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Purification and Characterization of the Membrane-Bound Complex of an ABC Transporter, the Histidine Permease[1]

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The bacterial histidine permease, an ABC transporter, from *Salmonella typhimurium* is composed of a membrane-bound complex, $HisQMP_2$, comprising two hydrophobic subunits (HisQ and HisM), two copies of an ATP-hydrolyzing subunit, HisP, and a soluble receptor, HisJ. We describe the purification and characterization of $HisQMP_2$ using a 6-histidines extension at the carboxy terminus of HisP [$HisQMP_{2(his6)}$]. The purification is rapid and effective, giving a seven–fold purification with a yield of 85 and 98% purity. Two procedures are described differing in the detergent used (decanoylsucrose and octylglucoside, respectively) and in the presence of phospholipid. $HisQMP_{2(his6)}$ has ATPase and transport activities upon reconstitution into proteoliposomes (PLS). $HisQMP_{2(his6)}$ has a low level ATPase activity (intrinsic activity), which is stimulated to a different extent by the receptor—liganded and unliganded. Its pH optimum is 7.8–8.0, it requires a cation for activity and it displays cooperativity for ATP. The effect of various ATP analogs was analyzed. Determination of the molecular size of $HisQMP_{2(his6)}$ indicates that it is a monomer. The permeability properties of two kinds of reconstituted PLS preparations are described.

KEY WORDS: ABC transporter; histidine permease; ABC purification.

INTRODUCTION

The superfamily of traffic ATPases (or ABC transporters) comprises both prokaryotic and eukaryotic transport proteins, which share a conserved nucleotide-binding domain (Ames *et al.*, 1992; Hyde *et al.*, 1990). The superfamily includes, among many 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), the mammalian heterodimeric transporter (TAP1/TAP2) involved in antigen processing, and a presumed cholesterol transport gene, ABC1 (Ames *et al.*, 1992; Hyde *et al.*, 1990; Doige and Ames, 1993; Higgins, 1992; Strauss, 1999; Young and Fielding, 1999; Young and Holland, 1999). 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 (Shyamala *et al.*, 1991; Liu and Ames, 1997; Liu *et al.*, 1997; Nikaido, 1994).

The histidine permease is composed of a soluble substrate-binding receptor, HisJ (the histidine-binding protein), and a membrane-bound complex, $HisQMP_2$, comprising two integral membrane proteins, HisQ and

[1] Key to abbreviations: $HisQMP_2$, and $HisQMP_{2(his6)}$ the membrane-bound complex containing HisQ, HisM, and two HisP subunits without and with the extension of eight amino acids (Leu-Glu-His-His-His-His-His-His) at the carboxyl terminus; PLS, proteoliposomes reconstituted with purified $HisQMP_2$ and subjected to LiposoFast treatment; PLS_{crude}, proteoliposomes not subjected to LiposoFast treatment; DS, *n*-decanoylsucrose; OG, octyl-β-D-glucopyranoside; DPA, dipicolinic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; NBD, nucleotide-binding domain; Plipid, phospholipid.

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HisM, and two copies of HisP, which carries the ATP-binding motif (Kerppola *et al.*, 1991; Mimura *et al.*, 1990, 1991). ATP hydrolysis provides the energy for the transport process (Ames, 1990). In a functional cycle, ligand translocation is strictly dependent on the presence of the liganded soluble receptor, which sends a transmembrane signal *via* HisQ/M to $HisP_2$ that results in the initiation of ATP hydrolysis and a series of conformational changes in $HisQMP_2$. (Petronilli and Ames, 1991; Liu and Ames, 1998; Kreimer *et al.*, 2000). Our general approach toward understanding the mechanism of action of these transporters has been to purify and characterize biochemically and structurally (1) the intact complex, (2) the isolated nucleotide-binding subunit, HisP (Nikaido *et al.*, 1997), and (3) a reassembled system in which the isolated nucleotide-binding subunit has been reconstituted into membrane vesicles and proteoliposomes containing only HisQ and HisM (Liu and Ames, 1998; Liu *et al.*, 1999). We have already achieved the purification to homogeneity and characterization of isolated soluble HisP (Nikaido *et al.*, 1997), followed by crystallization and resolution of its crystal structure (Hung *et al.*, 1998). In contrast, the purification of the intact $HisQMP_2$ complex presents considerable problems because of its membrane-bound nature and, in particular, because of its multisubunit composition, which requires care to be kept unaltered. The purification of functional membrane complexes of other ABC transporters has, in general, been difficult. In this communication we show that the $HisQMP_2$ can be solubilized and purified in an active form, able both to hydrolyze ATP and to translocate histidine. The final level of purity is estimated to be about 98%. Solubilized $HisQMP_2$ is shown to have a molecular weight of 120,000, indicating that it does not polymerize. $HisQMP_2$ can also be purified in the presence of added Plipid, which is demonstrated to enhance the level of its activities. This work contributes to our continuing characterization of this ABC transporter model system and provides additional information, in general, for the study of the ABC superfamily of transporters.

EXPERIMENTAL PROCEDURES

Production of $HisQMP_{2(his6)}$ and Preparation of Membrane Vesicles

A plasmid, pFA284 (Liu, 1996), carrying the *S. typhimurium* histidine permease genes, *hisQ*, *hisM*, and *hisP*, under the temperature-sensitive control of the λP_L promoter and carrying an eight-residue extension at the carboxy terminus of HisP including six histidines, was introduced into *E. coli* GA298 (Liu and Ames, 1997; Liu *et al.*, 1999) to form strain GA500. For the preparation of large amounts of membranes, GA500 is grown at 30°C in 200 L of L broth supplemented with ampicillin (50 μg/ml) until the culture reaches an $A_{650\,nm}$ of 0.4. The temperature is raised to 42°C and after 60 to 90 min, the cells are harvested and stored as a paste at −80°C. For smaller batches (1 to 4 L), the growth conditions are the same as above, but the harvested cells are washed once in 50 mM Tris/Cl buffer, pH 7.0, and stored frozen at −20°C overnight. Cell breakage and membrane vesicles preparation is done essentially as described (Liu *et al.*, 1999) for preparations containing Plipid, or with the following modifications for purification in the absence of Plipid. Approximately 20 g of cells (wet weight) are resuspended in 160 ml of ice-cold buffer A (50 mM MOPS/K^+ buffer, pH 7.0, containing 100 mM NaCl and a protease inhibitor cocktail, as indicated by the manufacturer; Boeringer Mannheim, Indianapolis, IN) and disrupted twice at 10,000 psi in a French press apparatus. After two successive centrifugations, for 10 min each at 1500 × *g* to eliminate intact cells, the supernatant is centrifuged in a Ti60 rotor at 37,000 rpm for 60 min in a Beckman ultracentrifuge and the supernatant discarded. Buffer A (20 ml) are added to the pellet (i.e., the membrane fraction), which is then divided into three portions that are thoroughly resuspended individually using a Teflon-coated tissue homogenizer. The resuspended membranes (usually containing 15–20 mg of protein/ml) are aliquoted and stored in liquid nitrogen, where they are stable for years.

Solubilization and Purification of $HisQMP_{2(his6)}$ in the Presence of DS

All operations are carried out at 4°C. A membrane preparation (about 16 mg) is slowly defrosted on ice, diluted with two volumes of 50 mM Tris/Cl, pH 8.0, containing 5% glycerol and a protease inhibitor cocktail (buffer B), and harvested by centrifugation in a TLA 100.4 rotor in the Beckman table-top ultracentrifuge at 100,000 rpm (=541,000 × *g*) for 30 min. The washed membranes are resuspended at a final protein concentration of 10 mg/ml in buffer B containing 1.2% DS and kept on ice for 45 min with occasional gentle mixing. The preparation is centrifuged as above at 100,000 rpm for 30 min and the clear supernatant is removed with a Pasteur pipette, being careful to exclude a loose yellow layer immediately above the tight pellet. The supernatant is made 20 and 10 mM in β-mercaptoethanol and ATP, respectively, and applied to a column (Bio-Rad BioSpin chromatography column, 1-ml capacity) containing 0.5 ml of a settled slurry of TALON metal-affinity resin (CLONTECH) equilibrated in buffer C (50 mM Tris/Cl, pH 8.0; 5% glycerol; protease inhibitor cocktail; 0.2% DS; 10 mM ATP; and 20 mM

β-mercaptoethanol). The material is passed through the column three times, followed by washing once with 2 ml of buffer C and elution is with 3.0 ml (in 0.5 ml batches) of buffer C to which 100 mM imidazole has been added and after readjusting its pH to 8.0 with 4 M KOH. Fractions (0.5 ml) are collected and their protein content determined qualitatively by the Bio-Rad dye-binding protein assay in a microtiter plate. The first three fractions usually contain the bulk of the protein and are pooled; if necessary, the protein can be concentrated up to at least 10 mg/ml using a Millipore Ultrafree Biomax 10K NMVL centrifugal concentrator. For structural studies, the purified protein is immediately used as such. In order to test its various activities, the purified protein is immediately reconstituted into PLS_{crude} or PLS as described below. An accurate protein assay is performed on all purification fractions using a modified Lowry procedure (Peterson, 1977). A typical experiment yields about 10% of the initial membrane proteins as purified $HisQMP_{2(his6)}$.

