One Intact ATP-binding Subunit Is Sufficient to Support ATP Hydrolysis and Translocation in an ABC Transporter, the Histidine Permease*

Kishiko Nikaido and Giovanna Ferro-Luzzi Ames†

From the Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology, University of California, Berkeley, California 94720-3202

The membrane-bound complex of the Salmonella typhimurium histidine permease, a member of the ABC transporters (or traffic ATPases) superfamily, is composed of two integral membrane proteins, HisQ and HisM, and two copies of an ATP-binding subunit, HisP, which hydrolyze ATP, thus supplying the energy for translocation. The three-dimensional structure of HisP has been resolved. Extensive evidence indicates that the HisP subunits form a dimer. We investigated the mechanism of action of such a dimer, both within the complex and in soluble form, by creating heterodimers between the wild type and mutant HisP proteins. The data strongly suggest that within the complex both subunits hydrolyze ATP and that one subunit is activated by the other. In a heterodimer containing one wild type and one hydrolysis defective subunit both hydrolysis and ligand translocation occur at half the rate of the wild type. Soluble HisP also hydrolyzes ATP if one subunit is inactive; its specific activity is identical to that of the wild type, indicating that only one of the subunits in a soluble dimer is involved in hydrolysis. We show that the activating ability varies depending on the nature of the substitution of a well conserved residue, His-211.

The superfamily of ABC transporters (or traffic ATPases) comprises both prokaryotic and eukaryotic proteins that share a conserved nucleotide-binding domain (1, 2) and a similar predicted secondary structure (3). The superfamily includes, among others, bacterial periplasmic permeases, the yeast STE6 gene product, the mammalian P-glycoprotein (or multidrug resistance protein, MDR)3, the human cystic fibrosis transmembrane conductance regulator (CFTR), the mammalian heterodimeric transporter (TAP1/TAP2) involved in antigen processing (3, 4), and the retina-specific transporter implicated (27) in the retina-specific transporter involved in several findings as follows: hydrolysis of ATP displays positive cooperativity with a Hill coefficient of n = 2 (8); chemical cross-linking of purified HisQMP2 yields a cross-linked HisP dimer4; reconstitution of the ATPase activity of HisQMP2 after disassembly of the complex and depletion of HisP requires the presence of two molecules of HisP (16). HisP has been purified and characterized also in an active soluble form, away from HisQ and HisM (21). Among its characteristics important for this study are the following: soluble HisP functions only as a dimer, it hydrolyzes ATP in the absence of liganded HisJ (i.e., in the absence of a signal), and it does not display cooperativity. Its three-dimensional structure has been resolved (22), and in agreement with the biochemical characteristics, it has a dimeric structure. Each subunit in the dimer has two arms in the shape of an “L.” Arm I contributes many of the ATP-binding pocket residues, and arm II appears to be embedded in the membrane by interaction with HisQM and is likely to be involved in the signaling mechanism.

Important questions concerning the precise function and relationship to each other of the two nucleotide-binding sites within ABC transporters have not yet been resolved. In the case of MDR, inactivation of only one of the two ATP-binding sites, either mutationally (23, 24) or chemically (25, 26), results in loss of activity by the other site. A similar finding was obtained for the bacterial transporter, the maltose permease (27). These findings are consistent with the notion that the two sites interact with each other. In the case of the histidine permease, the two HisP subunits are identical and might be expected to function identically. However, this is not necessar-

* This work was supported by National Institutes of Health Grant DK12121 (to G.F.-L.A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: Dept. of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology, 401 Barker Hall, University of California, Berkeley, CA 94720-3202. Tel.: 510-643-1979; Fax: 510-643-7935; E-mail: giovanna@dtc4.berkeley.edu
3 The abbreviations used are: MDR, multidrug resistance protein; CFTR, cystic fibrosis transmembrane conductance regulator; HisP, histidine permease of Salmonella typhimurium and the maltose permease of Escherichia coli have been characterized and provide excellent models for understanding the mechanism of action of this superfamily (6–9). The histidine permease, extensively studied in this laboratory, is composed of a soluble substrate-binding receptor, HisJ (the periplasmic histidine-binding protein), and a membrane-bound complex, HisQMP2, comprising two hydrophobic integral membrane-spanning proteins, HisQ and HisM, and two copies of HisP that carries a periplasmic dimer (10–13). ATP hydrolysis provides the energy for the transport process (8, 14, 15). HisP is strongly suggested to be a typical peripheral membrane protein, as shown by the poor release by urea (16) and other reagents (13), and its accessibility at both surfaces of the cytoplasmic membrane (17). ATP hydrolysis and ligand translocation are dependent on a signaling mechanism originating from the binding protein and traveling through HisQ/HisM (16). The entire HisQMP2 complex has been purified and characterized (20) and reconstituted into proteoliposomes (7). The HisP subunits interact with each other, as substantiated by several findings as follows: hydrolysis of ATP displays positive cooperativity with a Hill coefficient of n = 2 (8); chemical cross-linking of purified HisQMP2 yields a cross-linked HisP dimer4; reconstitution of the ATPase activity of HisQMP2 after disassembly of the complex and depletion of HisP requires the presence of two molecules of HisP (16). HisP has been purified and characterized also in an active soluble form, away from HisQ and HisM (21). Among its characteristics important for this study are the following: soluble HisP functions only as a dimer, it hydrolyzes ATP in the absence of liganded HisJ (i.e., in the absence of a signal), and it does not display cooperativity. Its three-dimensional structure has been resolved (22), and in agreement with the biochemical characteristics, it has a dimeric structure. Each subunit in the dimer has two arms in the shape of an “L.” Arm I contributes many of the ATP-binding pocket residues, and arm II appears to be embedded in the membrane by interaction with HisQM and is likely to be involved in the signaling mechanism.

