Modulation of ATPase Activity by Physical Disengagement of the ATP-binding Domains of an ABC Transporter, the Histidine Permease*

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The membrane-bound complex of the prokaryotic histidine permease, a periplasmic protein-dependent ABC transporter, is composed of two hydrophobic subunits, HisQ and HisM, and two identical ATP-binding subunits, HisP, and is energized by ATP hydrolysis. The soluble periplasmic binding protein, HisJ, creates a signal that induces ATP hydrolysis by HisP. The crystal structure of HisP has been resolved and shown to have an "L" shape, with one of its arms (arm I) being involved in ATP binding and the other one (arm II) being proposed to interact with the hydrophobic subunits (Hung, L.-W., Wang, I.-X., Nikaido, K., Liu, P.-Q., Ames, G. F.-L., and Kim, S.-H. (1998) Nature 396, 703-707). Here we study the basis for the defect of several HisP mutants that have an altered signaling pathway and hydrolyze ATP constitutively. We use biochemical approaches to show that they produce a loosely assembled membrane complex, in which the mutant HisP subunits are disengaged from HisQ and HisM, suggesting that the residues involved are important in the interaction between HisP and the hydrophobic subunits. In addition, the mutant HisP mutants are shown to have lower affinity for ADP and to display no cooperativity for ATP. All of the residues affected in these HisP mutants are located in arm II of the crystal structure of HisP, thus supporting the proposed function of arm II of HisP as interacting with HisQ and HisM. A revised model involving a cycle of disengagement and reengagement of HisP is proposed as a general mechanism of action for ABC transporters.

The ABC transporter superfamily (ATP-binding cassette transporters, also called traffic ATPases) comprises prokaryotic and eukaryotic ATP-energized transporters that share a conserved nucleotide-binding domain and have similar architectural organization (1, 2). It constitutes the largest paralogous protein family in the fully sequenced genome of Escherichia coli and Treponema pallidum (3, 4). The superfamily includes prokaryotic periplasmic binding protein-dependent transporters (5), such as the well characterized Salmonella typhimurium histidine permease, and many medically important transporters in humans, such as the cystic fibrosis transmembrane conductance regulator (CFTR) (6), the multidrug resistance protein (MDR or P-glycoprotein) (7), the transporters associated with antigen processing (TAP1/TAP2) (8), and the photoreceptor cell-specific transporter ABCR (9). Prokaryotic and eukaryotic ABC transporters have the same overall topological organization, with two hydrophobic domains and two nucleotide-binding domains (NBDs). In prokaryotes these domains are usually composed of separate protein subunits, whereas in eukaryotes they are usually fused into a single polypeptide.

The periplasmic histidine permease is an excellent model system to study the mechanism of action of the ABC transporter superfamily in general. It is composed of a soluble receptor, the periplasmic histidine-binding protein, HisJ, and a four-subunit membrane-bound complex (HisQMP4) composed of HisQ and HisM (the two hydrophobic subunits) and of two identical HisP subunits that carry the highly conserved nucleotide-binding domain (10, 11). The membrane-bound complex of the histidine permease has been purified and reconstituted into proteoliposomes (PLS), and shown to hydrolyze ATP and translocate histidine in a manner that is strictly dependent on the presence of the binding protein (12). The membrane-bound complex must interact with the liganded soluble receptor in order to achieve translocation (13, 14). In the absence of the binding protein, the complex hydrolyzes ATP at a low rate (intrinsically active), which is stimulated by the liganded or unliganded binding protein; the level of stimulation is highest when HisJ is liganded (15).

Numerous lines of evidence suggest that ATP binding and hydrolysis is performed by the NBDs, HisP in the case of the histidine permease, with the hydrophobic subunits controlling the rate of hydrolysis. HisP is photolabeled by azido-ATP analogs (16); mutations in the ATP-binding pocket lose the ability to bind azido-ATP (17); the membrane complex does not hydrolyze ATP when depleted of HisP, and hydrolysis is resumed after purified HisP is reconstituted back into the complex (18); and purified soluble HisP hydrolyzes ATP in the absence of HisQ and HisM (19). The recent resolution of the three-dimensional structure of HisP at 1.5 Å clearly shows the presence of an ATP-binding pocket containing an ATP molecule (20).

HisP has a hydrophilic sequence, yet it is tightly associated with the membrane; however, it behaves neither like a classical integral membrane protein nor like a peripheral membrane protein (21). HisP is accessible to proteases and impermeant biotinylating reagents from both the cytoplasmic and the periplasmic sides of the membrane, but not like a peripheral membrane protein (22). It is proposed that the HisP subunit is involved in the interaction between the liganded soluble receptor and the membrane complex (13).

