Reconstitution of the Histidine Periplasmic Transport System in Membrane Vesicles

ENERGY COUPLING AND INTERACTION BETWEEN THE BINDING PROTEIN AND THE MEMBRANE COMPLEX

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The periplasmic histidine transport system of *Salmonella typhimurium* has been reconstituted in isolated right-side-out membrane vesicles. The reconstituted system is entirely dependent on both the periplasmic protein, HisJ, and the membrane-bound complex, composed of proteins HisQ, HisM, and HisP. Transport is also dependent on the presence of ascorbate and phenazine methosulfate, which provide the energy for transport. Ascorbate oxidation generates a proton-motive-force, which allows ATP synthesis. ATP (or a co-generated molecule) appears to be the immediate energy donor. Dissipation of the proton-motive-force or reduction of the level of ATP by a variety of treatments results in inhibition of transport. Vanadate inhibits transport, indicating that ATP utilization is necessary to energize transport. The interaction between liganded HisJ and the membrane complex has been measured directly: it displays Michaelis-Menten type kinetics, with a $K_D$ of ~ 65 μM. The significance of this finding in terms of transport properties of whole cells is discussed.

Bacterial periplasmic transport systems (hereafter referred to as permeases (Rickenberg et al., 1956)) are complex transport systems, generally consisting of four protein components: three associated with the cytoplasmic membrane and one located in the space between the cytoplasmic and outer membrane, the periplasm (reviewed in Ames, 1986). As a model system, we are studying the histidine permease of *Salmonella typhimurium*. This permease has been thoroughly characterized, both in vivo, kinetically and genetically, and at the DNA sequence level (Higgins et al., 1982). The soluble periplasmic protein, the HisJ protein, binds substrate with high affinity ($K_D = 10^{-7}$ M) and is located in the periplasm at high concentration (on the order of 0.5-1 mM; Lever, 1972b). The combination of high protein concentration and high substrate-binding affinity results in a high concentration of liganded binding protein in the periplasm, thus increasing the concentration of substrate available for translocation across the cytoplasmic membrane. The membrane-bound components, HisQ, HisM, and HisP are responsible for translocating the substrate across the inner membrane and for the energy coupling process (Hobson et al., 1984). Cross-linking of HisJ to HisQ has recently provided biochemical evidence for a direct interaction between these two proteins (Prossnitz et al., 1988).

The energetics of periplasmic permeases have not been clearly understood. In this context, various investigators have proposed roles for, or argued against ATP (Berger, 1973; Berger and Heppel, 1974), acetylphosphate (Hong et al., 1979), several unidentified small molecules (Hunt and Hong, 1983), and the proton-motive-force (pmf) (Plate, 1979; Singh and Bragg, 1979). A recent detailed analysis of the nature of energy coupling in intact cells has excluded a role for pmf in energy coupling and has strongly suggested that ATP is the energy source (Joshi et al., 1989).

Right-side-out vesicles (Kaback, 1974) have been very valuable in the study of shock-resistant permeases. This system has been used extensively to analyze the molecular mechanism of action of the β-galactoside permease (Kaczorowski et al., 1980). However, in the past, this tool has been of limited use for periplasmic permeases because both the periplasmic component and the proper energization factor(s) are lost during preparation. A reconstituted vesicle system was developed by Hunt and Hong (1981) by the addition of periplasmic protein to vesicles obtained from a mutant strain that lacked completely the periplasmic protein. Energization of transport was initially achieved by the addition of d-lactate or pyruvate and inclusion of NAD within the vesicles. This method of reconstitution was also shown to be effective in the case of the methyl-β-D-galactoside permease (Rotman and Guzman, 1984). However, the characterization of the reconstituted system was incomplete, especially from the point of view of the energetics. Here we describe the reconstitution of the histidine permease in right-side-out vesicles and the extensive characterization of this *in vitro* system. We have used this reconstituted system to study the following aspects of transport: 1) the energy coupling mechanism; 2) the role of the binding protein in transport; 3) the physical interaction between the binding protein and the membrane complex (Prossnitz et al., 1988). These studies are possible because both the periplasm and the cytoplasm are replaced by media, the composition of which can be easily manipulated. Thus, events that occur on both sides of the cytoplasmic membrane can be studied.

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1 The abbreviations used are: pmf, proton-motive-force; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); PMS, phenazine methosulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CCCP, carbonyl cyanide p-chlorophenylhydrazone.

