The Nucleotide-binding Site of HisP, a Membrane Protein of the Histidine Permease

IDENTIFICATION OF AMINO ACID RESIDUES PHOTOOAFFINITY LABELED BY 8-AZIDO-ATP*

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The periplasmic histidine transport system (permease) of Escherichia coli and Salmonella typhimurium is composed of a soluble, histidine-binding receptor located in the periplasm and a complex of three membrane-bound proteins of which one, HisP, was shown previously to bind ATP. These permeases are energized by ATP. HisP is a member of a family of membrane transport proteins which is conserved in all periplasmic permeases and is presumed to be involved in coupling the energy of ATP to periplasmic transport. In this paper the nature of the ATP-binding site of HisP has been explored by identification of some of the residues that come into contact with ATP. HisP was derivatized with 8-azido-ATP (N3ATP). Both the undervatized and the derivatized forms of HisP were solubilized, purified, and digested with trypsin. The resulting tryptic peptides were resolved by high pressure liquid chromatography, and peptides modified by N3ATP were isolated and sequenced. Two peptides, X and Z, spanning amino acid residues 16–23 and 31–45, were found to contain sites of N3ATP attachment at His19 and Ser11, respectively. Both peptides are close to the amino-terminal end of HisP; peptide Z is located in one of the well conserved regions comprising the nucleotide-binding consensus motifs of the energy-coupling components of these permeases. These consensus motifs are found in many purine nucleotide-binding proteins. The relationship between the location of these residues and the overall structure of the ATP-binding site is discussed.

Periplasmic active transport systems (permeases) of Gram-negative organisms are complex systems functioning on a large variety of solutes: amino acids, sugars, peptides, vitamins, and ions (reviewed in Ames, 1986a; Ames et al., 1990; Dean et al., 1989). These recent results have been reviewed and discussed by several investigators (Ames, 1990; Higgins, 1990; Dean et al., 1990). The next unresolved problem is the process by which energy liberated in the hydrolysis of ATP is converted into accumulation of solute. Presumably signal(s) are transmitted between the site of the membrane complex which has been occupied by the liganded binding protein and the interior surface of the membrane where ATP is bound and hydrolyzed, resulting in conformational changes that allow passage of the substrate through the membrane. One of the biochemical approaches to this problem involves the characterization of the transport protein that binds and hydrolyzes ATP; as a first step we initiated the characterization of the protein domain(s) responsible for binding ATP. In this paper we present the results of our initial efforts in this direction. HisP peptides that are in close proximity to the bound nu-

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clotide have been identified by derivatization of the protein with a photoaffinity labeling analog of ATP, 8-azido ATP (N-ATP). Two peptides, spanning residues 16-23 and 33-47 and bearing homology to a nucleotide-binding motif (Walker et al., 1982), are derivatized by N-ATP, and the specific residues that had been modified have been identified.

### MATERIALS AND METHODS

#### Bacterial Cell Growth, Lysis, and Preparation of Membranes—  
*Escherichia coli* strain TA3662 containing pFA17 (Hobson et al., 1984) was grown in 4 liters of LB medium (Miller, 1972) containing ampicillin (50 µg/ml) at 30 °C to an A600 of 0.4 followed by 1 h at 42 °C. Cells were harvested at 16,000 × g for 10 min, washed once in 1 liter of 10 mM Tris/Cl, pH 7.4, resuspended to 40 ml in 10 mM Tris/Cl, pH 7.4, and disrupted by two passes in a French pressure cell at 14,000 psi. The membrane fraction was sedimented by centrifugation for 1 h at 150,000 xg. The pellet was resuspended in the latter detergent solution, and the last two supernatants were pooled and stored at -80 °C. The membrane protein was solubilized with a lyophilized derivatization analog of ATP, 8-azido ATP (N-ATP), in 1000 volumes of 10 mM Tris/Cl, pH 7.4, and O.2 ml aliquots were placed in the wells of a porcelain spot plate as indicated in the text. The reaction was stopped by the addition of 100 volumes of 100 mM Tris/Cl, pH 7.4; (ii) CaCl2 and MgCl2 were added to final concentrations of 10 and 50 mM, respectively.

HPLC-purified HisP (1 µg in 220 µl) was derivatized as for octyl glucoside-solubilized protein. In this case the reaction was carried out directly in the tube (Eppendorf, 1.5 ml) in which the protein was collected in order to avoid loss of protein by centrifugation. The lamp was placed directly over the opened tube at a distance of 2.5 cm above the sample.

