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Ligand-dependent Conformational Plasticity of the Periplasmic Histidine-binding Protein HisJ

INVOLVEMENT IN TRANSPORT SPECIFICITY*

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The periplasmic histidine permease of *Salmonella typhimurium* is composed of a membrane-bound complex and a soluble histidine-binding protein (the periplasmic receptor), HisJ. Liganded receptor interacts with the membrane-bound complex, inducing ATP hydrolysis and substrate translocation. Preliminary evidence had shown a lack of direct correlation between the affinity of HisJ for a ligand and translocation efficiency, suggesting that the precise form of the receptor is important in determining its interaction with the membrane-bound complex. We have investigated the nature of the conformations assumed by HisJ upon binding a variety of ligands by tryptophan fluorescence enhancement, reaction with a closed form-specific monoclonal antibody, and changes in UV absorption spectra. It is demonstrated that although HisJ binds all the ligands and undergoes a conformational change, it assumes measurably different conformations. We also show that the interaction between HisJ and the membrane-bound complex depends on the nature of the ligand. Transport specificity appears to be defined, at least in part, by the conformation of the bound receptor, manifested either by the effect of a given ligand on the closed structure *per se*, or by the effect of ligand association on the equilibrium constant relating the open and the closed liganded forms.

Bacterial periplasmic permeases are composed of a soluble receptor (the periplasmic substrate-binding protein) and a membrane-bound complex. Periplasmic receptors have several roles: ligand binding, positioning the ligand close to the translocation pathway by interacting with the membrane-bound complex, triggering the signal that results in the hydrolysis of ATP (which supplies the energy for transport), and delivering the ligand to the translocation passageway (reviewed in Refs. 1–3). The resolution of the structures of numerous receptors indicates that they have common characteristics, despite a general lack of sequence homology (4–11); for reviews on earlier papers, see Refs. 12 and 13. They are usually composed of two lobes connected via two or three peptide stretches, and separated by a cleft containing the ligand-binding site. When the binding site is empty, the lobes are far apart (open form); in the liganded form they are close to each other (closed form). The conformational change between the open and closed forms involves a large hinged domain movement; the closed liganded form is stabilized by numerous interactions of the ligand with side chain residues and the peptide backbone from both lobes, and by several lobe-lobe interactions (via water molecules). Although this conformational change has been usually considered to involve a rigid body movement (see, for example, Refs. 8 and 10), it appears that the residues in the individual lobes undergo significant changes during the rotation of one lobe relative to the other (14–16).

It has been proposed that receptors alternate between four forms: closed empty, open empty, open liganded, and closed liganded (17–19),[1] with the closed liganded form being the species initiating translocation through the membrane-bound complex. Although some receptors have been shown by x-ray crystallography to have identical overall conformations when liganded to different substrates (21–23), studies utilizing tryptophan fluorescence, UV difference spectroscopy, and some x-ray crystallographic evidence showed that they have different physical properties (18, 24–29). These results suggest that the interaction with different ligands may result either in different overall conformations of the respective liganded receptors or in differences in the equilibrium between the open and closed liganded forms.

While it has been tacitly assumed that the transport affinity and specificity of periplasmic permeases is modulated by the specificity of the receptor for ligands, several pieces of evidence suggest that additional parameters are involved. Here we use a well characterized model system for periplasmic permeases, the histidine permease of *Salmonella typhimurium*, to identify these parameters. This permease comprises the histidine-binding protein HisJ as the soluble receptor, and the membrane-bound complex (HisQ/M/P),[2] which is composed of four polypeptides, HisQ, HisM, and two copies of HisP. The correlation between the affinities and specificity of the transport process and of the receptor's binding activity is not always obvious; this was particularly clear in a study of numerous mutant HisJ receptors, in which a decrease in their substrate-binding affinity was not necessarily accompanied by a proportional decrease in the affinity for transport: the ratio between the K_d for binding and the apparent K_m for transport varied almost a 1000-

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[1] Although the existence of four forms was not specifically discussed, evidence that the galactose/glucose-binding protein assumes a closed empty form has been provided using a disulfide trapping method (20).

[2] The abbreviations used are: HisQ/M/P, HisQ/HisM/HisP membrane-bound complex; ELISA, enzyme-linked immunosorbent assay; MOPS, 4-morpholinepropanesulfonic acid; mAb, monoclonal antibody; 9D2, monoclonal antibody 9D2; LAO, lysine-, arginine-, ornithine-binding protein; D-HIPA, D-2-hydrazino-3-(4-imidazolyl)-propionic acid; L-azaserine, *O*-diazoacetyl-L-serine.

TABLE I
Kinetic and equilibria characteristics of free and liganded HisJ and its association with mAb 9D2

Ligand[a]	Ligand binding, K_d (μM)[b]	Fluorescence, liganded/empty[c]	mAb interaction			
			ELISA		Solution	
			Liganded/empty[d]	Exchange with L-[^{3}H]His relative[e]	k_{ass}[f] M^{-1} s^{-1} 10^{-6}	k_{dis}[g] s^{-1} 10^5
None	NA	1.0	1.0	1.0	1.6	1
L-Histidine	0.03	1.63	2.64	0.1	10	0.2
L-Arginine	0.7	1.43	2.16	0.3	8.3	ND
L-Azaserine	100	1.18	2.15	0.1	ND	ND
D-L-HIPA	100	1.35	1.59	0.3	ND	ND
N-Acetyl-L-histidine	60	1.55	2.21	0.2	ND	ND
L-Lysine	300	1.27	0.42	0.9	16	2.5
D-Histidine	500	1.57	1.74	0.3	12	ND
L-Histidinol	1400	1.47	3.32	0.1	ND	ND

[a] All the ligands shown in the table are substrates of the histidine permease or compete for transport. L-Histidine transport was measured by determining the transport rates of L-[^{3}H]histidine, both *in vivo*, and in the *in vitro* systems of right-side out membrane vesicles and in reconstituted proteoliposomes (45, 47). The involvement of HisJ and LAO in the transport of other compounds is supported by physiological and genetic evidence. HisJ and LAO mediates the transport of the toxic analogs D-2-hydrazino-3-(4-imidazolyl) propionic acid (D-HIPA) and L-azaserine (52, 63), arginine (64) (when used as the nitrogen source), and the histidine sources D-histidine, *N*-acetyl-L-histidine, imidazole propionic acid, and L-histidinol. L-Lysine is known to compete with D-histidine for transport via the histidine permease (65), but it is not known whether it is transported by HisJ.