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ATP Determinations—ATP was assayed essentially as described (Joshi et al., 1989) in samples taken from transport assay mixes lacking binding protein and substrate. Aliquots (100 μl) were removed at timed intervals after the addition of the ascorbate/PMS and added to 650 μl 5% perchloric acid on ice. After 10 min, 0.188 ml of 4 M KOH and 0.188 ml of 2 N HClO₄ were sequentially added, followed by incubation on ice for 10 min. After centrifugation at 15,000 × g for 10 min at 4 °C, the supernatant was assayed for ATP content (nmol/mg protein) by a luciferin/luciferase procedure using a Turner Designs model 20e luminometer. ATP (Sigma) was used as a standard.

Other Procedures—Protein concentrations were determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard. HisJ and hisG525 were obtained from TA4260 and TA5050, respectively (carrying the hisJ gene under Tac control), by osmotic shock and purified (~80% pure) by standard chromatographic techniques. Such protein is referred to as liganded HisJ, since it was found to contain bound histidine. The histidine content was analyzed by precipitating 2 nmol of HisJ in 20% trichloroacetic acid and analyzing the supernatant by paper chromatography, as described (Anees and Mitchell, 1952). Bound histidine was removed by two sequential dialyses, for 16 and 6 h, respectively, against 1 liter of 100 mm Tris/Cl, pH 7.2, containing 5 mm EDTA and 5 mM gusardine (Miller et al., 1983). HistJ was renatured by dialysis against 1 mm KCl, pH 7.0 (0.5 liter for 4 h, 1.0 liter for 4 h, 6 liters for 4 h, 4 times).

Histidine binding was assayed by equilibrium dialysis. The concentration of free histidine and free HisJ in a reaction mixture was calculated using the following equations:

\[
[HJ free] + [histidine free] = K_D = 10^{-7} M
\]

\[
[HJ free] = [HisJ total] - [HisJ bound]
\]

\[
[HJ bound] = [histidine bound] = [histidine free]
\]

RESULTS

Requirements for Reconstitution of the Histidine Permease—Vesicles were prepared from strains lacking HisJ, since it has been previously demonstrated that these vesicles derived from the wild type strain cannot be used in the reconstitution of binding protein-dependent transport systems (Hunt and Hong, 1981). Such vesicles transport proline upon addition of ascorbate/PMS as an exogenous energy source (data not shown), but not histidine. However, upon addition of both HisJ and ascorbate/PMS, histidine becomes associated with the vesicles in a time-dependent manner (Fig. 1). The nature of the energy-coupling mechanism will be discussed later. This result confirms previous evidence that periplasmic transport can be reconstituted in right-side-out vesicles. Since histidine transport also occurs through the aromatic permease (Anees and Roth, 1968), which apparently is nonperiplasmic in nature (Chye et al., 1986), phenylalanine was added to the assay mixture in order to inhibit histidine transport through this permease.

Transport was shown to occur through the histidine permease by its complete dependence on the membrane-bound components of the permease HisQ, HisM, and HisP (Fig. 1). Vesicles lacking these proteins are incapable of transporting histidine but are capable of energy-dependent proline uptake (0.5–1.0 nmol/min/mg), demonstrating that they are grossly defective and that they are capable of generating a pmf, since proline transport in vesicles is pmf energized (Cairney et al., 1984).

Orientiation of the vesicles, as expected, is critical for transport. Vesicles prepared by French pressure cell lysis, which results in predominantly inside-out vesicles (Rosen and Tsu- chiya, 1979), were incapable of histidine uptake under the conditions used here (less than 2% of control; data not shown). The amount of histidine associated with the vesicles at
equilibrium corresponds to an internal concentration of 4.3 mM (Fig. 1, inset). At the HisJ and histidine concentrations used (40 µM each), it can be calculated (from a KD for histidine binding of 10^-7 M) that the free histidine concentration in the assay mixture is approximately 2 µM, thus yielding a concentration factor of 2150-fold. The possibility that the vesicle-associated histidine is bound, but not transported, is unlikely due to the requirement for energization. In addition, we show that the equilibrium level of vesicle-associated histidine is directly related to the internal vesicle volume, as would be expected if the transported histidine were free inside the vesicles. Vesicle volume was altered by the addition of sucrose to the assay medium, with all other variables held constant (Fig. 2). As the osmolarity of the external medium increased, the total amount of histidine accumulated at equilibrium decreased, approaching the value obtained for unenergized vesicles. The initial rate of transport in the presence of sucrose is essentially unaffected (e.g. about 75% of normal at 0.3 M sucrose, at which concentration the equilibrium level is down to 25% of normal). These results indicate that the histidine associated with the vesicles is exposed to the vesicle interior and further supports the contention that reconstituted vesicles are actively transporting.