Derivatization with radiolabeled N-ATP was performed as described above, with the addition of 0.3-0.6 µCi of [γ-32P] ATP ([γ-32P] ATP, 25.8 Ci/mmol; N-[γ-32P] ATP, 5.2 Ci/mmol; ICN, Irvine, CA) and cold N-ATP to a final concentration of 100 nM.

#### Purification of Unmodified HisP from Cholate-solubilized Membranes—

The membrane fraction was solubilized with 1% sodium cholate, 0.5 mM EDTA, 200 mM NaHCO3, pH 8.1, and 0.02 ml of 45 mM dithiothreitol, and incubating at 50 °C for 30 min. Trypsinolysis was stopped by adding water to a final volume of 0.56 ml followed by 0.2 ml of 8 M urea in 0.4 M NH4HCO3, pH 8.1, and 0.02 ml of 45 mM dithiothreitol, and incubating at 50 °C for 15 min. Gel electrophoresis was carried out for a total of 20-24 h. Trypsin digestion of the tryptic peptides was carried out as described above, and the reaction was carried out in the same tube in which the protein was collected and derivatized. The gel was stained for 15 min in Coomassie Brilliant Blue and rinsed for 10 min in 5% acetic acid. The gel regions containing N-ATP-derivatized HisP and underivatized HisP were excised with a razor blade, added in 5 ml of elution buffer (50 mM Tris/Cl, pH 7.9, 0.1% SDS, 0.1 mM EDTA, 200 mM NH4HCO3) in a polycarbonate tube (15 ml), and boiled for 3 min. The gel was dissolved in sample buffer (Laemmli, 1970), and then centrifuged at 150,000 × g for 60 min. The pellet was washed once with 20 ml of 10 mM Tris/Cl, pH 8.1, and then centrifuged in the same buffer to a final volume of 13.2 ml (approximately 6.35 mg of protein/ml). An equal volume of 2.5% octyl β-D-glucopyranoside (Calbiochem) in 10 mM Tris/Cl, pH 8.1, was added with stirring, and stirred for 20 min longer at 4 °C. The detergent-insoluble material was removed by centrifugation as before. The supernatant (about 1 mg of protein/ml) was stored in aliquots at -80 °C.

Solubilization of HisP—Approximately 3 ml of membranes was thawed and resuspended by repeated passes through a 20-gauge bent needle after the addition of 10.2 ml of 10 mM Tris/Cl, pH 7.4 (final protein concentration, about 10 mg/ml). An equal volume of 10 mM NaCl, 10 mM Tris/Cl, pH 8.1, was added with stirring and stirred for 10 min, and then centrifuged at 150,000 × g for 60 min. The pellet was washed once with 20 ml of 10 mM Tris/Cl, pH 8.1, centrifuged under the same conditions, and resuspended in the wash buffer to a final volume of 13.2 ml (approximately 6.35 mg of protein/ml). An equal volume of 2.5% octyl β-D-glucopyranoside (Calbiochem) in 10 mM Tris/Cl, pH 8.1, was added with stirring, and stirred for 20 min longer at 4 °C. The detergent-insoluble material was removed by centrifugation as before. The supernatant (about 1 mg of protein/ml) was stored in aliquots at -80 °C. Essentially similar results were obtained if nonanoyl-N-methylglucamide (MEGA-9) (at 1%) replaced octyl glucoside. Alternatively, the washed membranes were suspended with a bent needle in 13.2 ml (10 mg of protein/ml) of 1% sodium cholate, 0.5 M NaCl, 10 mM Tris/Cl, pH 8.1; the suspension was stirred at 0 °C for 45 min and then centrifuged at 150,000 × g for 1 h. The supernatant that contained little or no HisP was discarded. The pellet was resuspended in 13.2 ml of 1% sodium cholate, 1 M NaCl, 10 mM Tris/Cl, pH 8.1, stirred, and centrifuged as above. The supernatant contained about 50% of the total HisP. The pellet was reextracted in the latter detergent solution, and the last two supernatants were pooled and stored at -80 °C. The concentration of the final pooled supernatants was typically about 0.7 mg of protein/ml.

