute transport in these permeases. It should be mentioned, however, that OTP could replace ATP reasonably well.

The reconstituted proteoliposome system functions with an efficiency comparable to that obtained with reconstituted right-side-out vesicles: 0.55 mol of histidine transported per min per mol of HisP, compared with 1 mol/min per mol of HisP in vesicles. Both of these values are comparable to those obtained for whole cells. Thus, the proteoliposome system must reflect reasonably well the conditions in vivo.

At the time of this writing, only the histidine permease has been reconstituted into proteoliposomes. The reconstitution of the maltose permease is apparently under way (quoted by D. A. Deans, A. L. Davidson, and H. Nikaido [Res. Microbiol., in press]). It will be useful to see whether the maltose permease behaves in a similar way and supports the results obtained with the histidine permease.

STOICHIOMETRY

An interesting point is the stoichiometry between ATP hydrolysis and transport. In the proteoliposome system this stoichiometry was preliminarily determined to be an average of five ATP molecules per histidine molecule. This value is not to be taken seriously, since the purification and reconstitution of the membrane complex could easily have altered its properties, resulting in slipping (uncoupling). A stoichiometry of one would be consistent with the energetics of in vivo substrate accumulation. A stoichiometry of as many as two ATP molecules per histidine molecule might be justified because several periplasmic permeases, or their eucaryotic equivalents, have two ATP-binding domains, either on the same protein or in the form of two separate ATP-binding proteins (3, 7, 14). The stoichiometry of ATPases has been notoriously hard to establish in a variety of cases, and it is to be expected that the periplasmic permease stoichiometry will not be any easier to obtain. It is only from careful studies in a well-tuned in vitro system, utilizing pure and undamaged proteins, and from a variety of perimerase that an acceptable stoichiometric value will be obtained. Interestingly, a recent set of data obtained for whole cells inhibited with iodoacetate indicates a stoichiometry of one to two for the maltose permease (28). This conclusion is reassuring, but the data should be interpreted with caution, since whole cells may have complicated metabolic routes for recycling ATP, despite and possibly because of the poisoning procedure. One problem might be the presence of adenylate kinase (27), the activity of which can be eliminated only by mutation.

UNIVERSALITY: TRAFFIC ATPases

Over the last 2 years an increasing number of eucaryotic proteins have been discovered to have structures strongly
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MINIREVIEWS have nature. The homology is not limited to two ATP-binding consensus motifs but extends to large sections of the proteins. The eucaryotic counterparts now known are the mammalian multidrug resistance (MDR) proteins involved in the development of drug resistance to cytotoxic agents in tumor cells; the multidrug resistance equivalent from the malarial parasite Plasmodium falciparum, which is responsible for its chloroquine resistance phenotype; the human cystic fibrosis transmembrane conductance regulator protein (CFTR), which is the product of the defective cystic fibrosis gene and which may be involved in the secretion of Cl- across the apical cell membrane; the white and brown gene loci in Drosophila melanogaster, which are presumed to be involved in the transport and deposit of pigments; and the Saccharomyces cerevisiae STE26 gene product, which is responsible for the secretion of the mating pheromone a-factor.

Members of this family of proteins, containing an ATP-binding domain and involved in transport phenomena, are likely to hydrolyze ATP as part of a mechanism of transport. Since the family includes eucaryotic and proacaryotic members that transport a vast variety of substrates in both directions across the membrane, it has been proposed that they be referred to as "traffic ATPases" (Ames, in press).

CONCLUSION

We now know that ATP energizes periplasmic permeases. What remains to be established? Of course, this knowledge has simply set the stage for experiments on how ATP hydrolysis ultimately results in solute translocation. For this purpose it will be necessary to establish which protein domains are essential for this process and to distinguish those involved in ATP binding and hydrolysis from those involved in transmitting the information to and from this protein and the other components of the permease. The respective domains should be defined by in vivo and in vitro mutagenesis. Similarly, the domains of the other components of the membrane-bound complex and of the binding protein that interact directly or indirectly with the energizing component can be defined by mutagenesis. It is hoped that eventually crystallization of the membrane complex will yield the three-dimensional structure of the transport machinery. Whichever way, interesting developments are ahead.

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LITERATURE CITED


