Purification and Characterization of HisP, the ATP-binding Subunit of a Traffic ATPase (ABC Transporter), the Histidine Permease of Salmonella typhimurium

SOLUBILITY, DIMERIZATION, AND ATPase ACTIVITY*

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The superfamily of traffic ATPases (or ABC transporters) comprises both prokaryotic and eukaryotic transport proteins that share a conserved nucleotide-binding domain (1, 2). The superfamily includes, among others, bacterial periplasmic permeases, the yeast STE6 gene product, the mammalian P-glycoprotein (multidrug resistance protein or MDR), the human cystic fibrosis transmembrane conductance regulator (CFTR), and the mammalian heterodimeric transporter (TAP1/TAP2) involved in antigen processing (3, 4). The histidine permease of Salmonella typhimurium and the maltose permease of Escherichia coli have been extensively characterized; both are good model systems for understanding the mechanism of action of this superfamily (5–8).

The histidine permease is composed of a soluble substrate-binding receptor, HisJ (the histidine-binding protein), and a membrane-bound complex, HisQMP2, comprising two integral membrane proteins, HisQ and HisM, and two copies of HisP that carries the ATP-binding motif (9–11). ATP hydrolysis provides the energy for the transport process (12). Our general approach toward understanding the mechanism of action of these transporters has been to purify and characterize biochemically (a) the intact complex, (b) the isolated nucleotide-binding subunit, HisP, and (c) a reassembled system in which the isolated nucleotide-binding subunit has been reconstituted into membrane vesicles and proteoliposomes containing only HisQ and HisM. Such an analysis will delineate the properties intrinsic to the individual subunits, relate them to those of the intact complex, and, in combination with the creation and analysis of mutant proteins, it will provide information of general use for understanding the mechanism of action of the traffic ATPases superfamily.

In this article we present the purification and properties of the ATP-binding subunit of the histidine permease, HisP. HisP binds and hydrolyzes ATP and, presumably, is the subunit responsible for energizing the transport process. We show that HisP functions as a dimer and that under optimal conditions the rate at which it hydrolyzes ATP is comparable to that of the intact complex, HisQMP2. The properties of HisP are compared with those of the intact complex and of related subunits from other traffic ATPases.

EXPERIMENTAL PROCEDURES

Production and Purification of HisP(his6)—The construction of pFA351, a plasmid carrying the S. typhimurium hisp(his6) gene under the control of an IPTG-inducible T7 polymerase gene (13), is described in the text. GA596, the E. coli strain containing plasmid pFA351 (freshly introduced by transformation) was grown at 30 °C until the culture reached an A_{600} value of 0.6. The slower growth at this temperature allows a higher percentage of HisP to remain in the soluble fraction. IPTG was added to a final concentration of 100 μM and growth continued for 2 h. Cells were harvested by centrifugation and stored at −80 °C as a paste. In a typical preparation, 8 g of cells harvested from 4 liters of culture were defrosted, washed with 40 ml of 50 mM TrisCl, pH 8.1, 1 mM MgCl_2, resuspended in 40 ml of 50 mM TrisCl, pH 8.1, and disrupted twice at 10,000 p.s.i. in a French press apparatus. After centrifugation for 10 min at 1,300 _g, ATP was added to a final concentration of 5 mM to the supernatant fraction (crude extract), which was then centrifuged in a TLA 100.4 rotor at 100,000 rpm for 30 min in a Beckman ultracentrifuge (the supernatant is the cytoplasmic fraction). GA596, the E. coli strain containing plasmid pFA351 (freshly introduced by transformation) was grown at 30 °C until the culture reached an A_{600} value of 0.6. The slower growth at this temperature allows a higher percentage of HisP to remain in the soluble fraction. IPTG was added to a final concentration of 100 μM and growth continued for 2 h. Cells were harvested by centrifugation and stored at −80 °C as a paste. In a typical preparation, 8 g of cells harvested from 4 liters of culture were defrosted, washed with 40 ml of 50 mM TrisCl, pH 8.1, 1 mM MgCl_2, resuspended in 40 ml of 50 mM TrisCl, pH 8.1, and disrupted twice at 10,000 p.s.i. in a French press apparatus. After centrifugation for 10 min at 1,300 _g, ATP was added to a final concentration of 5 mM to the supernatant fraction (crude extract), which was then centrifuged in a TLA 100.4 rotor at 100,000 rpm for 30 min in a Beckman ultracentrifuge (the supernatant is the cytoplasmic fraction). The cytoplasm-
mic fraction (35 ml) was applied to a column (Bio-Rad glass Econo column; 2.5 × 10 cm) prepared from 10 ml of a slurry of TALON metal affinity resin (CLONTECH) equilibrated with 50 mM Tris/Cl, pH 8.1, 1 mM MgCl₂, and gently shaken at 4 °C for 45 min. The column was allowed to run (flow-through fraction) and washed with 40 ml of cold buffer A (50 mM Tris/Cl, pH 8.1, 100 mM NaCl, 5 mM ATP, 20% glycerol) and then with 20 ml of cold buffer A containing 6 mM imidazole. The resin was then transferred to a narrower column (1.5 × 13 cm) and eluted with 10 ml of buffer A containing 100 mM imidazole and 0.1 mM EDTA. Ten fractions (1.0 ml each) were collected and analyzed for protein content by the micro-BCA protein assay (14) (Ferch kit with bovine serum albumin as standard). Over 90% of the HisP retained was eluted within one or two fractions. If necessary, fractions were concentrated using an Amicon Centricron 10 centrifugal concentrator. Pure protein was aliquoted and stored in liquid nitrogen. A typical experiment yields 8–10 mg of pure HisP₆₅₀₀. Whenever it was necessary to change the buffer, gel filtration through a PD-10 column (Pharmacia Biotech Inc.) equilibrated with the desired buffer was used.