Important questions concerning the precise function and relationship to each other of the two nucleotide-binding sites within ABC transporters have not yet been resolved. In the case of MDR, inactivation of only one of the two ATP-binding sites, either mutationally (23, 24) or chemically (25, 26), results in loss of activity by the other site. A similar finding was obtained for the bacterial transporter, the maltose permease (27). These findings are consistent with the notion that the two sites interact with each other. In the case of the histidine permease, the two HisP subunits are identical and might be expected to function identically. However, this is not necessar-
RESULTS

Abundant evidence indicates that HisP must dimerize in order to hydrolyze ATP, suggesting that the subunits assume an “activated” conformation upon dimerization. Two possibilities were considered for the basis of the activity as expressed by the wild type HisP dimer, both in the soluble and membrane-bound forms as follows: (i) both subunits hydrolyze ATP in each turnover cycle; (ii) only one subunit hydrolyzes ATP with the other one functioning as an “activator.” In the first case the turnover number per molecule of dimer would be twice as high as in the second case. If a heterodimer were constructed in which one of the two subunits were able to bind ATP but not hydrolyze it, the wild type subunit might still be able to carry on hydrolysis in either of the above cases. If this were true, the specific activity of the heterodimer would be half as much as that of the wild type homodimer if both subunits normally hydrolyze ATP, but it would be the same as that of the wild type if only one subunit normally hydrolyzes ATP, suggesting that the “activation” can proceed in the absence of hydrolysis. Alternatively, the heterodimer might be altogether unable to hydrolyze ATP, as has been reported for the maltose permease (27), which would suggest that activation of one subunit requires ATP hydrolysis and possibly binding by the other subunit. A comparison of the specific activities of the wild type homodimer and of heterodimers in which one of the subunits is unable to hydrolyze ATP would provide useful information for these questions. The characteristics of HisP mutants with respect to the ability to bind and hydrolyze ATP have been described (6). On this basis we selected HisP mutants that bind but do not hydrolyze ATP to test whether they have retained the presumed activation function. Since HisP is now available both in the soluble and in the complex-bound form, the properties of homodimers and heterodimers of HisP can be studied under both conditions.

Soluble HisP

We first analyzed the behavior of soluble HisP, which constitutes a simpler system because the formation of various heterodimers can be controlled by varying the amounts of the individual soluble species in appropriately calculated proportions. Wild type HisP carrying a 6-histidine residue carboxy-terminal extension was purified in soluble form as described (21). In agreement with previous findings (8, 16, 21), the ATPase activity of soluble wild type HisP is concentration-dependent because activity requires dimerization: at 50 μM protein the specific activity is very low, 160 nmol/min/mg, on average (Fig. 1A, open bars). As the concentration of HisP is increased, the concentration of the dimer increases and the specific activity increases correspondingly, reaching 515 nmol/min/mg when the protein concentration is 200 μg/ml.

Behavior of Mutations Eliminating ATP Hydrolysis—Several soluble mutant HisP proteins were similarly purified after introducing each mutant hisP gene of interest into a T7 promoter plasmid vector capable of overproducing the HisP protein containing a carboxy-terminal extension of 6 histidine residues. Mutations in the hisP gene which eliminate substrate translocation have been characterized according to their ability to bind ATP (6). A good candidate for testing the presumed activation ability is mutation hisP5551, in which residue His-211 has been replaced by arginine, coding for protein H211R, and is followed by the residue number and the replacing residue.
ATPase in ABC Transporter with One Defective Nucleotide Site

To support this conclusion, the activities of the wild type dimer and of the heterodimer were determined also under conditions where the ratio of H211R to wild type was maintained constant (1:1), whereas the total protein concentration was increased from 25 to 200 μg/ml. Fig. 2 (open circles) shows that, as expected, because of the dependence on dimerization, the activity obtained for the wild type alone increases faster than the increase in protein concentration; this relationship is linear when the activity is expressed relative to picomoles of dimer present (Fig. 2, inset). The activity of the mixture containing equal amounts of wild type and H211R similarly increases, although more slowly (solid diamonds), as would be expected from a fraction of the dimers being inactive (i.e. mm). If the activity of the mixture is corrected for that due to the formation of wild type homodimers and expressed relative to the number of picomoles of heterodimer, a linear relationship essentially identical to that for the wild type is obtained (Fig. 2, inset). Thus, these results support the conclusion that only one of the two subunits hydrolyzes ATP in both the wild type homodimer and the heterodimer.