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The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; NBD, nucleotide-binding domains; MOPS, 4-morpholinepropanesulfonic acid; HisQMP4, membrane-bound complex containing HisQ, HisM, and HisP; HisP*, constitutive mutant HisP; HisQMP, the integral membrane proteins HisQ and HisM; HisQMP*purified HisQMP4 with a carboxyl-terminal extension to HisP of 8 amino acids residues: Leu-Glu-His-His-His-His-His-PLS; PLS, reconstituted proteoliposomes; ATP-γS, adenosine 5′-O-(thiotriphosphate); AMP-PNP, adenosine 5′-β,γ-methylenetriphosphate; AMP-PCP, adenosine 5′-β-phosphate; MOPS, 4-morpholinepropanesulfonic acid; HisQMP4, membrane-bound complex containing HisQ, HisM, and HisP; HisP*, constitutive mutant HisP; HisQMP, the integral membrane proteins HisQ and HisM; HisQMP*purified HisQMP4 with a carboxyl-terminal extension to HisP of 8 amino acids residues: Leu-Glu-His-His-His-His-His-PLS; PLS, reconstituted proteoliposomes; ATP-γS, adenosine 5′-O-(thiotriphosphate); AMP-PNP, adenosine 5′-β,γ-methylenetriphosphate; AMP-PCP, adenosine 5′-β-phosphate.
periplasmic sides of the membrane, indicating that it spans the lipid bilayer (22). This membrane-spanning property has also been demonstrated for other ABC transporters, such as MalK (23), the ATP-binding domain of CFTR (24, 25), and KpmT (26). Because of the hydrophilic sequence of HisP, its membrane-spanning properties are likely to depend on a close interaction with the integral membrane subunits, HisQ and HisM (HisQM), with the strength of this interaction being important for regulating its ATPase activity. In this respect, there is evidence that HisQ and HisM are involved in the transmission of the transmembrane signal initiated by the soluble periplasmic receptor, HisJ, and in the stimulation of ATP hydrolysis by HisP (18, 27). Physical contact between HisJ and HisQ has been demonstrated by chemical cross-linking experiments (28). Direct contact between HisP and HisJ is not expected and was not found.

Permease mutations in the ATP-binding subunit (HisP*) were characterized that allow ligand translocation (29, 30) and ATP hydrolysis in the absence of the binding protein (27), indicating an altered signaling mechanism. To understand further these mechanisms and their regulation during a functional cycle, the biochemical properties of these HisP* mutants were analyzed in detail, and several novel characteristics were defined. HisQMP* complexes have a looser structure, with the mutant His* being “disengaged” from HisQM; they have lower affinity for ADP than the wild type; and their ATPase activity displays no cooperativity for ATP. Based on these observations, we propose a mechanistic model for ATP hydrolysis and ligand translocation which may be applicable to other ABC transporters.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions—**All E. coli strains used in this study were derivatives of E. coli G205 (uncA702, J43, Nam7-Nam53, aux-1, hisC5750-ai800, M72, Sm3, lacZam9350-bio-uvrB, thrE2, asnA::Tn10) which carries a chromosomal uncA702 deletion that eliminates the F-prime, ATase, and a C857ts gene which produces a heat-sensitive periplasmic protein. All plasmids carry either wild type or mutant S. typhimurium hisQ, hisM, and hisP genes under the control of the heat-inducible phase A P1 promoter (27) and the β-lactamase gene (16). A six-histidine tail was attached to the carboxyl terminus of HisP (HisP6His) (31). Such modification has no effect on either ATPase or transport activities (19).

**Preparation of Membrane Vesicles—**E. coli cells harboring plasmids carrying the hisQMP* genes under the control of the A promoter were grown at 30 °C in LB medium containing 200 μg/ml ampicillin with vigorous shaking until the A absorbance reached 0.4, when the growth temperature was shifted to 42 °C. Induction was for 2 h after the temperature of the medium had reached 42 °C. Cells were harvested by centrifugation, washed with buffer B (50 mM MOPS/K$_\text{H}_2$, pH 7.0, 100 mM NaCl), and resuspended in 10 ml (per liter of culture) of buffer A (50 mM MOPS/K$_\text{H}_2$, pH 7.0, 100 mM NaCl, 1 mM dithiothreitol, 1× concentration of the Complete$^\text{TM}$ protease inhibitor mixture (Roche Molecular Biochemicals)). Cells were disrupted by three passes through a French press cell at 10,000 pounds/square inch (68.9 MPa). The cell debris was removed by two successive 5-min centrifugations at 3000 rpm in an MSE 4000 rotor (Sorval, 1400 × g), and the membrane fraction was harvested by discontinuous sucrose gradient centrifugation as described (12). Cell extract (10 ml) was applied on top of a sucrose gradient (1 ml of 2 M sucrose at the bottom and 2 ml of 0.5 M sucrose on top, both in buffer A) and centrifuged in a SW41 rotor (Beckman) at 40,000 rpm for 1 h. Membranes, forming a layer at the interface between 2 and 0.5 M sucrose, were collected, resuspended in 2 ml of buffer A (per liter of culture) of buffer B containing a TE buffer/glucose homogenizer, aliquoted, and stored in liquid nitrogen until needed. The membrane protein concentration is about 10 mg/ml.