Solubilization and Purification of $HisQMP_{2(his6)}$ in the Presence of OG and Plipid

Membranes are prepared from about 2 L of cell culture, grown as described above, and solubilized, as described previously (Liu *et al.*, 1999), with protein, OG, and *E. coli* Plipid (Avanti Polar Lipids, Inc., Birmingham, AL) at concentrations of 1, 12.5, and 3.7 mg/ml, respectively; 50 mM Tris/Cl buffer (pH 8.0) is used instead of the MOPS/K^+, pH 7.0 buffer. Purification and reconstitution into PLS is essentially as described (Liu, 1997), with all operations conducted at 4°C. In brief, solubilized protein (1–2 mg) is applied to a BioSpin disposable column (Bio-Rad) containing about 0.3 ml of settled TALON beads equilibrated in the solubilization buffer. The flow-through is reapplied to the column twice, the column is washed twice with 2 ml each of solubilization buffer, and eluted with 2 ml of elution buffer (solubilization buffer containing 200 mM imidazole). The eluate is immediately passed through a PD-10 desalting column (Pharmacia, Inc.) equilibrated in 50 mM MOPS/K^+ buffer, pH 7.0, which removes imidazole and other small molecules. Additional Plipid is immediately added to the desalted protein to a final concentration of 10 mg/ml, together with 1.25% OG, 15 mM ATP, 5 mM $MgSO_4$, and the protein is immediately reconstituted into PLS_{crude} as described below.

Reconstitution into PLS

PLS_{crude} are prepared by dialysis (Liu and Ames, 1997) using protein prepared either in the presence or absence of Plipid and freshly eluted from the metal-affinity column at a concentration of about 0.2 mg/ml and after adding *E. coli* Plipid to a final concentration of 2 mg/ml for DS-solubilized protein and 10 mg/ml for OG-solubilized protein. The mixture is placed on ice for 30 min and then dialyzed against 100 volumes of 50 mM MOPS/K^+ buffer, pH 7.0, containing 1 mM DTT. Dialysis is at 4°C for 18 h, when the buffer is changed and dialysis continued for an additional 24 h. PLS_{crude} are aliquoted and stored in liquid nitrogen. PLS_{crude} can be used directly for ATPase activity. PLS for transport assays are prepared as follows: PLS_{crude}, obtained using protein purified by either method (0.2–0.3 mg protein/ml), are defrosted and ATP and $MgSo_4$ are added to final concentrations of 15 mM each; for DS-solubilized protein, sufficient Plipid is also added to bring it to a final concentration of 10 mg/ml. The mixture is then frozen and thawed five times, and extruded through polycarbonate filters using an extrusion device (LiposoFast, Avestin, Inc., Ottawa, ON, Canada), as described (Liu and Ames, 1997). The final protein concentration is assayed after treatment with LiposoFast, because it has been found that some protein is absorbed onto the polycarbonate filter (approximately a constant amount of 50 μg/filter, which would amount to a considerable percentage of the total protein if PLS_{crude} with low protein concentration are used). PLS are used immediately for transport assays.

Transport Assay

Histidine and arginine transport is assayed in PLS, with energy being supplied by the entrapped ATP/Mg. For some experiments, ATP is regenerated by inclusion of 20 mM creatine phosphate and 4 mg/ml of creatine kinase (both from Sigma) just before the freezing/thawing procedure. Due to creatine kinase instability, only two cycles of freezing/thawing are performed.

Determination of Molecular Size of $HisQMP_{2(his6)}$

Two methods were used. For molecular sieve chromatography, pure $HisQMP_{2(his6)}$ (2.5 mg/ml) in 1.5 ml of 50 mM Tris/Cl, pH 8.0, 5% glycerol, 0.2% DS, and 75 mM NaCl, is applied together with molecular weight standards to a Sephacryl S-300 HR (Sigma Chemical Corp.) in a Bio-Rad Econo-Column (2.5 $\times$ 50 cm) equilibrated with the same buffer. Elution is with the same buffer at a flow rate of 0.6 ml/min. Fractions (1 ml each) are collected and the protein content analyzed by SDS–PAGE and visualization with Coomassie Blue and immunoblotting.

For sucrose gradient centrifugation (Martin and Ames, 1961), 6 ml of a 5–25% sucrose gradient in 50 mM Tris/Cl, pH 8.0, 5% glycerol, 0.2% DS, containing NaCl

at the same concentration as that of the sample, is created in a 13-ml ultracentrifuge tube, with an overlay of 6 ml of the same buffer; 0.1–0.3 ml of sample, containing 0.25 to 0.5 mg/ml of pure $HisQMP_{2(his6)}$, several molecular weight standards (0.025 ml of 10 to 20 mg/ml each), and a slight excess of NaCl over that present in the overlayer, is applied beneath the overlayer. The tubes are centrifuged in a SW-41 rotor at 24,000 rpm, for 16.5 h at 4°C (57,000 rcf). Fractions are harvested by inserting a Pasteur pipette tip through the entire gradient and removing the gradient from the bottom up. About 15 fractions (0.4-ml each) are collected and their refractive index is measured using a Zeiss refractometer.

ATPase Assay

ATPase activity is assayed in PLS_{crude} essentially as described (Liu *et al.*, 1997) with minor modifications. In brief, the assay mixture (160 μl), which contains about 7 to 15 μg of protein, 50 mM MOPS/K^+ buffer, pH 7.5 (or 50 mM Tris/Cl, pH 8.0), 33 μM HisJ, and 34 μM L-histidine, is incubated for 3 min at 37°C in Eppendorf tubes in a water bath. A control is always performed in the absence of HisJ to determine the level of the intrinsic ATPase activity and of possible contaminating ATPases. The reaction is initiated by the addition of 3.2 μl of an ATP/$MgCl_2$ mixture (100 and 500 mM, respectively) and, at appropriate times, 25-μl samples are transferred to microtiter wells prepared containing 25 μl of 12% SDS. The amount of P_i liberated is determined by a colorimetric assay (Chifflet *et al.*, 1988), using Na_2HPO_4 as a standard. Rates are expressed as nanomoles P_i liberated/mg protein/min and, unless specified, are always corrected for the activity in the absence of HisJ.

Assay for Permeability of PLS

DPA (20 μM) is internally trapped by freeze/thawing and extrusion in a LiposoFast apparatus as described (Liu and Ames, 1997), using 0.3 mg of PLS_{crude} protein in 0.5 ml, in 50 mM Tris/HCl, pH 8.0, 100 mM NaCl. Unincorporated DPA is then removed by two sequential passages of this mixture through a prepacked PD-10 column of Sephadex G-25 M (Pharmacia Biotech, Uppsala, Sweden). Fifty microliters of such DPA-containing PLS are added to 1 ml of the same buffer containing 1 μM Tb^{3+} (Molecular Probes, Inc., Eugene, OR) preequilibrated at 25°C in a 1 × 1 cm cuvette, and the fluorescence emission spectrum determined from 460 to 530 nm with a Hitachi F-4000 fluorometer (with the excitation set at 280 nm and the slits set at 10 nm) equipped with a thermostat.