Another mutation analyzed is hisP6666, coding for protein D185E, which also binds but does not hydrolyze ATP.8 The behavior of mutant D185E is somewhat different from that of H211R. Fig. 1A (last bar) shows that it has an insignificant level of activity at a protein concentration of 200 μg/ml. Adding D185E to a fixed low concentration of wild type HisP results in an increase in activity (Fig. 1B, bars with horizontal lines), indicating that it also is capable of forming active heterodimers. However, Fig. 1B and Fig. 2 (crosses) show that D185E yields a lower level of activity than H211R when combined with wild type. If the measured activity is subjected to the same calculations as described for H211R, the calculated heterodimer specific activity is lower than that of the wild type homodimer and of the heterodimer with H211R (Table I). Thus, the nature of the residue being replaced affects the properties of the heterodimer. The possibility was also considered that the properties of the heterodimer depend also on the nature of the replacing residue. Therefore a hydrolysis-negative mutant HisP in which His-211 is replaced with aspartate (H211D) was also tested. Interestingly, H211D is unable to activate the wild type subunit when present in a heterodimer. These results are analyzed under “Discussion.”

A useful type of mutant would be one in which the ATP-binding ability is lost altogether. Such a mutant would allow the determination of whether the ATP-binding ability needs to be retained in order for the inactive subunit to activate hydrolysis by the wild type subunit. Unfortunately, although hisP mutants that do not bind ATP are available (6), this parameter cannot be tested at this time because these mutant proteins cannot be purified in useful amounts since soluble HisP precipitates out of solution if it is not liganded with ATP (21). It should be possible to mimic these results by inactivating HisP chemically and then forming a heterodimer between the inactivated subunit and an unaltered HisP. Soluble HisP was treated with 1 mM mercuric chloride which eliminates the ATPase activity in the complex.9 The activity was indeed lost upon this treatment; however, HisP precipitated out of solution (a possible explanation is that upon mercury treatment ATP is lost from HisP causing the protein to precipitate out of solution (21)). Since soluble HisP is insensitive to vanadate, neither of these chemical inactivation procedures could be tested.

---

7 Dimers are referred to as \textit{ww}, or \textit{mm}, or \textit{wm}, if they are composed of two wild type, or two mutant, or one wild type and one mutant subunit(s), respectively.

8 P.-Q. Liu, personal communication.

9 D. Kreimer, personal communication.
ATPase in ABC Transporter with One Defective Nucleotide Site

### Table I

| Subunit     | Dimer concentration | Activity units attributable to wmb | Measured activity | Specific activity of wmb |
|-------------|---------------------|-----------------------------------|-------------------|--------------------------|
| Wild type   | µg/ml               | pmol/ml                           | Total             | wmb                      |
| Mutant      | pmol/ml             | wm/ww/mm                          |                   |                          |
| 50          | 0                   | 32/0/0                            | 6.3               | 0                        |
| 50          | 25                  | 63/28/28                          | 12.4              | 5.1                      |
| 50          | 50                  | 103/51/51                         | 17.9              | 11.1                     |
| 50          | 100                 | 209/22/22                         | 31.6              | 25.6                     |
| 50          | 150                 | 356/22/22                         | 42.4              | 37.0                     |

The ATPase activity of soluble HisP was measured as described under “Experimental Procedures” and is expressed in units. One unit is the amount of activity that liberates 1 nmol of P inside ml of reaction mixture at 37 °C.

# Behavior of Signal-independent Mutations—A particularly interesting type of HisP (referred to as HisPbw), which possesses constitutive ATPase activity because it is released from signaling by HisJ and thus by regulation via HisQ/M, was also examined (18, 19, 33). Several such hisPbw mutants imparting this property are known; in the crystal structure of HisP they are clustered in arm II (22). Such a location is consistent with the hypothesis that arm II is involved in signaling and that these mutations have altered this process. As would be expected, the respective soluble HisPbw mutant proteins bind and hydrolyze ATP. One of these mutant proteins, P172T, was analyzed for its properties both as a homodimer and as a heterodimer in combination with H211R. Fig. 1A (bars with diagonal lines) shows that also P172T is dependent on the formation of a dimer for activity; however, the activity of its homodimer is higher than that of the wild type homodimer at all (total) protein concentrations tested. Its specific activity of 0.6 nmol/min/pmol dimer is about twice the specific activity of the wild type homodimer. The interpretation of this finding can be either that both dimer subunits hydrolyze ATP or that only one subunit hydrolyzes it but does so at double the rate of the wild type. If the first possibility were true, the specific activity of a heterodimer with an inactive subunit should be half that of the P172T homodimer, while it would be the same if the second possibility were true. Assay of the activity of a heterodimer formed between P172T and the hydrolysis-negative mutant H211R shows that such a heterodimer indeed is able to hydrolyze ATP (Fig. 1C). The specific activity of the heterodimer, calculated as described in the legend to Table I, is 0.27, which is essentially the same as that of the wild type homodimer and about half that of the P172T homodimer. The affinity of the P172T subunits for each other was measured, and it is the same as in the case of the wild type homodimer (data not shown) (21). Thus, it appears that both subunits hydrolyze ATP in the P172T homodimer, resulting in double activity; presumably the mutation has relieved HisP of regulation both by HisQ/M and by the other HisP subunit, resulting in unbridled ATPase activity by both subunits. These activation data also indicate that it is unlikely that the activity measured in the wild type/H211R heterodimer is due to a retrieval of the hydrolyzing activity of H211R. Support for this notion is also provided by experiments in which the activity of a heterodimer between wild type and P172T was shown to average 80% of the activity of the P172T homodimer.

