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Reconstitution of a bacterial periplasmic permease in proteoliposomes and demonstration of ATP hydrolysis concomitant with transport

(active transport/histidine permease/octyl glucoside/ATPase/*Escherichia coli* phospholipids)

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ABSTRACT **The histidine periplasmic permease of *Salmonella typhimurium* has been partially purified and reconstituted into proteoliposomes. In this *in vitro* preparation, transport activity is completely dependent on the presence of all four permease proteins (HisJ, HisQ, HisM, and HisP) and on internal ATP. The reconstituted system shows initial rates of transport that are comparable to those obtained with right-side-out membrane vesicles and it establishes a 100-fold concentration gradient for histidine. Proteoliposomes also transport histidine when GTP replaces ATP. Proteoliposomes do not catalyze significant ATP hydrolysis until histidine transport is initiated by addition of substrate along with HisJ, the water-soluble histidine-binding protein. Both initially and throughout the course of substrate transport there is a concomitant hydrolysis of ATP, with an apparent stoichiometry (ATP/histidine) of 5:1. These experiments demonstrate directly that ATP is the source of energy for periplasmic permeases, thus resolving previous controversies on this topic.**

Periplasmic transport systems are a family of frequently encountered bacterial permeases acting on vastly different substrates. Several periplasmic permeases have been characterized extensively by genetic and physiological approaches utilizing whole cells, and an overall pattern is starting to emerge (1). They are composed of a periplasmic substrate-binding protein and, usually, three membrane-bound proteins, all encoded within a single operon; several of these operons have been fully sequenced. Transport through these permeases can establish a large (10^4-fold) concentration gradient and it has been postulated that energization for this is derived from ATP (2, 3); however, this suggestion has been disputed (reviewed in ref. 1). In contrast with this complexity, shock-resistant permeases such as the β-galactoside permease are usually monocomponent and utilize the electrochemical gradient for energization (4).

While the nature and function of the water-soluble substrate-binding proteins are fairly well understood, the membrane-bound components have not been studied biochemically because it has been impossible to assay their activity. The sequences of the genes encoding the membrane proteins indicate that two of them are hydrophobic, and these are presumed to span the membrane; the sequence of the third one suggests a hydrophilic and presumably peripheral protein. A striking characteristic of this peripheral membrane component is that it belongs to a family of homologous proteins that contain sequences implicated in nucleotide binding (5). Indeed, several of these peripheral components have been shown to bind ATP, and therefore these subunits have been hypothesized to be involved in the coupling of ATP to substrate transport (6, 7). Interestingly, proteins homologous to this family have been discovered also in eukaryotic organisms where they are also involved in transport-related processes, such as in the multidrug-resistance excretion mechanism (MDR) and in the transport and deposit of the *Drosophila* eye pigment (8, 9).

Our studies utilize as a model system the well-characterized histidine permease of *Salmonella typhimurium* (10), which is composed of the histidine-binding protein, HisJ, two hydrophobic integral membrane proteins, HisQ and HisM, and the nucleotide-binding membrane protein, HisP. Data obtained using intact cells implicated ATP as the energy source, and it was shown incontrovertibly that the electrochemical gradient is not involved in energization (3). *In vitro* studies were also initiated in order to approach the question of the proximal energy source from a biochemical point of view and to advance our understanding of the molecular mechanism of action of these permeases. With an *in vitro* system that utilizes right-side-out membrane vesicles to which the histidine-binding protein is added externally (11, 12), additional evidence was obtained that energization is derived from the ATP generated from the electrochemical gradient via the proton-conducting F_0F_1 ATPase; it was also shown that the liganded binding protein is the true substrate in transport, interacting with the membrane complex with a K_m of 65 μM (12). A second *in vitro* system, which consists of inside-out membrane vesicles containing entrapped histidine-binding protein and energized by externally added ATP, but not by a nonhydrolyzable analog of ATP, also supplied evidence that ATP is the proximal energy source and that its hydrolysis is necessary (13). However, both above systems are unsatisfactory for further biochemical studies, since the only pure protein utilized is HisJ, the others being still embedded in membrane vesicles. We now describe a third system, reconstituted proteoliposomes.

