Bacterial periplasmic permeases belong to a family of transport proteins operating from Escherichia coli to human: Traffic ATPases

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1. SUMMARY

Bacterial periplasmic transport systems are complex permeases composed of a soluble substrate-binding receptor and a membrane-bound complex containing 2–4 proteins. Recent developments have clearly demonstrated that these permeases are energized by the hydrolysis of ATP. Several in vitro systems have allowed a detailed study of the essential parameters functioning in these permeases. Several of the component proteins have been shown to interact with each other and the actual substrate for the transport process has been shown to be the liganded soluble receptor. The affinity of this substrate for the membrane complex is approximately 10 μM. The involvement of ATP in energy coupling is mediated by one of the proteins in the membrane complex. For each specific permease, this protein is a member of a family of conserved proteins which bind ATP. The similarity between the members of this family is high and extends itself beyond the consensus motifs for ATP binding.

Interestingly, over the last few years, several eukaryotic membrane-bound proteins have been discovered which bear a high level of homology to the family of the conserved components of bacterial periplasmic permeases. Most of these proteins are known to, or can be inferred to participate in a transport process, such as in the case of the multidrug resistance protein (MDR), the STE6 gene product of yeast, and possibly the cystic fibrosis protein. This homology suggests a similarity in the mechanism of action and possibly a common evolutionary origin. This exciting development will stimulate progress in both the prokaryotic and eukaryotic areas of research by the use of overlapping procedures and model building. We propose that this universal class of permeases be called ‘Traffic ATPases’ to distinguish them from other types of transport systems, and to highlight their involvement in the transport of a variety of substrates in either direction relative to the cell interior and their use of ATP as energy source.

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2. INTRODUCTION

Gram-negative bacteria have a complex cell surface, consisting of an outer membrane, the cell wall or peptidoglycan, and a cytoplasmic membrane [1]. The compartment between the outer and the inner cell membranes is known as the periplasm. Small solutes cross the outer membrane by way of water-filled channels and diffuse readily through the peptidoglycan, a rigid layer permeable to most molecules. The cytoplasmic membrane, on the other hand, is impermeable to most water-soluble compounds, which therefore require specialized transport systems to cross it.

The transport systems (permeases) can be broadly divided into two classes depending on whether they use the electrochemical ion gradient or substrate level phosphorylation for energization. The distribution of the permeases according to this criterion reveals that they share also other fundamentally different characteristics. Electrochemical ion gradient-energized permeases are, as typified by the β-galactoside (lacY) permease [2], shock-resistant systems, usually composed of a single, very hydrophobic membrane protein that acts as a symport or an antiport, utilizing an ion or a proton gradient. In contrast, periplasmic permeases, which are energized by substrate level phosphorylation, have a complex composition. These transport systems are also referred to as shock-sensitive permeases because they are inactivated during osmotic shock, which releases proteins located in the periplasm amounting to about 15% of the total cell protein. In all periplasmic permeases studied, osmotic shock releases an essential component, i.e. a soluble protein that binds the transported solute with high affinity. Periplasmic permeases, acting on extremely disparate substrates (sugars, amino acids, phosphate esters, phosphonates, peptides, ions, and vitamins) have been characterized and their properties reviewed recently [3]. 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.

Periplasmic permeases are typically composed of one periplasmic substrate-binding protein and three membrane-bound components. This overall organization is schematically represented in Fig. 1, using the histidine permease as a model system. The outer membrane is represented as containing pores which allow entrance of the substrate into the periplasm, where it is bound by the binding protein (HisJ). The membrane-bound components (HisQ, HisM, and HisP) form a complex within the cytoplasmic membrane. Genetic evidence suggests a direct interaction between the periplasmic binding protein and the membrane-bound proteins; biochemical data to be discussed later support the genetic evidence. The genetic structure of the most extensively characterized permeases is very similar [3]. In all cases a single operon (or two divergent operons) contains all the genes cod-
ing for the known transport components. In some cases the operon contains additional genes whose function is either unknown at present (e.g. livL in the branched-chain amino acid permease, [4]) or is involved in further catabolism of the transported substrates (e.g. rbsK codes for a ribose kinase, [5]).

It seems then, that complexity is the norm for this particular mode of transport. These permeases typically concentrate substrates inside the cell against a very large gradient (e.g. 10^5-fold in the case of maltose; [6]); perhaps achieving and maintaining such large concentration gradients requires a complex mechanism, possibly in relation to energy coupling. Alternatively, the high efficiency of transport which these systems usually display requires a complex structure. They are in fact able to scavenge solutes from very low concentrations: the apparent K_m's for uptake range from 0.01 μM to 1 μM [6–8]. By comparison, the apparent K_m for the transport of lactose through the shock-resistant, monocomponent, β-galactoside permease (lacY) is 190 μM [9]. Such high efficiency may constitute an evolutionary advantage for these bacteria, at least where amino-acid transport is concerned, since biosynthetically produced amino acids can be lost from the cell and these high-affinity permeases are able to recapture the lost amino acid [10].