Additional evidence in this regard is derived from the use of a mutant HisJ protein, HisJ5625, that binds histidine normally, but does not support histidine transport in whole cells. The defect of HisJ5625 has been ascribed to its inability to interact properly with the membrane-bound complex (Kustu and Ames, 1974; Ames and Spudich, 1976; Prosnitz et al., 1988). HisJ5625 is unable to support histidine transport in reconstitution experiments (Fig. 1), specifically demonstrating that the time-dependent uptake is not the result of a nonspecific absorption of the HisJ-histidine complex to membrane vesicles.

These vesicles are generally competent in reconstitution as shown by reconstitution of another transport activity. Addition of 40 µM lysine-arginine-ornithine-binding protein resulted in an arginine transport rate of 0.3 nmol arg/min/mg protein (data not shown).

Effect of pH and Mg2+—Fig. 3A shows the pH activity profile of reconstituted histidine transport in membrane vesicles. Maximal transport occurs at pH 6.8. Maximal histidine binding to HisJ occurs at pH 5.5 (Lever, 1972b); therefore, factors other than substrate binding by HisJ predominate in determining maximal transport rates. Since the internal pH optimum for histidine transport in whole cells is 7.0 (Joshi et al., 1983), it is likely that altering the external pH in the vesicles system modifies accordingly the internal pH. In addition, the rate of oxidation of ascorbate/PMS (and, therefore, the size of pmf) varies with external pH (Ramos and Kaback, 1977) and might be one of these factors. The nature of the assay buffer has little effect.

Fig. 3B shows the effect of changing Mg2+ concentration. Mg2+ is required, since reducing its concentration by the addition of EDTA results in 75% inhibition of transport. The optimum concentration is about 50 mM and was used for all experiments. The dependence on Mg2+, together with the need for Pi inside the vesicles (see later), most probably reflect the requirements of the energy-coupling mechanism.

Effect of Histidine and HisJ Concentrations—In order to study the effect of changing the histidine and HisJ concentrations, it was necessary to ensure that no contaminating histidine was introduced into the assay together with HisJ, since purified HisJ is known to contain bound histidine (Miller et al., 1983). Our analysis of HisJ determined that it was more than 50% saturated with histidine. The ability of HisJ to support transport after removal of all the bound histidine (unliganded HisJ) was examined. Use of unliganded HisJ, prepared as described under “Experimental Procedures,” resulted in a 2-fold increase in transport, as would be expected if the specific activity of the labeled histidine were decreased during the assay by the unlabeled histidine present in liganded HisJ. All of the experiments in this section were performed with unliganded HisJ.
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Fig. 3. pH activity profile and dependence on Mg^{2+} concentration. Initial transport rates were normalized against the highest value. HisJ was unliganded. Vesicles were harvested after defrosting and resuspended, without washing, in the indicated buffer (A), or PIPES/Tris at the indicated MgSO_4 concentration (B). The sample with no MgSO_4 contained 5 mM EDTA and generated half as much ATP as any of the samples with MgSO_4. The amounts of ATP synthesized at 2 min were (nmol/mg protein): 0.75, 0.7, and 0.1 at pH values 5.2, 6.8, and 9.0, respectively. Buffers were used at 50 mM.

Fig. 4. Concentration dependence of histidine transport on HisJ and histidine concentrations. Top, the abscissa indicates the concentration of either the HisJ-histidine complex (curve A), or histidine (curve B), or HisJ (curve C). HisJ was unliganded. A, the concentration of HisJ and histidine were varied simultaneously, maintaining them at an equimolar ratio. B, the HisJ concentration was maintained at 50 μM, while the histidine concentration was varied. C, the histidine concentration was maintained at 40 μM while the HisJ concentration was varied. Bottom, Lineweaver-Burk plot of the data from curve A.

Conversely, increasing HisJ while maintaining a constant histidine concentration (40 μM) results in a maximum level of transport at about 40 μM HisJ, followed by an inhibition (curve C). Since the HisJ concentration equals and surpasses that of histidine, both the free histidine concentration drops and the free HisJ concentration increases. If the inhibition were due to a drop in the free histidine concentration, the decreasing rates should reflect the apparent K_m for transport of free histidine. From the calculated free histidine concentration, the affinity of the system for free histidine would be approximately 5 x 10^{-3} M. This affinity is about 10-fold worse than the apparent K_m value for histidine transport obtained in vivo (0.6 x 10^{-3} M, Kustu and Ames, 1974), rendering it unlikely that the dropping histidine concentration is responsible for the lowered transport rates. In support of this conclusion, curve B shows that transport is not affected by the free histidine concentration. The inhibition is most likely due to competition by excess unliganded HisJ and will be discussed later. On the whole, the results of Fig. 4 indicate that the HisJ-histidine complex is the actual substrate for transport. However, it should be remembered that the effect of
changing the free histidine concentration cannot be studied accurately in this system because the concentration cannot be adjusted with precision around the very low values of the known apparent K_m for transport, in vivo.