# Membrane-bound HisP

The possibility was considered that the HisP dimer incorporated into the membrane-bound complex behaves differently than when it is in soluble form, as has been shown for a number of other properties (16, 21, 30). Thus, the heterodimer activity within the membrane-bound complex was examined. This approach has been made possible by the recent development of a technique for disassembling and reassembling the membrane-bound complex in vitro (16). In brief, membrane vesicles containing wild type complex are stripped of HisP with urea, and the complex is reconstituted by the exposure of stripped vesicles to appropriate amounts of soluble wild type and mutant...
ATPase in ABC Transporter with One Defective Nucleotide Site

HisP. It has been shown that HisP subunits enter the complex one at the time and independently of each other (16). Fig. 3 shows a reassembly experiment using increasing identical concentrations of either wild type HisP (open circles) or of a mixture of wild type and H211R at the fixed ratio of 1:1 (solid diamonds). It can be seen that in the presence of the mixture the reassembly yields about half as much HisJ-stimulated ATPase activity as in the presence of only wild type HisP. Assuming that there is no preference in reassembly between the two types of subunits, the relative number of each of the possible dimers formed at each protein concentration in the mixture should remain the same, i.e., the homodimers, ww and mm (active and inactive, respectively), are each 25% of the total, and the heterodimer, wm, constitutes 50% of the total. If the only active species were ww, the overall activity should amount to 25% that of the sample containing only wild type. Because the resulting activity is higher than 25%, it must be concluded that the heterodimer contributes some of the activity. The specific activity of the wm heterodimer should be 50% that of the wild type homodimer ww, if both subunits in ww hydrolyze ATP, and its contribution would be 25% of the total activity (despite its constituting 50% of all dimers). If this were true, the total activity should be 50% as much as that of the ww homodimer. Fig. 3 shows that this is the case. On one hand it appears that, in contrast to the situation as found with soluble HisP, in the wild type membrane complex both HisP subunits hydrolyze ATP, and on the other hand, similar to the situation with soluble HisP, a wild type subunit can be activated to hydrolyze ATP by forming a heterodimer with an inactive subunit.

To support this conclusion, the relative amounts of wild type and mutant HisP in the reassembly mixture were varied, thus changing the proportion of the respective dimers and, consequently, varying the total activity proportionately. In the experiment shown in Table II, the total amount of HisP was maintained constant (20 μg/ml), with the ratios of wild type to H211R monomers varying. The conditions were chosen to ensure the incorporation of all of the HisP present in the dimer form (16). It can be seen that, as expected, the measured HisJ-stimulated activity decreases with the increasing percentage of H211R in the reassembly mixture (4th column). The distribution of the various dimers can be calculated (2nd and 3rd columns). The ATPase activity corrected for the contribution due to the mutant homodimer (mm), which has measurable activity if incorporated in the complex, is shown in column 4. The last two columns describe the activity attributable to the wm dimer (obtained by subtracting the activity of ww) and its specific activity at each ratio of subunit type. In agreement with the results described above, the specific activity of wm is half of the specific activity measured for ww as follows: 1.2 nmol of P/μg of HisP/min, versus 2.4. This supports the notion that in the wild type membrane complex both HisP subunits hydrolyze ATP.

The above conclusion would be strengthened considerably by demonstrating the physical existence of a heterodimer and comparing its characteristics once it is separated from a wild type homodimer. For this purpose, a heterodimer was created between wild type that does not carry a 6-histidine residue extension and H211R carrying such an extension. Wild type HisP was purified almost to homogeneity in two simple steps as follows: ammonium sulfate precipitation and molecular sieving. Wild type HisP, which does not carry a 6-histidine residue extension, can be easily distinguished by SDS-PAGE from H211R(his6), which, having a slightly higher molecular mass, migrates more slowly (Fig. 4A, lane 3). The heterodimer was formed by stripping membrane vesicles originating from strain TA1889, which overproduces wild type HisQMP2 (29), and incubating the stripped membranes with soluble wild type HisP and H211R(his6), present in equal amounts. Solubilization of the reassembled mixture and application of the complexes onto a TALON column (20, 30) resulted in the separation of two classes of complexes, eluting at different imidazole concentrations, 25 and 50 mM, respectively. The complex eluted at 25 mM imidazole contains two HisP forms, with mobilities corresponding to wild type and H211R(his6), respectively, whereas the complex eluting at 50 mM imidazole contains only H211R(his6) (Fig. 4A, lanes 1 and 2, respectively). This elution difference is due to the different content in 6-histidine residues present in these respective complexes. The complex containing wild type HisP homodimer is not retained by TALON, as expected, and appears in the flow-through fraction (not shown). This method is similar to one utilized for separating heterodimeric complexes of the maltose permease (27). All of these fractions contain HisQ and HisM, in addition to HisP, as seen faintly by Coomassie Blue staining of the SDS-PAGE in Fig. 4A, lanes 1 and 2, and clearly by immunoblotting (Fig. 4B, lanes 4 and 5). Thus, a complex containing a wm heterodimer is formed and can be purified. To demonstrate that the heterodimer-containing complex indeed possesses activity, the purified complex was reconstituted into proteoliposomes by the standard procedure (7) and its ATPase activity assayed; it was clearly shown to have ATPase activity, although because of the scarcity of this material it was not possible to determine accurately its specific activity. Therefore, an alternative procedure was devised that allows the production of larger amounts of purified heterodimer-containing complex.