Proteoliposome reconstitution of periplasmic permeases has not been previously attempted, presumably because of the complex multicomponent nature of these systems and of the uncertainty as to their mechanism of energization. In addition, the methodology for reconstituting bacterial permeases in general was not available until fairly recently (14–17). Armed with the availability of the overproduction of our permease membrane proteins, with the presumption that ATP is the energy source, and with the understanding of the HisJ–membrane complex interaction, we have now developed a method for reconstituting the histidine permease into proteoliposomes. We have used this technique to demon-

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strate directly that ATP is the energy source for this periplasmic permease. This approach is the first necessary step in developing an *in vitro* system that utilizes purified components.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The following *Escherichia coli* K-12 derivatives were used (6): TA1889, which harbors plasmid pFA17 containing the *S. typhimurium hisQ, hisM,* and *hisP* genes under the temperature-sensitive control of the λ P_L promoter and which produces a substantial fraction of total membrane protein as HisQ, HisM, and HisP; TA3874, isogenic to TA1889, which harbors plasmid pFA18 containing *hisQ, hisM,* and *hisP* in an inverted orientation relative to the direction of transcription from the P_L promoter, and therefore not producing these proteins. Both TA1889 and TA3874 produce chromosomal levels of *E. coli* HisQ, HisM, and HisP. To control for chromosomal-level activity we also used TA5093, which carries a deletion of the entire histidine-transport operon. All three strains carry also a deletion (*unc*Δ702) that eliminates the F_0F_1 ATPase (12).

Preparation of Membrane Vesicles. Bacteria were grown aerobically at 30°C in LB medium with ampicillin (50 μg/ml). When the culture OD_{650} reached 0.5, the temperature was raised to 42°C and growth was continued for 1 hr, after which cells were harvested by centrifugation, washed in 0.1 M potassium phosphate (pH 7), and resuspended in buffer A (0.05 M potassium phosphate/5 mM $MgSO_4$/1 mM dithiothreitol/1 mM phenylmethanesulfonyl fluoride, pH 7). The cells were disrupted by three passages through a French pressure cell at 10,000 psi (68.9 MPa). The debris was removed by two successive centrifugations at 1400 × *g* for 5 min, and the membranes were harvested by centrifugation at 160,000 × *g* for 45 min. The membranes were washed once with buffer A and resuspended by repeated extrusion through a syringe needle (20-gauge) in buffer A at a protein concentration of 30–50 mg/ml. The yield of membrane protein was typically 35–50 mg per liter of cell culture.

Solubilization of Membrane Vesicles and Reconstitution of Membrane Protein into Proteoliposomes. Solubilization and reconstitution were performed essentially as described (16, 17), with the following details. Solubilization was performed at 1.2 mg of protein per ml with 1.2% octyl β-D-glucopyranoside (octyl glucoside) in 100 mM potassium Mops, pH 7/20% (vol/vol) glycerol/5 mM $MgSO_4$, 1 mM dithiothreitol/1 mM phenylmethanesulfonyl fluoride/0.37% *E. coli* phospholipids (Avanti Polar Lipids); 25–30% of the input protein was extracted. Proteoliposomes were formed by dilution of 0.93 ml of a mixture containing protein (0.25–0.3 mg), 5 mg of bath-sonicated *E. coli* phospholipids (in 0.1 ml of 0.2 M potassium Mops, pH 7), and additional octyl glucoside to a final concentration of 1.24%, into 20 ml of buffer containing 50 mM potassium Mops (pH 7) and 50 mM potassium phosphate (or 20 ml of 100 mM potassium Mops), 1 mM dithiothreitol, 10 mM $MgSO_4$, and various concentrations of ATP (sodium salt, Sigma). Liposomes were prepared by the above protocol in the absence of protein. Liposomes or proteoliposomes were finally resuspended in 200 μl of buffer B (20 mM potassium Mops, pH 7/75 mM K_2SO_4/5 mM $MgSO_4$). For these conditions we expect a recovery of 4 mg of phospholipid (17).

Transport Assay. Proteoliposomes (25–30 μg of protein) or liposomes were added to buffer B with or without 20 μM HisJ (≈90% pure) at 23°C in a final volume of 1.0 ml. HisJ had been deprived of bound ligand as described (12). Transport was initiated 2 or 3 min later by the addition of 25 μM [^{3}H]histidine (Amersham), and 100-μl aliquots were filtered through 0.22-μm GSTF Millipore filters and washed with buffer B. For assays in the presence of ionophores the loading and assay buffers each had 100 mM potassium Mops (pH 7) and 10 mM $MgSO_4$, with 10 mM ATP and 20 μM HisJ, respectively. Ionophores in 50% ethanol were diluted 200-fold into assay buffer 1 min after the addition of proteoliposomes and 2–3 min prior to initiation of transport. The effect of externally added ATP was not examined.