**Determination of Dimer Form of HisP₆₅₀₀—Pure HisP₆₅₀₀ (2.1 mg/ml; 200 μl) was injected onto an HPLC column (TOSOHASG G3000SW, 7.8 mm × 30 cm) equilibrated with 50 mM MOPS, pH 7.4, 4% glycerol, 100 mM NaCl, with a flow rate of 1 ml/min; fractions (~0.5 ml each) were collected and the protein content analyzed by the BCA assay and by SDS-PAGE.** Carbonic anhydrase and bovine serum albumin (molecular masses, 29 and 66 kDa, Sigma) were used as molecular weight standards.

**ATPase Assay—ATPase activity was assayed essentially as described** (7) with the following modifications to miniaturize it. Sixty μl of a HisP₆₅₀₀ solution (final concentration: 0.7 mg/ml, 24 mM HisP₆₅₀₀) in assay buffer (60 mM MOPS, pH 7.0, 4% glycerol, 2 mM ATP, 0.1 mM EDTA) was equilibrated at 37 °C for 3 min, and the reaction was initiated by the addition of 2 μl of 30 mg/ml MgCl₂ (final concentration: 1 mM). Samples (10 μl) were taken at appropriate times (every 20 s at high enzyme concentrations) and placed into microtiter plate wells containing 40 μl of 7.5% SDS. The amount of Pᵢ liberated was determined by a colorimetric assay (15), using Na₂HPO₄ as a standard.

**CD Measurements—** HisP₆₅₀₀ (150 μl, 6.8 mg/ml) was first applied to a Sephadex G-50 column (0.5 × 3.2 cm) equilibrated with 10 mM NaPi, pH 7.0, 4% glycerol, 200 mM NaCl, and gently shaken at 4 °C for 45 min. The column was washed with the same buffer; 0.2-ml fractions were collected, and all of HisP₆₅₀₀ appeared in two fractions, the first of which was used for the CD measurements, after dilution in the same buffer to a final concentration of 0.8 mg/ml. HisP₆₅₀₀ prepared under these conditions stays in solution for several hours. The CD spectrum was determined at 25 °C using an AVIV 62DS spectropolarimeter, using a 0.01-cm path length cuvette. The final spectrum, representing an average of six scans, was corrected for the corresponding buffer and measured. Estimation of percentage of secondary structure was performed by unrestricted least squares fitting of the experimental spectrum to the sum of pure-component CD spectra characteristic of α-helical, β-sheet, turn, and non-regular structural elements (the LINCOMB algorithm (16–20)).