This procedure, which involves an exchange of soluble HisP with native complex-bound HisP, results in larger amounts of purified material, eliminates possible artifacts arising during urea treatment that might alter the specific activities of the complex, and it eliminates the need to purify HisP in the absence of a his6 tail extension. In brief, membrane vesicles containing native overproduced complex are incubated in the presence of soluble H211R(his6) followed by solubilization and TALON purification as usual. The dimer and the heterodimer are eluted from TALON exactly as described for the reassembled complex. About 0.5 mg of heterodimer can be obtained from 40 mg of total membrane in this way, which can be...
utilized for reconstitution into proteoliposomes and for the determination of the ATPase specific activity. The abundance of the material recovered by this method also permits assay of histidine transport and chemical cross-linking of the subunits. Table III shows both the ATPase and the transport activities. It can be seen that, in agreement with the results obtained above, the heterodimer has half the ATPase activity of the homodimer. Interestingly, the rate of histidine transport in the heterodimer has half the ATPase activity of the homodimer. Therefore, proteoliposomes reconstituted either with the homodimeric HisP or with the heterodimeric complex were cross-linked using formaldehyde (34) and the products resolved by SDS-PAGE. Visualization by immunoblotting with antibodies raised against HisP (Fig. 4C) and against the his 10 tail (data not shown) reacted with both cross-linked dimers; a cross-linked dimer of HisP wild type (i.e. HisP with no his 6 tail) (lanes 1) or a homodimer of wild type HisP with HisP with no his 6 tail (lanes 1) or a homodimer of wild type HisP with a HisP molecule with a HisP molecule with a his 6 tail (H211R) and a lower molecular weight one (wild type without a his 6 tail). These results eliminate the possibility that the complexes have rearranged themselves during the manipulations, thus losing the heterodimer.

For the purpose of understanding the mechanism of action, it is interesting to determine whether the heterodimer displays positive cooperativity. The ATPase activities of the wild type homodimer and of the heterodimer containing H211R were tested at various ATP concentrations. Although the homodimer displayed, as expected, positive cooperativity (8), the heterodimer did not (Fig. 5). This result is discussed later.

In conclusion, these results support the notion that in the membrane-bound complex both HisP subunits in a dimer hydrolyze ATP, that only one of the two ATP-binding sites needs to be active for hydrolysis and translocation to proceed, and that dimerization with an inactive subunit can activate the wild type subunit.

**DISCUSSION**

The data presented demonstrate that both nucleotide-binding subunits need not be intact to allow hydrolysis of ATP, in the case of both the membrane-bound complex and soluble HisP. How do the data presented fit with what is known about the mechanism of ATP hydrolysis in ABC transporters? By combining these with previous data, the following overall picture emerges. The ATP-binding subunits interact with each other forming a dimer within the membrane-bound complex. The subunits interact in a functional sense because they must both be present for ATP hydrolysis to occur, i.e. one subunit activates the other. Activation and hydrolysis are separate
functions. Binding or hydrolysis of ATP by one of these subunits increases the affinity of the other for ATP, as indicated by the fact that the ATPase activity displays positive cooperativity. Since the complex hydrolyzes ATP at half the specific activity if one of the two ATP-binding subunits is inactive, it appears that in the wild type homodimer both subunits hydrolyze ATP, i.e., two ATP molecules are hydrolyzed within one cycle of activity. The loss of cooperativity in a heterodimer is consistent with the notions that the subunits talk to each other and that one subunit affects the ATP affinity of the other. Finally, each subunit is separately responsible for a specific ratio of hydrolysis to translocation events because transport activity is also halved in a heterodimer (although we have no information relative to the stoichiometry between hydrolysis and translocation).