#### Purification of Unmodified HisP from Cholate-solubilized Membrane Protein by Microbore HPLC—One hundred µl of cholate-solubilized protein was injected onto a Brownlee C8 microbore 5-µm silica cartridge (2.1 x 30 mm) and resolved in a gradient of acetonitrile from 0 to 80% in 0.1% trifluoroacetic acid at 100 µl/min using an Applied Biosystems HPLC instrument model 130A. HisP was eluted at about 50% acetonitrile and was more than 90% pure as estimated by SDS-PAGE.

### Photoaffinity Derivatization of HisP with N-ATP—Intact membranes, octyl glucoside- and cholate-solubilized membranes, or HPLC-purified HisP were used as described above. Nine ml of cholate-solubilized membranes was dialyzed against 1000 volumes of 10 mM Tris/Cl, pH 8.1, 5 mM CaCl2, 10 mM MgCl2, 0.2% sodium cholate for 2 h at 4 °C. To 10 ml of the dialyzed protein (0.63 mg of protein/ml) N-ATP was added to a final concentration of 100 nM, and the reaction was carried out as described above. Eight ml of octyl glucoside-solubilized membranes was treated as for cholate-solubilized membranes except for omitting of the dialysis step. The pH of the reaction mixture was brought to 7.4 by the addition of 1 M Tris/Cl, pH 7.4; (iii) CaCl2 and MgCl2 were added to final concentrations of 5 and 10 mM, respectively.

HPLC-purified HisP (1 µg in 220 µl) was derivatized as for octyl glucoside-solubilized protein. In this case the reaction was carried out directly in the tube (Eppendorf, 1.5 ml) in which the protein was collected in order to avoid loss of protein by centrifugation. The lamp was placed directly over the opened tube at a distance of 2.5 cm above the sample.
Separation of Tryptic Peptides by Two-dimensional Thin Layer Electrophoresis and Chromatography—Trypsin-digested protein (2 nmol) was lyophilized and resuspended in H2O in a final volume of 0.03 ml. General peptide handling techniques were performed essentially as described (Graae, 1977), allowing its easy identification. The optimum concentration of the membranes (from 25 to 500 μm), the concentration of the membranes (from 0.2 to 4 mg of protein/ml), the length of UV irradiation time (from 1 to 12 min, in 30-s to 5-min intervals, with cooling in between) with and without the repetitive addition of N3ATP between irradiations. The progress of the reaction was estimated by SDS-PAGE (data not shown). The derivatized form of HisP is present in the octyl glucoside-soluble fraction, representing the major band, as is apparent in lane C (quantitation data not shown). HisQ and HisM also are major bands in this fraction. This is to be expected because these proteins form a complex that is stable to this solubilization procedure. The other major membrane components, the porins, are conveniently partitioned exclusively in the octyl glucoside-insoluble fraction. Essentially similar results were obtained upon solubilization with cholate or nonanoyl-N-methylglucamide (data not shown).

Derivatization with N3ATP—N3ATP has been shown previously to interact specifically with the nucleotide-binding site of HisP in membranes and to form a covalent bond, resulting in a derivatized form of HisP (Hobson et al., 1984) and to inactivate specifically the transport function (Ames et al., 1989). The conditions for derivatization in membranes were optimized by varying the concentration of N3ATP (from 25 to 500 μm), the concentration of the membranes (from 0.2 to 4 mg of protein/ml), the length of UV irradiation time (from 1 to 12 min, in 30-s to 5-min intervals, with cooling in between) with and without the repetitive addition of N3ATP between irradiations. The progress of the reaction was estimated by SDS-PAGE (data not shown). The derivatized form of HisP has lower mobility in SDS-PAGE than HisP (Hobson et al., 1984), allowing its easy identification. The optimum conditions were established to be 100 μm N3ATP, 1 mg/ml protein, 1–2-min irradiation at 1-min intervals. Irradiation for periods longer than 6 min resulted in blurred bands, indicating damage to proteins as a result of extensive UV exposure.