[b] K_d values for HisJ-ligand complexes estimated by equilibrium dialysis with radiolabeled L-histidine and L-arginine or by competing for radiolabeled L-histidine binding by L-lysine, L-azaserine, HIPA, *N*-acetyl-L-histidine, L-histidinol, and D-histidine (34). The reaction with L-azaserine, which is photoreactive, was carried out in the dark. The HIPA and *N*-acetyl-L-histidine samples were shown to contain a small amount of contaminating L-histidine (less than 1%); thus their dissociation constants underestimate the real values which does not affect the general conclusion.

[c] See Fig. 1, legend; the ratio is calculated at the emission wavelength of 330 nm.

[d] See Fig. 2*a*, legend. Measured in the presence of 10 ng/ml 9D2.

[e] The effect of 9D2 on the exchange between free L-[^{3}H[histidine and bound ligands was determined as described under "Experimental Procedures." Unliganded HisJ (0.4 μM, in 10 mM MOPS buffer, pH 7.0) was incubated for 5 min with ligands (0.5, 2.0, 1.0, 100, 100, 100, 1000, and 1000 μM for L-histidine, L-arginine, L-lysine, L-azaserine, D-HIPA, *N*-acetyl-L-histidine, L-histidinol, and D-histidine, respectively), followed by the addition of 9D2 (40 nM) and 1 h incubation at room temperature to permit completion of the association of the mAb with HisJ-ligand complexes. This was followed by addition of 10 μM L-[^{3}H]histidine, and dialysis against ligand-free buffer (24 h at 4 °C) to remove free L-[^{3}H]histidine. The complexes of the liganded HisJs form a high affinity ternary complex with 9D2 and therefore suppress the rate of [^{3}H]histidine incorporation into the complex. The results are normalized to the amount of L-[^{3}H]histidine bound after preincubation of unliganded HisJ with 9D2, when about 80% of HisJ was liganded. The low extent of inhibition of histidine binding by 9D2 is a result of its limiting concentration (relative to unliganded HisJ). It should be noted that the concentrations of the unlabeled ligands used in these experiments were close to the K_d values for ligand binding. However, similar results were obtained when the ligand concentrations were 3-fold lower or 10-fold higher. This is because 9D2 associates with all the liganded forms faster than with the unliganded forms and sequesters these forms even at low (relative to the respective K_d for ligand binding) ligand concentrations (data not shown).

[f] See Fig. 4*a*, legend. Unliganded or unlabeled histidine-liganded HisJ (40 nM in 10 mM MOPS buffer, pH 7.0) was mixed with 9D2 (40 nM) at 25 °C and L-[^{3}H]histidine was added to a final concentration of 0.1 μM at the time indicated. After incubation for 10 min at room temperature aliquots (100 μl) were filtered and washed.

[g] See Fig. 4*b*, legend.

fold (15). Mutant receptors have also been found in the histidine permease that impart an altered transport specificity (30, 31), although their binding specificity is normal;[3] a similar situation appears to exist for the maltose permease (32). We demonstrate here that, as suggested above, receptors either assume different overall conformations with different ligands, or that the equilibrium between the open liganded and the closed liganded forms is a function of the ligand structure.

EXPERIMENTAL PROCEDURES

Binding Proteins Preparations—Wild type HisJ, LAO, and the HisJ mutant protein Y14H[4] were obtained by osmotic shock (34) from *S. typhimurium* strains carrying, respectively, plasmids pFA54, pFA104, and pFA247, which contain the *hisJ*, *argT*, and *hisJ5626* genes, under *tac* promoter control. They were purified to over 90% purity (as judged by SDS-polyacrylamide gel electrophoresis) by a two-step ammonium sulfate precipitation (35) and diethylaminoethyl-cellulose high performance liquid chromatography (24). They were determined to be ligand-free (17, 24).

Fluorescence Measurements—Measurements were performed at 25 °C with a Perkin-Elmer LS50B Luminescence spectrometer, using 2.8 μM wild type HisJ or LAO in 10 mM MOPS buffer, pH 7.0. The excitation wavelength was 296 nm, which excites selectively tryptophan residues, and the widths of the excitation and emission slits were 5 mm.

Enzyme-linked Immunosorbent Assay (ELISA)—The antibody used for all experiments was mAb 9D2, previously shown to be specific for the closed form of HisJ (17). The assay was performed essentially as described (36). Plastic plates were coated overnight at 4 °C with unliganded HisJ (10 ng/well) in 5 mM sodium carbonate buffer, pH 9.6. The incubations with the first and the second antibodies were performed at 37 °C for 2 h each, which was found to be enough time for the interactions to go to completion. When ligands were present, they were mixed with 9D2 before addition to the microtiter wells. Visualization was by the alkaline phosphatase reaction, using as second antibody a 500-fold dilution of goat-anti mouse IgG coupled to alkaline phosphatase and a conjugated phosphatase assay kit (Bio-Rad). The optimum pH for this assay is 7.5; sodium chloride in concentrations up to 1.0 M had no effect.

Exchange between Bound Ligand and Free L-[^{3}H]Histidine—Unliganded HisJ (10 μg/ml in 10 mM MOPS buffer, pH 7.0) was incubated for 5 min with various ligands followed by the addition of 9D2 (3 μg/ml) and, after 1 h incubation, by 10 μM L-[^{3}H]histidine (which is a 300-fold excess over the K_d for L-histidine binding to HisJ) to replace the unlabeled ligands. After 1 more hour of incubation the reaction mixture was dialyzed for 24 h at 4 °C against 500 volumes of ligand-free buffer. After dialysis, the concentration of free L-[^{3}H]histidine was 70 nM. All steps except dialysis were performed at room temperature.

Determination of the Rate of Association of 9D2 with HisJ by Filtration—Unliganded or histidine-liganded HisJ (40 nM in 10 mM MOPS buffer, pH 7.0) was mixed with 9D2 (40 nM) at 25 °C and L-[^{3}H]histidine was added at the indicated times. After incubation for 10 min at room temperature which allows completion of the interaction with 9D2, aliquots (100 μl) were filtered through nitrocellulose membrane filters (BA85, Schleicher & Schuell), which were washed 3 times with 0.7 ml of the same buffer; this washing procedure was shown to remove all the free L-[^{3}H]histidine while retaining that trapped in the complex with HisJ and 9D2.

Determination of the Rate of Dissociation of 9D2 from Unliganded

[3] A. Wolf, unpublished results.

[4] The first letter indicates the wild type residue (in the single-letter amino acid code) and is followed by the residue number and the replacing residue (33).