Activation is an intrinsic property of the HisP molecule and is not dictated by interaction with HisQM, because soluble HisP dimers also require activation. In addition, it appears that the ability of one subunit to activate the other is separate from the ability to increase the affinity for ATP, since soluble HisP does not display positive cooperativity (21). In soluble HisP there appears to be only one hydrolysis event in each cycle, because a soluble heterodimer has the same specific activity as the wild type homodimer. This suggests that the presence of HisQM imparts special properties to one or both of the HisP subunits, such that in the complex both of them hydrolyze ATP within one cycle. It is also possible that asymmetric interactions between the identical ATP-binding subunits and the two dissimilar proteins, HisQ and HisM, are responsible for imparting positive cooperativity. Conversely, the lack of these interactions in soluble HisP results in a single hydrolysis event per cycle and loss of cooperativity. A possible explanation is that a soluble dimer falls apart after one hydrolysis event because it is not held together by HisQM. Consistent with the notion that HisQM contributes to cooperativity is the finding that constitutive HisP mutants, which do not display cooperativity but still require activation within the dimer, form a loosely assembled complex from which HisP is easily released (a phenomenon referred to as “disengagement” (37)). The view that disengagement of HisP* from HisQM leads to lack of cooperativity is also consistent with the lack of cooperativity in soluble HisP, which could be viewed as being completely disengaged. In this respect, it might be useful to view the HisP* mutants as a better model for eukaryotic systems than the wild type since, like them, they do not require a soluble receptor.

What does the process of activation within a dimer involve? Since we have shown that there is a difference in activation ability between H211R and H211D, a comparison of the structural characteristics of these two proteins should be valuable. First, what is the function of His-211, a strongly conserved residue in periplasmic permeases (11) and in the ABC superfamily in general? From the crystal structure of HisP (22), it can be seen that His-211 must be intimately involved in the mechanism of hydrolysis since it forms a hydrogen bond with the γ-phosphate of ATP via a water molecule. This hydrogen bond is likely to be important for stabilizing the γ-phosphate for successful hydrolysis. Resolution of the crystal structure of both H211R and H211D indicates that in H211R the hydrogen bond to the γ-phosphate is slightly different, possibly making it weaker; this same bond is completely missing in H211D. Although this observation might explain the hydrolysis defect of H211R and H211D, it does not help to understand the difference in their subunit activation property because His-211 and its immediate area are far removed from the interface between the monomers (22). Interestingly, in the case of the maltose permease, it was shown that a heterodimer containing mutant H192R as the inactive subunit is completely unable to hydrolyze ATP (27). Because His-192 in MalK and the replacing residue in that study, arginine, are exactly the same as the ones we have analyzed here, this result is surprising, also considering that this histidine residue is very well conserved. We have no explanation for this difference.

Another residue analyzed, Asp-185, is exposed and located on the surface of arm II and has no obvious interaction with the bound ATP (22). Therefore, it is not clear why D185E is defective in ATP hydrolysis and has a poor ability to activate the wild type subunit. The crystal structure of D185E is in progress, which will give some clue as to the nature of its defect.

It is interesting to compare our findings using the histidine permease with those obtained for other ABC transporters. In the case of MDR, there is strong evidence that the MDR nucleotide-binding sites interact with each other. It has been found that both sites hydrolyze ATP and that both sites must be intact for any hydrolysis to occur. However, they do not display

---

10 Although this interpretation is the simplest, the possibility should be considered that within the complex one HisP molecule interacts specifically with HisQ, whereas the other interacts with HisM, resulting in hydrolysis competence in only one of these combinations (e.g., only the HisP-HisQ combination can hydrolyze ATP in each cycle). If this were true, the finding that the rate of hydrolysis is halved in the heterodimer could be explained by lack of activity in the heterodimers in which the mutant HisP has interacted with HisQ. We consider that this possibility is less likely also because in other ABC transporters both nucleotide-binding domains have been shown to hydrolyze ATP (e.g., MDR (35) and CPTR (reviewed in Ref. 36)).

11 It should be noted that in the maltose system equivalent signal-independent mutant complexes maintain cooperativity (38). The reason for this discrepancy with the histidine permease is not clear, although it is possibly due to the fact that the maltose mutations are multiple and located in the hydrophobic subunits, whereas those in the histidine permease are all single events and located in arm II of HisP (19, 22).

12 L.-W. Hung, personal communication.

13 L.-W. Hung, unpublished data.
positive cooperativity, and it has been proposed that they hydrolyze ATP separately and alternately (catalytic cooperativity) (26, 39). Positive cooperativity was also not detected in the case of another eukaryotic ABC transporter, CFTR. On the other hand, positive cooperativity was observed in a different prokaryotic transporter, the maltose permease (38). Thus, it may be that prokaryotic ABC transporters, or at least the periplasmic binding protein-dependent ones, behave differently than the eukaryotic ones with respect to the activity of the two ATP-binding sites. The finding that in MDR both ATP-binding sites must be intact for any hydrolysis to occur (26) is also different from the results presented here. It is possible that this latter difference is accidental, reflecting mostly the respective choice of mutants. In this context, it may be relevant that many of the mutants studied in MDR are located within the ATP-binding pocket and that the vanadate treatment used to inactivate one of the subunits would also have affected directly the ATP-binding pocket. This would not be the case with the mutants used in our study, which bind ATP but do not hydrolyze it.