Miscellaneous Assays

SDS-PAGE and immunoblotting are performed as described (Wolf *et al.*, 1995), using polyclonal antibody raised against HisP or against the carboxyl terminal end of HisQ (Kerppola *et al.*, 1991), and quantitating the bands with an Alpha Imager (IS-1000; Alpha Innotech Corp., San Leandro, CA). Whenever indicated, the pH of the resolving gel is adjusted to 8.55 to allow the resolution of HisM from HisQ (Hobson *et al.*, 1984). The following methods are performed essentially as described: protein assay (Peterson, 1977) and purification of HisJ (Nikaido and Ames, 1992). Nucleotides and ATPase inhibitors are obtained and prepared as described (Liu *et al.*, 1997).

RESULTS AND DISCUSSION

It has been previously shown that the 6-histidine carboxyl terminal extension does not affect the physiological and biochemical properties of $HisQMP_2$, both *in vivo* and *in vitro* (Liu *et al.*, 1997; Nikaido *et al.*, 1997). The temperature-sensitive lambda P_L promoter allows the production of large, but controllable amounts of $HisQMP_{2(his6)}$, which helps avoid the formation of inclusion bodies. Two purification procedures, which differ in the nature of the detergent and in the presence of added Plipid, were developed. One involves solubilization and purification in DS in the absence of any added Plipid and was developed with the specific intention of using the purified material for crystallization purposes. The other procedure involves solubilization with OG in the presence of added *E. coli* Plipid, which is present also during all of the purification steps. A full biochemical characterization was performed on both preparations.

Purification of $HisQMP_{2(his6)}$ Solubilized in DS, in the Absence of Plipid

$HisQMP_{2(his6)}$ is synthesized abundantly upon induction of strain GA500; the subunits can be visualized as obvious proteins bands, even in whole cells, by Coomassie Blue staining following SDS-PAGE resolution (data not shown). The amount and purity of $HisQMP_{2(his6)}$ in several fractions after a typical purification sequence are analyzed by SDS–PAGE and Coomassie Blue staining (Fig. 1, left) and Table I summarizes various quantitations. About one half of the total membrane protein and over 90% of the complex (as defined by the presence of HisP stained with Coomassie Blue) are solubilized by DS (lanes 2 and 3). Upon application of the soluble fraction

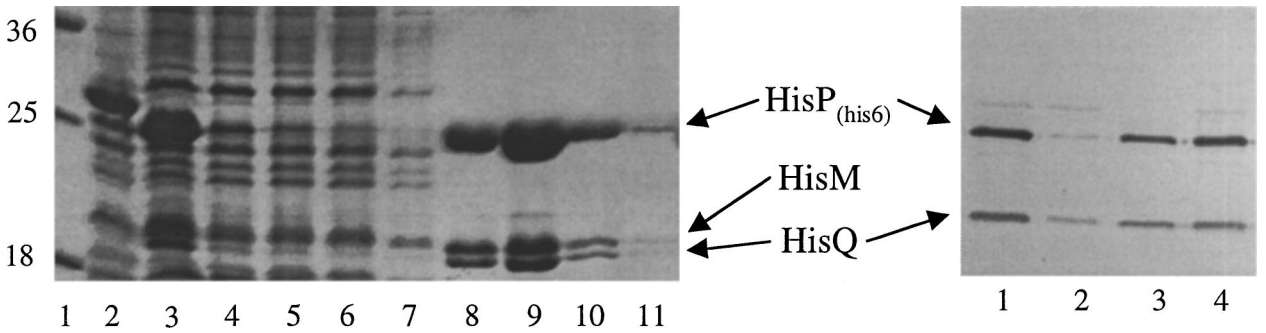

Fig. 1. Purification of $HisQMP_{2(his6)}$. Membranes were prepared and solubilized as described in the Section Experimental Procedures. Left, a typical SDS-PAGE (15%, with the pH of the resolving gel adjusted to 8.5; Hobson *et al.*, 1984) of fractions obtained at various stages of purification, stained with Coomassie Blue, is shown. The pH of the separating gel is 8.55. Lane 1, molecular mass standard (the molecular masses of the standards used, in kDa, are indicated); lane 2, membrane proteins that were not solubilized; lane 3, solubilized membrane proteins (33 μg protein); lane 4–6; flow-through fractions of the three successive passages on TALON column; lane 7, wash fraction; lanes 8–11, imidazole eluate. Lanes 2–5 and 8–10 contain similar percentages of the total preparation. Right, an immunoblot of fractions from the preparations shown in A is shown. The resolution gel of the SDS-PAGE was 12.5% and its pH was 8.80. Lane 1, Solubilized membrane proteins (0.6 μg); lane 2, flow-through fraction (1.4 μg); lane 3, pooled fractions from lanes 8–11 in A (0.3 μg); lane 4, original membrane preparation (1 μg). The antibodies used were raised against HisP and HisQ. The positions of $HisP_{(his6)}$, HisQ, and HisM are indicated.

to a TALON column, most of the protein is not retained (flow-through, lanes 4–6, and wash, lane 7). Essentially all of $HisQMP_{2(his6)}$ is eluted with 100 mM imidazole in the first three fractions (lanes 8–10), with a very small amount appearing in the flow-through. The complex is estimated visually to be of a high level of purity, as also seen in overloaded gels (data not shown). Quantitation of an immunoblot prepared with anti-HisP and anti-HisQ antibodies also indicates that more than 90% of HisP and of HisQ is solubilized (Fig. 1, right) (HisM antibody is of poor quality and could not be used). The HisP/HisQ/HisM subunits are present in native membranes in the ratio of 2/1/1 (Kerppola *et al.*, 1991); from Coomassie Blue-stained PAGE it is clear that HisP is more easily stained by Coomassie Blue than HisQ or HisM, in accordance with the fact that the latter two proteins are very hydrophobic (Kerppola and Ames, 1992). By immunoblot it is possible to determine that the complex is stable throughout the entire procedure, from the crude membrane to the purified complex, since the relative amounts of HisP and HisQ do not change. It should be noticed, however, that disassembly into subunits of a few percentages of the complex would not be detected by this assay.

Table I shows a typical purification scheme. The ATPase activity cannot be assayed in the presence of the levels of detergents commonly used for solubilization and purification (Liu *et al.*, 1997) and, therefore, it is shown only for the crude membrane preparation and for PLS_{crude}

Table I. Typical Purification

Fraction	Volume (ml)	Protein		ATPase		Recovery (%)
		mg/ml	Total (mg)	Specific activity (nmol/min/mg)	Total activity (nmol/min)	
Total membrane	1.0	16	16	129	2064	100
Soluble fraction[a]	1.4	6.0	8.5			
Insoluble fraction[b]	1.4	4.9	7.0			
TALON flow-through	1.4	2.8	3.9			
Wash	2.0	1.5	3.1			
Pooled eluate[c]	1.5	1.4	2.0			
PLS_{crude}	1.4	1.4	1.9	928	1763	85

[a] Fraction containing protein soluble in 1.2% DS.
[b] Fraction insoluble in 1.2% DS.
[c] Fractions eluted with 100 mM imidazole.

reconstituted with the purified complex. The final specific activity of $HisQMP_{2(his6)}$ is 928 nmol/min/mg protein and the final purity is estimated to be about 98% by inspection of SDS-PAGE resolutions. The high yield (recovery of 85%) is in agreement with the finding that very small amounts of $HisQMP_{2(his6)}$ appear in other fractions. From immunoblots, using a standard curve with purified HisP protein, it can be estimated that $HisQMP_{2(his6)}$ constitutes about 11% of the crude membrane protein, which is in reasonable agreement with the seven-fold purification achieved.