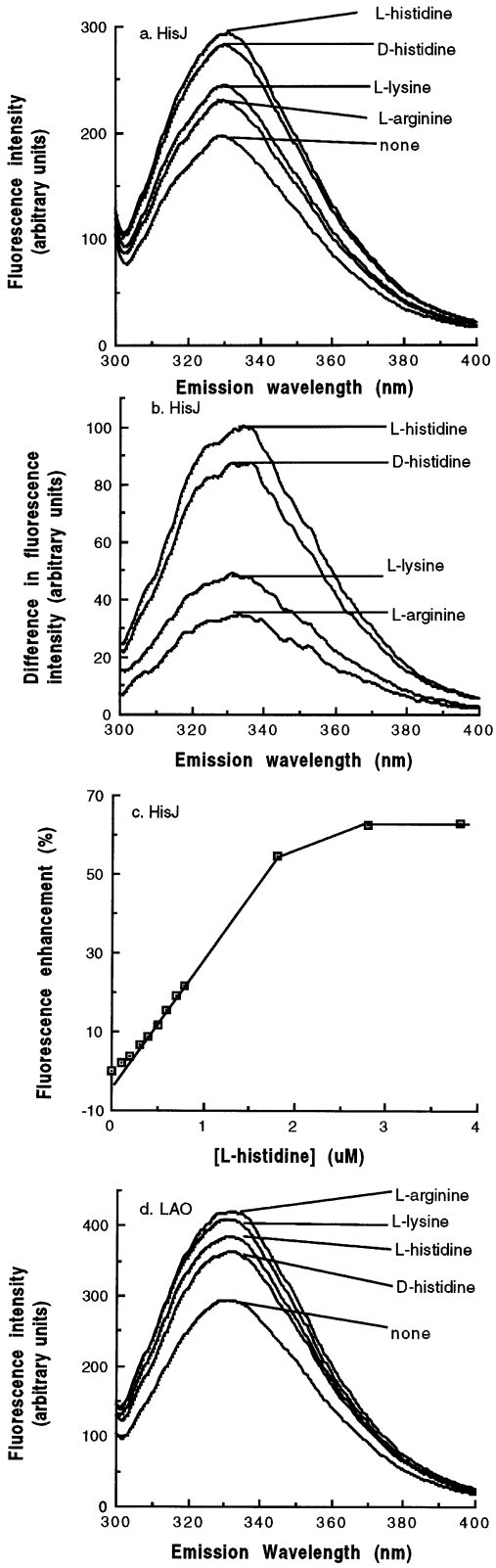

FIG. 1. **Effect of ligands on fluorescence emission spectra of HisJ.** The change in HisJ and LAO (2.8 μM in 10 mM MOPS buffer, pH 7.0 at 25 °C) fluorescence emission spectra upon ligand addition were determined as described under "Experimental Procedures." *a*, HisJ in the presence of: 4 μM L-histidine, 2 mM D-histidine, 4 μM L-arginine, 0.4 mM L-lysine, and no ligand. Higher ligand concentrations do not increase the fluorescence intensity. *b*, difference spectra for HisJ. The data were obtained from *panel a* by subtracting the unliganded HisJ spectrum from those of the various liganded HisJ forms. *c*, effect of varying the L-histidine concentration on the fluorescence intensity of HisJ. The results are expressed as percent of the fluorescence emission enhancement at 330 nm as compared to the unliganded protein. *d*, emission spectra for LAO in the presence of: 10 μM L-arginine, 10 μM L-lysine, 10 μM L-histidine, 1 mM D-histidine, and no ligand. Higher ligand concentrations do not increase the fluorescence intensity. LAO, unlike HisJ, contains a tryptophan residue at position 47 (Trp-47, in lobe I), in addition to Trp-130, which may contribute to fluorescence in the absence of ligand.

and L-[^{3}H]Histidine-liganded HisJ—Unliganded 40 nM HisJ in 10 mM MOPS buffer, pH 7.0, containing 0.1% sodium azide, was incubated for 10 min at room temperature with 40 nM 9D2 to allow formation of the 9D2-HisJ complex. Then 1.0 μM L-[^{3}H]histidine was added and incubation continued for 10 more minutes. The samples were shifted to 4 °C and, at the times indicated, aliquots were filtered and washed. The dissociation of 9D2 from L-histidine-liganded HisJ was determined by incubating it with 40 nM 9D2 at room temperature for 3 h to form the L-[^{3}H]histidine-HisJ-9D2 complex. Free histidine was removed by dialyzing the samples at 4 °C against 10 mM MOPS buffer, pH 7.0, containing 0.1% sodium azide, for 17 h, which decreases the concentration of free L-[^{3}H]histidine to 1 nM or less. The samples were then incubated further at 4 °C, aliquots were taken and filtered at the indicated times, and the radioactivity determined. During the latter incubation, the dissociation of 9D2 from the complex would result also in the release of histidine, which, because of its very low concentration, cannot be bound again; this was shown in control experiments, in which addition of a large excess of unlabeled L-histidine to the dialysis buffer did not change the observed rate of dissociation. Remaining radioactive material was shown by paper chromatography (37) to be [^{3}H]histidine.

Difference in UV Absorption Spectra—Absorption measurements were performed at 25 °C with a Perkin-Elmer λ6 spectrometer, in 0.2-nm intervals, using 20 μM unliganded wild type HisJ or Y14H in 10 mM MOPS buffer, pH 7.0. The absorption spectra in the absence and presence of a ligand were recorded and the difference between them was calculated. The maximum absorption at OD_{280} nm was 0.4.

Cross-linking Experiments with Formaldehyde in Whole Cells—The cross-linking measurements were performed as described previously (38), with the modification that the cells were not preincubated with the ligand prior to formaldehyde addition and that after stopping the reaction with glycine the cells were diluted directly into Laemmli gel sample buffer.

Histidine Binding Assay—L-[^{3}H]Histidine binding was measured in 10 mM MOPS, pH 7.0, by equilibrium dialysis (39). The K_d values for ligand binding were calculated from Scatchard and Lineweaver-Burk plots.

RESULTS

The structures of HisJ in the liganded form and of LAO (lysine-, arginine-, and ornithine-binding protein, another histidine permease receptor that is closely related to HisJ (40)) both in the liganded and unliganded forms, have been solved (8, 33, 41, 42). HisJ binds several ligands that are substrates or competitors of the histidine permease with dissociation constants (K_d) that vary between 30 nM and 1.4 mM (Table I (43)). It has higher affinity for L-histidine than for any of the other ligands, and modification of the functional groups (*i.e.* side chain, carboxyl, or amino) decreases affinity. The higher K_d values displayed by the other ligands can be explained by the destabilization of the respective liganded forms due to: (i) fewer direct ligand-protein bonds; (ii) fewer interactions not involving the ligand directly, such as lobe-lobe interactions; or (iii) steric interference by the ligand with lobe closure. The nature of the liganded forms of HisJ was investigated by four techniques: the fluorescence of the single tryptophan present in HisJ (Trp-130) (15, 27, 44), the interaction of HisJ with the closed form-specific mAb, 9D2 (15, 17), the UV absorption spectrum (mainly due to a tyrosine residue located in the binding pocket, Tyr-14) (24, 26), and cross-linking between the various forms of HisJ and HisQ/M/P (38).