How do these findings fit into a model for the mechanism of action of these systems? The most recent version of the current

---

**Fig. 6. Model representing ATP hydrolysis and ligand translocation.** HisP is represented in light gray. HisQ and HisM are represented in black; there is no reason presently to distinguish between functions specific for HisQ or HisM; thus they are represented as indistinguishable at the moment. The receptor, HisJ, is shown in darker gray. The ligand is a black circle. A small black rectangle indicates the phenomenon of intradimer activation and may function only if ATP is bound. A full cycle can be schematically and hypothetically described as follows. Within the membrane-bound complex the HisP dimer is tightly bound to HisQM. *Step 1,* ATP binds to one of the subunits, and the HisP dimer is disengaged from HisQM. *Step 2,* the affinity of the second ATP-binding site is increased (positive cooperativity), and this site binds ATP (alternatively, the affinity of the first site is increased by the second site binding the second ATP molecule). *Step 3,* liganded HisJ, the periplasmic receptor, interacts with HisQM causing them to change conformation and “release” HisP, thus activating its hydrolyzing activity. *Step 4,* as a consequence of ATP hydrolysis and possibly the creation of a high energy conformational state at one of the sites (including the release of Pi by the site?), a translocation pathway is formed, and the released ligand diffuses unidirectionally (possibly utilizing also ligand-binding sites in the complex (7)). *Step 5,* the first ADP is released (if not released at step 4), and hydrolysis occurs at the second ATP-binding site, and a second ligand bound to the receptor is released and translocated. *Step 6,* the second ADP and Pi are released, leading to reengagement and a new cycle. The dashed lines (*steps 7 and 7’*) indicate two possible routes that might be taken by a heterodimer containing, for example, H211R; in *step 7’* the defective HisP subunit loses the unhydrolyzed ATP and starts a new cycle; in *step 7* the bound ATP is transferred directly to the active subunit and the cycle proceeds. In either case, a cycle with only one hydrolysis event should occupy the same length of time as a normal cycle. At this time it is not possible to speculate effectively about the nature of the limiting step. The alternating nature of the normal cycle as depicted bears a clear resemblance to the alternating catalytic cycle proposed for MDR (39). It is important to be aware that, despite the appearances in this scheme because of the limitations in describing a complex structure two-dimensionally, we consider it very unlikely that the HisP subunits form a channel.
mechanism scheme proposes that HisP undergoes a cycle of physical disengagement from and reengagement with the hydrophobic subunits (as part of its activation/hydrolysis cycle), concomitantly with the hydrophobic subunits assuming alternatingly suppressing and releasing modes (37). In brief, the model proposed the following sequence of events for the wild type: (i) Within the membrane-bound complex the HisP dimer is tightly bound to HisQM; (ii) when ATP binds to one of the subunits, the HisP dimer is disengaged from HisQM; (iii) the affinity of the second ATP-binding site is increased (positive cooperativity), and this site binds ATP; (iv) liganded HisJ, the periplasmic receptor, interacts with HisQM causing them to change conformation and “release” HisP, thus activating its hydrolyzing activity; as a consequence of ATP hydrolysis (possibly after release of P_i and/or ADP by one of the sites), a translocation pathway is formed and the released ligand diffuses unidirectionally; (v) ADP and P_i are released by the other ATP-binding site, leading to reengagement and a new cycle. Most of the steps as proposed in this model are consistent with the results presented here, according to the simplest interpretation. That the disengagement requires ATP binding but not hydrolysis is compatible with our finding that a hydrolysis-incompetent subunit can activate the normal subunit. Indeed, this situation is equivalent to that in which non-hydrolyzable ATP analogs were also shown to induce HisP disengagement (37).

However, a modification of this model has to be introduced to explain the finding that both hydrolysis and translocation activities are halved in the heterodimer. Fig. 6 shows such a modified cycle. After one ATP hydrolysis in step 3, one molecule of ligand is released and translocated in step 4. (Possibly the binding of ATP to the second site prohibits hydrolysis at the other site, as has been proposed for an MDR cycle of alternating ATPase activity (39).) A second molecule of ligand binds to the receptor (without the receptor necessarily leaving the complex, as has been postulated previously (40)) and is released and translocated following the second ATP hydrolysis by the other HisP subunit (steps 5 and 6). This modification allows for the separate coupling of each ligand translocated to a hydrolysis event. A heterodimer would be unable to perform steps 5 and 6, and the cycle may be short-circuited to steps 1 or 2 (dashed arrows 7 and 7').

In conclusion, our in vitro system using purified proteins has allowed us to focus on biochemical aspects specifically relevant to the mechanisms of ATP hydrolysis and of ligand translocation. Regarding the former, our results display both differences and similarities with other ABC transporters. The availability of the crystal structure of HisP together with our extensive genetic analysis, using both spontaneous and site-directed mutations in all of the subunits of this transporter, will allow further probing of these aspects. The histidine permease may be considered a classical representative of the periplasmic permeases, and these results should be directly applicable to the study of other members of the periplasmic permeases family and of the many medically important eukaryotic members of the ABC superfamily.