**Solubilization of Crude Membrane Proteins and Reconstitution into PLS—**Membranes were diluted to a final protein concentration of 1 mg/ml into solubilization buffer (50 mM MOPS/K$_\text{H}_2$, pH 7.0, 1.25% octyl-$n$-glucopyranoside, 20% glycerol, 15 mM ATP, 5 mM MgSO$_4$, 1 mM dithiothreitol, and 3.7 μg/ml E. coli total phospholipids (Avanti Polar Lipids Inc., Birmingham, AL)). The mixture was incubated on ice for 40 min, gently shaken occasionally, and then centrifuged at 47,000 rpm (T60 rotor, Beckman) for 1 h at 4 °C. For reconstitution into PLS, additional E. coli phospholipids were added to a final concentration of 10 mg/ml immediately to the supernatant (solubilized membrane protein), and the mixture was dialyzed at 4 °C for about 24 h against 100 volumes of dialysis buffer (50 mM MOPS/K$_\text{H}_2$, pH 7.0, 1 mM dithiothreitol) with one change of buffer after the first 9 h as described (12). The resulting PLS were assayed immediately for ATPase activity, aliquoted, and stored in liquid nitrogen.

**ATPase Activity Assay—**The ATPase assay was performed as described (15) in the presence of 50 mM MOPS/K$_\text{H}_2$, pH 7.5, 12 μM HisJ, and 100 μM λ-histidine, unless specified differently. The reaction was started by the addition of ATP and MgSO$_4$, simultaneously to final concentrations of 2 and 10 mM, respectively.

**Miscellaneous Material and Methods—**HisJ was purified essentially as described (32). HisQMP$_\text{sm}$ was purified using metal affinity chromatography (31). Urea extraction was performed as described (18). Fluorescence emission spectra were determined in a Perkin-Elmer spectrophotometer (LS50) at 25 °C, using an excitation wavelength of 295 nm and fluorescence emission wavelengths varying from 300 to 500 nm with slits 5 nm in the presence of 1.2% octyl glucoside, which minimizes light scattering by solubilizing the PLS. Background fluorescence caused by octyl glucoside was subtracted in all experiments. Trypsin was of sequencing grade obtained from Roche Molecular Biochemicals.

**RESULTS**

**Mutant HisP* Is Disengaged from the Integral Transmembrane Subunits HisQ and HisM—**The possibility that the signal processing in these mutant HisQMP$_\text{sm}$ complexes is modified because the physical interaction between HisP* and HisQM has been altered by the mutations was specifically explored by testing the strength of this interaction. Urea-extractability, which is useful in distinguishing integral from peripheral membrane proteins (33), was examined first. As shown in Fig. 1 after 1 h incubation on ice, 3.6 μm urea extracts very little of the wild type HisP from the membrane, whereas it extracts 4, 31, and 53%, respectively, of mutant HisP* proteins T205A, P172T, and P172L; 7.3 μm urea solubilizes 30–40% of

**FIG. 1. HisP* is disengaged in the constitutive mutants as detected by urea extraction.** Bacterial membranes were prepared from various E. coli strains which overproduce the 6×His tag version of HisP*, in an unc~ background: wild type, P172T, P172L, or T205A. Urea extraction was performed with either 3.6 μm urea in buffer A (final concentration) or 7.3 μm urea as described (18). The HisP and HisQ protein present in the total starting membrane vesicles (T, total), in the urea-solubilized fractions (S, supernatant), and in urea-insoluble fractions (P, pellet) were resolved by 10% SDS-PAGE, visualized by immunoblotting using antibodies, and detected by ECL, and the band density was quantitated using densitometry. The positions of HisP and HisQ are indicated by arrows. The quantitation indicating the percent of HisP extracted is summarized in Table I. HisM was not tested due to the lack of high titer antibodies but is likely to behave similarly to HisQ.