Properties of the ATPase Activity of $HisQMP_{2(his6)}$

Various properties of the ATPase activity of purified $HisQMP_{2(his6)}$ in PLS_{crude} were characterized. Under standard assay conditions, the activity is linear for about 5 min, after which the rate starts to decline (Fig. 2A). This decline may be due to a combination of the decrease in ATP concentration and the increase in ADP, which is a good inhibitor of this activity (Liu *et al.*, 1997). The initial rate is proportional to protein concentration up to at least 150 μg of protein/ml (Fig. 2B). The pH optimum is fairly broad, at 7.5–8.0, with a 50% decrease at pH 9.5 and complete loss of activity at pH 6.0 (Fig. 2C). This pH optimum is the same as that measured in intact membranes (Liu *et al.*, 1997). Mg^{2+} stimulates the activity, reaching the highest level at a concentration of 4 mM and maintaining it at the same level thereafter (Fig. 2D). This effect may reflect a combination of stimulation of enzymic activity and of permeabilization of PLS, the latter being an essential requirement (Liu *et al.*, 1997). Mn^{2+} also results in an initial increase to an activity level similar to the maximum achieved with Mg^{2+}, followed by severe inhibition above 2 mM. Similarly to Mg^{2+}, the increase may reflect a combination of permeabilizing and stimulating actions. Co^{2+} has no stimulatory effect and it is strongly inhibitory in the presence of Mg^{2+}, with 50 μM Co^{2+} giving an 80% inhibition. The Co^{2+} inhibition does not effect signaling by HisJ, because a binding protein-independent HisP mutant is inhibited by Co^{2+} to the same extent as the wild type; Co^{2+} does not inhibit by binding to the carboxyl-terminal extension of six histidine residues because a strain that does not carry the extension is equally inhibited (data obtained in membrane preparations and not shown). Thus, it is probable that Co^{2+} affects directly the ATP-binding pocket. In this respect, it is interesting and not well understood why up to 1 m*M* Co^{2+} stimulates the soluble form of HisP (Nikaido *et al.*, 1997); presumably the binding pocket is somewhat altered by interaction of HisP with HisQ/M.

Finally, NaCl is not required and has an inhibitory effect at concentrations higher than 50 mM, inhibiting the activity completely at 1.0 M (Fig. 2E).

The activity is stable if the unreconstituted protein is stored in liquid nitrogen immediately after it is eluted from TALON, maintaining full activity for at least 4 months, even with some defrosting and refreezing. It can also be stored at 4°C for at least a week without losing activity if EDTA (3 mM for a protein concentration of 13 mg/ml) is added immediately upon elution from TALON. If stored at 4°C in the absence of EDTA, it loses about 30 and 60% of the activity in 2 days 1 week, respectively.

The dependence of activity on ATP concentration is shown in Fig. 3. In the absence of the receptor HisJ, there is a low level of activity (0.1 μmol/min/mg) ascribable to an intrinsic ATPase activity of $HisQMP_2$ (Liu *et al.*, 1997) (Fig. 3A). The activity is stimulated about 20-fold by the liganded receptor, HisJ-his (Fig. 3B), and by unliganded receptor, although considerably less (Fig. 4). A positive cooperative effect, both in the absence of HisJ (intrinsic activity) and in the presence of liganded HisJ (fully induced activity) is apparent, with Hill coefficients (n_{app}) of 1.97 and 1.90, respectively, and approximate $K_{0.5}$ of 0.5 to 1.0 mM. These values indicate that two sites are involved, whether or not the activity is induced, confirming previous results (Liu *et al.*, 1997). This rate of ATP hydrolysis is similar to that reported consistently for the eukaryotic transporter, the MDR P-glycoprotein. However, the latter does not show any cooperativity for ATP (reviewed in Sharom *et al.*, 1999); interestingly, mutant forms of $HisQMP_2$ that do not require signaling by the receptor to hydrolyze ATP ($HisQMP_2^*$), also do not display cooperativity (Liu *et al.*, 1999). It is possible that the structure of P-glycoprotein, at least as purified, is "loose," as has been described for the $HisQMP_2^*$ mutants (Liu *et al.*, 1999).

The effect of ADP and of various ATP analogs (AMP–PNP, AMP–PCP, and TNP–ATP) on the ATPase activity was tested. IC_{50} values of 0.8, 1.4, 1.8, and 0.08 mM, respectively, were obtained for ADP, AMP–PNP, AMP–PCP and TNP–ATP, respectively. These values are very similar to those reported for the ATPase activity in membrane vesicles (Liu *et al.*, 1997). Also, in agreement with previous results, ouabain and NEM, up to 2 and 10 mM, respectively, have no effect. The lack of inhibition by NEM suggests that cysteine residues are not essential for activity (as also shown using sulfhydryl reagents; Kreimer *et al.*, 2000).

The effect of varying the concentration of the soluble receptor HisJ, both liganded and unliganded, is shown in Fig. 4A. The corresponding affinities for $HisQMP_{2(his6)}$ are 6.4 and 10 μM, respectively (Fig. 4B). These values are essentially indistinguishable from each other and are

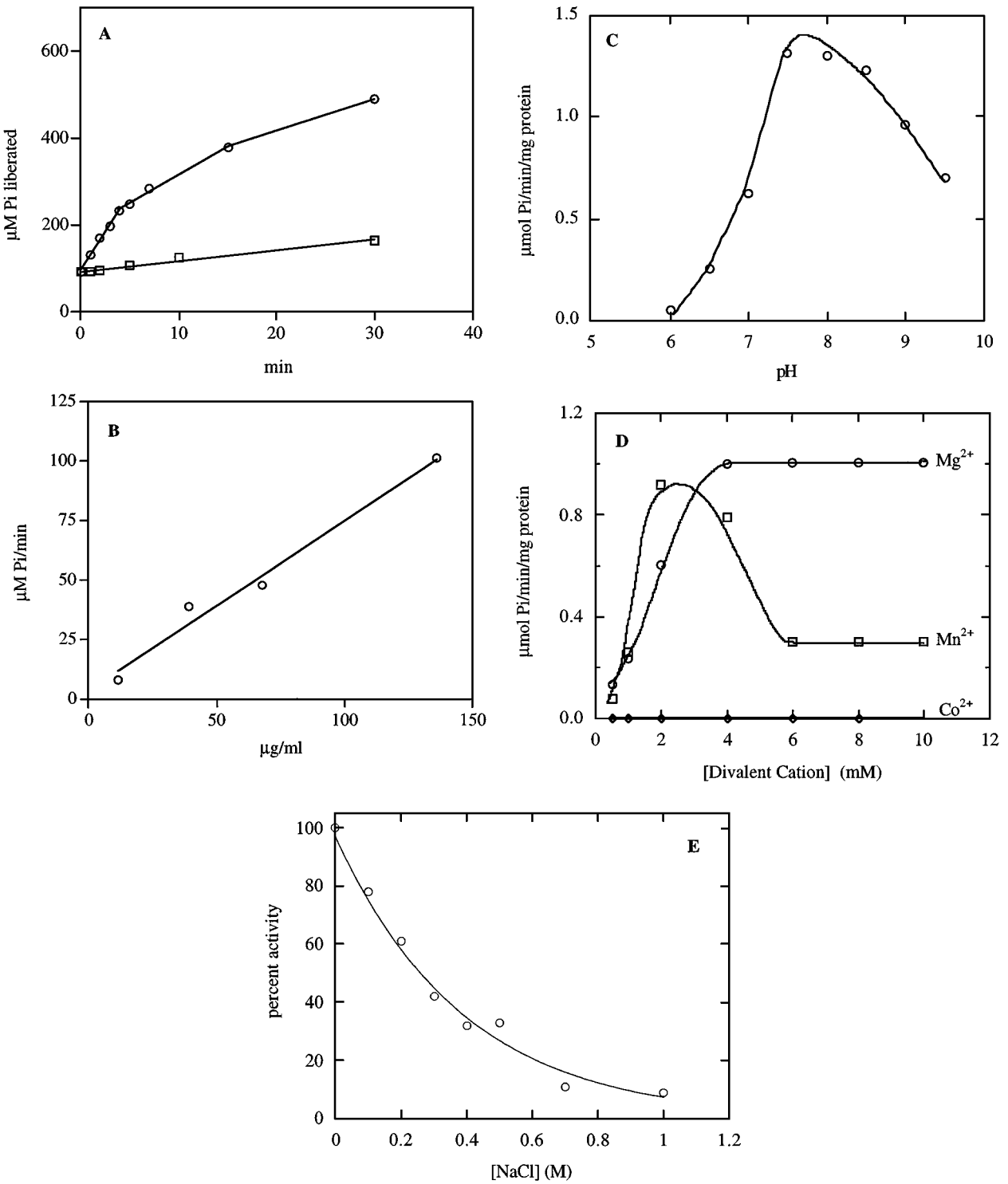

Fig. 2. Properties of $HisQMP_{2(his6)}$. ATPase activity was assayed under a variety of conditions. A, ATPase activity versus time. The assay was performed as described in the Section Experimental Procedures, with a $HisQMP_{2(his6)}$ concentration of 38 μg/ml and in the absence (squares) or presence of 34 μM liganded HisJ (circles). B, ATPase activity versus $HisQMP_{2(his6)}$ concentration. C, $HisQMP_{2(his6)}$ solution was diluted tenfold into buffers at various pH values (indicated on the abscissa) and the ATPase activity measured. The buffers and their respective pH values are: MES/Na, 6.0 and 6.5; MOPS/Na, 7 and 7.5; Tris/Cl, 8.12 and 8.5; and ethanolamine/Cl, 9.0 and 9.5. D, The assay was performed as described in the Section Experimental Procedures, with the replacement of $MgCl_2$ by the indicated cations at the concentrations indicated on the abscissa. E, NaCl was added to the assay buffer at the concentrations indicated on the abscissa and the ATPase assayed as usual (expressed in percentage); this assay was performed with PLS_{crude} reconstituted by the rapid procedure (see Section on Results).