Acknowledgments—We thank Drs. D. I. Kreimer, L.-W. Hung, and P.-Q. Liu for extensive discussions. We also thank Erick Lin for constructing a plasmid for the production of H211D protein.

REFERENCES

1. Ames, G. F.-L., Mimura, C., Holbrook, S., and Shyamala, V. (1992) Adv. Enzymol. 65, 1–47
2. Hyde, S. C., Emsley, P., Hartshorn, M. J., Mimmaek, M. M., Gileadi, U., Pearson, S. R., Gallagher, M. P., Gill, D. R., Hubbard, R. E., and Higgin, C. F. (1990) Nature 346, 362–365
3. Doige, C. A., and Ames, G. F.-L. (1993) Annu. Rev. Microbiol. 47, 291–319
4. Higgin, C. F. (1992) Annu. Rev. Cell Biol. 8, 67–113
5. Spengler, D. R., and Singh, N. S. (1991) J. Bacteriol. 173, 1444–1451
6. Shyamala, V., Baichwai, V., Beall, E., and Ames, G. F.-L. (1991) J. Biol. Chem. 266, 18714–18719
7. Liu, C. E., and Ames, G. F.-L. (1997) J. Biol. Chem. 272, 859–866
8. Liu, C. E., Liu, P.-Q., and Ames, G. F.-L. (1997) J. Biol. Chem. 272, 21883–21891
9. Nikaido, H. (1994) FEBS Lett. 346, 55–58
10. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) J. Biol. Chem. 257, 5857–5865
11. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 8857–8865
12. Mimura, C. S., Admon, A., Hurt, K. A., and Ames, G. F.-L. (1990) J. Biol. Chem. 265, 19535–19542
13. Kerppola, R. E., Shyamala, V., Kleppa, P., and Ames, G. F.-L. (1991) J. Biol. Chem. 266, 14859–14864
14. Joshi, A. K., Ahmed, S., and Ames, G. F.-L. (1989) J. Biol. Chem. 264, 2126–2133
15. Ames, G. F.-L., Nikaido, K., Gnearke, J., and Petithory, J. (1989) J. Biol. Chem. 264, 3998–4002
16. Liu, P.-Q., and Ames, G. F.-L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3985–3990
17. Baichwai, V., Liu, D., and Ames, G. F.-L. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 620–624
18. Petrini, V., and Ames, G. F.-L. (1991) J. Biol. Chem. 266, 16293–16296
19. Liu, C. E., Liu, P.-Q., and Ames, G. F.-L. (1997) J. Biol. Chem. 272, 27245–27252
20. Hung, L.-W., Wang, I. X., Nikaido, K., Liu, P.-Q., Ames, G. F.-L., and Kim, S.-H. (1998) Nature 396, 703–707
21. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 21449–21452
22. Beaudet, L., Urbatsch, I. L., and Goss, P. (1998) Biochimie 67, 9073–9082
23. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 22957–22961
24. Senior, A. E., and Bhagat, S. (1998) Biochemistry 37, 831–836
25. Davidson, A. L., and Sharma, S. (1997) J. Bacteriol. 179, 5458–5464
26. Ames, G. F.-L. (1985) Adv. Genet. 23, 103–119
27. Ames, G. F.-L., Nikaido, K., Hobson, A., and Malcolm, B. (1985) Biochimie (Paris) 67, 149–154
28. Liu, P.-Q. (1997) Molecular Mechanism of Action of the Periplasmic Histidine Permease, Ph.D. thesis, University of California, Berkeley
29. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 22957–22961
30. Liu, P.-Q., and Ames, G. F.-L. (1997) J. Biol. Chem. 272, 27245–27252
31. Hung, L.-W., Wang, I. X., Nikaido, K., Liu, P.-Q., Ames, G. F.-L., and Kim, S.-H. (1998) Nature 396, 703–707
32. Luo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 21449–21452
33. Amesz, E., and Spudich, E. N. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 1877–1881
34. Prossen, E., Nikaido, K., Ulbrich, S. J., and Ames, G. F.-L. (1988) J. Biol. Chem. 263, 17917–17920
35. Urbatsch, I. L., Sankaran, B., Bhagat, S., and Senior, A. E. (1995) J. Biol. Chem. 270, 26956–26961
36. Bear, C. E., Li, C., Galley, K., Wang, Y., Garami, E., and Ramjeesingh, M. (1997) J. Bioenerg. Biomembr. 29, 465–473
37. Liu, P.-Q., Liu, C. E., and Ames, G. F.-L. (1999) J. Biol. Chem. 274, 18310–18318
38. Davidson, A. L., Laghaiean, S. S., and Manering, D. E. (1996) J. Biol. Chem. 271, 4858–4863
39. Senior, A. E., Al-Shawi, M. K., and Urbatsch, I. L. (1995) FEBS Lett. 377, 285–289
40. Ames, G. F.-L., Liu, C. E., Joshi, A. K., and Nikaido, K. (1996) J. Biol. Chem. 271, 14264–14270