HisP can be derivatized with N3ATP also following octyl glucoside solubilization of the membranes as described under "Materials and Methods." Quantitation of gels by densitometric scanning indicated that about 40% of HisP is usually derivatized under our conditions (quantitation data not shown). Fig. 2 shows a Coomassie Blue-stained gel of the octyl glucoside-soluble proteins (lane A) and the corresponding N3ATP-treated proteins (lane B). HisP, clearly visible as a major band in lane A, decreases to about half its amount in lane B.
sites of photoaffinity labeling on HisP with \( N_3 \)ATP

Fig. 2. \( N_3 \)ATP labeling of HisP results in a mobility shift on SDS-PAGE. SDS-PAGE was on 12.5% acrylamide, with the pH of the resolving gel adjusted to 8.61. Lanes A, B, and C were stained with Coomassie Blue. Lane MW, molecular weight standards; lane A, octyl glucoside-soluble protein; lane B, \( N_3[\gamma-^3P] \)ATP-derivatized octyl glucoside-soluble protein; lane C, \( N_3[\gamma-^3P] \)ATP-derivatized, HPLC-purified HisP; lanes D and E, autoradiograms of lanes B and C, respectively. \( P \) and \( P^* \) indicate the positions of HisP and derivatized HisP, respectively.

Fig. 3. \( N_3 \)ATP labeling of membranes and of detergent-solubilized membrane proteins. SDS-PAGE was on 12% acrylamide, with the pH of the resolving gel adjusted to 8.61. Lane I, molecular weight standards; lanes 2-4, E. coli membrane protein; lanes 5-7, cholate-soluble membrane protein; lanes 8-10, octyl glucoside-soluble membrane protein. Lanes 3, 6, and 9, protein subjected to UV irradiation in the absence of \( N_3 \)ATP; lanes 4, 7, and 10, protein subjected to UV irradiation in the presence of \( N_3 \)ATP. The positions at which HisP and \( N_3 \)ATP-derivatized HisP run are indicated with \( P \) and \( P^* \), respectively.

Lane B, and a new major band appears immediately above it. This upper band is derivatized HisP, as confirmed by its reaction with antibodies raised against HisP when analyzed by immunoblot (data not shown). Lane C shows HPLC-purified HisP derivatized with \( N_3 \)ATP. In both lanes B and C the \( N_3 \)ATP contained \( \gamma-^3P \) label. Lanes D and E are autoradiograms of B and C, respectively. The radioactivity corresponds to the upper HisP band. In no case does UV irradiation in the absence of \( N_3 \)ATP, or addition of \( N_3 \)ATP in the absence of UV irradiation, result in the formation of the upper band (Fig. 3 and data not shown). This excludes the possibility that the upper band is the result of phosphorylation by \( N_3 \)ATP or of UV-induced modifications of the protein. Fig. 3 shows a comparison of the extent of derivatization of HisP in intact membranes and in detergent-solubilized proteins; solubilization in either octyl glucoside, cholate, or nonanoyl-N-methylglucamide (data not shown) is comparable to that obtained in intact membranes, indicating that the ability of HisP to bind \( N_3 \)ATP is unaltered by these detergents.

Tryptic Digestion of HisP and Peptide Resolution—The different mobility of \( N_3 \)ATP-derivatized and undervatized HisP on SDS-PAGE provided a convenient method for the purification of derivatized protein. Proteins treated with \( N_3 \)ATP were resolved on preparative gels, and the bands corresponding to the two forms of HisP were excised and treated as described under "Materials and Methods." We have developed a method for the elution of protein from SDS-PAGE slabs which is both quantitative and yields protein that is amenable to trypsin digestion. A detailed description and discussion of this method are in preparation. In brief, protein was eluted from crushed gels using an ammonium bicarbonate:SDS:EDTA buffer. Alternative elution procedures such as electroelution, elution concomitant with trypsinolysis, or substitution of NaCl for the ammonium bicarbonate in the elution buffer resulted in low yields or nondigestible protein (data not shown). The reasons for this may be multiple, including absorption of the protein onto a variety of surfaces and irreversible precipitation. In situ protease digestion of HisP transferred to nitrocellulose (Aebersold et al., 1987) or to polyvinylidine difluoride (Immobilon P) (Legendre and Matsudaira, 1988) produced more complete digests, as evidenced by HPLC peptide mapping. However, sequences of these peptides showed high background contaminants (data not shown). The technique finally chosen gives consistently efficient digestion and utilizes many of the desirable aspects of the unsuccessful techniques. HisP and derivatized HisP were each eluted with a yield of 80-100%. Routine conditions were as follows. Four mg of membrane protein (20% of which is HisP, and 10% is derivatized HisP) was applied to two preparative gels and yielded about 0.6 mg of HisP and 0.2-0.3 mg of derivatized HisP after elution, aceton precipitation, and resuspension in trypsin digestion buffer. The acetone precipitation removed SDS, which would interfere with the digestion.