Tryptophan Fluorescence Measurements—The single tryptophan residue, Trp-130, is located far from the ligand-binding pocket (17 Å between the α carbons of the ligand, histidine, and Trp-130 (15)), and is not on the surface (the solvent accessibil-

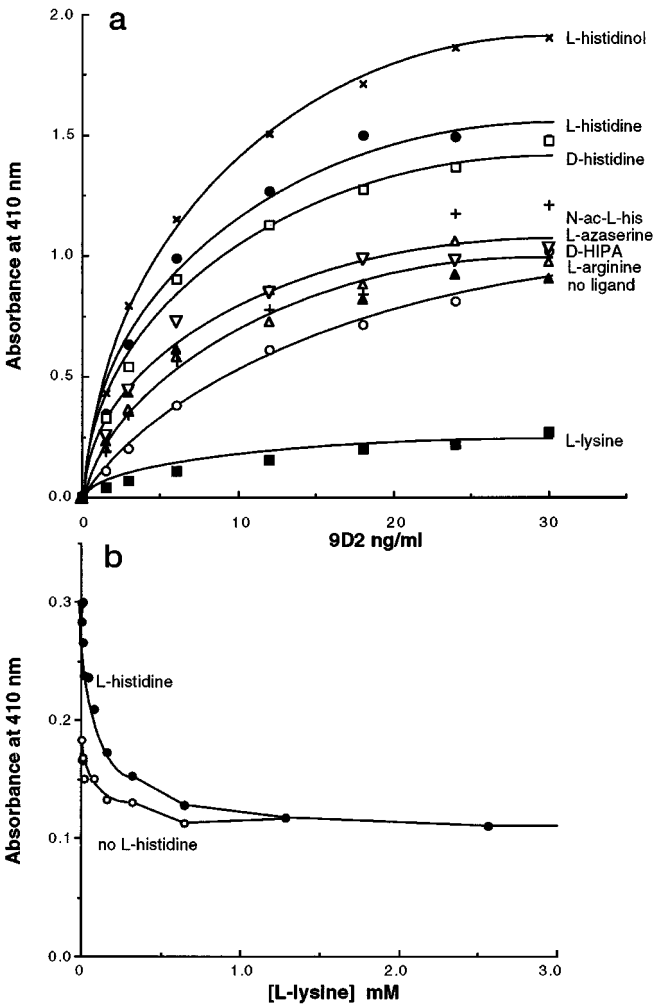

FIG. 2. **ELISA measurements of the interaction of 9D2 with HisJ in the presence of ligands.** The experiments were performed as described under "Experimental Procedures." Plastic plates were coated overnight at 4 °C with unliganded HisJ (10 ng/well); the incubations with the first and the second antibodies were performed at 37 °C for 2 h each. *a*, effect of increasing concentrations of 9D2 in the presence of: no ligand (*open circles*), 1.0 μM L-histidine (*solid circles*), 10 μM L-arginine (*solid triangles*), 1.0 mM D-HIPA (*open triangles*), 1.0 mM L-lysine (*solid squares*), 10 mM D-histidine (*open squares*), 10 mM L-histidinol (×), 2 mM *N*-acetyl-L-histidine (+), and 1 mM azaserine (*inverted open triangles*). All ligands are at saturating concentrations. *b*, effect of L-lysine on the interaction of HisJ with 9D2. The 9D2 concentration was kept constant at 10 ng/ml (70 pM), in the absence or presence of L-histidine (30 nM) (*open* and *solid circles*, respectively). Immobilized HisJ behaves similarly to HisJ in solution because: (i) HisJ in solution competes with immobilized HisJ for interaction with 9D2 (with a K_i of 2 nM with wells coated in the presence of 4 nM HisJ; data not shown); (ii) immobilized HisJ undergoes the expected conformational change upon liganding of histidine because liganded soluble HisJ competes with it for 9D2 (with the same K_i as above; data not shown); (iii) the histidine concentration resulting in half the maximal enhancement of the interaction of immobilized HisJ with 9D2 is the same as the K_d value for histidine binding (15); (iv) the interaction of immobilized mutant proteins with 9D2 is similar to that obtained with them in solution (15).

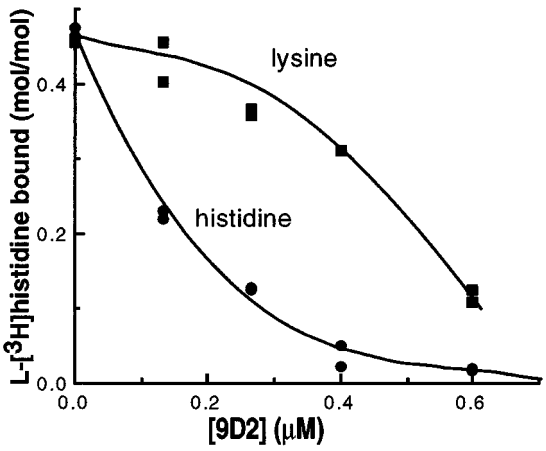

FIG. 3. **mAb 9D2 decreases the rate of dissociation of bound ligand from the HisJ complex.** HisJ (10 μg/ml, in 10 mM MOPS buffer, pH 7.0) liganded either with 1 μM L-histidine (*solid circles*) or 0.3 mM L-lysine (*solid squares*) was incubated with increasing concentrations of 9D2 for 1.0 h at room temperature, then dialyzed against buffer containing 30 nM L-[^{3}H]histidine (= K_d of HisJ/L-histidine complex) for 17 h, at 4 °C. The results are expressed as mol of L-[^{3}H]histidine bound per mol of HisJ.

ity is <4%, and its fluorescence is quenched <5% by 0.1 M KI (data not shown)). A change in fluorescence of Trp-130 upon ligand binding (27, 44) indicates that its environment is sensitive to events in the binding pocket which is distant (15). Therefore, variation in fluorescence enhancement upon association with different ligands should reflect differences in the global conformation of HisJ.