The Plateau Level Is Due to an Equilibrium between Entry and Exit—Fig. 5 shows the time-dependent uptake of histidine at equimolar HisJ/histidine concentrations of 40 and 100 μM. As expected, the initial rate of transport at 100 μM (2.4 nmol/min/mg) is approximately 2-fold the initial rate of transport at 40 μM (1.0 nmol/min/mg). Similarly, the equilibrium level at 100 μM is about 2-fold that at 40 μM. The plateau levels could be due to either a lack of further uptake or to the attainment of a steady state in which the rate of histidine efflux from the vesicles equals its rate of uptake. The latter is demonstrated to be the case by the following experiment. Transport is initiated with HisJ plus unlabeled histidine and allowed to reach equilibrium; if a trace quantity of labeled histidine is then added, label is taken up at the same rate that occurring when transport is initiated at time 0 with labeled histidine. Therefore, since no net uptake is occurring, despite the fact that histidine is still entering the vesicles, histidine must exit at the same rate as it enters. Additionally, decreasing the specific activity of the external histidine by the addition of a large excess of unlabeled histidine at equilibrium results in an efflux of labeled histidine from the vesicles at rates similar to the initial rates of uptake at 100 and 40 μM HisJ/histidine (2.6 and 1.1 nmol/min/mg, respectively) and proportional to the intravesicular histidine concentration, supporting the contention that the histidine present in the vesicles at equilibrium is exiting.

Energetics of Histidine Transport in Membrane Vesicles—Since it has been postulated that periplasmic permeases derive their energy from substrate level phosphorylation, and since membrane vesicles are essentially free of cytoplasmic components, and thus presumably unable to synthesize ATP, it was puzzling to find that ascrobate/PMS energizes histidine transport. Ascorbate/PMS is known to energize proline transport in membrane vesicles (Konings et al., 1971) by generating pmf (Ramos et al., 1976). We show that ascorbate/PMS energizes proline transport in our system as well. Table I shows that with energization by ascorbate/PMS, addition of CCCP, a protonophore, completely abolishes histidine transport, thus suggesting a role for pmf as an energy donor for histidine transport in reconstituted membrane vesicles. Oxygen is not limiting for transport, since oxygenation of the reaction did not increase the rate of transport, or prolong histidine uptake (data not shown). Other energy sources, such as glucose and lactate, are only 10% as effective as ascorbate/PMS.

However, the ability of ascorbate/PMS to energize histidine transport remains to be explained at the molecular level. In particular, the possibility exists that some F_0F_1 ATPase complexes remain associated with the vesicles and convert residual ADP into ATP utilizing the pmf generated from ascorbate/PMS (Tsuchiya, 1976, 1977). This possibility was tested by preparing vesicles from an isogenic strain lacking all components of the F_0F_1 ATPase. These vesicles, given ascorbate/PMS as an energy source, are able to transport proline, but incapable of histidine transport (Fig. 6, A and B) suggesting that, in this system, histidine transport is energized by ATP generated from the pmf.

We confirmed that ATP is indeed synthesized in vesicles from the unc^+ strain upon energization with ascorbate/PMS (Fig. 6C). Unenergized vesicles contain a basal level of ATP (about 0.5 nmol of ATP/mg of protein) that cannot be eliminated by either varying the volume of the spheroplast-shocking medium (between 100 and 1000 ml) or by repeated washing of the final vesicle preparation (data not shown). This ATP, however, is not accessible for energizing transport, since neither proline nor histidine are transported in the absence of an added energy source (Fig. 6, A and B). Since the ATP level in whole cells is 6.5 nmol/mg of protein (in agreement with other published values; Bochner and Ames, 1982), and since membrane protein amounts to about 16% of the total (Osborn et al., 1972), the ATP content expressed in terms of membrane protein is 41 nmol/mg. The residual level of ATP in unenergized vesicles (0.65 nmol/mg of membrane protein) is about 1.6% of that present in whole cells, in agreement with the extent of vesicle contamination by cytoplasmic components.