Reduction and alkylation of the protein prior to proteolysis were necessary for efficient digestion, and the conditions were chosen so as not to require dialysis, which often causes protein loss. The presence of urea (8 M) and heating at 50 °C for 15 min were necessary to redissolve the protein after precipitation. Subsequent dilution to a final concentration of 2 M urea resulted in efficient digestion. Digestion was most effective when carried out for 24 h with two additions of trypsin. The optimal ratio of trypsin to HisP was 5:100 (w/w); higher or lower ratios were not as effective, as determined by SDS-PAGE of the reaction products (data not shown). Protein purified by HPLC was equally amenable to trypsin digestion and peptide resolution; the capacity of this technique is lower.

Resolution of Peptides by HPLC—Tryptic peptides were resolved by reverse phase microbore HPLC and could be applied directly to the Sequencer without concentration or further purification. A typical peptide elution profile obtained by reverse phase HPLC of the underivatized HisP is shown in Fig. 4A. The separation was designed to produce sharp, clearly defined peaks in the region in which small and hydrophilic peptides elute (between 20 and 40% acetonitrile) because one peptide of interest is found in this region. Twenty-

\[ C. S. Mimura, A. Admon, and G. F.-L. Ames, in preparation. \]
Sites of Photoaffinity Labeling on HisP with N$_3$ATP

Fig. 4. Microbore HPLC separation of tryptic peptides from underivatized and derivatized HisP. The trypsinolysis was performed on SDS-PAGE-purified protein. The dotted line indicates the acetonitrile gradient. A, underivatized HisP (approximately 0.4 nmol); B, HisP derivatized with N$_3$[γ-$^{32}$P]ATP (approximately 0.4 nmol; 5500 $^{32}$P cpm); peak X is marked; vertical lines indicate $^{32}$P radioactivity above background (40 cpm). Radioactivity in the void peak and in peak X is 2000 cpm.

Four tryptic peptides were expected from complete digestion of HisP. About 20 peaks appear in this region, and the later region is presumed to contain partial digestion products. Fig. 4B is the corresponding peptide elution profile of the N$_3$[γ-$^{32}$P]ATP-derivatized protein. An arrow indicates the position of a new peak (peak X) comparable in size to that of other peaks. Radioactivity was found corresponding to peak X and was also present in the unretained volume (void volume). This can be ascribed to breakdown products from the derivatized form of N$_3$ATP (see “Discussion”). Fig. 4B shows a typical elution profile. The flow rate at which the chromatography is performed affects the recovery. The flow rate used here (120 µl/min) results in better recovery than is typically reported (Haley, 1990). Recovery of radioactivity from the column, and it was observed regardless of the source of HisP for the derivatization (HPLC-purified HisP, cholate-solubilized membranes, and octyl glucoside-solubilized membranes (data not shown)), indicating that not only the ability to bind the nucleotide is maintained but also that the same specific geometry is maintained at the ATP-binding site under all these conditions. The radioactivity in peak X is unstable since rechromatography causes loss of counts from peak X with their concomitant appearance in the void peak.

When N$_3$[α-$^{32}$P]ATP was used to derivatize HisP, two radioactive peaks (in addition to the one corresponding to the void volume) were found after HPLC separation (Fig. 5). One of the radioactive peaks is in the same position as peak X. The second one, labeled Z, elutes later in the gradient, and it accounts for about 50% of the total counts recovered from the column. Presumably peak Z is not apparent with N$_3$[γ-$^{32}$P] ATP because it loses at least the terminal phosphate.

Sequence Analysis of Peak X—The sequence of peak X was determined to be Tyr-Gly-Gly-no residue-Glu-Val-Leu-Lys, which corresponds to amino acids 16-23 of HisP, lacking histidine in the 4th position. The yields at each cycle and carryovers are shown in Table I. This is one of the expected tryptic peptides. The fact that the fourth sequencing step did not yield a product suggests that the histidine at this position is modified. The peak immediately preceding peak X does not contain any radioactivity and is consistently reduced to varying extents upon derivatization; this peak was also sequenced and shown to contain Tyr-Gly-Gly-His-Val-Leu-Lys (Table I). Therefore, this must be the parent (underivatized) peptide, and its derivatization by N$_3$ATP apparently results in only a small shift in retention time under the HPLC conditions used. This may be because of the high hydrophilicity of this peptide even without modification. Thus we conclude that since histidine can be detected readily by our sequencing procedure, its absence in the sequence of peak X means that it is indeed derivatized by N$_3$ATP.