A comparison of the fluorescence spectra in the presence of various ligands is shown in Fig. 1*a* and the quantitation of the respective changes at 330 nm is shown in Table I (third column). The emission spectra of unliganded HisJ and HisJ fully liganded with L-histidine are shown as the bottom and top lines, respectively. The fluorescence intensity is enhanced 1.63-fold upon liganding. The difference between the two emission spectra has a peak at 336 nm with a shoulder at 325 nm (Fig. 1*b*), which suggests that in the liganded form Trp-130 may exist in at least two environments. Fig. 1*c* shows the titration of HisJ with L-histidine as measured by fluorescence enhancement.

D-Histidine, L-lysine, and L-arginine also enhance the fluorescence of HisJ (Fig. 1*a*), but to a lesser extent than does L-histidine, between 1.18- and 1.57-fold at 330 nm (Table I), without changing the position of the emission wavelength peaks (Fig. 1*b*). It can be seen that there is no correlation between the K_d for ligand binding and the extent of enhancement: poor affinity ligands such as D-histidine and L-histidinol produce relatively large increases in fluorescence, while intermediate affinity ligands, such as L-arginine, yield smaller effects (Table I). The concentrations at which half the maximum fluorescence change occurs for the various ligands match the respective K_d values for receptor binding (data not shown). The unlikely possibility that the various ligands interact with different binding pockets is excluded because the closely related protein LAO has been shown by x-ray crystallography studies to bind L-arginine, L-lysine, and L-histidine at the same site (21). That these results are not a peculiarity of HisJ is excluded by showing that LAO behaves similarly with respect to fluorescence enhancement. LAO's fluorescence emission spectrum also has a maximum at 330 nm, the intensity of which increases upon binding different ligands (Fig. 1*d*). However, the extent of tryptophan enhancement in LAO is different than that obtained in HisJ for the same ligands: 1.23-, 1.30-, 1.39-, and 1.42-fold for D-histidine, L-histidine, L-lysine, and L-arginine, respectively. In contrast to the data with HisJ, L-arginine and L-lysine have larger enhancement effects in LAO than L- and D-histidine.

Thus, the different levels of enhancement indicate differences in the respective physical properties when the various ligands bind to the same site. Although the differences are too subtle to be detected by x-ray crystallography (8, 16), they can be detected by fluorescence measurements.

Interaction with a Closed Form-specific mAb; ELISA Experiments—mAb 9D2, which is specific for the closed form of HisJ

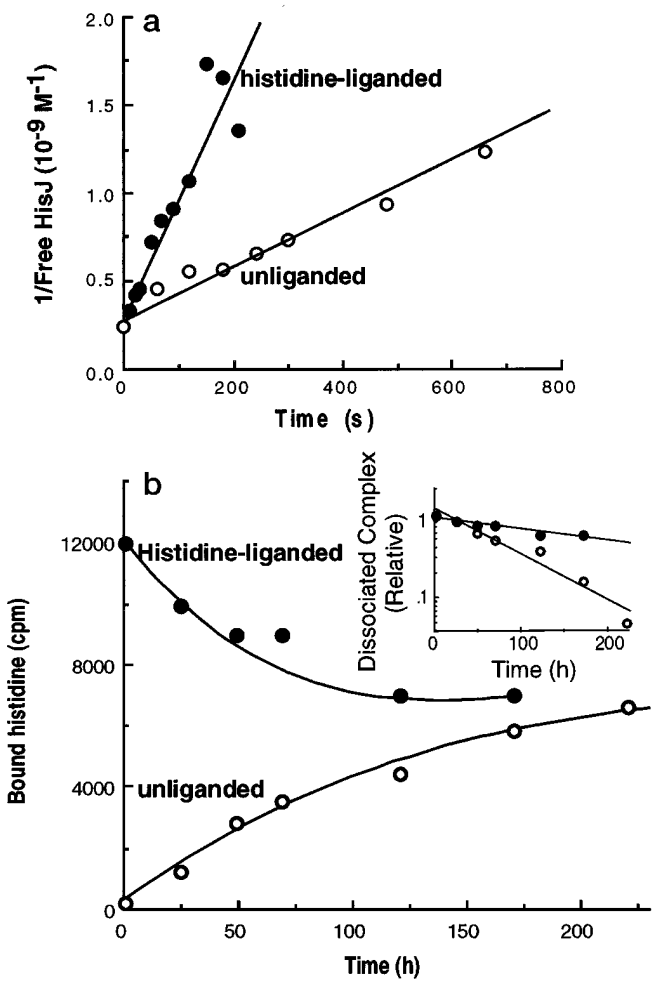

FIG. 4. **Rate of reaction of 9D2 with liganded and unliganded HisJ.** The association and dissociation rates with unliganded and histidine-liganded forms were determined as described under "Experimental Procedures." *Panel a*, association rate with unliganded and histidine-liganded forms of HisJ. Unliganded or L-[^{3}H]histidine-liganded HisJ (40 nM in 10 mM MOPS buffer, pH 7.0) was mixed with 9D2 (40 nM) at 25 °C. At the times indicated, L-[^{3}H]histidine was added to unliganded HisJ to a final concentration of 0.1 μM, or unlabeled L-histidine was added to L-[^{3}H]histidine-liganded HisJ to a final concentration of 10 μM. The rate of association and dissociation of histidine with HisJ is very fast compared to the rate of 9D2 association with HisJ, and the latter is practically irreversible under the condition of the experiment (17). After incubation for 10 min at room temperature, to permit completion of the association of the mAb with HisJ, aliquots (100 μl) were filtered and washed. *Open circles*, unliganded HisJ; *solid circles*, histidine-liganded HisJ. The experimental points at time 0 were determined when mAb 9D2 was added immediately after L-[^{3}H]histidine addition, the condition in which all HisJ is in complex with L-[^{3}H]histidine and 9D2. The concentration of remaining HisJ that is not associated with 9D2 is a direct measure of bound L-[^{3}H]histidine in the case of unliganded HisJ, and is calculated by subtracting the concentration of bound L-[^{3}H]histidine from the total HisJ, in the case of L-[^{3}H]histidine-liganded HisJ. The results are expressed in a linear plot of second-order reaction and the rate constants are shown in Table I. *Panel b*, dissociation rate of 9D2 from the unliganded and L-histidine-liganded forms of HisJ; dissociation of 9D2 from the unliganded forms (*open circles*). Unliganded HisJ (40 nM in 10 mM MOPS buffer, pH 7.0, containing 0.1% sodium azide) was incubated for 10 min at room temperature with 40 nM 9D2, to complete the formation of the complex, then 1.0 μM L-[^{3}H]histidine was added, and incubation continued for additional 10 min. The samples were shifted to 4 °C and, at the times indicated, aliquots were filtered, washed, and the radioactivity was determined. Since 9D2 inhibits lobe opening, the rate of L-[^{3}H]histidine binding reflects dissociation of 9D2 from the 9D2-HisJ complex. The dissociation of 9D2 from L-histidine-liganded HisJ (*solid circles*) was determined by first mixing 40 nM HisJ with 1 μM L-[^{3}H]histidine followed by the addition of 40 nM 9D2 and incubation at room temperature for 3 h to form the L-[^{3}H]histidine-HisJ-9D2 complex. Free [^{3}H]histidine was removed by dialysis against the same buffer, at 4 °C for 17 h, and the samples were then incubated further at 4 °C, and at the indicated times aliquots were taken. Only 50% of the additional bound radioactivity is ultimately released possibly as a result of slow protein denaturation during the long incubation times at low protein concentration. *Inset*, the relative amount of L-[^{3}H]histidine-liganded HisJ which never dissociated was calculated as a fraction of the concentration of the complex in time 0. The quantity of unliganded HisJ which never dissociated from the complex is the difference between the total concentration of the complex and that of the L-[^{3}H]histidine-HisJ-9D2 complex. The total concentration of the complex was determined from the maximal amount of L-[^{3}H]histidine-HisJ-9D2 obtained after 13 days of incubation when the system reached equilibrium. Rate constants are shown in Table I.