**Miscellaneous Assays—** SDS-PAGE and immunoblotting were performed as described (21); using polyclonal antibody raised against HisP, bovine serum albumin as standard. Over 90% of the HisP retained was eluted within one or two fractions. If necessary, fractions were concentrated using an Amicon Centricron 10 centrifugal concentrator. Pure protein was aliquoted and stored in liquid nitrogen. A typical experiment yields 8–10 mg of pure HisP₆₅₀₀. Whenever it was necessary to change the buffer, gel filtration through a PD-10 column (Pharmacia Biotech Inc.) equilibrated with the desired buffer was used.

**RESULTS**

**Construction of a Plasmid Producing HisP₆₅₀₀—** To study the properties of HisP it is most convenient to have it in a soluble form. It had been established previously that a sizable portion of HisP overproduced in the absence of HisQ and HisM is located in the cytoplasmic fraction (9). Among several possible promoter systems, those producing large amounts of protein (such as the combined lambda P₁/P₂ promoter) were discarded to avoid the risk of creating insoluble forms of HisP. Although inclusion bodies protein can be solubilized, e.g. by dissolving them in urea, and renatured (22), a more gentle purification process was deemed more appropriate for future crystallization attempts. Reliably moderate amounts of soluble protein can be produced using the chosen T7 promoter system (23) by controlling the induction time. A peptide extension containing leucine, glutamate, and six histidines (His₆₅₀₀) tail was engineered into the carboxyl terminus of HisP. Plasmid pFA351 was constructed by introducing a hisP₆₅₀₀-containing DNA fragment obtained by polymerase chain reaction from

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2 Plasmid pFA284 carries the hisQ, hisM, and hisP₆₅₀₀ genes under the temperature-sensitive control of the lambda P₁ promoter (strain GA506; laboratory strain collection). 2 To exclude the possibility that this material did not stick to the column because of an overloading effect, it was reapplied to a new TALON column. None of it was retained. Therefore, it is assumed that this material has undergone some form of denaturation. 2 A small amount of HisP₆₅₀₀ (11%) is retained by the column after elution.
Properties of Soluble HisP, an ATP-binding Permease Subunit

Table I

| Fraction                  | Total protein\(^a\) | HisP\(_{\text{His6}}\)\(^b\) |
|--------------------------|----------------------|-----------------------------|
| Crude extract            | 741 \(99\%\)        | 22 (3)                      |
| Cytoplasmic fraction     | 574                  | 17.2 (3)                    |
| Flow-through              | 518                  | 3.4 (0.6)                   |
| Wash I                   | 34.2                 | 1.5 (4.4)                   |
| Wash II                  | 3.2                  | ND\(^d\)                    |
| Imidazole eluate         | 11.2                 | 10.6 (96)\(^e\)            |

\(^a\) Determined using the BCA assay.
\(^b\) Quantitated from an immunoblot using antibody raised against HisP and normalized against a standard curve of pure HisP\(_{\text{His6}}\), the concentration of which had been determined by the BCA assay. In parentheses, % of total protein.
\(^c\) ND, not determined.
\(^d\) As determined from a Coomassie Blue-stained gel.

solubility. Therefore, EDTA (0.1 mM) is also added to eliminate a slow, continuous hydrolysis of ATP during storage.\(^5\)

The activity and solubility of HisP\(_{\text{His6}}\) at 5–10 mg/ml in storage buffer and in liquid nitrogen do not change for at least several months and can withstand repeated freezing and thawing.