Sequence Analysis of Peak Z—The sequence of peak Z was determined to be Ala-Gly-Asp-Val-Ile-Ser-Ser-Ile-Ile-Gly-Ser-no residue-Gly-Ser-Gly-Lys, which corresponds to amino acids 31-45 of HisP, with the empty cycle corresponding to Ser$^41$ (see Table I for yields). The amount of serine detected in cycle 1 can be entirely accounted for by the carryover from the previous cycle. That this cycle failed to detect any newly

from the column, and it was observed regardless of the source of HisP for the derivatization (HPLC-purified HisP, cholate-solubilized membranes, and octyl glucoside-solubilized membranes (data not shown)), indicating that not only the ability to bind the nucleotide is maintained but also that the same specific geometry is maintained at the ATP-binding site under all these conditions. The radioactivity in peak X is unstable since rechromatography causes loss of counts from peak X with their concomitant appearance in the void peak.

FIG. 5. Microbore HPLC separation of tryptic peptides from HisP derivatized with N$_3$[α-$^{32}$P]ATP. Conditions and symbols are the same as in Fig. 4. Peptides X and Z are marked. The amount of radioactivity in the void peak and in peaks X and Z is 1500, 140, and 1400 cpm, respectively.
Sites of Photoaffinity Labeling on HisP with N₆ATP

**Table I**

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<th>Peptide X</th>
<th>Un-derivatized Peptide X</th>
<th>Amino Acid</th>
<th>Peptide Z</th>
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<td>85*</td>
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</table>

* Since the next cycle contains the same amino acid, the carryover cannot be calculated.

1°S and S° represent PTH-serine and PTH-dehydroserine, respectively. Occasionally a contaminant migrates with PTH-serine, which is a common occurrence in this kind of analysis. A dash indicates uncertainty in distinguishing PTH-serine from the contaminant and therefore the calculation is omitted.

The estimated carryover would be 24% (as averaged from carryovers for serine-containing cycles in the undervatized peptide), resulting in 0.4 pmol in cycle 11, thus accounting for virtually all of the detected signal.

Not assayed.

liberated serine is supported by the finding of larger quantities of serine in a later cycle (cycle 13). The peak immediately following peak Z was also sequenced and shown to contain Ala Gly Asp Val Ile Ser Ile Gln Gly Ser Gly Lys, thus identifying this peak as the un derivatized peptide identified by HPLC and/or to peptide charge heterogeneity.

**DISCUSSION**

Azido-purine analogs bind to nucleotide-binding proteins at the purine-binding sites because they are nearly identical to the normal substrates (Potter and Haley, 1983). Upon activation by UV light the azide group is converted to a nitrene that then bonds covalently to amino acids in or near the nucleotide-binding pocket. We have identified two peptides (X and Z) that are covalently modified by photoaffinity labeling with N₆ATP and therefore must be in or near the nucleotide-binding site of HisP. Presumably, when ATP is bound, portions of these peptides are in close proximity to the C-8 carbon of the adenine ring of ATP since this is the site of attachment of the reactive group in N₆ATP. Additional peptides may contribute to form the spatial boundaries of the nucleotide-binding pocket; these peptides could be identified through the use of other affinity-labeling reagents.

One important parameter in our study was the maintenance of the nucleotide-binding site in a native state. The detergents octyl glucoside, sodium cholate, and nonanoyl-N-methylglucamid were chosen because they are generally known to be favorable for retention of biological activity (Helenius and Simons, 1974; Helenius et al., 1979) including ATPase activity (Jorgensen, 1982) and to preserve protein-protein interactions (Kagawa, 1972; Tzagoloff and Penefsky, 1971). This has been demonstrated in the case of the histidine permease since the HisQ, HisM, and HisP proteins solubilized with these detergents are known to be preserved as an active complex by coimmunoprecipitation studies and from its activity toward reconstitution into proteoliposomes (Bishop et al., 1989). An additional useful attribute of these detergents is that they solubilize the permease proteins effectively while leaving other abundant membrane proteins such as porins unsolubilized.