Upon energization with ascrobate/PMS, unc^- vesicles synthesize ATP (total amount: about 1.5 nmol ATP/mg membrane protein), while unc^- vesicles, as expected, do not synthesize any ATP. The amount of ATP synthesized in unc^- vesicles corresponds to 3.7% of the ATP in whole cells expressed/mg of membrane protein. Concomitant measurements of transport show that in the presence of ascorbate/PMS unc^- vesicles transport both histidine and proline, but unc^- vesicles are unable to transport histidine, even though Table I

| Treatment | Initial transport rate (% of control) | ATP (% of control) |
|-----------|--------------------------------------|-------------------|
| Standard* | 100                                  | 100               |
| + 5 μM CCCP | <2                                    | <2                |
| + Oxygenation of transport reaction | 100 | 97 |
| + 20 mM glucose (instead of ascorbate/PMS) | 12 | 9 |
| + 5 mM D-lactate (instead of ascorbate/PMS) | 10 | 9 |

*Transport assays were carried out as described under “Experimental Procedures” using unliganded HisJ. Control transport rates (100%) is 1.5 nmol of histidine/min/mg protein. ATP synthesis was measured as in Fig. 6C and the 2 min ATP levels, corrected for the ATP level in unenergized vesicles, are expressed in % of control; 100% is 1.8 nmol/mg protein.

![Fig. 5. Accumulation and exit of histidine. Histidine uptake was measured as described under “Experimental Procedures,” using a total assay volume of 1 ml. Equimolar HisJ (unliganded) and histidine concentrations were: 40 (○, ○) and 100 μM (▲, ▲). At 16 min, either water (●, ●) or histidine (8 mM final concentration; ○, ○) was added. Transport was initiated at time 0 with 40 μM unlabeled histidine, and at 15 min 4 μCi of [3H]histidine (0.2 μM final) were added (+).]
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The ability to vary both the HisJ and histidine concentrations has allowed the measurement of the affinity of each, and of liganded HisJ (as calculated from a K<sub>v</sub> for histidine of 10<sup>-7</sup> M), for the membrane complex. The results in Fig. 4 indicate that liganded HisJ, rather than free histidine, is the effective substrate in the transport reaction by the membrane complex, with an apparent K<sub>a</sub> for transport of about 65 μM. This result must be reconciled with the in vivo situation, where the apparent K<sub>a</sub> for histidine transport is 6 nM (Kustu and Ames, 1974; Ames and Lever, 1970). This apparent discrepancy is resolved when we calculate that at an external histidine concentration of 6 μM (the K<sub>a</sub> value), the concent-

their capacity to transport proline is unchanged (Fig. 6, A and B).

If ATP generation is required for histidine transport, altering the ATP levels within vesicles should affect the rate of transport. The following two approaches were taken to achieve this: 1) lysis of spheroplasts in the presence of apyrase, which specifically degrades ATP to AMP, thus lowering the level of ATP generated by the F<sub>0</sub>F<sub>1</sub>-ATPase, and 2) lysis of spheroplasts in the presence of 5 mM ATP, increasing the ATP levels within vesicles should affect the rate of transport.

In this report we describe the properties of the periplasmic histidine permease as reconstituted in right-side-out membrane vesicles. Our results clearly show that the in vitro reconstituted permease faithfully reflects the activity of the permease in vivo, as shown by the following facts. (i) Histidine is concentrated intravesicularly, since accumulation is sensitive to vesicle volume. The possibility that the vesicle-associated histidine is bound to HisJ, which is unspecifically bound to the surface of the vesicles, is also excluded by the fact that no detectable amount of HisJ remains bound to the vesicles when they are harvested after reconstitution (data not shown). (ii) Histidine transport is entirely dependent on the presence of all components of the histidine permease, both membrane-bound and periplasmic. (iii) Transport is dependent on the addition of an energy source. (iv) A mutant protein, HisJ<sub>5625</sub>, that binds histidine but does not transport it, does not support transport in vitro either. (v) The concentration of liganded HisJ needed to give maximal transport is about 0.4 μM, which is comparable to the calculated periplasmic HisJ concentration in vivo (0.5 μM).

Vanadate is known to inhibit several enzymes involved in phosphotransferase or phosphohydrolase reactions, and in the case of the (Na,K)-ATPase, it is thought to inhibit by binding to the P<sub>i</sub>-binding site (Cantley, 1981). As an indication that hydrolysis of ATP is necessary for its energization function, we tested whether reconstituted histidine transport in membrane vesicles is sensitive to vanadate. The presence of 0.5 mM sodium orthovanadate in the standard lysis buffer inhibited histidine transport by 70%, but had no effect on either proline transport or ATP levels, as expected.

It has been suggested that the enzymes acetate kinase and phosphotransacetylase (encoded by ack and pta, respectively) are required to energize binding protein-dependent transport in membrane vesicles (Hong et al., 1979). In the case of the histidine permease we have found no such requirement. Vesicles from TA3964 (a deletion mutant of ack and pta) were found to transport histidine at rates identical to vesicles from TA4211 (wild type with respect to ack and pta) under the standard conditions of assay (data not shown).