![Diagram](image-url)
HisQ contents of wild type membranes (as judged by immunoblotting and densitometry quantitation). The fold stimulation by liganded HisJ over densitometry after immunoblotting. The initial rate of ATP hydrolysis is expressed per mg of membrane protein normalized against the HisP and HisQ in each membrane preparation was quantitated by phospholipids using membrane vesicles prepared under the same conditions. The total protein concentration of the crude membrane preparations is defined as 1 for the wild type, the ratios in the material not retained by TALON in the case of HisP* 6His mutants (E191K, T205M, and E202K) and are active data on urea extraction were obtained for these and several other HisQMP*2 mutants (E191K, T205M, and E202K) and are summarized in Table I. All of the HisQMP*2 mutants show a greater ease of HisP* release from HisQM; P172L has the loosest structure of all. The increased ease of HisP* release by urea indicates that integrity of the complex is compromised by the presence of the HisP* mutation, presumably by weakening the interaction between HisP6His and the integral membrane components. The disengagement of HisP* from the integral membrane subunits. This phenomenon is hereafter referred to as "disengagement" of HisP involved in the transmembrane subunits of the HisQM complex than in the wild type and supports the notion that wild type HisP, yet it releases 76, 90, and 95, respectively, of T205A, P172T, and P172L. In all cases, all of HisQ is found in the urea-insoluble pellet after ultracentrifugation. Quantitative data on urea extraction were obtained for these and several other HisQMP*2 mutants (E191K, T205M, and E202K) and are summarized in Table I. All of the HisQMP*2 mutants show a greater ease of HisP* release from HisQM; P172L has the loosest structure of all. 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![Fig. 2. Different HisQMP2 complex stability detected using TALON co-retention assays.](Image)

Bacterial membranes were prepared from various *E. coli* strains in a *unc-1* background overproducing wild type HisP*6His* mutant T205A, P172T, or P172L as indicated. Membranes were solubilized as described. TALON resin was added to the solubilized material and incubated overnight at 4 °C with gentle shaking. The unbound material and the bound proteins were separated by centrifugation and resolved by 10% SDS-PAGE. HisP and HisQ were visualized by ECL after immunoblotting and quantitated by densitometry. The positions of HisP and HisQ are indicated by arrows. The quantitation data indicating the relative amounts of HisP and HisQ in each fraction are shown at the bottom of the gels.
HisP* is disengaged.

This notion was further supported by studies on the fluorescence spectra of purified mutant and wild type complexes. Each complex contains 9 tryptophan residues, 1 in each HisP6His, 2 in HisQ, and 5 in HisM. As shown in Fig. 3A, both HisQMP6His* T205A (top line) and P172T (middle line) have a higher fluorescence intensity (per mg of protein) than the wild type complex (bottom line) at the fluorescence maximum wavelength (335 nm), indicating a different organization in the microenvironment surrounding one or more of these tryptophan residues. As a test for the structural stability of the complex, the effect of SDS on the fluorescence spectrum was examined. SDS micelles appear to stabilize certain helical secondary structures (34, 35); and peptides appear to exist in SDS in a conformation that is similar to that assumed in a lipid environment (36, 37). Thus, SDS can be used at appropriate concentrations as a partial complex-disrupting agent in studying the packing stability of HisQMP2. The mutants and the wild type respond to SDS differently (Fig. 3, B–D). SDS (1%) dramatically decreases the tryptophan fluorescence intensity of both T205A and P172T, whereas it has no significant effect on the wild type. This result suggests that the wild type complex is not unfolded in 1% SDS, or is so to a lesser extent, and thus reflecting a tighter and more stable structure than that of the mutants. Although the interpretation of these data at the molecular level is impossible due to the great number of tryptophans and possible energy transfer, this differential response to SDS is consistent with the notion that the mutants have a more easily disturbed complex structure than the wild type.

Finally, the structure of the complex was also studied by protease degradation. At a trypsin/protein ratio of 1:160, only 20% of HisQ in the wild type complex is sensitive to trypsin digestion, whereas over 80% of HisQ in the mutant complex is degraded (Fig. 4). The higher trypsin sensitivity of HisQ in HisQMP* than in the wild type is compatible with the notion that mutant HisQMP*2 has a looser complex structure.

Comparison of Disengagement with ATPase Activities—If the constitutivity (i.e. the activity in the absence of HisJ) of the ATPase in HisP* mutants is due to disengagement, a correlation might be expected between the level of disengagement and the level of ATPase in the absence of HisJ. The degree of HisP disengagement, expressed either by urea extractability or by the level of HisQ co-retention on TALON, correlates well with the respective intrinsic ATPase activities (Table I), with the HisQMP* mutant complexes that are more disengaged displaying higher levels of ATPase activity (between 1.4 and 3.5 μmol of Pi liberated per min/mg of membrane protein, as compared with 0.086 μmol/min/mg for the wild type). The P172L mutant complex is the most disengaged, has the least co-retention on TALON, and the highest intrinsic ATPase activity (3.4 μmol/min/mg). Replacement of Pro-172 with threonine instead of leucine (P172T) results in a lower level of disengagement and an intermediate level of intrinsic ATPase activity (2.8 μmol/min/mg). Complex T205A has a modest level of HisP* disengagement and a correspondingly modest intrinsic ATPase activity (1.4 μmol/min/mg). This supports the notion that the ATPase activity of HisP is controlled by its association with the integral membrane components HisQM such that ATP hydrolysis is suppressed by a tight association and that the physical disengagement of HisP is at least one factor leading to ATPase activation.
The level of stimulation of the ATPase activity by the receptor HisJ is roughly inversely correlated with the degree of HisP disengagement (Table I). The ATPase activity of the wild type complex is greatly stimulated (23-fold) by HisJ (15), whereas the activity of the mutants is stimulated no more than 3-fold, and sometimes it is inhibited (up to 64%). The T205A complex is at least one of the least disengaged, and its ATPase activity is further stimulated by HisJ about 3-fold, to a level higher than that of the fully stimulated wild type, suggesting that the HisJ-transmembrane signaling pathway is at least partially maintained in this mutant. The ATPase activity of mutants with intermediate levels of disengagement (E202K, E191K, P172T, T205M) have intermediate levels of stimulation or inhibition and that of the most disengaged mutant complex (P172L) is inhibited the most, 60% by 100 μM HisJ. Therefore, as the level of HisP disengagement increases, the ATPase activity tends to be stimulated less (and may even be inhibited) by the receptor. These data are consistent with the hypothesis that the ATPase activation mechanism involves HisP disengagement.