ATPase Activity of HisP\(_{\text{His6}}\)—HisP is known to bind ATP (and other nucleotides), and it has been shown that the intact complex, HisQMP\(_2\), hydrolyzes ATP under a variety of conditions (7), and it has an intrinsic, low level ATPase activity that is stimulated somewhat by unliganded HisJ and to a high level by liganded HisJ. It was proposed that HisQ and/or HisM normally constrain the ability of HisP to hydrolyze ATP and that signaling by liganded HisJ allows hydrolysis to proceed by relieving such a constraint (7). If this were true, isolated HisP should be able to hydrolyze ATP at a level considerably higher that the intrinsic activity of HisQMP\(_2\) in the absence of external stimuli. Fig. 3A (solid dots) shows that purified HisP\(_{\text{His6}}\) indeed hydrolyzes ATP\(^6\) at a high linear rate in the presence of Mg\(^{2+}\). The activity is dependent on the presence of a cation, and the efficiency of stimulation by various cations varies with their concentration (Fig. 3B); Mn\(^{2+}\) is the best stimulator at higher concentrations (above 1.5 mM) and Co\(^{2+}\) is the best at lower concentrations (optimum at 1 mM, followed by an inhibition).\(^7\) Ca\(^{2+}\) has a very poor stimulatory ability and Zn\(^{2+}\) has none (data not shown). Fig. 3C shows that the pH optimum for activity is around 7, with a slow decline at higher pH values. Although HisP\(_{\text{His6}}\) is dependent on the presence of 20% glycerol to stay in solution, the presence of glycerol above 7.5% during the assay inhibits the activity (Fig. 3D), possibly by disruption of hydrophobic interactions. This inhibition is reversible, as shown by the fact that even though the protein is eluted from the TALON column in the presence of 20% glycerol, it is fully active upon dilution to glycerol concentrations lower than 7.5%. The activity of a preparation diluted to 0.7 mg/ml protein and 4% glycerol is stable at 0 °C for at least 1 h.

The Active Form of HisP\(_{\text{His6}}\) Is a Dimer—The ATPase activity was observed to be non-linearly dependent on protein concentration (Fig. 4, circles), suggesting that the active form of the enzyme is a multimer. The specific activity, as calculated along the concentration curve in Fig. 4, indicates that the activity of the monomer, if any, would be extremely low. Be-

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\(^5\) However, exhaustive removal of Mg\(^{2+}\) by the addition of EDTA in several column washing steps before elution with 100 mM imidazole results in the disappearance of HisP\(_{\text{His6}}\) from all column fractions (presumably it precipitates within the column matrix).

\(^6\) That this activity is due to HisP\(_{\text{His6}}\) rather than to a contaminating ATPase is demonstrated by the fact that preparations obtained by an identical purification scheme from several hisP mutant strains defective in transport do not display any ATPase activity.

\(^7\) Ca\(^{2+}\) may stimulate the activity by interacting with the (His\(_6\)) tail and inducing a conformational change.

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Fig. 2. Purification of HisP\(_{\text{His6}}\)—SDS-PAGE of fractions obtained at various stages of purification, stained with Coomassie Blue is shown. Molecular mass standard, lane 1 (the molecular masses of the standards used, in kDa, are indicated); crude extract, lane 2; membrane and cytoplasmic fractions derived from the crude extract, lanes 3 and 4; column flow-through, lane 5; wash I, lane 6; imidazole eluate, lane 7. The arrow indicates the position of HisP\(_{\text{His6}}\). Lanes 2–7 contain 10 and 2 µg of protein, respectively. For this and all other experiments were cultured in a 50-liter fermentor and induced for 2 h.
FIG. 3. Properties of the ATPase activity of HisP(his6). A, linearity of activity in time. The assay was performed as described under "Experimental Procedures" (solid circles) or with the omission of MgCl2 (open circles). The concentration of HisP$_{\text{his6}}$ was 200 μg/ml. B, the assay was performed as described under "Experimental Procedures," with the replacement of MgCl2 by the indicated cations at the concentrations indicated on the abscissa. The ordinate indicates specific ATPase activity. C, a HisP$_{\text{his6}}$ solution was diluted 10-fold into buffers at various pH values (indicated on the abscissa) and the ATPase activity measured. The buffers and their respective pH values are as follows: MES/Na, 6.0–6.5; MOPS/Na, 6.7–7.5; Tris/Cl, 8.0 and 8.5; ethanolamine/Cl, 9.5. D, a HisP$_{\text{his6}}$ solution (8.3 mg/ml) containing 20% glycerol was diluted in assay buffer containing glycerol in amounts such that the final concentrations were as indicated on the abscissa; the ATPase activity is on the ordinate.