**TABLE II**

| Lysis buffer<sup>a</sup> | Initial rates of transport<sup>b</sup> | ATP |
|--------------------------|-----------------------------------|-----|
|                          | Histidine Proline at 15 s | at 2 min | % Control |
| None (control)           | 100 | 100 | 100 | 100 |
| 5 mM ATP                 | 170 | 108 | 256 | 267 |
| Apyrase (2 units/ml)     | 58  | 122 | 46  | 50  |
| 50 mM HEPES, pH 7.0 (replacing KP<sub>i</sub>) | 20  | 57  | 49  | 38  |
| 0.5 mM Na<sub>2</sub>VO<sub>4</sub> | 33  | 96  | 92  | 91  |

<sup>a</sup>Vesicles were prepared from TA3964; liganded HisJ was used.
<sup>b</sup>Spheroplasts were divided into equal parts and lysed in 50 mM KPi, pH 7.0 (reconstituted permease faithfully reflects the activity of the permease in vivo).

In a 2-fold increase. Neither of these treatments had a significant effect on proline transport, indicating that pmf generation from ascorbate/PMS was unaffected and that vesicle integrity was preserved. Furthermore, since ATP generation is a phosphate-requiring process, lysis of spheroplasts in a phosphate-free buffer should severely impede this process and hence histidine transport. Lysis in 50 mM HEPES buffer shows that indeed both ATP levels and histidine transport are inhibited; proline transport is also inhibited, although to a lesser degree. Confirming the correlation between transport and ATP levels is the inhibition of both by CCCP (Table I) and by EDTA (Fig. 3B). As expected, proline transport was also inhibited by CCCP (data not shown).
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toration of liganded HisJ in the periplasm is approximately 57 μM, using a value for the periplasmic HisJ concentration of 1.0 mM. Thus, if the measured $K_a$ value in vivo is translated in terms of liganded HisJ, it is compatible with the value measured in the reconstituted system. Additional results support our contention that liganded HisJ is the real substrate. (i) Histidine is not transported in the absence of HisJ (Fig. 1). (ii) If the concentration of histidine is in excess over that of HisJ, transport does not reflect the increasing histidine concentration past the point of saturation for HisJ (Fig. 4B). Thus, free histidine does not seem to have direct access to the membrane components. (iii) Under conditions where either HisJ or histidine are limiting, transport is directly dependent on the concentration of the limiting component (Fig. 4, B and C), and (iv) it reaches a maximum when the two components become equimolar, indicating the one to one stoichiometry (Lever, 1972b). The high value determined for the affinity of liganded HisJ for the membrane complex (65 μM) is comparable to a value calculated from in vivo measurements for the maltose-binding protein in its function in both maltose periplasmic transport and chemotaxis (~90 and ~250 μM respectively; Manson et al., 1985).

With the understanding that liganded HisJ is the transport substrate, we can speculate on the factors responsible for the high affinity displayed by periplasmic transport systems in vivo. Using the histidine permease as an example, it is apparent that if the periplasmic HisJ concentration were very low relative to that of the membrane complex, transport rates would reflect the limiting levels of liganded HisJ, as dictated by the binding affinity of HisJ for histidine. In this case, the overall $K_a$ for transport would approach the value of the $K_d$ of HisJ for binding to histidine. On the other hand, at higher periplasmic HisJ concentrations, the fixed level of the membrane components becomes the limiting factor and dictates the highest achievable transport rate. Under these conditions, the $K_a$ with respect to histidine will also depend on the periplasmic HisJ concentration because increasing amounts of periplasmic HisJ would result in increasing amounts of liganded HisJ, and therefore, in higher transport rates at any specific histidine concentration. The combined effect of a constant $V_{max}$ together with varying transport rates at a specific external histidine concentration is that of lowering the apparent $K_a$, as the HisJ concentration increases. That the overall transport affinity is indeed affected by the concentration of the periplasmic component has been seen in the case of the histidine permease, where increasing the HisJ concentration through a promoter-up mutation results in a lowering of the apparent $K_a$ from 26 to 6 nM with a very small change in $V_{max}$ (Ames and Lever, 1970). Conversely, increasing the level of the membrane components keeping HisJ constant, results in an increase in the apparent $K_a$ (a 30-fold increase in the amount of membrane proteins resulting in a 10-fold increase in the $K_a$). Since periplasmic permeases have usually much higher levels of the periplasmic binding protein than of the membrane components, it is reasonable to speculate that this fact may be responsible for the generally high apparent affinities for the substrate displayed by these systems (Ames, 1986).