An interesting aspect of some periplasmic systems is the fact that the membrane-bound complex can be multifunctional, i.e., it can be used for transport of several unrelated substrates and for that purpose it is utilized by more than one binding protein. For example, in the case of the histidine permease, the membrane-bound proteins are also essential for transport of arginines via the lysine arginine ornithine-binding protein (LAO protein, coded for by the argT gene [3,11]). This means that whatever mechanism is deduced from the available data, it will have to include for each of the different periplasmic components their alternating interaction with and removal from the same set of membrane-bound components. The existence of alternative periplasmic binding proteins utilizing the same set of membrane-bound components has also been shown for the branched-chain amino-acid permease [4,12].

3. GENERAL STRUCTURE

The periplasmic binding proteins are the most thoroughly analyzed of these transport components. Figure 1 depicts the histidine-binding protein, HisJ. The properties periplasmic binding proteins share as a group are summarized here (reviewed in [3,13], see references for individual cases therein). They are monomeric with molecular weights varying between about 25,000 and 56,000; several are stable to heat; they have high affinity for their substrates; they undergo a conformational change upon binding of substrate; and they have two functionally and genetically separable active domains. The latter three properties, discussed below, are the most enlightening with respect to the mechanism of action of these proteins.

The binding affinity (K_D) is between 0.1 and 1 μM for sugar substrates and around 0.1 μM for amino acids. Presumably because of this high affinity, some of these proteins have been purified with tightly bound substrates, which can be removed by reversible denaturation with guanidine-HCl [14,15]. Binding proteins undergo a conformational change upon binding of substrate, as has been measured in the case of the histidine-, maltose-, arabinose-, ribose-, galactose-, glutamine- and leucine-isoleucine-valine-binding proteins by a variety of methods (see [3,13] for references). Since a substrate-induced conformational change occurs in all binding proteins analyzed, it is likely to be an essential aspect of their mechanism of action.

X-ray crystallographic studies of several binding proteins, have shown that they are organized into two globular domains (lobes) forming a cleft and connected by a flexible hinge. The binding site for the substrate is located in the concave region between the two lobes. The molecule is flexible, the cleft becoming somewhat narrower upon binding of substrate [16,17], thus ‘trapping’ the substrate deep within the protein (hence the name of ‘Venus’s flytrap’ for this type of binding mechanism) [18–22]. Thus, these X-ray results corroborate the evidence that binding proteins undergo a conformational change upon interaction with substrate. Once the substrate is trapped in the liganded form of the protein, it is likely that
this more stable form requires an external stimulus to release the substrate, presumably supplied by the interaction with the membrane-bound transport components.

Thus, the first step in the action of these permeases involving the liganding of the substrate to the periplasmic protein in the periplasm, results in the formation of the actual transport substrate, the liganded binding protein [23]. 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 (in the order of mM) of the binding protein in the periplasm. The affinity of the liganded binding protein for the membrane complex has been measured for the histidine permease and shown to be around 10 \( \mu \)M. This value is considerably higher than that measured for the transport process as a whole (apparent \( K_m \): 6 nM). This disparity is accounted for by the high periplasmic concentration of the binding protein and the relative amounts of the binding protein versus the membrane complex [23].

The biochemical study of the membrane-bound components has lagged far behind that of the binding proteins, since they are more difficult to study. Their number, between two and four, has been determined for several permeases by genetic or recombinant DNA techniques [3]. Two of the components (when the total number is three or four) are usually very hydrophobic [24-29]. In the case of the histidine permease they are named HisQ and HisM (Fig. 1). The remaining one(s) (named HisP in Fig. 1) has an amino acid sequence that is not recognizably hydrophobic [26,27,30], despite the fact that it is clearly, and sometimes tightly, membrane-bound [31-33]. More will be said later about this component (Section 5). The hydrophobic HisQ and HisM proteins are accessible to protease digestion from both the outer and the inner surface of the cytoplasmic membrane, thus indicating that they span the membrane, as expected for integral membrane proteins. Preliminary evidence suggests that both proteins cross the membrane several times, with the amino and carboxy termini located outside and inside the membrane respectively, as established by antibody and protease accessibility and by Tn5-phoA fusion studies. Preliminary results by crosslinking and antibody coprecipitation indicate that the histidine permease membrane complex is composed of one molecule each of HisQ and HisM and two molecules of HisP (Kerppola and Ames, in preparation). In cases where only 1 hydrophobic membrane component is known, the complex may contain a homodimer of this protein. This view is supported by the finding that in several cases the two hydrophobic components are similar to each other, indicating a common evolutionary origin by duplication. This is the case for example, for HisQ and HisM, whose predicted topology is so similar that they have been postulated to form a heterodimer [34]. Along the same lines, in cases where two hydrophilic membrane components are known (e.g. in the oligopeptide permease, [29]) rather than one, they may exist as a heterodimer in the membrane complex, thus maintaining the overall proportions in the complex of two hydrophobic protein molecules for each pair of hydrophilic membrane protein molecules.