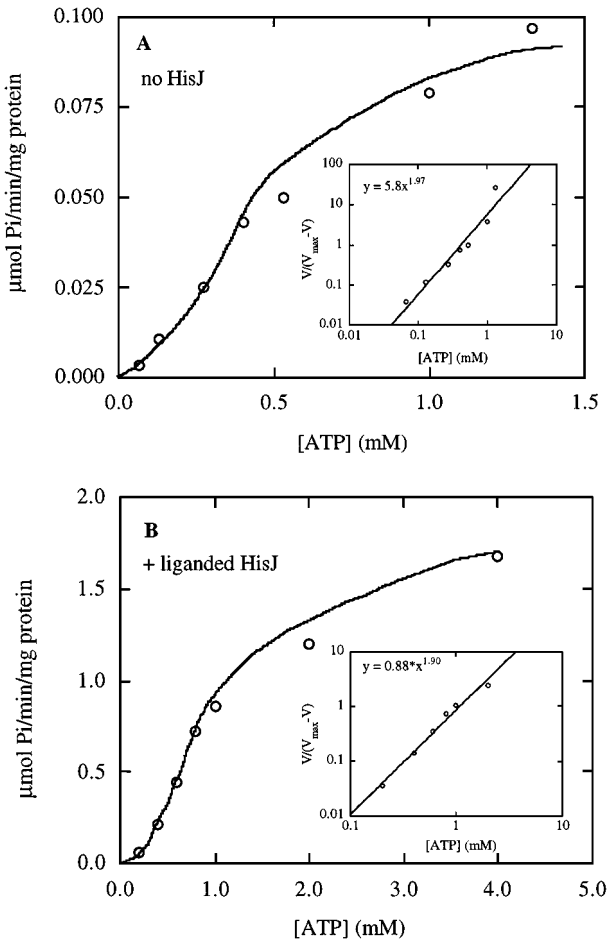

Fig. 3. Dependence of activity on ATP concentration. A PLS_{crude} preparation was diluted to 100 μg/ml $HisQMP_{2(his6)}$ and the ATPase activity was assayed as described in the Section Experimental Procedures (using Tris/Cl buffer), either in the absence of HisJ (A) or in the presence of 10 μM histidine-liganded HisJ (B). The insets show the same data plotted according to Hill's equation. The ordinates show the initial rates of ATPase activity.

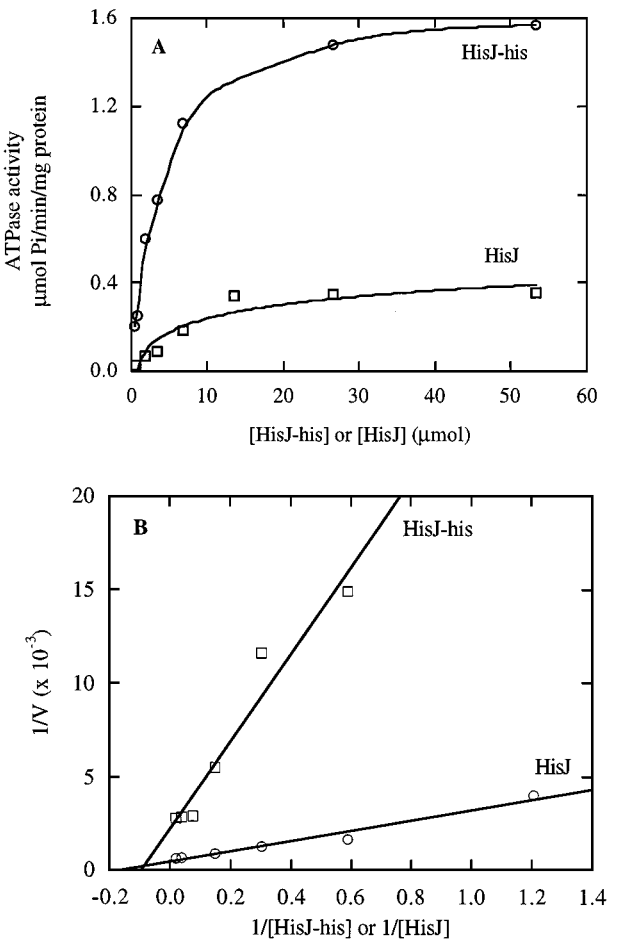

Fig. 4. Affinity of $HisQMP_{2(his6)}$ for liganded and unliganded HisJ. Increasing amounts of either histidine-liganded or unliganded HisJ were present during the ATPase assay and the activity was determined as described in the Section Experimental Procedures (using Tris-Cl buffer). The specific activities at each receptor concentration is shown in A. B shows the same data in a Lineweaver–Burk plot.

similar to those established previously in membrane vesicles (Ames *et al.*, 1996).

Molecular Size of $HisQMP_{2(his6)}$

Two procedures were used to determine the oligomeric structure of $HisQMP_{2(his6)}$: sucrose density-gradient centrifugation and molecular sieve chromatography. By sucrose gradient centrifugation $HisQMP_{2(his6)}$ positions itself between bovine serum albumin and alcohol dehydrogenase (M_r: 67,000 and 150,000, respectively), as shown in Fig. 5A. This corresponds to an M_r of about 120,000, which matches reasonably well the value of 110,627, as calculated mathematically for $HisQMP_{2(his6)}$, including the carboxyl-terminal extension. The results are the same in the presence of either 100 or 200 mM NaCl.

The molecular size was also determined using a molecular sieve column fractionation. By this technique $HisQMP_{2(his6)}$ tends to give a wide peak if the molecular weight standard proteins are present in the same run and the concentration of $HisQMP_{2(his6)}$ is high, presumably indicating the existence of nonspecific interactions at high protein concentrations. The presence of 200 mM NaCl does not improve the sharpness. However, a considerably sharper peak is obtained if the concentration of $HisQMP_{2(his6)}$ is low (applying 0.9 mg in 0.7 ml and visualizing the protein by immunoblotting) and if molecular weight standards are absent from the actual run. Figure 5B shows the result of one such experiment, where it can be