(15, 17), was used to analyze the effect of ligands on the efficiency of its interaction with HisJ. Qualitative information was first obtained using variously liganded HisJ and increasing concentrations of 9D2, in ELISA reactions (Fig. 2, *panel A*). The ratios of the ELISA readings obtained with liganded *versus* unliganded HisJ are shown in Table I (fourth column). At a limiting 9D2 concentration (10 ng/ml) the interaction is enhanced 2.6-fold by L-histidine, confirming previous results (17, 45). All other ligands, except for L-lysine (see below), enhance the reaction with 9D2 by various extents, mostly less than L-histidine, but considerably more in the case of L-histidinol, which enhances the reaction better than L-histidine (3.3-fold). In agreement with the fluorescence measurements, the concentrations of the various ligands at which about half the maximal enhancement is observed reflect the respective K_d values for ligand binding (data not shown). Thus, upon association with all ligands, except lysine, HisJ undergoes a conformational change, and therefore it excludes the possibility that the ligands are bound to a frozen open form.

L-Lysine, in contrast with the other ligands, inhibits the reaction of 9D2 with HisJ. The ELISA results reflect a conformation normally taken by HisJ when liganded with L-lysine in solution, as shown in Fig. 2*b*. The lower curve shows that, at a fixed concentration of 9D2, L-lysine inhibits the interaction with a K_d = 100 μM, which is comparable to the K_d of HisJ for L-lysine binding (Table I). In agreement with this result, the top curve shows that L-histidine reverses the inhibitory effect of L-lysine with a K_i for L-lysine of 100 μM. These data taken together indicate that L-lysine binds to HisJ, but that the conformational change is significantly different from that occurring in the presence of L-histidine.

Interaction with 9D2 in Solution—The effect of 9D2 on the exchange between bound and free ligand in solution was also investigated. 9D2 inhibits the release of bound ligand by interacting with the liganded form and therefore locking it in the closed liganded form (17). HisJ, fully liganded with the various ligands, was preincubated with increasing concentrations of 9D2 and then equilibrated with L-[^{3}H]histidine by dialysis. Fig. 3 (*bottom curve*) shows that, as expected (17), 9D2 inhibits the exchange between bound L-histidine and free L-[^{3}H]histidine. A fixed concentration of 9D2 (saturating with respect to L-histidine-liganded HisJ) was then chosen to test the exchange of all other ligands. Table I (fifth column) shows that under these conditions 9D2 inhibits the exchange of all ligands, except for L-lysine, with the extent of inhibition ranging between 70 and 90%. The exchange between bound L-lysine and free L-[^{3}H]histidine is inhibited by 10% only. An experiment in which the 9D2 concentration was varied (Fig. 3, *upper curve*) shows that three times higher 9D2 concentrations are needed to inhibit the exchange of L-[^{3}H]histidine with bound L-lysine by 50%, as compared to bound L-histidine. Thus, L-lysine-liganded HisJ is poorly recognized by 9D2.

Kinetics of Formation and Dissociation of the 9D2-HisJ Complex—The basis for the particular interaction between various

liganded HisJs and 9D2 may be due to different rate constants of association and/or dissociation. The association rate constants were obtained by measuring the decrease in free HisJ with time after addition of 9D2 (see "Experimental Procedures"). Ligand must be present at saturating concentration when determining the association rate for the liganded form, otherwise 9D2 would recognize and trap any closed empty form present in unliganded HisJ solutions (17). Fig. 4*a* shows that the association rate is faster for L-histidine-liganded HisJ than for unliganded HisJ, confirming qualitative previous results (17). The second-order rate constants (at 25 °C) are 1×10^7 and 1.6×10^6 M^{-1} s^{-1}, for the L-histidine-liganded and the unliganded forms, respectively (Table I).

The measurement of the rate constants for dissociation of 9D2 from L-histidine-liganded HisJ is based on the fact that, upon release of 9D2 from L-[^{3}H]histidine-liganded HisJ, the radioactivity is rapidly lost from the complex by exchange with free unlabeled histidine in the medium (see "Experimental Procedures"). Fig. 4*b* (*solid symbols*) shows that 9D2 dissociates very slowly from liganded HisJ following a single exponential decay pattern with a first-order rate constant of 2.0×10^{-6} s^{-1} (inset in Fig. 4*b*). The rate of dissociation from unliganded HisJ was obtained by adding excess L-[^{3}H]histidine to a solution of unliganded HisJ fully complexed with 9D2, and incubating for various lengths of time before harvesting the complex by filtration. HisJ binds [^{3}H]histidine and therefore is trapped by 9D2 as a radiolabeled complex only upon dissociation of the 9D2-HisJ complex. The dissociation rate constant from unliganded HisJ is 6-fold greater than that from liganded HisJ: 1×10^{-5} s^{-1} (Fig. 6*b*, *inset*).

Only the method shown in Fig. 4*a* could be used to measure the rate constants of association in the presence of other ligands because many of them are not available in radiolabeled form. Table I shows that the association rate constants are not significantly different from that for L-histidine-liganded HisJ. Because large K_d differences are not expected, except in the case of L-lysine (see ELISA and the exchange experiments), and the more accurate direct assay with radiolabeled ligands is not uniformly possible, the dissociation rate constant was measured only for L-lysine-liganded HisJ and found to be 12-fold greater than that for L-histidine-liganded HisJ. Thus, the poor reaction of 9D2 with L-lysine-liganded HisJ, as detected by the ELISA and exchange experiments, is due to a larger rate constant for the dissociation of 9D2 from the complex.