4. PROTEIN–PROTEIN INTERACTIONS WITHIN THE PERMEASE COMPLEX

Genetic data have been obtained which indicate the existence of direct interaction between the binding protein and the membrane-bound complex, for both the histidine and the maltose permeases. In the case of the histidine permease a mutant histidine-binding protein was isolated which cannot function in transport despite the fact that it has an intact histidine-binding site [35]. This suggested the existence of a region of the protein which is essential for transport but not necessary for binding histidine; this region was postulated to be involved in interacting with the membrane complex. Several mutations have been characterized which suppress the mutation in the binding protein, all located in the membrane-bound HisP protein ([36,37]; Speiser and Ames, in preparation). This can be interpreted most easily, but not exclusively, as a result of a direct interaction between the histidine-binding protein HisJ and HisP. A comparison of the sequence of HisJ...
with that of the closely related lysine-arginine-
orinthine-binding protein (LAO, the \textit{argT} gene product) lends support to the hypothesis that a domain of the binding protein is specifically involved in interacting with the membrane complex. Since both of these proteins require the same membrane complex for function, they must be very similar in their respective domains involved in this interaction. Indeed, regions of these two proteins are better than 90% homologous, which is significantly higher than their overall level of homology, thus suggesting that these regions are involved in forming the interaction domain. The \textit{hisJ} mutation mentioned above, which alters the interaction domain of HisJ, is indeed located in one of these regions of very high homology [37,38].

Genetic evidence has also been obtained for the existence of an interaction between the maltose-binding protein and the MalF and MalG proteins of the maltose permease. Mutations were characterized in these genes which allow transport of maltose in the absence of the binding protein; the introduction into these mutants of a wild-type \textit{malE} gene (encoding the binding protein) inhibits maltose uptake [39,40]. These results have been explained by postulating an interaction between the maltose-binding protein, MalE, and MalF or MalG. The binding protein-independent function of the mutated membrane components has been hypothesized to be inhibited by the presence of wild-type binding protein, because it would result in a non-productive and interfering interaction with the altered membrane components. Evidence that the maltose-binding protein has separate domains, for binding maltose and for interacting with MalF and MalG, has not yet been obtained.

The direct interaction between binding proteins and the membrane complex has been demonstrated biochemically in the case of the histidine permease, both in vivo and in vitro by two entirely different crosslinking methods [41]. The in vivo method utilizes formaldehyde and has been applied to whole cells, in order to investigate protein-protein interactions as they occur in vivo, thus decreasing the possibility of artifactual architectural alterations. A crosslinked product was formed and shown by immunological methods to contain both HisJ and HisQ. An entirely different method was utilized in vitro to provide additional evidence of protein-protein interactions. A photolyzable crosslinking reagent (Sulfo-SANPAH) was covalently reacted with pure HisJ. Addition of this derivatized protein to an in vitro reconstituted vesicle system under conditions known to allow active transport [23], followed by UV irradiation resulted in a covalent bond between the HisJ and HisQ proteins ([41]; Prossnitz and Ames, in preparation). It was also confirmed by crosslinking experiments that the mutant HisJ postulated on the basis of genetic data to be defective in its interaction site indeed was crosslinked very poorly to HisQ. Additional mutations have been introduced into HisJ by site-directed mutagenesis of residues postulated to be located in the domain that interacts with the membrane complex; the decreased ability of the mutated proteins to interact with the membrane complex has been assessed, thus forming the basis for the initial characterization of the interaction domain in HisJ (Prossnitz and Ames, in preparation). Interestingly, unliganded HisJ could also be crosslinked to HisQ, though at least ten times worse than the liganded form, indicating that the unliganded protein has a conformation that still allows it to interact with HisQ. Thus, with the availability of this biochemical evidence supporting the genetic implications, it is possible to conclude firmly that HisJ and HisQ interact directly.

It is entirely possible that HisJ makes direct contact also with HisM and HisP, but direct evidence is not yet available. The lack of a measurable crosslinked product between HisJ and HisP is somewhat puzzling, in view of the genetic data presented above, which strongly suggested such an interaction. If the interaction occurred in a hydrophobic environment it might not be accessible to our crosslinking reagents; alternatively, the genetic evidence may reflect an indirect interaction between HisJ and HisP.

5. A FAMILY OF CONSERVED TRANSPORT PROTEINS INVOLVED IN ENERGY COUPLING

Besides the hydrophobic components, all periplasmic permeases contain a protein which is usu-
ally thought to be peripheral. These peripheral membrane components form a family of related proteins that share extensive homology. We will refer to these proteins as the 'conserved' components. An alignment of several of the sequences of these conserved proteins has been presented in a review [3]. Additional sequences which appeared since are: \textit{oppF} [29], \textit{ugpC} [42], \textit{araG} [43], \textit{fhuC} [44, 45], \textit{chlD} [46], \textit{glnQ} [47], \textit{sfuC} [48], \textit{proV} [49, 50], \textit{btuD} [51, 120]. The similarity amongst these sequences is extensive, including several long stretches of amino acid residues, presumably involved in the performance of functions which are common to the entire family. In particular, two regions of extensive similarity share homology with several other proteins known to bind ATP, such as the \alpha- and \beta-subunits of the proton-translocating ATPase, myosin, adenylate kinase, RecA protein and others [52, 53]. This homology strongly suggested that the transport proteins also bind ATP. Indeed, both \textit{HisP} and \textit{MalK} have been shown to carry a nucleotide-binding site by using the photoaffinity label 8-azido-ATP [54]. Similar results were obtained with the \textit{RbsA} protein (Hermodson, personal communication). Competition of 8-azido-ATP labelling with a variety of nucleotide-containing compounds suggested that ATP and/or GTP are the natural substrates of \textit{HisP} [54]. The ATP analog 5'-p-fluorosulfonylbenzoyladenosine was also shown to react with the \textit{HisP} protein (Hobson and Ames, unpublished results) and with a chimeric \textit{lac} fusion derivative of \textit{OppD} [55]. Since nucleotide-binding activity has been demonstrated for several of the permease proteins, it was reasonable to generalize that all conserved membrane components bind a nucleotide and that such function is essential for all periplasmic systems, probably involving the energy-coupling mechanism [54, 55]. Indeed, this finding revived the interest and stimulated research in the mode of energy coupling of these permeases, as reported below.

