# Nuclear Magnetic Resonance and Fluorescence Studies of Substrate-Induced Conformational Changes of Histidine-Binding Protein J of *Salmonella typhimurium*†

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ABSTRACT: The histidine-binding protein J of *Salmonella typhimurium* binds L-histidine as a first step in the high-affinity active transport of this amino acid across the cytoplasmic membrane. High-resolution nuclear magnetic resonance spectroscopy has been used to monitor the conformation of histidine-binding protein J in the presence and absence of substrate. Evidence is presented to show that this binding protein undergoes a conformational change involving a substantial number of amino acid residues (including tryptophans) in the presence of L-histidine and that this change is specific for L-histidine. In order to monitor the involvement of tryptophan residues in the substrate-induced conformational change, 5-fluorotryptophan has been incorporated biosynthetically into the histidine-binding protein J using a tryptophan autotroph of *Salmonella typhimurium*. There are no significant differences in the conformation and binding activity between the 5-fluorotryptophan-labeled and the normal histidine-binding protein J. Proton and fluorine-19 nuclear magnetic resonance studies of the 5-fluorotryptophan-labeled binding protein show that at least one (and possibly two) of the tryptophan residues undergo(es) a change toward a more hydrophobic environment in the presence of L-histidine. These observations are supported by fluorescence data and by differences in the reactivity of the tryptophan residues of this protein toward *N*-bromosuccinimide in the presence and absence of substrate. The present results are consistent with models for the action of periplasmic-binding proteins in shock-sensitive transport systems of gram-negative bacteria which require a substrate-induced conformational change prior to the energy-dependent translocation of substrates.

When Gram-negative bacteria are subjected to the osmotic-shock procedure of Neu and Heppel (1965), several transport activities normally present in intact cells are lost. The loss in transport activities is paralleled by the release of a number of periplasmic proteins which are known as binding proteins. These proteins, which have highly specific affinities for certain amino acids, sugars, or inorganic ions, have molecular weights ranging from 25 000 to 45 000. For a recent review on binding proteins, refer to Oxender (1972). Based on the effects of osmotic shock, two categories of the active-transport system have been shown to exist in gram-negative bacteria: (1) the shock-sensitive systems, which are associated with periplasmic binding proteins and which are absent from isolated membrane vesicles, and (2) the shock-resistant systems, whose carrier proteins are not released by osmotic shock and which are active in membrane vesicles (Berger and Heppel, 1974). Berger and Heppel (1974) have presented strong evidence that the mechanisms of energy coupling in these two classes of transport systems are fundamentally different.

It has been assumed by several investigators that a direct and specific interaction of a given binding protein with the cytoplasmic membrane (membrane-bound proteins and/or lipids) is a necessary step in the osmotic shock-sensitive transport systems. It has also been suggested that this interaction is preceded by a substrate-induced conformational change of the binding protein, which creates an appropriate stereochemistry for the interaction. For recent reviews on this subject, refer to Boos (1974) and Singer (1974). Evidence in support of such conformational changes has been presented only for a few binding proteins. For example, fluorescence and polyacrylamide gel electrophoresis studies have suggested that the galactose-binding protein of *Escherichia coli* undergoes a change in its tertiary structure upon substrate binding, which alters the environment around the active site as well as its total surface charge (Boos et al., 1972; Silhavy et al., 1974). Fluorescence studies of the sulfate-binding protein of *Salmonella typhimurium* (Langridge et al., 1970) and of the glutamine- (Weiner and Heppel, 1971), galactose- (Silhavy et al., 1974), and maltose (Szmelcman et al., 1976) binding proteins of *E. coli*, and proton nuclear magnetic resonance ($^1$H NMR) studies of the glutamine-binding protein (Kreishman et al., 1973) have provided further evidence for the existence of substrate-induced conformational changes in periplasmic-binding proteins.

The most convincing evidence for binding-protein mediation of transport has been presented for the high-affinity histidine transport system in *S. typhimurium* (Ames and Lever, 1970, 1972). This system requires the presence of at least two proteins, the histidine-binding protein J (J protein) (the product of the *hisJ* gene) and a second protein, the P protein (the product of the *hisP* gene), which is believed to be membrane bound (Ames and Lever, 1970). Recent genetic evidence (Ames and Spudich, 1976) has implicated a direct and specific interaction between the J and P proteins as an essential condition for the high-affinity transport of L-histidine ($K_m \sim 6 \times 10^{-9}$ M). Genetic evidence has also shown that the sub-

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strate-binding site of J protein is quite distinct from the site responsible for the interaction with the P protein (Kustu and Ames, 1974; Ames and Spudich, 1976). Thus, if the substrate induces a conformational change in the J protein molecule to permit the J–P interaction, it must extend well beyond a simple alteration of the substrate-binding site.