**Nucleotide Binding Induces Disengagement of Wild Type**

HisP—To investigate how disengagement of HisP is involved in the normal ATPase activation mechanism, various agents were tested for their ability to induce HisP disengagement in the wild type. The presence of ATP at physiological concentrations greatly increases the level of wild type HisP extracted by urea (Fig. 5), with 10 mM ATP resulting in almost complete release of HisP, a level similar to that observed for the most disengaged mutant complex (P172L). The Lineweaver-Burk reciprocal plot and the Hanes-Woolf plot yield a value of 1.8 ± 0.2 mM for ATP (not shown), and there is no indication of cooperativity. Thus, interaction with ATP appears to alter the conformation of HisP, resulting in disengagement. ATP analogues ATPγS, AMP-PNP, and AMP-PCP were shown to behave identically to ATP in disengaging wild type HisP, as measured by the level of HisP being released by urea in their presence; Mg²⁺ has a marginal effect on HisP disengagement, either by itself or in combination with ATP or ATP analogues; finally, the addition of HisP in the absence of ATP has no significant effect on the level of disengagement of wild type HisP (not shown). Therefore, nucleotide binding appears to be sufficient to disengage HisP.

Consistent with the notion that ATP binding induces disengagement, thus weakening the structure of the wild type complex, HisQ is more accessible to trypsin digestion in the presence of ATP, under conditions of no hydrolysis (Fig. 6, triangles), as shown by a decrease in the amount of the HisQ band upon SDS-PAGE resolution and by the disappearance of a 6-kDa fragment containing the carboxyl terminus of HisQ (Fig. 6, inset). That HisP undergoes a conformational change upon ATP binding is also supported by experiments using chemical sulfhydryl modification.² ³

What is the physiological relevance of the nucleotide binding-induced HisP disengagement? Could it be a prerequisite or a priming mechanism for activating ATP hydrolysis? In agreement with this hypothesis, ATP hydrolysis by the wild type was found to involve two different ATP-binding events as evidenced by the fact that the ATPase activity of the wild type HisQMP₂ (both intrinsic and HisJ-stimulated) displays positive cooperativity for ATP with a Hill coefficient n = 2 and a K₀.₅ value of 0.5 mM (15). The affinity of the poorer ATP-binding site was calculated to have an approximate K₀.₅ value of 2 mM similar to that of ATP-induced HisP disengagement. Hence, it is possible that the positive cooperativity observed in the wild type reflects an allosteric ATP binding, in which binding of the first ATP induces HisP disengagement which subsequently increases the affinity of the second site for ATP. If this were true, since mutant HisQMPᵀᴹ already has a disengaged structure, it should not display cooperativity for ATP. Indeed, ATP hydrolysis by HisQMPᵀᴹ mutant P172T shows no cooperativity for ATP with a Hill coefficient n = 0.93, and the rate of ATP hydrolysis follows regular Michaelis-Menten kinetics with respect to ATP concentration (Fig. 7). The same result was obtained for the least disengaged mutant, T205A. The K_m values for ATP, as determined from Lineweaver-Burk plots, are 0.9 and 1.2 mM for mutants P172T and T205A, respectively. Consistent with the

² D. I. Kreimer, personal communication.
³ It should be noted that a paper that appeared during the preparation of this manuscript reported that in the case of the maltose permease the ATP-binding subunit, MalK, is more tightly associated with the membrane-spanning components in the presence of ATP/Mg than in its absence (Moore, M., Jehanno, M., Schneider, E., and Dassa, E. (1998) *Mol. Microbiol.* **30**, 353–363). The fact that MalK serves a regulatory function in addition to being a component of the permease may be at the basis of this difference.
concept that cooperativity reflects an ATP-dependent disengagement step, we observed that when HisP is purified away from the membrane components HisQM in a soluble form, and therefore can be viewed as being completely disengaged from HisQM, it displays no cooperativity for ATP binding (19); soluble HisP also hydrolyzes ATP in the absence of HisJ with a turnover rate of & 2 s⁻¹ (19), which is significantly higher than the intrinsic HisP activity in a wild type HisQMP₂ complex (0.3 s⁻¹) (15). Table II summarizes and compares the properties of wild type and mutant, both soluble and in the complex, with respect to cooperativity and affinity for ATP and ADP. These findings support the hypothesis that nucleotide binding induces HisP disengagement as a priming mechanism for activating ATP hydrolysis.