Besides the ATP-binding motifs, several additional regions of homology are also present. No information is yet available regarding their function. Several individual residues, in particular glycine, are highly conserved. A secondary structure prediction of \textit{HisP} [56] indicates that the conserved glycines occur at join points between \alpha-helices and \beta-sheets or at predicted turn points. A consensus sequence obtained from an alignment of the known conserved components (16 sequences) was used to predict secondary structure. This prediction indicates that the secondary structure has been well preserved. The structure features the nucleotide-binding fold characterized by five $\beta \rightarrow \text{turn} \rightarrow \alpha$ motifs found in many nucleotide-binding proteins (C.S. Mimura et al., in preparation).

The location of the conserved components within the membrane is of particular interest. Their ability to bind ATP strongly suggests that they face the cytoplasm. Indeed \textit{HisP} was shown to present at least one domain to the cytoplasmic side as shown by protease accessibility experiments (Kerppola and Ames, in preparation). On the other hand, in the case of the histidine permease, the existence of several \textit{hisP} mutations that correct the defect in the interaction domain of \textit{HisJ} strongly suggests that \textit{HisP} also has a domain accessible to the periplasm. Preliminary evidence that \textit{HisP} may be accessible to protease digestion from the exterior surface of the membrane supports this hypothesis (Kerppola and Ames, in preparation). It is interesting that \textit{HisP} is relatively resistant to protease digestion from the cytoplasmic side of the membrane, as compared to \textit{HisQ} and \textit{HisM}. This may indicate that \textit{HisP} is physically protected from digestion by \textit{HisQ} and \textit{HisM}. In the case of the corresponding conserved protein, \textit{OppF}, from the oligopeptide permease, accessibility from the periplasmic face could not be demonstrated [33]. Since the converse, i.e. the demonstration of the accessibility of \textit{OppF} to protease digestion from the cytoplasmic side was performed in the presence of detergent, its cytoplasmic orientation remains in question in this case [33].

6. ENERGY COUPLING

6.1. Conflicting evidence

Until recently there has been considerable disagreement concerning this particular aspect of transport (reviewed in [57, 58]). It had been postulated that ATP or some form of phosphate-bond
energy is responsible for powering periplasmic permeases [59,60]. This conclusion had been achieved on the basis of experiments performed with intact cells which had been starved to eliminate endogenous energy sources and which, in addition, were either treated with a variety of metabolic poisons or were defective in the proton-translocating ATPase. The various treatments, meant to affect differentially and specifically various metabolic steps, aiming at lowering either the ATP level or the proton motive force, gave conflicting results. 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, and thus explains the conflicting results obtained in this early work.

More confusion was generated by results directly conflicting with the hypothesis that ATP is the energy source, and rather implicating the proton motive force as the energy coupling mechanism. One set of experiments demonstrated that under conditions where 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 (a periplasmic permease) is decreased. This was taken to indicate (1) that ATP is not sufficient to power transport, and (2) that the proton motive force plays a role (directly or indirectly) in periplasmic transport [61]. In agreement with Plate's results, Singh and Bragg showed that periplasmic permeases are functional only under conditions in which a proton motive force is expected to be generated [62]. However, some of these experiments should be reinterpreted today taking into consideration the fact that a proton motive force could have been built up by way of the proton-conducting ATPase activity [63]. On the other hand, against an involvement of the proton motive force it was shown that proton translocation during transport by several periplasmic systems does not occur [64].

An interesting set of data implicated acetylphosphate [65] or a compound derived from it as energy source for periplasmic permeases [66]. These data suffered from the same sources of ambiguity as the above experiments, since the correlation between transport and acetylphosphate concentrations was indirect. Unfortunately, the mutants used in these experiments were still capable of partial acetyl phosphate synthesis and therefore capable of producing ATP. Later it was shown 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 [66].

Additional critical discussions concerning energy coupling in shock-sensitive systems are available [3,57,58,67–70].

6.2. Energization is by ATP hydrolysis

The notion that ATP is responsible for energy coupling received some support from the finding that several members of the conserved family bind ATP and its analogs, as shown by affinity labelling of several of these proteins with ATP analogs. These data, while not proving the involvement of ATP in energy coupling, gave a strong impetus into this aspect of research. Four different experimental systems, one in vivo and three in vitro, were used in our laboratory to prove this point.