FIG. 4. Dependence of activity on protein concentration. ATPase assays (circles) were performed as described under "Experimental Procedures," except that the temperature of the assay was 22 °C. The concentration of HisP$_{\text{his6}}$ (abscissa) was varied by diluting a 3.4 mg/ml preparation into assay buffer and maintaining the final glycerol concentration between 4 and 7%. The molarity of HisP$_{\text{his6}}$ was calculated from the measured protein concentration using a molecular mass of 29.8 kDa. A curve fitting the data (solid line) was obtained using the following equations: $V = k[D]$, where $V$ is the rate of ATP hydrolysis, $k$ is its rate constant, and $[D]$ is the molarity of the dimer (Eq. 1)

$$[D] = \frac{K_d + 4[E]}{-K_d^2 + 8K_d[E]}$$

and where $[E]$ is the total molarity of enzyme (in the case of HisP$_{\text{his6}}$, calculated as if it were all monomeric), and $K_d$ is the affinity of single monomers for each other. The curve best fitting the experimental data gives a $K_d$ value of 100 μM and a $k$ of 26 min$^{-1}$. The inset shows the relationship between HisP$_{\text{his6}}$ concentration and the fraction (w/w) of the protein present as a dimer, as calculated from the above formula after converting $[D]$ and $[E]$ to mg/ml and using a $K_d$ of 100 μM.

Because HisP is present in two copies in HisQMP$_2$ and its ATPase activity displays positive cooperativity for ATP with a Hill coefficient of 2 (7), the most likely form of the active enzyme would be expected to be a dimer. The membrane-bound complex of the maltose permease, which contains two copies of the ATP-binding subunit, MalK, also displays positive cooperativity for ATP (26).

The dimeric state of HisP$_{\text{his6}}$ could also be demonstrated by physical methods. A preparation of HisP$_{\text{his6}}$ (1 ml, 1 mg/ml) was applied to a molecular sieve column (0.5 × 75 cm; ACA54, LKB). A large peak of HisP was eluted at the position corresponding approximately to a molecular mass of 29.8 kDa (which is the calculated molecular mass of a single copy of HisP (23) containing the 8-residue extension), and a small peak appeared in the position corresponding to about 55 kDa, indicating that both monomer and dimer forms might be present.

The small amount of the presumed dimer form suggested that the two forms are in rapid equilibrium with each other. Therefore, a more rapid separation was performed using HPLC (Fig. 5A). HisP$_{\text{his6}}$ was present both in the large peak corresponding to the monomer position and in small amount (about 3% total protein) in the position corresponding to about 60 kDa. An additional small peak appeared in the void volume; considering the propensity of HisP$_{\text{his6}}$ to precipitate, it was assumed that this latter peak contains higher aggregates and was not investigated further. A fraction from the presumed dimer peak was rechromatographed on the same column, yielding a peak at the position of the monomer, which is consistent with its being a dimer in equilibrium with the monomer.

Evidence that the equilibrium between the monomer and the dimer is rapid was obtained by diluting a solution of 5.6 mg/ml HisP$_{\text{his6}}$ (which is estimated to contain 60% of the protein as a dimer; see inset in Fig. 4) 8-fold in assay buffer to a final concentration of 0.7 mg/ml (which contains 26% of the protein as dimer); one aliquot was assayed immediately and another aliquot was kept at 0 °C, and the ATPase activity was assayed at several time intervals (up to 1 h). The specific activity did not change in time, indicating that the protein reaches final equilibrium within the earliest time interval tested (5 min at 0 °C). A more rapid procedure was utilized by diluting 30- and 80-fold the same HisP$_{\text{his6}}$ solution into prewarmed assay buffer containing Mg$_2^+$ and immediately (within 5 s) assaying for activity. Fig. 5B shows that the rates do not change significantly in time after dilution and that they correspond to what is expected for the amount of HisP$_{\text{his6}}$ dimer that is present at those final concentrations (10 and 4%, respectively). This indicates that the protein reaches equilibrium within seconds after dilution.

Nucleotide Specificity and Inhibitors—The affinity for ATP, measured following dilution of a concentrated preparation to
final concentrations of HisP(his6) and ATP of 167 µg/ml and 100 µM, respectively, gave a K_d value for ATP of 205 µM (Fig. 6). In contrast to the results obtained with the intact complex, HisQMP2 (7), there is no evidence of cooperativity. Although Fig. 4 showed that the measurable activity is ascribable to the dimeric form and that the monomeric form, if active at all, has a very low specific activity, it is possible that the low level, non-cooperative, (hypothetical) activity of monomeric HisP(his6) contributed slightly to the measurements. Therefore, the K_d value was also measured at high HisP(his6) concentration. The ATPase activity of such a preparation (containing 0.8 mg/ml HisP(his6), which is calculated to contain about 29% dimer) displays no cooperativity for ATP and gives a K_d value of 250 µM, which is not significantly different from the one obtained at low HisP(his6) concentration. Thus, by combining these results with those in Fig. 4 and with those obtained from phospholipid inhibition (see below), it appears likely that cooperativity is not a property of the dimer of HisP(his6).