HisP presumably undergoes disengagement and re-engagement during a normal activity cycle, since it can be either tightly associated with HisQM in the absence of ATP or loosely associated in its presence. Under these conditions, while HisP is in the disengaged state, another HisP molecule might be able to displace it. Taking advantage of the fundamental difference between wild type HisQMP₂ and HisQMP* (P172L), whose ATPase activity is inhibited by HisJ, we show here that mutant HisP* can indeed be exchanged with an externally supplied wild type soluble HisP₆₆₆. The activity of HisQMP* (P172L) membranes (Fig. 8, 1st and 2nd column) was compared with that obtained after mixing these membranes with a 15-fold excess of soluble wild type HisP₆₆₆ (Fig. 8, 5th and 6th columns). In the absence of HisJ, the mixture has a reduced initial rate of intrinsic ATP hydrolysis (less than half that of the original membrane; compare column 5 with 1), and its ATPase activity is stimulated 2.5-fold by 18 μM liganded binding protein HisJ (6th column), i.e. the membrane in the mixture has acquired properties typical of wild type HisQMP₂ complex. This result can be explained by an exchange between the HisP*(P172L) in the original membrane vesicles and the externally added soluble wild type HisP₆₆₆.

**FIG. 6.** Effect of ATP on trypsin digestion of the membrane-bound complex. Trypsin digestion was performed with varying protein/trypsin ratios in the presence of 50 mM MOPS/K⁺, pH 7.0, in the presence (triangles) or absence (circles) of ATP, using membrane vesicles containing wild type HisQMP₂. Inset, trypsin digestion was performed with a 1.01 protein/trypsin ratio and analyzed as described above, except that samples were overloaded in order to see the digestion product, which put the HisQ full-length bands out of the linear range for quantitation.

**DISCUSSION**

We show that the interaction between the ATP-binding subunit (HisP) and the integral transmembrane subunits (HisQ and HisM) is weaker in mutant HisQMP*₂ complexes than in the wild type, indicating that the integrity of the complex is compromised by these mutations. We refer to this phenomenon as “disengagement of HisP from HisQM.” Concomitantly, these mutant complexes display a higher rate of constitutive ATP hydrolysis. The behavior of these mutant HisP*’s resembles that of peripheral membrane proteins, so that they could be viewed as interaction mutants. Such a notion is consistent with the recent resolution of the crystal structure of HisP, which was shown to have an “L” shape, with two separate arms: arm I is mostly dedicated to forming the ATP-binding pocket, whereas arm II is likely to form the domain that interacts physically with HisQM (20). The formation of the correct quaternary structure of the complex would be expected to be dependent upon the nature of the interaction between arm II of HisP and HisQM. Such a quaternary structure is likely to be essential not only for the correct enzymatic activity of the complex but also for creating the signaling pathway that regulates its ATPase activity. The fact that all of the mutations considered in this paper, which simultaneously result in a loosener complex structure and in signal-independent ATPase activity, are clustered and located in arm II is consistent with the idea that arm II interacts with HisQM and that the mutated residues are involved either directly or indirectly in an interaction with HisQ and/or HisM.

If the hypothesis of an interaction between arm II and HisQM is correct, it should be possible to identify mutants in arm II that have completely eliminated the interaction between HisP and HisQM, resulting in a complex that is altogether unable to insert into the membrane. Mutants of this type are likely to exist but would have gone unrecognized through our selection procedures because they would be inactive in transport activity. It is worthwhile stressing the fact that all of the mutations analyzed here had been isolated with the requirement that transport activity be maintained (in the absence of the periplasmic binding protein). Thus, they all have modified the function of the complex but have not inactivated it. It also should be mentioned that mutations with properties equivalent to those of HisP* mutants but located in the hydrophobic subunits should also exist. Likely candidates indeed have been
ATPase Regulation of ABC Transporters by NBD Disengagement

| Membrane-bound proteins | Cooperativity for ATP | Affinity for ATP | Affinity for ADP | Initial rate of ATP hydrolysis |
|-------------------------|----------------------|-----------------|-----------------|-----------------------------|
| Wild type HisQMP_2      | Yes                  | \( K_c = 0.6 \) | \( IC_{so} = 0.1 \) | 0.13 \( \mu \text{mol/min/mg} \) |
| HisQMP*_P(T127T)        | No                   | \( K_c = 0.9 \) | \( IC_{so} = 0.6 \) | 3.3 \( \mu \text{mol/min/mg} \) |
| HisQMP*_M(T205A)        | No                   | \( K_c = 1.2 \) | \( IC_{so} = 0.5 \) | 2.5 \( \mu \text{mol/min/mg} \) |