(i) The first one [71] used whole cells lacking the entire proton-conducting ATPase (unc deletion mutants) and thus unable to interconvert the two important pools of energy, ATP and proton motive force, to manipulate separately the levels of ATP and of the 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 maintaining a high ATP pool, transport was unaffected. This result clearly demonstrates that the proton motive force is unnecessary. Starved cells with low ATP and high proton motive force were unable to transport, thus showing that the proton motive force, besides being unnecessary, is also not sufficient and supporting the contention that ATP is the energy source. Importantly, the whole cells 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) was artifactual, since there was no such effect on maltose transport. The artifact is probably due to specific inhibitory effects on the histidine and glutamine permeases resulting from an increase in the internal pH following the valinomycin treatment. Indeed, agents such as valinomycin/K⁺ and TPP⁺ are known to cause side effects [72]. Thus, dissipation of the membrane potential by these particular methods cannot be used for studying the energetic mechanism of periplasmic permeases.

To demonstrate the involvement of ATP, its level needs to be reduced. However, lowering the ATP pool in whole cells with arsenate [73] unavoidably also lowers the pool of several other energy-rich molecules. 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 non-involvement of the proton motive force) [71]. Thus, these data are suggestive, but cannot be considered satisfactory to draw a final conclusion, since the arsenate treatment is likely to have additional effects, such as a direct inhibition of permeases. Indeed, an alternative method of lowering the ATP level, gave interesting results. The ATP pool was depleted drastically by creating an idle cycle of ATP consumption. This is a very mild treatment involving no addition of poisons [74]: no proportionality could be seen between ATP levels and transport and there was essentially no change in transport levels, despite a decrease in ATP to less than 10% of the normal level. These results indicate that a very low concentration of ATP is sufficient to energize transport, and that arsenate has additional effects.

(ii) The second system consists of right-side-out membrane vesicles reconstituted with added binding protein and energized with ascorbate and phenazine methosulfate, or β-lactate [23]. This system was initially developed using the glutamine [67] and the galactose periplasmic [75] permeases. We demonstrated that addition of ascorbate/phenazine methosulfate or lactate allows the creation of a proton motive force which can be converted into ATP by the residual proton ATPase activity. As a consequence, periplasmic transport is energized. It was shown that unc mutants could not transport and various treatments lowering or raising the ATP pool correspondingly inhibited and increased transport. The postulated involvement of acetylphosphate was excluded by the use of mutants completely lacking acetylkinase and phosphotransacetylase, which were shown to be unaffected in their transport ability [23]. 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 [76]. 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 [23] and those with the maltose permease [76] can be taken as strongly suggestive that ATP is the energy source, but they cannot in any way be considered conclusive.

(iii) 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 vesicles system that contained the histidine-binding protein trapped internally [77]. This system allows the controlled and direct presentation of the normally impermeable energy substrate to the energy-coupling side of the membrane. Addition of ATP caused histidine translocation from inside out; this movement corresponds to inward transport in intact cells. A non-hydrolyzable analog of ATP (AMPPNP) 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 [54], 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, which renders it about 300 μM [71].
A reconstituted proteoliposome system finally resulted in the incontrovertible demonstration that ATP is the energy source. Proteoliposomes were formed utilizing a solubilized partially purified histidine permease membrane complex and *E. coli* phospholipids [78]. 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* [79] and of anion-exchange permeases [80]. Also essential was the understanding of the important parameters of periplasmic permeases function, such as the nature of the liganded binding protein as the true transport substrate [23], the elimination of the proton motive force and acetyl phosphate as energy sources [23,71], and the knowledge of the appropriate concentrations of liganded binding protein and of ATP needed [23,77].

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 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, however, mentioned that GTP could replace ATP reasonably well.

The proteoliposome reconstituted system functions with an efficiency that is comparable to that obtained in reconstituted right-side-out vesicles: 0.55 mol of histidine transported per min, per mol of HisP, as compared to 1 mol per min per mol of HisP in vesicles. Both 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 writing, only the histidine permease has been reconstituted into proteoliposomes. The reconstitution of the maltose permease has been also achieved (quoted in [70]). It will be useful to see whether the maltose permease behaves similarly to the histidine permease.

### 6.3. Stoichiometry of ATP hydrolysis

The stoichiometry between ATP hydrolysis and transport was preliminarily determined in the proteoliposomes system to be an average of 5 ATPs per histidine. This value is most likely to be artifactual and to be the result of damage inflicted upon the membrane complex during purification and reconstitution resulting in slipping (uncoupling). A stoichiometry of one or two ATP molecules per histidine is more likely to be correct. Several periplasmic permeases, or their eukaryotic equivalents, have two ATP-binding domains, either on the same protein or in the form of two separate ATP-binding proteins [3,27,33], thus indicating that two may be the correct value. Only from careful studies in a well-tuned in vitro system, utilizing pure and undamaged proteins, and from a variety of permeases, will an acceptable stoichiometric value be obtained. Data obtained in whole cells inhibited with iodoacetate indicate a stoichiometry of one to two for the maltose permease [81]. This observation is reassuring, but the data should be interpreted with caution, since, as discussed above, whole cells may have complicated metabolic routes for recycling ATP, despite and possibly because of the poisoning procedure. One possible problem is the known presence of adenylate kinase [82], the activity of which can only be eliminated by mutation.