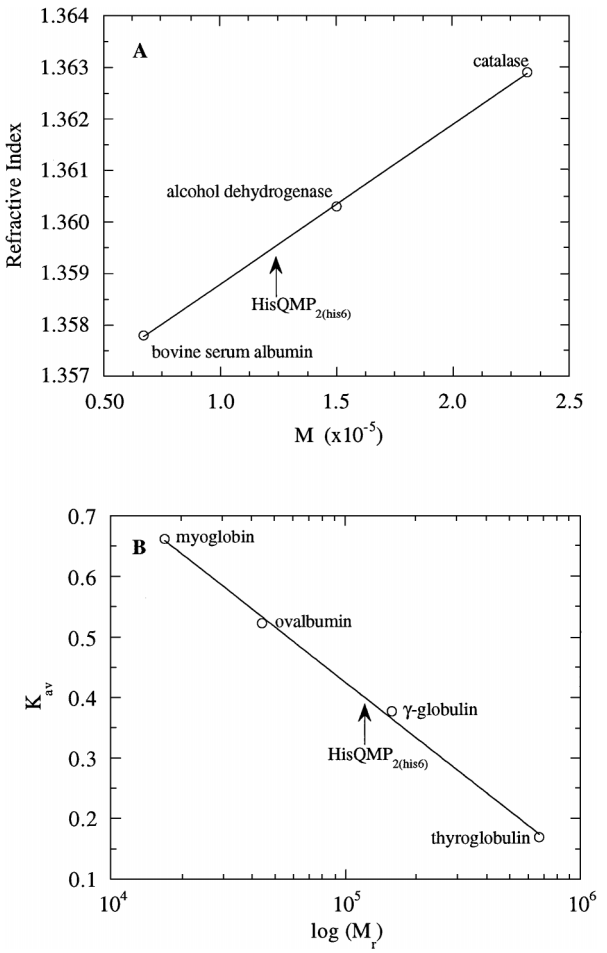

Fig. 5. Estimation of molecular size of $HisQMP_{2(his6)}$. A. Estimation by sucrose density centrifugation. A solution containing 50 μg of $HisQMP_{2(his6)}$ in 0.3 ml was applied to the top of a 5–25% sucrose gradient and centrifuged as described in the Section Experimental Procedures. The positions of protein standards and of $HisQMP_{2(his6)}$ are marked, with the abscissa and the ordinate indicating the M_r values and the refractive index of the gradient fractions, respectively. The M_r values of the proteins used as standards are: bovine serum albumin, 67,000; alcohol dehydrogenase, 150,000; and catalase, 232,000. B. Estimation by molecular sieve resolution. $HisQMP_{2(his6)}$ (2.5 mg in 1 ml) was applied to a Sephacryl S-300 HR column and eluted as described in the Section Experimental Procedures. The K_{av} $[(V_e-V_0)/(V_t-V_0)]$ and the M_r values are plotted on the ordinate and abscissa, respectively. The positions of standard proteins and of $HisQMP_{2(his6)}$ are indicated. The M_r values of the proteins used as standards are: thyroglobulin, 670,000; γ-globulin, 158,000; ovalbumin, 44,000; and myoglobin, 17,000.

seen that an M_r value of 121,000 is obtained. The ratio of HisQ to HisP was estimated in each of the fractions by an immunoblot using antibodies raised against HisQ and HisP and was found to be essentially the same throughout the resolution. Despite the fact that both of these M_r estimates are approximate, because the complex is presumably associated with detergent, it can be reasonably concluded that $HisQMP_{2(his6)}$ maintains its integrity and exists only as a four-subunit monomer in solution. Presumably this is its functional unit.

Reconstitution into Proteoliposomes (PLS_{crude} and PLS)

Measurements of transport require complete vesicle impermeability, whereas many biochemical experiments, such as using covalent modification reagents, require some permeability. Therefore, we developed two types of PLS preparations, which have different permeability. The dialysis of purified $HisQMP_{2(his6)}$ in the presence of Plipid yields PLS_{crude}, in which detergent has been removed and the protein complex has interacted with the externally added Plipid to form proteoliposomes. The concentration of protein and of Plipid and the ratio of protein to Plipid during reconstitution affect the ATPase activity of the protein. It was established that protein and Plipid concentrations of 200 μg/ml and 2 mg/ml, respectively, are optimal for the reconstitution of DS-solubilized $HisQMP_{2(his6)}$, with changes in either the protein/Plipid ratio or their absolute concentrations lowering the final level of ATPase activity (data not shown). PLS_{crude}, as they emerge from dialysis, can be converted into PLS by extrusion *via* LiposoFast, leading to the formation of larger and more uniform proteoliposomes.

Comparison of Permeabilities

The permeability level of PLS_{crude} and PLS were compared. PLS are shown to be completely impermeable using a very sensitive fluorescent method as follows: DPA is trapped internally during LiposoFast treatment (see Section Experimental Procedures) and its leakage is monitored using externally added terbium as an indicator. Tb^{3+} forms a strongly fluorescent complex with DPA, with sensitivity in the nanomolar range (Barela and Sherry, 1976) and an emission maximum at 495 nm. When PLS-containing DPA are exposed to 1 μM, Tb^{3+}, only a marginal increase in fluorescence intensity at 495 nm is observed (data not shown); this indicates that Tb^{3+} does not have access to the interior of the PLS. There is very little leakage of DPA if the PLS are stored at 0°C, with less than 30% of DPA leaking out upon incubation of PLS at 0°C for 24 h. However, upon incubation at 25°C, DPA leaks out with a half-time of 30 min (data not shown). Therefore, PLS are fully impermeable if stored on ice, maintaining impermeability for several hours, as

also shown previously by their ability to allow solute accumulation (Liu and Ames, 1997).

In contrast, PLS_{crude} were shown to be somewhat permeable. Since about one half of the reconstituted $HisQMP_{2(his6)}$ complexes are oriented with HisP facing the exterior, with the other half in the opposite orientation (Liu and Ames, 1997), ATP hydrolysis could occur if ATP and HisJ were both added externally or internally only if either of them could cross the proteoliposome surface. PLS, being fully impermeable, require that HisJ be on the opposite side of the surface from ATP (Liu *et al.*, 1997). PLS_{crude}, however, hydrolyze ATP even if it is added externally, together with HisJ (Fig. 6, solid triangles), indicating that PLS_{crude} are permeable, to either or both ATP and HisJ; permeability to ATP is the most likely situation. External HisJ should activate only those complexes in which HisP faces the interior; if ATP freely penetrates the interior, the activity should be one half as high as the full potential activity. (Full activity is obtained by the addition of excess Mg^{2+}, which completely permeabilizes PLS and PLS_{crude}; Liu *et al.*, 1997). Indeed, if excess Mg^{2+} is added, the activity is stimulated twofold (Fig. 6, solid circles), indicating that both ATP and HisJ gain access to both surfaces, activating all complexes. As a control, PLS were simultaneously shown to be completely unable to activate ATP hydrolysis in the absence of excess Mg^{2+} (or to transport L-histidine), unless ATP was trapped internally during extrusion *via* LiposoFast (data not shown). Figure 6 also shows that varying the amount of Plipid during dialysis, up to 20 mg/ml, does not affect the permeability of PLS_{crude}. Thus, Plipid exposure does not seem to increase the impermeability of these structures. In conclusion, PLS are completely impermeable and PLS_{crude} are partially permeable.[7]

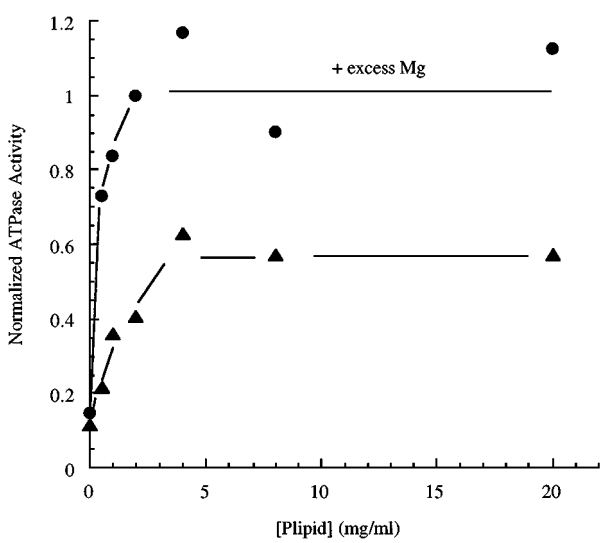

Fig. 6. Permeability of PLS_{crude}; effect of $MgCl_2$ and of Plipid. The ATPase activity was assayed in the presence of histidine-liganded HisJ and of either 2 mM $MgCl_2$ (solid triangles) or 12 mM $MgCl_2$ (solid circles). The Plipid concentration present during dialysis [in the presence of 0.4 mg/ml of $HisQMP_{2(his6)}$] is shown on the abscissa. The activity is normalized to that obtained in the presence of excess Mg^{2+} at 2 mg/ml Plipid.

Rapid PLS_{crude} Reconstitution for Immediate ATPase Assay

One of the problems encountered in the purification of $HisQMP_{2(his6)}$ has been that the ATPase activity is inhibited by detergents, requiring their removal by dialysis in order to assess the quality of purified preparations. Since dialysis is a slow process, a rapid dilution method was developed. The concentration of DS compatible with activity was determined by adding various DS concentrations to PLS_{crude}. The presence of 0.01, 0.02, and 0.04% DS gave 0, 10, and 30% inhibition, respectively. The following procedure, which combines molecular sieving with dilution, decreases the concentration of DS to 0.02% or less. DS-purified $HisQMP_{2(his6)}$ is immediately passed on a molecular sieve PD-10 column (prepacked Sephadex G-25; Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated and eluted in buffer B, which removes imidazole, any cobalt (which is inhibitory) possibly released by the TALON column, and other small molecules. The protein is immediately concentrated to 5–10 mg/ml using a Millipore Ultrafree Biomax concentrator (10K cut-off), during which the initial DS is simultaneously concentrated from 0.2 to about 0.5%. Concentrated $HisQMP_{2(his6)}$ (5 μl; 25–50 μg) are placed in an Eppendorf tube and 5 μl of Plipid (250 μg, briefly sonicated while under nitrogen) is added and vigorously vortexed for a few seconds; then 115 μl of MOPS/K^+ buffer, 50 mM, pH 7.5, is added followed by vortexing. After incubation at 0°C for 30 min, the ATPase assay is performed as usual (which involves a further four–fold dilution).