- Assayed by inhibition of ATP hydrolysis by ADP in 1 mM ATP.
- Potential ATP hydrolysis turnover numbers for soluble HisP in dimeric form. The rate for wild type-soluble HisP has been described previously (19).

ATPase activity suggests that engagement of HisP leads to suppression of the ATPase. Such a suppression (when the complex is in the resting stage, see below) could be exercised in a variety of ways. HisP might come in direct contact with phospholipids that inhibit the ATPase activity (19), or its nucleotide-binding pocket may be disrupted or not be fully accessible, or HisQM might increase the affinity for ADP, thus slowing down the rate of ADP release (HisQMP_2 indeed has higher affinity for ADP than soluble HisP, with IC_{50} values of 0.1 \text{versus} 0.7 \text{mM} ADP, respectively).

The proposed cycle of membrane disengagement/reengagement is reminiscent of SecA, the E. coli translocation ATPase, which undergoes ATP-dependent membrane insertion and deinsertion (39). However, ATP binding and hydrolysis have been modeled to achieve different goals in the process of membrane cycling. Upon ATP hydrolysis, SecA releases the preprotein peptide and deinserts from the membrane, whereas in the case of HisP, ATP binding causes the disengagement of HisP and reengagement occurs after hydrolysis. The fundamental difference between HisP and SecA may be related to the difference in transport direction: SecA translocates peptides from inside to outside (39–41), whereas the histidine permease translocates substrate from outside to inside.

The fact that the ATPase activity of wild type HisQMP_2 shows positive cooperativity for ATP, whereas that of the HisQMP* mutants does not, provides some insight into the mechanism of ATP hydrolysis. Since such cooperativity is observed both for the HisJ-stimulated and intrinsic activities (15), it must be independent of HisJ signaling. Because mutant HisQMP*_P is already disengaged in the absence of ATP and shows no cooperativity for ATP, the positive cooperativity observed for the wild type may be explained by an initial binding of ATP to a low affinity site that induces HisP disengagement, with the disengaged complex subsequently binding the second ATP with higher affinity. It is also relevant that HisP dimer in the soluble form, which can be viewed as fully disengaged, also shows no cooperativity (19). The two identical HisP molecules within a complex may acquire different ATP-binding and -hydrolyzing properties because of an asymmetric interaction with the two different subunits, HisQ and HisM. It should be noted, however, that the two cooperative binding sites could also be one in HisQ and the other in one of the HisP subunits, since HisQ is known to bind ATP (16, 42). The lack of cooperativity in HisQMP*_P is in contrast with the results presented in the equivalent type of binding protein-independent mutants in the maltose system, which retain cooperativity (43). The reason for this discrepancy is not clear. It is possible, although we consider it unlikely, that the two permeases differ in the mechanism of transport; alternatively, the fact that the mutations

**Fig. 8.** Active HisP membrane dissociation detected by an exchange reaction. Membrane vesicles containing 2 \( \mu \)g of HisQMP*_P (as determined by densitometric quantitation using pure HisQMP_2 as standard) were incubated together with 15 \( \mu \)g of purified wild type soluble HisP_{dimer}, in the presence of 2 mM ATP, 4% glycerol, 50 mM MOPS/K+, pH 7.0, in a volume of 200 \( \mu \)l, on ice for 5 min before assaying for ATP hydrolysis. The ATPase assay was then initiated by the addition of MgSO_4 to a final concentration of 10 mM. The initial rate of ATP hydrolysis was determined in the absence (5th column) or presence of 15 \( \mu \)g of purified HisP_{dimer}. Each assay point contained 25 \( \mu \)l of the mixture. The same HisQMP*_P (P172T) membrane vesicles were also incubated with the same storage buffer (without HisP protein) in assay buffer, and the initial rate of ATP hydrolysis was assayed in the absence or presence of liganded HisJ. ATP hydrolysis by the same purified soluble HisP_{dimer} was also assayed with or without HisJ. * indicates the absence of HisJ; + indicates its presence. HisP* represents wild type HisP_{dimer}.

identified both in HisQ and in the hydrophobic components of the maltose permease (38).