UV Absorption Spectra—UV absorption changes upon liganding had been noticed previously in HisJ from *Escherichia coli* (26), in LAO from *S. typhimurium* (24), and in the maltose-binding protein from *E. coli* (29). In the case of LAO it was suggested that the absorption change is due to Tyr-14 (located in lobe I), that is in direct contact with the ligand and changes orientation dramatically upon ligand binding (8, 24). It had been suggested that, because the difference spectrum varied with the ligand, the orientation of Tyr-14 depends on the nature of the ligand bound (24). Because Tyr-14 is also involved in interactions with residues in the other lobe (lobe II) (15), it is possible that its orientation also affects the global conformation. Differences in the shapes assumed by HisJ upon binding different ligands may reflect different orientations of Tyr-14, and, therefore, the analysis of UV absorption spectra would be a useful probe for this study. Upon L-histidine binding, the UV spectrum of HisJ from *S. typhimurium* displays two peaks of increased absorption, at 288 and 295 nm (Fig. 5). A HisJ mutant protein in which Tyr-14 is replaced by histidine (Y14H) does not display these peaks, even when fully saturated with L-histidine (500-fold higher than its K_d for Y14H), thus demonstrating directly that this is indeed the UV-absorbing chromatophore[5] (Fig. 5, *top spectrum*). It can be seen that the UV spectra changes of HisJ associated with various ligands are all different from each other and from the one obtained with L-histidine.

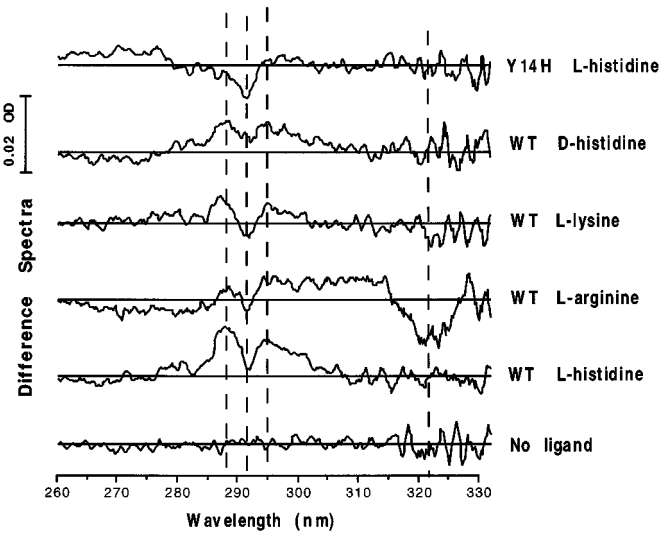

FIG. 5. **The effect of different ligands on the UV difference spectra of wild type HisJ and Y14H.** Absorption measurements were performed as described under "Experimental Procedures" using 20 μM unliganded wild type HisJ or Y14H in 10 mM MOPS buffer, pH 7.0. The maximum absorption at 280 nm was 0.4 OD units. *WT*, wild type HisJ; *Y14H*, mutant receptor with Tyr-14 replaced by histidine. Ligand was added to wild type HisJ in the following concentrations: 0.1 mM L-histidine, 0.1 mM L-arginine, 1.0 mM L-lysine, 1.0 mM D-histidine; Y14H was liganded with 1.0 mM L-histidine. All ligands are at saturating concentrations (at least 10-fold higher than their K_d values for HisJ complexes, except for D-histidine which is only 2-fold higher than its K_d values for HisJ complexes). The *straight line* indicates the theoretical zero absorbance difference.

Interaction of HisJ with HisQ/M/P—It has been shown that the affinity of interaction between HisJ and the membrane-bound complex can be established by quantitating the appearance of a cross-linked product between HisJ and HisQ, J~Q (38). Thus, measuring the efficiency of cross-linking between various liganded HisJ forms and HisQ is useful both as an additional evaluation of conformation and as an indication that one of the known functions of the receptor is affected. Formaldehyde was chosen as the cross-linking reagent *in vivo* because it had been demonstrated to be sensitive to conformation (38). Fig. 6 shows that, as expected, no cross-linking occurs when HisJ is unliganded (*lane 1*). *Lanes 2–5* were obtained by cross-linking in the presence of a large excess of D-histidine, L-lysine, L-arginine, or L-histidine, respectively. The ligand concentrations were chosen to ensure in each case full saturation of the receptor (at least 5-fold higher than the K_d). It can be seen that the amount of J~Q produced is significantly different depending on the ligand: L-histidine yields the highest level, with L-lysine and D-histidine producing little if any J~Q, and L-arginine producing intermediate amounts.

Another function of the receptor is trans-membrane signaling and induction of ATPase activity of the membrane-bound complex (46–48). Assays of the extent of stimulation of ATPase activity in reconstituted proteoliposomes by HisJ fully liganded with each of the above amino acids showed that the stimulation decreased in intensity, with L-histidine > D-histidine > L-argi-

[5] A strong negative peak at 292 nm that appears in Y14H is presumably due to one of the other seven tyrosine residues present in HisJ. The environment of this residue is changed by ligand binding, either directly or indirectly; its absence in the wild type is presumably due to masking by the two positive peaks.

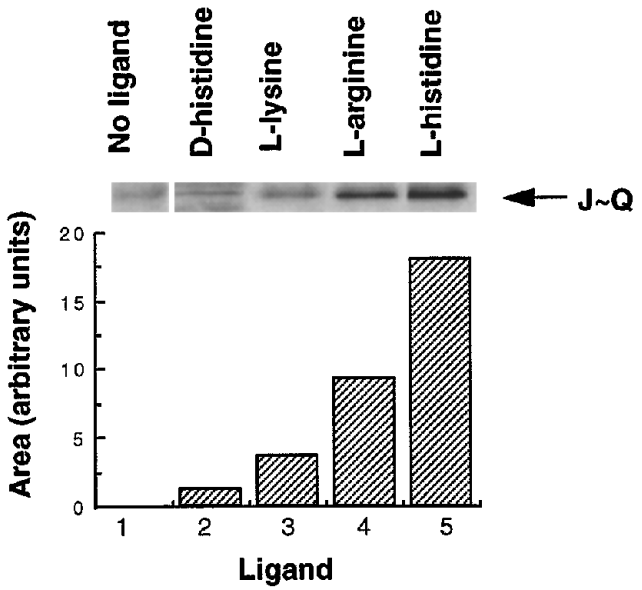

FIG. 6. **The effect of ligands on the cross-linking of HisJ with HisQ.** The cross-linking measurements were performed *in vivo,* as described under "Experimental Procedures." *Lanes 1–5* shows the effect of: no ligand, 2.5 mM D-histidine, 2.5 mM L-lysine, 0.25 mM L-arginine, 0.025 mM L-histidine. All ligands are at near saturating concentrations (at least 5-fold higher than their K_d for binding by HisJ).

nine > L-lysine, the latter being about one-fifth as active as L-histidine.[6] This finding is in agreement with the cross-linking results.