### 7. TRANSPORT MODELS

From the overall information obtained with all periplasmic permeases to date a general model can be drawn using the histidine permease as an example and building upon previous models [3] (Fig. 2). The substrate (histidine) enters the periplasm and encounters the binding protein (HisJ), that binds it reversibly. The concentration of free substrate is the same inside and outside the periplasm, but the total concentration (bound plus free) is higher inside and dependent on the binding protein concentration and on its affinity for the substrate. Upon interaction with histidine, HisJ undergoes a conformational change which increases its affinity for the membrane-bound complex (composed of proteins HisQ, HisM, and HisP). This interaction (certainly with HisQ, possibly also with HisM and HisP) triggers conformational changes in the membrane-bound apparatus. The substrate is re-
leased from the binding protein and a pore is formed through the membrane-bound component(s), allowing passage of the substrate into the cell. The ATP is hydrolyzed concomitantly, possibly as a consequence of the liganded binding protein interaction with the membrane complex. The unliganded binding protein is released because of its decreased affinity for the membrane. Alternatively, ATP hydrolysis may be needed to cause the release of the binding protein and/or to close the pore. Since it has not been established that substrates cross the membrane through a pore, an analogous model can be designed that allows the formation or activation of substrate-binding sites on the membrane complex [83]. The loaded site of the binding protein must be very close (juxtaposed) to the pore or to the next binding site, thus not allowing release of the substrate into the periplasm, which otherwise would negate any special function of the binding protein itself.

8. UNIVERSALITY OF THE CONSERVED COMPONENT: RELATIONSHIP TO ENERGY COUPLING

Interestingly, it has emerged lately that numerous proteins, not necessarily involved with transport of small molecules, belong to the family of the conserved components of periplasmic permeases [53, 84–86]. Among them, several eukaryotic proteins are particularly interesting and will be discussed individually.

8.1. Multidrug resistance

In 1986 the gene encoding the MDR protein (P-glycoprotein) was sequenced (in two different eukaryotic organisms: human and mouse) and portions of it displayed a striking homology to the sequence of the ATP-binding component of periplasmic permeases. What business does a respec-

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Fig. 2. A speculative model for periplasmic transport. The components undergo a series of conformational changes initiated by the binding of histidine to the periplasmic binding protein HisJ. The liganded HisJ binds to the membrane complex. This interaction occurs presumably with both the hydrophobic membrane components (HisQ and HisM) and elicits a conformational change in the ATP-binding membrane protein (HisP), thus causing ATP hydrolysis. Hydrolysis of ATP leads to the opening of a pore that allows the unidirectional diffusion of the substrate to the interior. After the substrate has been released to the interior, an additional conformational change (release of ADP?) closes the pore. The unliganded binding protein, having a poorer affinity for the membrane complex, dissociates from it. The membrane complex again binds ATP, ready to start the cycle. Alternatively, the substrate might be transferred from the binding protein to specific binding site(s) on the membrane complex, from where it would then be finally transferred to the interior, the rest of the cycle remaining the same.
table bacterial protein have being in humans? Since that finding several other eukaryotic proteins have been observed to display the same homology. They all seem to have a transport function in common as summarized below. The development of resistance to cytotoxic agents in tumor cells (Multi Drug Resistance: MDR; recent reviews cite the relevant literature: [87,88]) is one of the causes of failure in cancer chemotherapy. The problem is compounded by the fact that tumors become resistant simultaneously to many of the commonly used antineoplastic agents, most of which are structurally unrelated. It is clear that at least some aspects of this resistance have their basis in an altered ability to accumulate the drugs intracellularly, specifically in the fact that resistant cells actively expel the internalized drug at rates higher than the sensitive parent cells. The biochemical basis of MDR lies in the overproduction of a large (170 kDa) phosphorylated membrane protein named P-glycoprotein (or gp 170) which is presumed to be responsible for the active, ATP-dependent excretion of the toxic agents. The evidence for this model is strong, though still somewhat indirect since the activity of the protein in a purified in vitro system, such as reconstituted proteoliposomes has not yet been demonstrated. The MDR protein binds several of the toxic drugs [89,90], it hydrolizes ATP [91], and membrane vesicles containing elevated levels of P-glycoprotein transport the drugs in an ATP-dependent manner [92]. Thus, amplification of the mdr gene and its genetic modification following exposure to chemotherapeutic agents result in a lower internal level of drugs and, therefore, in their relative ineffectiveness. The P-glycoprotein amino-acid sequence shows two homologous halves, each including a hydrophobic region and a hydrophilic region containing an ATP-binding motif [84,93,94]. The homology suggests that the two halves possibly arose by a gene duplication event followed by fusion. Surprisingly, the hydrophilic region was found to share strong homology with the bacterial ATP-binding component of periplasmic permeases, including and extending beyond the consensus ATP-binding motif, thus implying similar mechanisms of action. Interestingly, even the existence of two ATP-binding moieties per transport component is shared with the bacterial systems. However, there is no homology between the prokaryotic hydrophobic transport components and the hydrophobic moieties of P-glycoprotein. This is not particularly surprising since these components share minimal homology even when the comparison is limited to the prokaryotic counterparts. On the other hand, the proteins may share secondary structure homology as predicted by computer modelling. Presumably these moieties have extensively diverged during evolution towards specialized functions, most likely recognition of different substrates and/or substrate-binding proteins. It seems that P-glycoprotein might have incorporated into one protein (as eukaryotes often do) what are separate proteins in prokaryotes: the genes for the two hydrophobic components and for the ATP-binding protein of bacterial permeases have been fused into one gene, which has then been duplicated and fused again. However, an obvious problem is that of the relationship between the orientation of P-glycoprotein, the site of ATP hydrolysis, and the direction of transport. The analogy with the periplasmic permeases does not appear to include a receptor equivalent to the substrate-binding protein, nor does it include the same directionality of transport. While a bacterial permease transports inwards utilizing the liganded binding protein as the true substrate, the eukaryotic system transports substrates outwards and there is no evidence for a binding protein. Eukaryotic binding proteins might exist [84]; an important function for such proteins might be that of imparting specificity, as is the case for the bacterial counterparts. In fact, particularly puzzling and pharmacologically troublesome is the fact that cells resistant to one drug to which they have been exposed, are also resistant to seemingly unrelated drugs, thus resulting in the multidrug aspect of the resistance phenomenon. By analogy with the bacterial counterpart, it is possible that receptors (soluble binding proteins?) for different substrates channel them through the same membrane machinery, as is the case for several permeases in bacteria [3]. Extensive searches by various laboratories have not yet turned up possible candidates. The identification of such hypothetical receptor proteins is im-
important since it might give a clue as to the physiological substrate of the eukaryotic transport systems. In fact, since the drug transport may belong to the class known as ‘illicit transport’, i.e. it utilizes a permease designed for another purpose [95], an unresolved issue is the function of these transport systems in normal, untreated cells. Two obvious possibilities are the secretion of physiological substrates [88,96–98] and a defense mechanism against toxic compounds naturally found in the environment. Needless to say, understanding the mechanism of action of P-glycoprotein would be extremely helpful in devising strategies to overcome the problem of chemotherapeutic failures due to overexpression of this protein.