The specific residues modified within peptides X and Z are His° and Ser°, respectively. This identification was based on
the following considerations. In both cases, the only empty cycles in the Edman degradation series in an otherwise clearly identifiable series were those corresponding to these 2 residues. In both cases, unmodified histidine and serine residues could be identified easily either in the parent peptide or at a different position within the same peptide, indicating that there is no problem with our sequencing technology. This is particularly relevant in the case of Ser41 since the later serine, Ser48, is detected in larger amounts, and both are clearly detected in comparable amounts in the parent peptide. A likely explanation for the empty cycle in lieu of the residues is that the respective ATP adducts are too polar to become uncoupled from the glass filter during sequencing (Hollemans et al., 1983). Other amino acids bearing modifications by photoaffinity labeling probes have also been resistant to automated Edman sequencing (Knight and McEntee, 1985b; Russo et al., 1985; Rao et al., 1985; Chen et al., 1986; Yue and Schimmel, 1977).

Our data provide strong evidence against the derivatization of HisP being caused by phosphorylation since at either site modification does not occur unless the reaction mix is irradiated with UV light. In addition, in the case of His19, its modification by way of phosphorylation may be discounted because phosphohistidine would be hydrolyzed to free histidine when exposed to the acidic conditions (100% trifluoroacetic acid, 40-50°C) of one of the sequencing steps.

We have no information at present on the precise nature of the modification of His19 and Ser41. When the derivatization was carried out with N3ATP, a significant and variable amount of radioactivity was found in the void peak when the tryptic peptides were separated by HPLC (Figs. 4B and 5). Since this occurred when the label was either in the α- or the γ-position it implies that the nature of the instability is not simply hydrolysis of the γ-phosphate. One possibility is that the glycosidic bond of photoinserted nucleotide is labile, especially when HPLC is used as a method of analysis (Haley, 1990).

Although the usefulness of 8-azido-purine analogs to probe the nucleotide-binding sites in proteins is indisputable (Potter and Haley, 1983), caution should be exercised in interpreting the data since the specificity of these compounds for the true binding site decreases linearly with increased concentration of the photoaffinity reagent (Potter and Ialey, 1983). In our system, the specificity of the reaction has been demonstrated previously by competition with ATP itself (Hobson et al., 1984); for the present work the relatively low concentration used (100 μM) is consistent with maintaining binding site specificity (Hobson et al., 1984). The finding that only two peptides are labeled using N3ATP indicates a relatively short half-life of the activated nitrone, further supporting our contention that specific labeling has occurred.

Peptide Z Is Located in a Highly Conserved Nucleotide-binding Domain—Purine nucleotide-binding proteins share structural and sequence homologies that have been identified over the past several years. Two motifs stand out, having been found in many ATP-binding proteins; their respective consensuses are Gly-X-X-X-Gly-Lys (consensus A) and H-H-H-H-Asp (with H meaning hydrophobic; consensus B) (Walker et al., 1982). Both of these motifs are found in the nucleotide-binding component(s) of each periplasmic permease (Higgins et al., 1986; Ames, 1986a). Several of these transport components have been shown to bind ATP and its affinity labeling analogs and GTP (Hobson et al., 1984; Higgins et al., 1985); the regions of homology between them are much more extensive than the above consensuses. This family of membrane-bound permease components has been postulated to be involved directly in the utilization of ATP for the energization process (Ames, 1986b, Higgins et al., 1986). Analysis of the homologous regions of these transport proteins by simple inspection, coupled with judicious in vivo and in vitro mutagenesis experimentation, should yield useful information as to their structural and biochemical function. In our model system, the HisP sequence homologous to consensus A includes Ser41, thus indicating that Ser41 must be in close contact with the bound nucleotide. This conclusion is supported by evidence obtained with several other nucleotide-binding proteins, which positions this consensus region in close contact with their nucleotide-binding pockets. The positioning of consensus A has been deduced from the crystal structure of three purine nucleotide-binding proteins: adenylate kinase, elongation factor Tu, and p21(r) (Fry et al., 1986; Woolley and Clark, 1989; de Vos et al., 1988). On the basis of these structures several common features were clearly seen to emerge. The residues in and around consensus A form a glycine-rich loop, which has been shown to be flexible and has been postulated to control access of ATP to the binding pocket. The terminal lysine of consensus A is close to the α-phosphate of ATP in adenylate kinase (Fry et al., 1986) and to the β-phosphate in elongation factor Tu (Woolley and Clark, 1989), and the entire loop is described as a phosphate-binding loop in p21(r) (Kim et al., 1988). On the basis of these structures we can surmise that if the same loop exists in HisP, as is likely, Ser41 would indeed find itself in close proximity to the nucleotide-binding site, thus resulting in its covalent modification by the activated nitrone derivative of N3ATP.