DISCUSSION

The notion that periplasmic receptors assume different physical properties depending on the nature of the ligand is an important aspect of the mechanism of action of these proteins. Such a notion is supported by results obtained using tryptophan fluorescence and UV absorption spectra in studies of the association of receptors with various ligands, and from x-ray crystallography (see Introduction). However, the differences obtained by the spectroscopic measurements using different ligands might simply reflect local changes in the ligand-binding site (24, 26–29) or in residues of unknown location (18, 49)). In the case of the maltose receptor, the difference in its structure upon association with β-cyclodextrin, as compared with maltose, may be due to steric interference with lobe closure due to the large size of β-cyclodextrin (25). To avoid these problems, we have used probes sensitive to conformational changes and ligands of comparable sizes. The experimental results obtained in this work show that the physical properties of the liganded forms of HisJ are indeed dependent on the structure of the ligand. Two physical models that cannot be resolved on the time scale of these experiments are consistent with these observations: (i) the equilibrium constant for the conversion between the open liganded and closed liganded forms is ligand dependent; (ii) the equilibrium constant is unchanged, and the shapes of the respective closed liganded forms are ligand dependent. Therefore, we propose that the description of receptors in the closed form should be modified to include a family of liganded forms.

These findings help to clarify results that conflict with the working hypothesis for the mechanism of action of periplasmic transporters, that predicts a correlation between the transport affinity and specificity of the permease and the ligand-binding affinity and specificity of the receptor. They also provide a new approach to understanding the transport process, in which more attention is paid to the interaction between receptor and membrane-bound complex. The conformation of the liganded receptor is a critical factor in modulating the response of the complex, *i.e.* the signaling pathway that results in the hydrolysis of ATP and the consequent release of the ligand from the receptor (upon lobe opening). It would be expected that some liganded forms would be better than others in stimulating these activities; therefore, the K_m and/or V_{max} values for the transport of the respective substrates should reflect the nature of the interaction (see Refs. 45 and 50 for a discussion of the factors involved in the estimation of these parameters).

The behavior of several HisJ mutant proteins, in which the ratios between the K_d values for L-histidine binding and the respective apparent K_m values for its transport have been shown to vary over a 1000-fold, can be understood on the basis of the data presented here. Among such proteins, several display normal, or slightly higher, K_d values for histidine binding, but very poor transport (*e.g.* proteins S72P, T120M, and R154C); they are also poorly recognized by 9D2, which led to the conclusion that they have altered closed conformations (8, 15, 30, 31). Therefore, the poor K_m values for transport, despite the good K_d values for binding, may reflect the particular interaction between an abnormal closed liganded shape of these proteins and the membrane-bound complex. In agreement with this hypothesis, these mutant proteins cross-link poorly with HisQ.[7] Work with other mutant proteins with different properties also supports the concept that the overall shape can determine the specificity of transport. Mutant proteins D144N and D149N have normal K_d values for all ligands, but impart an altered spectrum of transport specificity: transport of L- and D-histidine is normal, but azaserine transport is dramatically reduced (30, 31).[3]

It would be expected that the interaction of HisJ with the membrane-bound complex should also be sensitive to the exact nature of HisQ, HisM, and HisP. In fact, a number of strains carrying mutations in the membrane-bound complex have altered substrate specificity for transport (51). These include a large group of strains that carry a small in-frame deletion in *hisM* and are unable to transport L-histidine, D-histidine, or the inhibitory analogs azaserine and HIPA, but transport L-histidinol better than the wild type (52, 53). Another type is mutant *hisQ6699* (P60L), which transports L-histidine normally, but does not transport D-histidine or HIPA (51). These findings could be taken to indicate that these mutant membrane proteins are defective in their interaction with HisJ when associated with those ligands that are poorly transported, but interact properly, or even better, with HisJ bound with other ligands that are transported well. Alternatively, the behavior of these mutants could simply reflect the alteration of a hypothetical ligand-binding site in the membrane-bound complex (46, 51, 54, 55). Such a site might also interact differently with different ligands in the wild type and contribute to transport specificity. Until an assay for this binding site becomes available, the two hypotheses cannot be distinguished from each other.

The finding that the conformation of L-lysine-liganded HisJ is structurally distinct is puzzling. The size and charge of L-lysine is not very different from that of several other ligands. The high resolution structure of L-lysine-liganded LAO does not indicate any unusual properties (21). Therefore, we have no explanation for this phenomenon. The high resolution structure of L-lysine-liganded HisJ would resolve this question.

We speculate that the ability of periplasmic receptors to discriminate between different ligands, and therefore dictate

[6] C. E. Liu, unpublished results.

[7] G. F.-L. Ames, K. Nikaido, and C. Chi, unpublished results.

the subsequent response, may be shared by eukaryotic receptors, which may modulate their agonist selectivity in this fashion. A good example is the glutamate receptor (GluR3) for the neurotransmitter glutamate, which has a large extracellular ligand-binding domain that is homologous to LAO (56, 57) and which is responsible for agonist binding (Ref. 58, and references cited therein). The notion that this domain functions similarly to periplasmic receptors is supported by the finding that antibodies directed against it activate its response in the absence of ligand (59), presumably by stabilizing a closed empty form (which would be analogous to 9D2 stabilizing the closed (empty) form of HisJ). The selectivity of the ligand-binding domain of the glutamate receptor might be achieved by its assuming different shapes for the various agonists and antagonists (for example, an antagonist-liganded form might not permit signaling). It is possible that extracellular domains in other receptors, such as the Ca^{2+} sensor (60), or in eukaryotic members of the traffic ATPases family, such as the multidrug resistance protein (Mdr or P-glycoprotein) (61), also have a ligand-binding function. Evidence for such sites is available in the case of P-glycoprotein (Ref. 62 and references therein); it has been shown that its ATPase activity is stimulated to different extents, depending on the particular compound used, which might reflect a sensitivity to the nature of the ligand.

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