An analysis of the effects of varying the concentration of the periplasmic protein was performed in whole cells for the maltose permease (Manson et al., 1985). In agreement with our calculations and results, the apparent $K_a$ for maltose was shown to increase with decreasing maltose-binding protein concentration. However, the authors chose to interpret the relatively small change in the $K_a$ (from 0.77 to 1.3 μM) as not significant. We feel that, since the variation in the concentration of the binding protein was not extensive and since the $K_d$ value for binding (3 μM) is not vastly different from that of the liganded binding protein for the membrane complex (calculated to be 90 μM), it is reasonable to expect that the change would be small.

It is known that periplasmic binding proteins, including HisJ, undergo a conformational change upon binding of substrate (Ames, 1986). It has therefore been postulated, and generally assumed, that only the liganded binding protein is capable of interacting with the membrane complex. If this were true, it would be expected that excess unliganded HisJ should have no effect on transport by a fixed amount of liganded HisJ, much as excess histidine has no effect on transport rates. However, excess unliganded HisJ strongly inhibits transport (Fig. 4C), suggesting that unliganded HisJ competes with liganded HisJ for a binding site on the membrane complex. This is in agreement with cross-linking experiments that show that unliganded HisJ can indeed interact with the membrane complex, although less well than liganded HisJ. In addition, preliminary experiments indicated that the unliganded lysine-arginine-histidine-binding protein, which interacts with the membrane-bound complex, but binds histidine very poorly (Higgins and Ames, 1981), inhibits histidine transport by HisJ in reconstituted vesicles.

It is important to determine the efficiency of the reconstituted system as compared with that of whole cells. The $V_{max}$ for transport in vitro is 6 nmol/min/mg membrane protein. The $V_{max}$ for transport in *S. typhimurium* whole cells (in a promoter-up mutant, *dhua1*) is 3.1 nmol/min/mg membrane protein (i.e. 0.5 nmol/min/mg of cell protein [Kustu and Ames, 1974; Ames and Lever, 1970]) and thus, comparable to transport in vesicles. Since vesicles are prepared from strains with overproduced levels of the membrane transport proteins (due to the lambda Pr promoter control), the activity of the reconstituted system should be compared with that of such strains. However, such comparison cannot be done because the same strain cannot be used for assaying transport in *vivo* and in vesicles derived from it: vesicles are prepared from a hisJ mutant which therefore does not transport. A *dhua1* strain carrying pFAS3 has a $V_{max}$ about 5- to 9-fold higher after induction of the plasmid-encoded membrane proteins (about 15-27 nmol/min/mg of membrane protein; data not shown). Using this value, transport in the reconstituted system is calculated to be between 40 and 22% as efficient as in intact cells. It should be noted that in strains overproducing the membrane complex, the increase in transport is not proportional to the increasing amounts of membrane transport proteins, possibly because not all the overproduced protein is functional and/or HisJ becomes limiting (as discussed earlier).

Among the parameters which were explored for optimizing transport, the effect of pH should also be reconciled to the *in vivo* situation. Transport in whole cells is highest at an external pH around 5.5 and decreases to about 20% at pH close to 8. The low external pH optimum for transport *in vivo* (Joshi et al., 1988) reflects the known low pH optimum for histidine binding to HisJ (Lever, 1972b), since the periplasmic pH is presumably the same as the pH of the suspension medium. On the other hand, because the cytoplasmic pH is maintained at about 7.6 despite drastic changes in the external pH, the internal pH has to be modified artificially: its pH optimum for histidine transport is about 7.0 and transport is essentially nonexistent at pH 5.5. This internal pH profile is similar to that of vesicles (Fig. 3A). In contrast to whole cells,

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1 B. Davis, E. Prossnitz, and G. F.-L. Ames, unpublished data.
2 D. Speiser and G. F.-L. Ames, manuscript in preparation.
the internal pH of vesicles presumably equilibrates with the external pH. Thus, the pH profile for vesicle transport may reflect the same pH-sensitive parameters as those affected by the internal pH in whole cells.

An important use of this reconstituted system is the examination of the mode of energy coupling in periplasmic permeases, which has been a subject of controversy for many years. Since the highest histidine transport rates are obtained with ascorbate/PMS as an energy source, it might be concluded that pmf provides the energy for histidine transport. However, measurements of ATP in vesicles demonstrates that residual ATP is present and that upon energization with ascorbate/PMS higher levels of ATP are generated. Presumably, the newly synthesized ATP is formed from residual ADP in the vesicles and P, present in the lysis buffer. The presence of residual ATP in vesicles prepared by osmotic lysis upon energization with an artificially generated pmf has been previously reported. However, no significant generation of ATP was observed unless spheroplast lysis was carried out in a solution containing ADP (Tsutsui and Rosen, 1976a, 1976b).