The modification would occur either if Ser41 is quite close to the 8 position of adenine or if the half-life and mobility of the activated group allow it to react at a small distance.

One residue in the glycine-rich loop has received considerable attention. In the p21(r) protein, a GTPase, the 2nd residue downstream from the 1st glycine of the A consensus, is occupied by a glycine (Gly12) whereas the corresponding position is occupied by valine (Val29) in elongation factor Tu, another GTPase, and by Ser41 in HisP. Mutation of Gly12 in p21(r) to a valine greatly decreases GTPase activity; this mutant protein (and other p21(r) proteins mutated at the same site) are involved in cell transformation (Tabin et al., 1982). These findings point additionally to the central function of the glycine-rich loop in the binding and hydrolysis of purine nucleotides. The importance of the glycine-rich loop is also shown by mutation analysis of the A consensus in some of the transport proteins. In vivo and site-specific mutation of Gly32 in HisP, a strongly conserved residue in this region, diminishes N3ATP binding, indicating that this residue is indeed crucial for ATP binding; presumably, by analogy to p21(r), it is also crucial to the ATP-hydrolyzing activity of HisP. Replacement of Gly32 with serine and Lys32 with asparagine in HisP also considerably reduced its ability to interact with N3ATP. The corresponding residues in the PetB protein of the periplasmic phosphate permease were shown by site-specific mutagenesis to be essential for phosphate transport (Cox et al., 1989). The very same Gly-Lys pair has been shown by site-specific mutagenesis to be essential for ATPase activity in the β subunit of the F1Fo, proton translocating ATPase from E. coli, strengthening the link between these residues and hydrolysis (Parsonage et al., 1987).

Recently a growing family of homologous eukaryotic proteins, presumed to be involved in energy coupling, often associated with a known transport activity, has been discovered (reviewed in Ames et al., 1990; Ames, 1986b). These proteins bear extensive regions of homology to the nucleotide-

* V. Shyamala, in preparation.
binding components of periplasmic permeases, particularly in and around the regions of consensuses A and B, and are hypothesized to share some of their functional characteristics with the prokaryotic permeases (Ames, 1986b). The importance of the glycine-rich loop for the function also of this family of proteins has been demonstrated by site-directed mutagenesis of the multidrug resistance protein, which confers resistance to multiple antineoplastic drugs, most likely by active expulsion of the drug at the expense of ATP (Endicott and Ling, 1989; Gottesman and Pastan, 1988).

Peptide X—The underlying reason for the affinity labeling of peptide X in HisP is less clear. Even though it is not located within the A or B consensus, portions of it must be in close contact with the bound nucleotide. Interestingly, it bears some homology to the A consensus, being rich in glycine and bearing a lysine residue at a distance from a glycine similar to the A consensus. The modified residue, His79, is in the same position relative to the 1st glycine and to the lysine as Ser41 in the A consensus. If HisP undergoes extensive conformational changes it is possible that the region including peptide Z performs a function similar to, and alternatively with, that of the A consensus. The importance of peptide X for ATP binding has been shown by the existence of mutants in which Tyr16 has been changed to serine or Val17 has been changed to glutamic acid, resulting in a loss of binding activity.4

Conclusions—Our data represent the initial steps toward an understanding of the function of the family of nucleotide-binding proteins in periplasmic permeases as related to their involvement in energy coupling. Further biochemical characterization of the nucleotide-binding site in HisP, including also the analysis of HisP mutants defective in various aspects of energy coupling, should significantly advance our understanding of this process. An alignment of the entire family of the nucleotide-binding components of periplasmic permeases with the known crystal structures of adenylate kinase, p21C", and elongation factor Tu has provided the framework from which a tertiary structural model has been proposed for these components and for HisP in particular. This model has provided important insights into the structure and function of these proteins.5

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REFERENCES


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C S Mimura, A Admon, K A Hurt and G F Ames


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