Transport Properties of Purified $HisQMP_{2(his6)}$

Having established that PLS prepared with purified $HisQMP_{2(his6)}$ are impermeable, their transport ability was

[7] PLS_{crude} were also shown to be permeable to another charged and moderately large compound, the sulfhydryl specific reagent 2-(4′-maleimidylanilino)naphtalene-6-sulfonic acid (MIANS), as shown by its ability to modify HisP in the absence of Mg^{2+} (D. I. Kreimer, K. P. Chai, and G. F.-L. Ames, 2000).

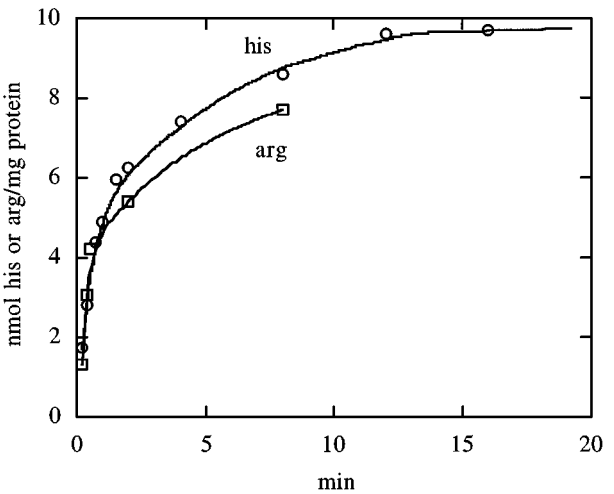

Fig. 7. Histidine and arginine transport. The mixture contains PLS (corresponding to 0.3 mg/ml of purified $HisQMP_{2(his6)}$), 10 mM ATP, 10 mg/ml Plipid, 100 mM NaCl, 50 mM Tris/Cl pH 8.0 buffer, and either 20 μM apo-HisJ and 20 μM [^{3}H] L-histidine (open circles), or apo-LAO and 20 μM [^{3}H] L-arginine (open squares). The assay is performed as described in the Section Experimental Procedures.

measured. Figure 7 shows that transport of L-histidine follows normal kinetics, with an initial linear rate of 8 nmol/min/mg (i.e., 0.09 nmol/min/nmol $HisQMP_{2(his6)}$), followed by a plateau with a value of about 10 nmol/mg of $HisQMP_{2(his6)}$ that is reached within about 10 to 15 min; these results are similar to those obtained with PLS reconstituted from nonpurified $HisQMP_2$ (Liu and Ames, 1997). Figure 7 shows that these PLS also translocate L-arginine, which is transported through the same permease, but utilizing a different receptor, the LAO protein (Kustu and Ames, 1973; Higgins and Ames, 1981). Preliminary results had shown that the incorporation of an ATP-regenerating system yields increased rates of transport (Liu *et al.*, 1997). This was confirmed using PLS containing purified $HisQMP_{2(his6)}$ and incorporating an ATP-regenerating system as described in the Section Experimental Procedures; increases in transport rates between 50 and 100% were obtained (data not shown).

Purification of $HisQMP_{2(his6)}$ Solubilized in OG in the Presence of Plipid

A variety of data had suggested that the presence of total *E. coli* Plipid improves the performance of the $HisQMP_2$ complex, with respect to both transport and ATPase activity. Thus, the optimal conditions for purifying $HisQMP_2$ in the presence of Plipid were also established. Various detergents were initially tested and OG was shown to be effective for both solubilizing and maintaining activity (Liu, 1996; Liu and Ames, 1997). The properties of $HisQMP_{2(his6)}$, purified in the presence of Plipid and OG, were analyzed and compared to those of the complex prepared in the absence of Plipid.

Effect of Plipid on ATPase Activity and Complex Integrity

The effect of Plipid during solubilization was tested, assaying for HisP as an indication of $HisQMP_{2(his6)}$ levels. The amount of HisP solubilized is the same whether Plipid is absent or present during solubilization up to a concentration of 3 to 3.7 mg/ml, but it decreases at higher Plipid concentrations. The total protein solubilized remains essentially the same (about 50% of the total) at all Plipid concentrations; therefore, the specific activity of $HisQMP_{2(his6)}$ decreases at Plipid concentrations higher than about 4 mg/ml (data not shown).

The effect of Plipid during purification was also tested. Membranes were solubilized in the presence of 3.7 mg/ml Plipid and $HisQMP_{2(his6)}$ was purified in the presence of varying concentrations of Plipid. The purified $HisQMP_{2(his6)}$ was then reconstituted using the standard final Plipid concentration (10 mg/ml). Figure 8A shows that varying the Plipid concentration has no effect on the efficiency of retention/elution of $HisQMP_{2(his6)}$ from the TALON column, whereas the specific ATPase activity increases with increasing concentrations of Plipid, reaching 150% at a Plipid concentration of 3 mg/ml. Taking these various findings into consideration, an optimal purification protocol was adopted (see Section Experimental Procedures). Figure 8B (left) shows an SDS–PAGE of such a typical purification experiment and the corresponding immunoblots for HisP and HisQ (right). The level of purity is above 90% (lane 4, left) and the ratio of HisP to HisQ does not change significantly during the purification. Thus, $HisQMP_{2(his6)}$ maintains its integrity during the entire procedure. The lower ATPase activity observed when the Plipid concentration is lower during purification may reflect, at least in part, the fact that Plipid appears to protect the integrity of the complex when solubilized in OG. In fact, if Plipid is absent from the column equilibration, wash, and elution buffers, the subunits of the complex become dissociated, with all of the HisP being retained by the column, and variable amounts of HisQ appearing in the flow-through and wash (data not shown).

Properties of $HisQMP_{2(his6)}$ in the Presence of Plipid

Various general properties were tested and found to be mostly similar to those of $HisQMP_{2(his6)}$ purified in the

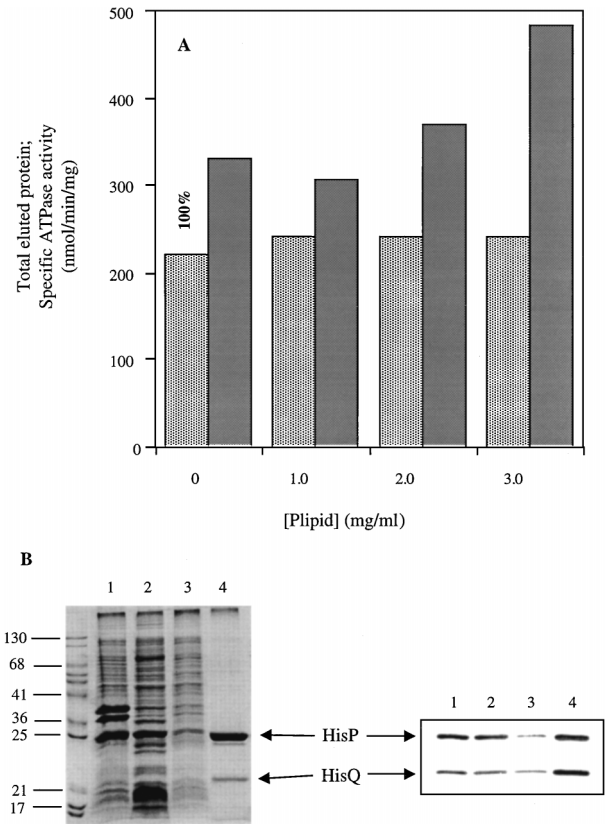

Fig. 8. Effect of Plipid on purification and preservation of ATPase activity. $HisQMP_{2(his6)}$ was solubilized as described in the Section Experimental Procedures in the presence of 3.7 mg/ml Plipid and then purified in the presence of varying amount of Plipid as indicated, added in the TALON column buffer and in the wash and elution buffers. Reconstitution was under standard conditions, i.e., in the presence of 10 mg/ml Plipid for all samples. (A) Total amount of eluted $HisQMP_{2(his6)}$ (light columns; expressed as percentage of the amount of protein obtained in the absence of Plipid) and corresponding ATPase activity (dark columns) at each of the indicated concentrations of Plipid. (B) (Left). SDS–PAGE of fractions from a typical TALON purification. Lanes 1–4 contain: intact membranes, OG-soluble protein in the presence of 3 mg/ml Plipid, flow-through, and material eluted with imidazole, respectively. (Right) Immunoblot of PAGE samples 1–4 with antibodies raised against HisP and HisQ.