Assay of ATP. Aliquots (50 μl) were taken in parallel during transport assays and the ATP present was determined as described (3).

Other Methods. Membranes, octyl glucoside-soluble and -insoluble protein, and proteoliposomes were analyzed by sodium dodecyl sulfate/15% polyacrylamide gel electrophoresis (18) with the pH of the resolving gel adjusted to 8.65 (6). The Coomassie blue-stained gels were scanned with a Kratos SD300 densitometer. The resolved proteins were electroblotted onto Immobilon membrane (Millipore) and probed with antibodies to HisQ, HisM, and HisP. Protein was measured by a modified Lowry procedure (19) using bovine serum albumin as standard.

RESULTS

Extraction and Reconstitution of Membrane Proteins. As a source of protein for standard experiments we used membranes derived from TA1889, which carries a plasmid that contains the genes encoding the three membrane-bound components of the histidine permease, *hisQ, hisM,* and *hisP,* under the temperature-inducible control of the λ P_L promoter (6). Fig. 1*A* (lane 1) shows the protein composition of membranes after heat induction: HisP becomes a major component of the membrane protein (6), contributing about 13% of the stainable material. HisQ and HisM are also evident, though they are not well stained and appear as less abundant bands than HisP (6% and 7% of stainable material, respectively). However, the molar abundance of HisQ and

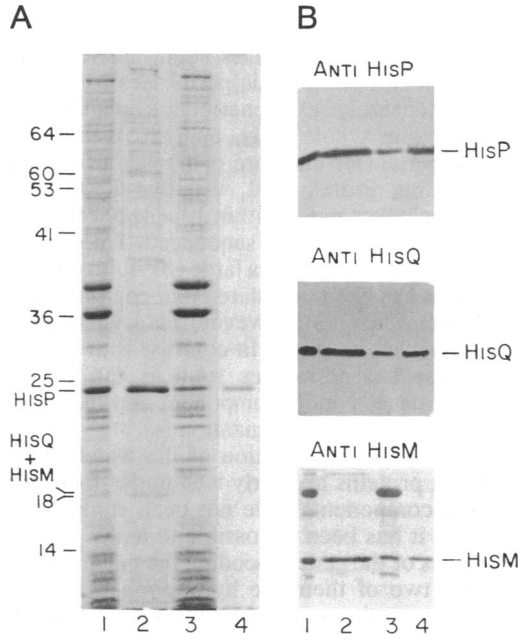

FIG. 1. Distribution of protein in membranes and proteoliposomes. TA1889 membranes (lanes 1) and proteoliposomes (lanes 4) were prepared as described in *Materials and Methods*. Octyl glucoside-soluble (lanes 2) and -insoluble (lanes 3) protein is also shown. Proteins were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and stained with Coomassie blue (*A*) or immunoblotted (*B*) against antibody to HisQ, HisM, or HisP, as indicated. The antiserum against HisM crossreacts with an additional protein of unknown nature. Molecular weight standards are indicated (M_r × 10^{-3} at left). HisQ and HisM run as a single band in this system.

HisM is comparable to that of HisP (A. Hobson and G.F.-L.A., unpublished data).

The composition of the solubilized and insoluble material is shown in Fig. 1*A* (lanes 2 and 3): 25–30% of the total membrane protein is extracted, and HisP accounts for 25–29% of the total stainable protein. About 35–40% of the solubilized protein is incorporated into proteoliposomes (Fig. 1*A*, lane 4), of which 22–28% is HisP. HisQ and HisM are not well visualized by Coomassie blue in proteoliposomes, but Fig. 1*B* shows the presence of all three of these proteins by immunoblotting. Both dye staining and immunoblot analysis indicate that the relative proportions of HisP, HisQ, and HisM are maintained through the various manipulations, suggesting that these proteins form a complex. Evidence for the existence of this complex has been obtained independently by coimmunoprecipitation and by crosslinking studies (R. Kerppola and G.F.-L.A., unpublished results).