Table II shows the relative affinities of HisP(his6) for several nucleotides. The results are in agreement with the fact that GTP, CTP, and TTP have all been shown to energize transport (27, 28) and to be bound and hydrolyzed by HisQMP2 (7, 29). ADP is not hydrolyzed and is a strong inhibitor, which is also consistent with previous results obtained with HisQMP2, both for transport and ATPase activity (6, 7, 30). Among the other ATP analogs, AMP-PCP displays poor affinity and TNP-ATP has a very good one.

The ATPase activity of HisQMP2 is inhibited by vanadate, with 6.5 µM vanadate giving 50% inhibition in the presence of 2 mM ATP (7); vanadate also inhibits transport in reconstituted right-side out vesicles (31). However, no inhibition of HisP(his6) was observed up to a concentration of 0.5 mM vanadate (in the presence of 2 mM ATP). Vice versa, although NEM does not inhibit the activity of HisQMP2 (up to 10 mM) (7), 0.05 mM NEM inhibits 50% of the activity of HisP(his6). Similar to its effect on HisQMP2 (7), NaCl inhibits the activity of HisP(his6) (by 50 and 75% at 200 and 600 mM, respectively), indicating that the NaCl effect observed in the complex does not involve the interaction of HisP with HisQ and HisM. The soluble receptor, HisJ, has no effect (up to 180 mM) on the activity of HisP(his6), which is in agreement with the inability to demonstrate a direct interaction between HisJ and HisP by chemical cross-linking. Histidine also has no effect (up to 20 mM), indicating that a histidine-binding site (6) either is not located on HisP or that

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9 This measurement requires the elimination of the ATP obligatorily present in solutions of HisP(his6) at high concentrations. The ATP analog, TNP-ATP, which has a high affinity for the enzyme and is hydrolyzed very slowly, maintains high concentrations of HisP(his6) in solution when present at 10 µM, for 14 days at 4 °C. HisP(his6) was purified as usual and the ATP was replaced with TNP-ATP by passage through a gel filtration column containing 10 µM TNP-ATP. This concentration of TNP-ATP does not significantly interfere with the assay for ATPase activity (Table II).

10 G. F.-L. Ames and K. Nikaido, unpublished data.
histidine binding is unrelated to the ATP hydrolysis mechanism.

Total E. coli phospholipids inhibit the activity of HisP(his6) and the extent varies with the concentration of HisP(his6) (Fig. 7). The Ki values are calculated to be 14, 0.6, and 0.14 mM, respectively. At these concentrations HisP(his6) is calculated to be about 26, 11, and 3% in the dimeric form, respectively. It is possible that the phospholipids associate with the monomeric form of HisP(his6) and prevent it from forming the active dimer. If this were true, the fact that even at the highest level of inhibition there is residual ATPase activity may indicate that monomeric HisP has an intrinsic, very low level of activity (10 nmol/min/mg of protein).

Physical Properties of HisP(his6)—The fluorescence emission spectrum of the single tryptophan residue (Trp-105) present in HisP has a peak at 348 nm (Fig. 8A, top line), indicating that the tryptophan is in a polar environment. The spectrum does not change with the concentration of HisP(his6) (as measured between 0.04 and 1.5 mg/ml), suggesting that the tryptophan residue is insensitive to the dimerization status. The spectrum is also insensitive to ATP concentration (from 0.25 to 1 mM). No dramatic differences are observed between the ADP- and ATP-ligated forms, suggesting that the tryptophan-containing domain does not change with nucleotide binding, and therefore, it is physically removed from conformational changes which might occur in the nucleotide-binding pocket. This finding is in agreement with the proposed tertiary structure of HisP (11).