presence of DS and absence of Plipid. $HisQMP_{2(his6)}$ displays positive cooperativity and the values of the affinity for liganded and unliganded HisJ are 5 and 11 μM, respectively. However, an important difference is that the level of ATPase activity and transport are consistently higher than in DS-purified $HisQMP_{2(his6)}$, with average values of 2.1 μmol P_i/min/mg and 9.3 nmol His/min/mg, respectively. This is to be compared to average values of ATPase activity of about 1.2 μmol P_i/min/mg and of transport of 5 nmol His/min/mg for DS-purified complex in the absence of Plipid. Presumably the presence of Plipid protects the activity by maintaining the correct complex structure.

Effect of Varying the Plipid Concentration on ATPase Activity

Because the major difference between the two $HisQMP_{2(his6)}$ preparations relates to the presence of Plipid, their effect on ATPase activity was tested. Figure 9 shows that in OG-solubilized/Plipid-containing preparations the ATPase activity varies with the Plipid concentration in the assay mixture. The optimal total Plipid concentration varies with $HisQMP_{2(his6)}$ concentration, being 0.8, 1.3, and 2.7 mg/ml, for $HisQMP_{2(his6)}$ concentrations of 6, 12, and 36 μg/ml, respectively. Plipid concentrations higher than the optimal amounts are inhibitory at all three $HisQMP_{2(his6)}$ concentrations. The inhibitory effect observed for OG-solubilized/Plipid-containing $HisQMP_{2(his6)}$ might be ascribed to an expanded surface area in PLS upon addition of Plipid, a phenomenon described as "surface dilution kinetics" (Lill and Wickner, 1990), which might reflect and have an

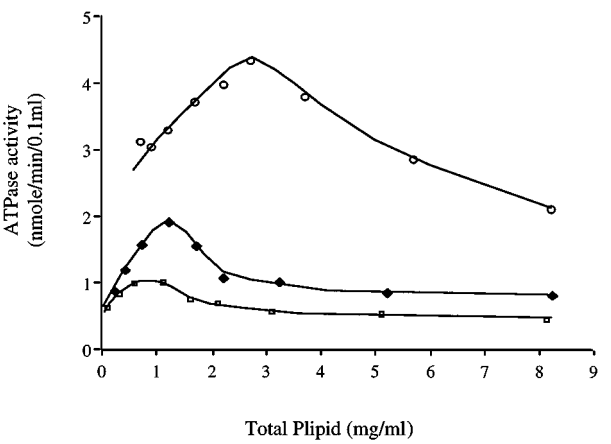

Fig. 9. Effect of the presence of Plipid during the ATPase assay. The ATPase activity was assayed in the presence of total Plipid as indicated on the abscissa. Each of the Plipid concentrations includes the residual Plipid calculated to be present in each of the $HisQMP_{2(his6)}$ samples. $HisQMP_{2(his6)}$ was solubilized in the presence of OG and Plipid, purified, and reconstituted. Final $HisQMP_{2(his6)}$ concentrations were 6, 12, and 36 μg/ml, respectively (squares, diamonds, and circles), obtained upon dilution in assay buffer containing varying amounts of Plipid (abscissa); the Plipid present in the each of the $HisQMP_{2(his6)}$ samples (as derived from solubilization and reconstitution) is 0.12, 0.24, and 0.72 mg/ml, respectively, with Plipid/protein ratios being 133:1, 108:1, and 75:1, (w/w) respectively; the respective maximum specific activities are 1.7, 1.7, and 1.3 μmol/min/mg.

effect on a two-dimensional movement of HisP in the planar lipid bilayer during ATP hydrolysis. A similar inhibitory effect of Plipid was observed in the case of a binding protein-independent mutant complex (Liu, 1997; Liu *et al.*, 1999), which suggests that the inhibitory effect does not involve the signaling mechanism by the receptor (HisJ). The possibility that HisP moves within the lipid bilayer during ATP hydrolysis is consistent with the previously proposed notion that HisP undergoes a membrane insertion/deinsertion process during ATP hydrolysis and histidine transport (Liu and Ames, 1998; Liu *et al.*, 1999; Nikaido and Ames, 1999) and with the known ability of HisP to be exchanged between complexes (Liu and Ames, 1998). It should be noted that in membrane vesicles, added Plipid stimulates the ATPase activity up to a plateau level, without causing inhibition (Liu *et al.*, 1997), i.e., without displaying the hypothetical phenomenon of surface dilution kinetics. Thus, it appears that the mobility of HisP is relatively restricted in intact membranes, presumably because of the higher protein/Plipid ratio.

It should, however, be noted that in the case of DS-solubilized $HisQMP_{2(his6)}$, increasing the Plipid concentration in the assay had little effect (data not shown). This behavior might reflect a substantially different composition and/or organization of $HisQMP_2$ as prepared under these conditions, such as the presence of native Plipid molecules remaining tightly associated with the complex throughout the purification. This might lead to an increased stability during solubilization and purification in the absence of added Plipid. It should also be noted that the the ratio of protein to DS is tenfold higher than in the case of OG, which also might have an effect on the nature of the solubilized complex. In agreement with the notion of an increased stability of DS-purified $HisQMP_{2(his6)}$ is the finding that it does not readily dissociate into its components; thus, it is reasonable to postulate that such preparations are resistant to surface dilution kinetics.

CONCLUSIONS

In conclusion, we have purified and characterized $HisQMP_{2(his6)}$ under two sets of conditions that give preparations differing mainly in the level of both of its activities and in the ability to maintain the integrity of the complex. The availability of these purification procedures and the understanding of the biochemical properties of this complex is essential for its continuing analysis. $HisQMP_{2(his6)}$ purified using DS might have a structure closer to that of the native complex. For this reason and because of the absence of exogenous Plipid the DS preparation will be more useful for crystallographic studies. $HisQMP_{2(his6)}$ purified in OG and containing Plipid is be the system of choice for disassembly and reassembly of the individual components and for a number of basic biochemical studies.

The question of stoichiometry in these transporters is an important aspect of their mechanism of action, requiring precise measurements of both activities. Under the conditions of the present purification and ATPase assay, measurement of the full hydrolytic activity is straightforward, giving reproducibly a rate of about 2000 nmol/min/mg.[8] However, in transport assays, the rate is affected by numerous factors that influence the measurements, as already discussed (Liu *et al.*, 1997). In brief, transport rates are limited by the orientation of the complexes in the PLS (∼50% in each direction; Liu and Ames, 1997), by the rapid accumulation of inhibitory ATP hydrolysis products such as ADP, by the diminution of the ATP concentration, and, possibly, by the inhibitory effect of the accumulated substrate. In addition, transport assays are very sensitive to the exact manipulations during the purification procedure and particularly to the treatment with LiposoFast. Previous calculations involving a number of extrapolations to take these factors into account gave a maximum transport rate of about 100 nmol/min/mg (Liu and Ames, 1997), which corresponds to a stoichiometry of ATP hydrolyzed to substrate translocated of about 20, which seems unlikely to be the true number. Different tools need to be developed to resolve this complex problem.

Having developed two rapid and effective purification methods, we are presently making extensive efforts to crystallize the complex. Solution of the structure of this model system, together with the analysis of the many mutant proteins available in each of the subunits, would be a great advancement in the study of other members of this important ABC transporters family.

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[8] This rate is considerably higher than the value of 20 nmol/min/mg reported for the purified maltose transport complex (Davidson and Nikaido, 1991). A possible explanation is that the purification procedure used for the latter, which involves exposure to OG, has caused dissociation of the complex, similarly to what has been observed with $HisQMP_2$.

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