Reconstitution of Histidine Transport. Fig. 2 shows that proteoliposomes containing trapped ATP transport histidine in the presence of the periplasmic histidine-binding protein, HisJ. Transport requires both ATP and HisJ, since their omission results in no transport. The three membrane-bound components, HisQ, HisM, and HisP, are also required since no accumulation occurs in proteoliposomes containing membrane proteins from TA3874. TA3874 is isogenic with TA1889, except for the recombinant plasmid pFA18, which does not express the transport proteins (6). Since this strain produces chromosomal levels of the *E. coli* histidine permease components, these levels must be too low to elicit transport under these conditions. Proteoliposomes from TA5093, which has a deletion of the host histidine permease operon, are also unable to transport histidine (Fig. 2), confirming the absolute requirement for HisQ, HisM, and HisP.

The observed histidine incorporation cannot be attributed to a simple binding reaction, since it requires the presence of both internal ATP and HisJ, and since it is not found when histidine and HisJ are incubated with ATP-loaded liposomes (no membrane protein) (data not given). The concentrations of HisJ and histidine were chosen to be 20 μM and 25 μM respectively, reflecting a convenient compromise between the measured affinity of liganded HisJ for membrane vesicles, 65 μM (12), and the availability of purified HisJ. A slight excess of histidine was used to ensure full saturation of HisJ. Preliminary experiments varying the molarity of HisJ and/or histidine resulted in variation of initial rate (data not shown) in agreement with the K_m value measured for right-side-out vesicles (65 μM), indicating that the concentration of liganded HisJ is below saturation for proteoliposomes. In several experiments of the sort shown in Fig. 2, histidine transport was measured at early times (0–4 min) for estimates of initial rate. These data gave a value of 1.3 ± 0.3 pmol/min per μg of protein and background levels that ranged from 0.5 to 2 pmol/μg of protein (1.7 ± 0.5 pmol/μg of protein; means ± SE, five experiments). Steady-state levels of substrate accumulation ranged from 10 to 30 pmol/μg of protein, with a mean value of 20 ± 3.3 pmol/μg of protein (seven experiments).

Taken together, these findings establish that we have reconstituted an active transport process that reflects operation of the *S. typhimurium* periplasmic histidine permease.

The Source of Energy. Because reconstitution of histidine transport requires the presence of internal ATP, it seems probable that ATP serves as the immediate energy source in proteoliposomes. These data are consistent with early suggestions (2) and with recent evidence implicating ATP as the energy source for this permease (3, 12, 13). The related nucleotide GTP has been shown to energize transport almost as effectively as ATP (13). GTP is effective in proteoliposomes as well, resulting in a rate about 70% that of ATP at equal molarity (10 mM); entrapped CTP also results in a low level of transport (40% of ATP; data not shown).

Nevertheless, the possibility that ATP is converted to another, proximal energy source under these conditions was not entirely excluded, since we did not use pure components. In particular, the possibility that ATP establishes an electrochemical potential (by a system other than the known F_0F_1 ATPase) that might energize histidine transport had to be excluded. Accordingly, we studied the effect of several ionophores: valinomycin, an electrogenic K^+ carrier, nigericin, a neutral K^+/H^+ exchanger, and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), a protonophore. To ensure disruption of K^+- and H^+-motive gradients, each ionophore was used at 0.1, 0.5, and 1 μM, in conditions of equal internal and external K^+. These manipulations had no effect on the initial rate of transport or on the final accumulation level (Fig. 3 and data not shown), eliminating the possibility that histidine transport is energized by a membrane potential or a K^+ or H^+ electrochemical gradient.

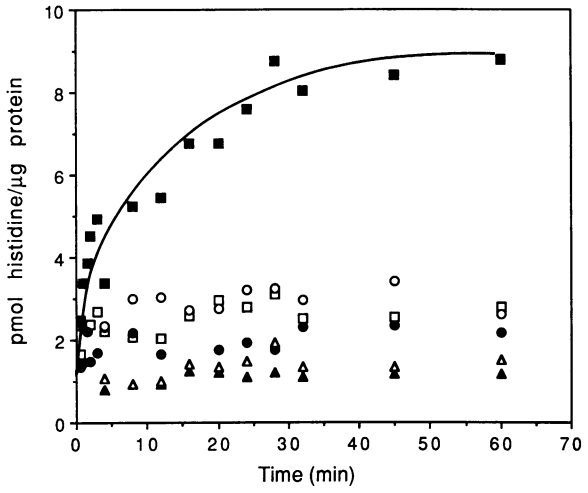

FIG. 2. Reconstitution of histidine transport in proteoliposomes. Uptake was assayed in proteoliposomes derived from TA1889 in the presence of 10 mM internal ATP and 20 μM external HisJ (■); ATP, no HisJ (△); HisJ, no ATP (○); no ATP, no HisJ (▲). Proteoliposomes derived from TA3874 (□) or TA5093 (●) were assayed in the presence of internal ATP and external HisJ; the results for these two strains were the same in the absence of either HisJ and/or ATP.