The CD spectrum of HisP(his6) (Fig. 8B) is typical of a protein containing a substantial content in α-helical structure. Deconvolution of the spectrum using four different sets of pure component CD spectra characteristic of α-helical, β-sheet, turn, and non-regular structural elements yields percentages of α-

11 The molarity of phospholipids was calculated using 700 as an average molecular weight.
transporters) is that they comprise the several domains of action, i.e. the hydrophobic membrane-spanning and the cytoplasmic, membrane-associated nucleotide-binding domains, as separate entities, thus allowing the purification and characterization of the individual components.

HisP, to which a carboxyl-terminal (His$_6$) tail has been added for ease of purification, is soluble in the absence of the hydrophobic components throughout the purification procedure. The purification procedure is very simple and rapid and gives an excellent yield of high purity HisP$_{\text{his6}}$. In vivo (i.e. as part of the intact membrane-bound complex), HisP$_{\text{his6}}$ has physiological properties indistinguishable from those of HisP; thus, the (His$_6$) tail does not alter the functionality of the protein. The enzymatic properties of HisP$_{\text{his6}}$ in the absence of HisQ and HisM are somewhat different from those of HisP in the complex, i.e. HisP$_{\text{his6}}$ does not display cooperativity for ATP, goes important structural rearrangements, leading to alterations in some of its enzymatic properties. Despite being active as a dimer, HisP$_{\text{his6}}$ does not display cooperativity for ATP, suggesting that the two subunits function independently. This finding is in contrast with the properties of the intact complex (7). Similar results have been obtained for the maltose permease (7, 28, 31). Usually inhibition by vanadate is a characteristic of "P"-type ATPases, by blocking the formation of a phosphorylated intermediate (34). However, since no evidence has ever been obtained for the malsee permease (26, 33). The cooperativity of HisQMP$_{\text{his6}}$, together with chemical cross-linking$^{13}$ and reassembly experiments,$^{14}$ indicates that the two identical HisP subunits present in the complex behave differently because of being complexed with HisQ and HisM.

HisP$_{\text{his6}}$, in not inhibited by vanadate, although both transport and ATPase activities of the intact complex are inhibited by it (7, 28, 31). Usually inhibition by vanadate is a characteristic of "P"-type ATPases, by blocking the formation of a phosphorylated intermediate (34). However, since no evidence has ever been obtained for the existence of a phosphorylated intermediate of HisP (35),$^{15}$ it is unlikely that vanadate acts in this fashion in this system. The higher sensitivity of HisP$_{\text{his6}}$ to NEM as compared with the complex presumably indicates an increased accessibility of the reagent to the 2 cysteine residues present in the HisP dimer (25). It should be noted that, although there is no conservation of this cysteine residue within the family of the conserved nucleotide-binding domains (2, 11), the cysteine residue in HisP$_{\text{his6}}$ is located in the middle of the predicted a-helix immediately carboxyl-terminal to the P (or glycine-rich) loop structure (36) of the nucleotide-binding pocket. The response to phospholipids is also different from that of the intact complex, with both the total and individual phospholipids (other than PE) inhibiting HisP$_{\text{his6}}$ whereas total phospholipids are actually necessary and stimulatory in the case of the complex (as assayed in membrane vesicles) (7). This may be due to a specific protective effect in membrane vesicles by the hydrophobic HisQ and HisM integral membrane proteins and/or by the native phospholipids, rather than to the different conformation assumed by HisP$_{\text{his6}}$ in its soluble form. Also in contrast to the behavior of the complex, HisP$_{\text{his6}}$ can hydrolyze ATP in the absence of the obligatory stimulus normally supplied by the soluble receptor, HisJ (6, 7), supporting the notion that the activity of HisP in the complex is repressed (and thereby regulated via the interaction with HisJ) by HisQ and HisM. This is also in agreement with the finding that signal-independent hisP mutants, which are presumed to