The observation that HisP* is disengaged has important implications for the mechanism of ATPase activation. ATP hydrolysis by HisP during the transport cycle is regulated by HisQ and HisM through a balanced counterforce of stimulation and suppression (15, 18, 19). HisQM, through its physical association with HisP, would exercise such a dual role and regulate the ATPase activity, thus preventing useless ATP consumption and promoting a swift response when a ligand is presented. During a cycle of ATP hydrolysis, HisQ and HisM may alternate between an activating and a suppressing mode, with HisQM assuming the activating mode when liganded HisJ is present and the suppressing mode in the absence of HisJ. The fact that a tight complex structure is associated with low ATPase activity suggests that engagement of HisP leads to suppression of the ATPase. Such a suppression (when the complex is in the resting stage, see below) could be exercised in a variety of ways. HisP might come in direct contact with phospholipids that inhibit the ATPase activity (19), or its nucleotide-binding pocket may be disrupted or not be fully accessible, or HisQM might increase the affinity for ADP, thus slowing down the rate of ADP release (HisQMP_2 indeed has higher affinity for ADP than soluble HisP, with IC_{50} values of 0.1 \text{versus} 0.7 \text{mM} ADP, respectively).

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causing constitutivity occur in a hydrophobic subunit in the maltose system and in the ATP-binding subunit in the histidine system might result in different behavior.

Although HisP* disengagement appears to be a priming mechanism for ATPase activation, it is not sufficient to activate ATP hydrolysis. The presence of ATP alone disengages wild type HisP but does not result in ATP hydrolysis in the absence of HisJ. Hydrolysis is not necessary for disengagement because several mutant complexes that bind ATP normally, but cannot hydrolyze it, display an ATP-induced disengagement (as measured by urea extraction) identical to that of wild type HisP (data not shown). Although HisJ stimulates ATP hydrolysis, it was not found to induce HisP disengagement by itself. Soluble HisP* (i.e. fully disengaged) does not hydrolyze ATP at a rate as high as its potential, i.e. as high as that of fully stimulated HisQP2* (19). Therefore, there must be other events or determining factors regulating the rate of ATP hydrolysis by the wild type complex. The fact that soluble HisP* mutants hydrolyze ATP at a higher rate than soluble wild type HisP (Table II) suggests that this other factor(s) reflects an intrinsic property of HisP. A possible factor is suggested by the finding that the mutants have a poorer affinity for ADP than the wild type, both in the soluble and complex form (Table II); thus, their higher level of ATP hydrolysis may result from an accelerated rate of ADP release.

In light of our results, we propose a revised model for binding protein-dependent ABC transporters (Fig. 9). In the resting stage (stage I) HisP is engaged and inactive. Upon binding ATP, the complex assumes a different quaternary structure, in which the ATP-binding subunit becomes loosely attached (disengaged; stage II). ATP hydrolysis follows upon signaling by the soluble receptor, which alters the conformation of HisQM (stage III), followed by the opening of a passageway within the complex thus allowing substrate translocation (stage IV). In stage II, despite disengagement of HisP, ATP is hydrolyzed at a low rate (intrinsic ATPase activity), because HisQM is still in the suppression mode and HisP has a high affinity for ADP, which is therefore released slowly. In stage III, the liganded binding protein converts HisQM to an activating mode, which enables rapid ADP release. HisQMP2* mutants already have a disengaged structure in the resting stage, in which HisP* mimics stage III of the wild type even in the absence of the binding protein and with HisQM in the stage I/II conformation. These mutants are always in a primed state for hydrolysis, because HisP* is constantly in an activated form and has a lower affinity for ADP.

Although this model provides a mechanism specific for the binding protein-dependent ABC transporters, these results could be extrapolated to other members of the superfamily. This may be particularly true because the structure of the HisQMP2* mutants is reminiscent of that of eukaryotic ABC transporters in that neither relies on a soluble receptor for ATP hydrolysis and transport. In this respect it is interesting to compare the properties of HisQMP2* mutants with those of the most common mutation, ΔPhe-508, in CFTR in which a phenylalanine residue (Phe-508 in NBD1) has been deleted. Upon alignment of the sequence of CFTR with that of HisP, Phe-508 is found to be located in a position in the crystal structure very similar to that of the HisP* mutants described here (20). Thus, a reasonable hypothesis is that ΔPhe-508 is defective in the interaction between NBD1 and its respective hydrophobic domain(s), and as a consequence its overall folded structure is incorrect. This proposal is compatible with the finding that among the characteristics of ΔPhe-508 are its inability to reach its final site of action and the fact that the defect can be obviated by protein-stabilizing conditions, such as the presence of glycerol or growing the cells producing ΔPhe-508 at low temperature (44-47). Presumably, the formation of a defective folded structure during CFTR synthesis affects its insertion into the membrane and results in mislocation of the protein. It should be stressed that the defect of ΔPhe-508 is not in its activity and that this situation is similar to that of HisP* mutants which, although defective in the structure of the complex, are still functional in ATP hydrolysis and transport (27, 30).

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