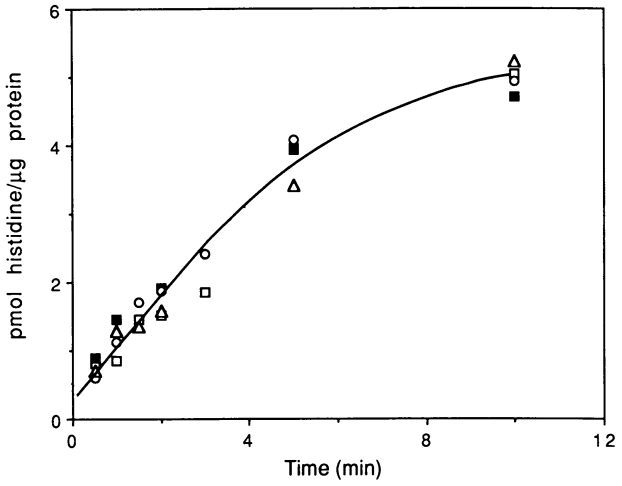

FIG. 3. Effect of ionophores on transport. Proteoliposomes derived from TA1889 were loaded with 10 mM ATP and assayed in the presence of 20 μM HisJ. Additions: ethanol (vehicle; final concentration, 0.25%; △), 0.5 μM valinomycin (○), 1.0 μM nigericin (■), 0.5 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (□).

ATP Is Hydrolyzed During Transport. If ATP is the proximal energy source for histidine transport it should be hydrolyzed concomitantly with transport. In the present work the use of *unc*-carrying strains and the partial loss of unrelated ATPases during solubilization and reconstitution have made it feasible to identify ATP hydrolysis specifically associated with histidine transport. For example, Fig. 4 *Upper* shows that the rates of ATP hydrolysis are 1.8 and 8.6 pmol/min per μg of protein, respectively, in the absence and presence of liganded HisJ. Thus, a rate of 6.8 pmol of ATP per min per μg of protein occurs specifically with histidine transport. This increased hydrolysis is absent in proteoliposomes prepared from TA3874 or TA5093 and is not found for liposomes (no membrane protein) (data not shown). Fig. 4 *Lower* shows a plot of the rate of histidine transport as measured in the same experiment. The initial rate of uptake is 1.4 pmol of histidine per min per μg of protein. Thus, the apparent stoichiometry of ATP hydrolysis to histidine transport is 4.9. Possible explanations for this stoichiometry will be discussed below.

DISCUSSION

This paper describes the reconstitution of an active periplasmic permease into proteoliposomes. The activity is completely dependent on the presence of the four known protein components of the histidine permease, HisJ, HisQ, HisM, and HisP, and on the entrapment of ATP in the interior of the proteoliposomes. Hydrolysis of ATP concomitant with histidine transport has been clearly demonstrated. In addition, a diagnostic use of ionophores excludes the possibility that the role of ATP is secondary to the generation of a membrane potential and of an electrochemical H^+ or K^+ gradient. Thus, our data demonstrate directly that ATP is the energy source for these permeases and finally resolve the controversial question of the mechanism of energy coupling in periplasmic systems (1).