\begin{table}[h]
\centering
\caption{Effect of phospholipids on ATPase activity}
\begin{tabular}{lccc}
\hline
Phospholipid & \% ATPase activity & \text{K}^+ & \\
\hline
Total \textit{E. coli} phospholipids & 89 & 58 & 42 & 0.6 \\
PE & 87 & 58 & 76 & 10 \\
Phosphatidic acid & 98 & 60 & 53 & 0.45 \\
Phosphatidylserine & 76 & 48 & 33 & 0.26 \\
Phosphatidylglycerol & 48 & 34 & 15 & 0.13 \\
Cardiolipin & 85 & 22 & 11 & 0.04 \\
Lyso phosphatidic acid & 49 & 13 & 7 & 0.06 \\
\hline
\end{tabular}
\begin{flushleft}
\textsuperscript{a} The activity was measured as described under “Experimental Procedures,” using 0.250 mg/ml HisP$_{\text{his6}}$. 100% activity is the one obtained in the absence of added phospholipids. \\
\textsuperscript{b} The \text{K}^+ is obtained from a Dixon plot assuming that the inhibition is noncompetitive and using an average molecular weight for total phospholipids of 700 and for cardiolipin of 1400.
\end{flushleft}
\end{table}

$^{12}$ That this would be so had already been deduced from the higher sensitivity to proteolytic degradation of HisP produced in the absence of the hydrophobic subunits. Although HisP can exist in a soluble form and its sequence gives no indication of hydrophobicity, it has a strong tendency to associate with the membrane (9, 32).

$^{13}$ G. F.-L. Ames and K. Nikaido, unpublished results.

$^{14}$ P.-Q. Liu and G. F.-L. Ames, submitted for publication.

$^{15}$ G. F.-L. Ames and K. Nikaido, unpublished data.

$^{16}$ The inhibition of HisP$_{\text{his6}}$ by total phospholipids can be fully accounted for by the inhibitory effects of phosphatidylglycerol and cardiolipin, which, after PE, are the most abundant phospholipids in \textit{E. coli} (about 25% of the total phospholipids (37)).
have an altered interaction between HisP and HisQ/HisM, which exhibit high constitutive ATPase activity (30) and with the properties of signal-independent mutants in the maltose permease system (38).

HisP$_{\text{NBD}}$ hydrolyzes and interacts with ATP and other nucleotides with rates that are comparable to those of the complex. The affinity of HisP$_{\text{NBD}}$ for ATP is 205 $\mu$M, which is compatible with the measured $K_m$ value of 640 $\mu$M in the complex (7). Under standard assay conditions, when the protein concentration is 0.7 mg/ml, the maximum rate of ATP hydrolysis is generally 500 nmol/min/mg protein. At this concentration, 26% of the protein is in the dimer form (as calculated at 22 °C; Fig. 4), from which it can be calculated that the specific activity of the dimer is 2000 nmol/min/mg protein, corresponding to a turnover rate of 2 s$^{-1}$. This value is higher than that observed for the intrinsic activity of the complex (about 0.3 s$^{-1}$ (7)), which is consistent with the notion that in the absence of HisQ and HisM the activity of HisP is relieved. It is likely that under optimal conditions soluble HisP$_{\text{NBD}}$ would hydrolyze ATP as effectively as the fully stimulated complex (8 s$^{-1}$; calculated for the complex at maximum receptor stimulation (7)).

A comparison of the properties of nucleotide-binding subunits of other traffic ATPases is useful to start delineating a general picture of the properties of these enzymes. The nucleotide-binding domains of other bacterial traffic ATPases have been purified and characterized. MalK, the nucleotide-binding component of the maltose permease, has been purified via a denaturation-renaturation of inclusion bodies (22); it has a turnover rate of 0.9 s$^{-1}$ (calculated from Ref. 33, assuming that it functions as a monomer) and an affinity for ATP of 70 $\mu$M, both of which are comparable to those of HisP$_{\text{NBD}}$. Also similar to HisP$_{\text{NBD}}$, MalK’s activity is not inhibited by vanadate and is sensitive to NEM; the latter would be expected from the location of a cysteine residue right in the middle of the P loop of MalK. The nucleotide-binding domain of the E. coli hemolysin exporter, HlyB, purified as a soluble fusion protein containing its amino-terminal one) which has been characterized in vitro as an ATPase with one of the nucleotide-binding domains of CFTR (the main which is itself a dimer.

$K_m$ glutathione exporte, HlyB, purified as a soluble fusion protein containing the isolated nucleotide-binding subunit will provide invaluable information on its mechanism of action.

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