8.2. Chloroquine resistance in the malarial parasite

Importantly, a P-glycoprotein-related phenomenon is the one responsible for the appearance of chloroquine-resistant derivatives of the malarial protozoan parasite, *Plasmodium falciparum*. This is an area of mammoth concern since 200 million cases of malaria occur every year, accompanied by 2 million deaths per year [99]. The evolvement of resistant parasites has strongly interfered with efforts at controlling malaria. Recently it has been shown that the phenotype of chloroquine resistance shares features with that of MDR, by exhibiting a lower level of accumulation of chloroquine [100]. Indeed, the sequence of a gene called *pfmdr*, the expression of which is amplified in some resistant strains, is so similar to *mdr*, including the hydrophobic membrane-spanning regions, that it has been classified as belonging to the *mdr* family (as opposed to the larger family of these related transport proteins) [101,102]. Thus, the family also includes protozoan proteins.

8.3. Cystic fibrosis

The surprise over the discovery of the MDR homology to bacterial proteins had barely abated when similar evidence emerged concerning other eukaryotic proteins. The most dramatic of these is undoubtedly the recent identification of the cystic fibrosis gene [103–105]. Cystic fibrosis is the most common recessive caucasian disease (one in 2500 live births) which results in early death due to multiple problems as a consequence of the abnormal composition of the secreted mucus, especially in the lungs. Damaged lung function leads to repeated infections that eventually cause death. It has been postulated that impaired secretion of Cl\(^-\) across the apical membrane of epithelial cells results in the production of thick mucus and is responsible for the symptoms of cystic fibrosis [106]. The isolation and sequencing of the cDNA for the gene carrying the defect has now supplied important information in this respect, since the product of this gene is clearly another eukaryotic counterpart of periplasmic permeases. The predicted protein structure (referred to as CFTR: cystic fibrosis transmembrane conductance regulator) has a mass of 169 kDa, with the familiar motif of two repeated halves each of which consists of a hydrophobic portion comprising several membrane spanners, and a hydrophilic portion carrying the ATP-binding consensus. These, of course, are also the characteristics of the P-glycoprotein. The cystic fibrosis defect was identified as a deletion of 3 bp resulting in the deletion of a phenylalanine residue in the first hydrophilic domain of CFTR. This alteration occurs in 68% of cystic fibrosis patients, thus constituting the major mutation group [103]. From genetic analysis, at least 7 additional mutations can be expected in the subgroup of the pancreatic insufficient patients, none of which has yet been identified at the molecular level.