Because energization of vesicles with ascorbate/PMS results in both the generation of a pmf, as demonstrated by the ability to transport proline, and ATP, measured directly, an unisogenic strain (which cannot convert pmf into ATP), was used to distinguish between these two energy sources for histidine transport. The unc vesicles are unable to transport histidine when energized with ascorbate/PMS, while their proline transport is normal, clearly demonstrating that pmf alone is insufficient to support histidine transport and that generation of ATP (or of another cogenerated molecule) is required. This conclusion is supported by experiments with unc- whole cells, that demonstrate histidine transport to be unaffected by the dissipation of the membrane potential with CCP (Joshi et al., 1989). Dissipation of pmf by CCP in vesicles of course results in loss of histidine transport because ATP synthesis is eliminated (Table 1).

The following correlations between the level of ATP generated and the rate of transport supports the contention that ATP is the immediate energy source. (i) Apyrase inclusion lowers both the level of ATP and transport activity. (ii) ATP inclusion raises both parameters. (iii) Removal of Mg++ by EDTA lowers both parameters, presumably because Mg++ is required for ATP synthesis. (v) Lysis in phosphate-free buffer (HEPES) has the same effect, presumably because of the P requirement for ATP synthesis. (vi) The use of D-lactate or glucose as energy source, results in low ATP and transport levels. (vi) Exposure to high pH lowers both the level of ATP and transport activity. (ii) ATP is the immediate energy source. This conclusion is supported by experiments with unc- whole cells, that demonstrate histidine transport to be unaffected by the dissipation of the membrane potential with CCP (Joshi et al., 1989). Dissipation of pmf by CCP in vesicles of course results in loss of histidine transport because ATP synthesis is eliminated (Table 1).

The direct involvement of ATP in the energization of histidine transport in membrane vesicles is further indicated by the inhibition of histidine uptake by the presence of sodium orthovanadate during vesicle formation. Vanadate is thought to inhibit the hydrolytic activity of the (Na,K)-ATPase by occupying the dephosphorylation site (Cantley, 1981). Its inhibition of histidine transport supports the contention that ATP hydrolysis is necessary for energization, possibly through a mechanism similar to that of the (Na,K)-ATPase. Since the level of ATP is unaffected by the presence of vanadate, the F,F-ATPase must be insensitive to this compound.

The ATP concentration generated by ascorbate/PMS is 0.75 mM, calculated using 2 µl/mg membrane protein as the internal volume of membrane vesicles (Kaback and Barnes, 1971). This concentration is of the same order of magnitude as that of whole cells (3 mM, Bochner and Ames, 1972). Such high concentration can presumably be achieved because of the much smaller internal vesicle volume as compared with whole cells (20 µl/mg of membrane protein, as calculated from a value of 1.5 µl/mg dry weight (Ahmed and Booth, 1983), assuming the membrane protein to be 16% of the total protein). Thus, it is not surprising that the much lower ATP synthetic ability of these vesicles is sufficient to power transport. Assuming that 1 molecule of ATP is hydrolyzed for each molecule of histidine transported, a transport rate of 1 nmol of histidine/min/mg of membrane protein should result in a measurable decrease in the pool of accessible ATP, which is about 1 nmol/mg. No such decrease was observed, indicating that the ATP must be rapidly replaced (data not shown). It is unclear, however, how this can occur, since the initial rate of synthesis of ATP is of a magnitude comparable to the rate of transport (Fig. 6). Clearly, because of the extensive synthetic capabilities of the vesicles, it is not possible to define unambiguously ATP as the proximal energy source. More refined in vitro systems, such as proteoliposomes formed by purified transport components, are needed to provide a final answer to this problem.

The working models for periplasmic transport systems have usually indicated that the interaction between the liganded HisJ and the membrane-bound complex triggers the release of the substrate and its passage through the membrane, either by way of a pore or of membrane-binding sites, using the energy of ATP (Ames, 1986, 1989). The unliganded HisJ has usually been assumed not to interact with the membrane. The present work lends support to the model, insofar as it confirms that ATP is the energy source, that liganded HisJ is the active substrate, and that the free substrate cannot enter directly through the membrane complex. However, we also show that unliganded HisJ must also bind to the membrane complex since it competes with liganded HisJ. It is not known whether bound, unliganded HisJ is capable of binding substrate (and transporting it) and what its affinity would be. It is also not yet known whether a pore or membrane-binding site is responsible for the actual membrane-crossing step of transport. The reconstituted membrane vesicles system is a very useful tool that can be used to further the understanding of many of these unresolved problems in the molecular mechanism of periplasmic transport. In particular, it constitutes an important step to a more complete understanding of the signaling events that take place across the membrane, since it gives access to experimentation at both periplasmic and cytoplasmic faces of the inner membrane.

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