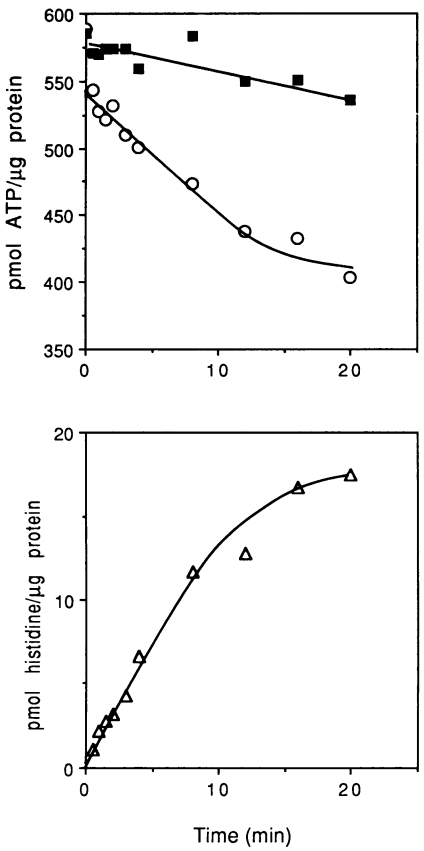

FIG. 4. Histidine uptake and concomitant ATP hydrolysis. (*Upper*) Proteoliposomes derived from TA1889 and containing 15 mM ATP were assayed for ATP content in the presence (○) or absence (■) of HisJ, before (time 0) and after addition of histidine. (*Lower*) Histidine transport was measured in a parallel tube in the same experiment. No measurable transport of histidine occurs in the absence of HisJ. An initial burst of ATP hydrolysis at zero time is unexplained.

We can estimate the efficiency of the reconstituted permease by relating it to the amount of HisP incorporated into the liposomes. HisP comprises roughly 25% of proteoliposomal protein (Fig. 1), and this level of HisP corresponds to about 10 nmol/mg of protein. Under the subsaturating conditions of the assay (20 μM liganded HisJ) the rate of transport is 1.3 pmol/min per μg of protein, which indicates a V_{max} of 5.5 pmol/min per μg of protein—i.e., a turnover number of 0.55 mol/min per mol of HisP. This is probably a minimal estimate, since the transport complex may be randomly oriented in the proteoliposomes, since not all complexes may be functional, and since the measured rate of uptake may be underestimated if it represents a balance between true uptake and an exit process. Nevertheless, the turnover number in proteoliposomes is comparable to that obtained in right-side-out vesicles, 1 mol/min per mol of HisP, which is itself reasonably close to the value estimated for intact cells that overproduce these transport proteins (12).

In proteoliposomes the accumulation of histidine reaches a plateau at a time when only 30% of the ATP has been hydrolyzed in a HisJ-dependent manner (Fig. 4). At this plateau, assuming an internal volume of 1 μl/mg of phospholipid (17) we calculate that the concentration of internal histidine corresponds to about 0.5 mM and that a concentration gradient of 100-fold has been established; this gradient is sigificantly smaller than may be obtained *in vivo* (1). Since the amount of ATP still associated with the proteoliposomes at the plateau should be sufficient to saturate the transport complex (apparent K_m = 200 μM; ref 13), the failure to continue to accumulate histidine may indicate that this residual ATP is not available for transport. ATP may be inaccessible because it is on the exterior of the proteoliposomes (17) or inside smaller proteoliposomes contained within larger ones. Thus, it is reasonable to assume that the plateau could reflect exhaustion of accessible ATP.

An important mechanistic question concerns the stoichiometry of ATP hydrolyzed to histidine transported. The apparent stoichiometry of 5:1 (Fig. 4) is not readily understood in mechanistic terms. A 1:1 ratio is consistent with the energetics of *in vivo* substrate accumulation. As many as 2 ATP molecules per histidine might be justified, because 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 (20, 21). However, higher values are unrealistic at a molecular level. One possible explanation for the observed stoichiometry is that ATP hydrolysis has become uncoupled from histidine transport. By analogy to the uncoupling observed in the F_0F_1 (22) and E_1E_2 (23) ATPases, the ATP hydrolysis by HisP may occur despite the lack of, or decrease in, histidine translocation if transport complexes are damaged or improperly inserted in proteoliposomes. A second possible explanation for the high stoichiometry is a leak of histidine either through the histidine permease or through an unrelated one. Derivation of an accurate stoichiometry requires obtaining the pure transport complex and ensuring its reconstitution in a native conformation. This task has proven difficult in a number of other systems (23, 24).

We have used the reconstitution of the histidine permease to demonstrate directly that ATP hydrolysis drives solute transport in this bacterial system, but our results have wider significance. Thus, the biochemical methods introduced here

will form the basis for purification of this transport ATPase in a functional state, opening the way to the productive use of both biophysical and chemical techniques. Perhaps more important, ATPases resembling the histidine permease constitute a widespread class of membrane proteins involved in a variety of biological processes (25, 26), and understanding the molecular biology of these proteins is an increasingly important goal in fields as distinct as oncology (8), cell division, and protein secretion (26). Based on work reported here, we suggest that each example in this class couples ATP hydrolysis to some transport event and that the histidine permease serves as a useful model for the entire group.

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