Since it is not yet known what is the precise mechanism of action of CFTR, we do not understand why deleting the phenylalanine residue affects the activity of CFTR. If CFTR is directly involved in the transport of Cl\(^-\) (and/or water) it should be possible to study this phenomenon in a simplified in vitro system such as reconstituted proteoliposomes [78]. On the other hand, CFTR may be involved in transport indirectly, by regulating ion channel activity of a transport protein(s), since ion conductance is thought to be regulated by a cyclic AMP-dependent protein kinase (protein kinase A) or by protein kinase [107,108]. In agreement with this latter possibility is the fact that recently purified proteins functioning as chlo-
ride channels appear dissimilar from CFTR [109]. However, since the related P-glycoproteins and, in particular, the prokaryotic periplasmic permeases are clearly involved in transport, it seems most reasonable that CFTR is also a transport protein. The phenylalanine deletion may have impaired the binding and/or hydrolysis of ATP or have altered the overall structure sufficiently to interfere with its activation by the kinases, or both. The region where the phenylalanine resides is not located within either of the two ATP-binding consensus sequences, and is in fact located in a region of relatively poorer homology with comparable regions of other proteins from the same family (see e.g., alignments in [Fig. 2; [101,110]). This could be taken as an indication that it is a specialized region that has evolved uniquely in CFTR for dealing with ion transport; in this case, the phenylalanine deletion may affect exclusively this function. It should be mentioned that in the case of periplasmic permeases it has been clearly shown that these systems do not use or require a membrane potential or a K⁺ or H⁺ electrochemical gradient [71,78] and therefore do not involve ion movement [64]. Nothing is yet known of the coinvolvement of ion movement in the activity of P-glycoprotein or of the STE6 protein from yeast (see below).

Independently from the usefulness of having identified the most common defect in CFTR for an understanding of its mechanism of action, its usefulness for genetic diagnosis in cases without a previous family history is also enormous, since anyone can now be screened for being a carrier of the most common mutation, and hopefully in the near future, for the other mutations. Newer therapeutic approaches, once the biochemical nature of the defect is understood, might be possible through the design of specific drugs and may be within sight.

8.4. Insect proteins

Another eukaryotic protein, the product of the white locus in Drosophila melanogaster (and the closely related brown protein; [111]) also clearly belongs to this family, having a hydrophobic and a hydrophilic moiety, the latter clearly related to the ATP-binding family of transport proteins [112]. The location and function of its product is not known with certainty, but it is probably membrane-bound and involves the transport of pigments [113]. Thus, one more eukaryotic transport-related group of proteins, this time found in insects, can be assigned to this family.

8.5. Yeast mating pheromone

But wonders are not over: the yeast Saccharomyces cerevisiae produces at least one protein that belongs to the family and this time it secretes a lipopeptide, the mating pheromone, α-factor. Two recent publications describe the sequence of the gene, STE6, encoding this transporter [110,114]. This is the first eukaryotic case in which an essential physiological function for a transporter is known. Thus, it is an exciting addition to the family. It is also particularly exciting because it contrasts with the known mechanism of secretion of its counterpart pheromone α-factor, which is secreted through the classical secretory pathway as a much larger precursor that is proteolyzed in transit to its final size, a tridecapeptide. On the other hand, production of α-factor, which is 12 amino acids long and carries a farnesyl moiety and a methyl group, involves intracellular processing from a 36 or 38 amino acid precursor, and secretion independently of the classical protein secretory pathway (see; [115]). Yeast MATa cells depend on a functional STE6 gene to secrete the α-factor: ste6 mutants cannot mate ('sterile') because they cannot secrete α-factor. The deduced sequence of the STE6 protein has the unmistakable characteristics of the transport family: two homologous halves, each half including a hydrophobic domain and a hydrophilic ATP-binding domain. The homology with P-glycoprotein is extensive, including portions of the hydrophobic domains, besides the usually extensively homologous hydrophilic domain. Since α-factor appears to be synthesized and processed in the cytosol [116], it is most likely that the STE6 protein carries out the actual translocation of α-factor. The finding that an MDR-like transporter translocates a poly-
peptide raises again the question of the physiological function of MDR and of the uniqueness of the classic secretory pathway for proteins. As suggested by the sequencers of STE6, the strong possibility arises that members of the MDR family may indeed be involved in the secretion of some proteins or peptides, via a route that is independent of the classical secretory pathway, especially in the case of those secreted proteins that lack recognizable signal sequences or other obvious hydrophobic domains (for example, interleukin 1). A more detailed discussion of this proposal appears in [110].

Finally, it should be mentioned that bacterial transport systems responsible for polypeptide secretion (hemolysin in *E. coli*, hlyB: [117]; cyclolysin in *Bordetella pertussis*, cya B: [118]; and microcin B17 in *E. coli*, mcbE and mcbF: [119]) can be viewed as a link between the family of prokaryotic permeases for small substrates and that of the eukaryotic transporters for outward transport of high molecular weight substrates.

9. CONCLUSIONS

It is certain that we will see more and more examples of members of this family of transporters. Since all their structures have included an ATP-binding domain, since it is likely that they all hydrolyze ATP, and since they are involved in extensive traffic through the membrane in either direction and of a vast variety of substrates, we propose that they be named 'Traffic ATPases'. The general term ‘traffic’ helps distinguish them from other transport, usually ion ATPases (such as Ca, Mg ATPase or proton-conducting ATPase) and eliminates the problem of making reference to a specific substrate or specific accessory characteristics, such as periplasmic components, which may be unique only to some members of the family.

Exciting developments are certainly in store as this remarkable example of the unforeseen merger of vastly different areas of basic research enters the next step of cross-fertilization.

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REFERENCES

The mechanism of sugar binding to the periplasmic receptor for galactose chemotaxis and transport in *Escherichia coli*. J. Biol. Chem. 255, 2465–2471.


component of the histidine transport system of *Salmonella typhimurium*. J. Biol. Chem. 263, 17917–17920.