Ames and her colleagues have carried out a number of elegant experiments on the genetic and biochemical characterizations of the histidine-binding protein J. This protein has been purified and has a molecular weight of approximately 26 000 (Lever, 1972). According to Lever (1972), L-histidine binding is reversible with a dissociation constant for the J protein–histidine complex of 0.15 $\mu$M. The isoelectric point for J protein is pH 5.5 and the L-histidine binding decreases slightly at high ionic strength and with increasing pH. All the usual amino acids are present in J protein (Lever, 1972). In addition, Lever (1972) has compared the amino acid compositions of J protein with those of other binding proteins from *S. typhimurium* and *E. coli* and has found that there is a remarkable similarity. Of special interest to this work is that there are approximately 3–4 tryptophans and 3 histidines per molecule of J protein (Lever, 1972). On the other hand, the P component has only been defined genetically and physiologically, but not biochemically (Ames, 1972; Kustu and Ames, 1973). It has been found that the P protein molecule is multifunctional. In addition to being an essential component for the high-affinity histidine transport in the J–P system, P protein is also required in the K–P system for the transport of L-histidine (K protein which works in parallel to J protein requires the P protein in order to be operative in transport) and is also an essential component of an arginine transport system in *S. typhimurium* (Kustu and Ames, 1973).

This communication presents a study of the conformation and conformational changes of J protein in the absence and presence of substrate under a variety of experimental conditions. $^1$H and $^{19}$F NMR[1] techniques are used. $^{19}$F NMR spectroscopy is particularly well suited for an investigation of protein conformation in bacterial systems when an appropriate $^{19}$F-labeled amino acid, in this case 5-fluorotryptophan, can be incorporated biosynthetically into the protein molecule. In the case of J protein, the substitution of 5-fluorotryptophan for L-tryptophan does not appear to alter the protein structure significantly and, therefore, provides an excellent $^{19}$F probe for the fluorotryptophan environments in the protein molecule. The advantages of using $^{19}$F NMR are its high natural abundance (100%), its comparable sensitivity to protons (83% at the same magnetic field), and its chemical-shift range which is nearly an order of magnitude larger than that for protons. (For a recent discussion on the advantages of applying $^{19}$F NMR spectroscopy to fluorotyrosine alkaline phosphatase, refer to Sykes et al. (1974).) The environment of the tryptophan residues in J protein has also been further characterized by fluorescence spectroscopy and by the reactivity of the tryptophan residues toward *N*-bromosuccinimide in the presence and absence of L-histidine.

## Experimental Procedures

*Chemicals.* All chemicals used in this investigation were reagent grade and were used without further purification, unless otherwise stated. L-[3,5-$^3$H]Histidine (specific activity 55 Ci/mmol) was obtained from Amersham/Searle. Amino acids were purchased from Sigma. *N*-Bromosuccinimide, which was purchased from Pierce, was recrystallized twice from deionized water.

*Bacterial Strains and Media.* The strains of *S. typhimurium* used in this work were generously donated by Dr. G. F-L. Ames. TA1859 (with a *dhuA1* mutation), produces five to ten times the wild-type level of J protein (Ames, 1972). TA1010 (*his*$\Delta$*2327, trpA8, purE801 mtl, dhuA42, hisP5505*) is a tryptophan auxotroph which requires histidine, purine, and tryptophan to support growth (G. F-L. Ames, personal communication). The TA1859 strain was grown in A salts (Kelmers et al., 1971) with 5% glycerol as the carbon source. The high-yield growth procedure of Willis et al. (1974) was used to obtain cell densities of approximately $10^{10}$ cells/mL. Cultures were grown with vigorous aeration either in 15-L polypropylene carboys or in a F-50 Fermacell fermentor (New Brunswick Scientific). The cultures were titrated with concentrated $NH_4OH$ to maintain a pH of 7. Cells were harvested during the stationary phase of growth. The TA1010 strain was grown in a medium consisting of modified medium A (Willis et al., 1974) plus a growth-limiting concentration of L-tryptophan ($10^{-5}$ M) and an excess of 5-fluoro-DL-tryptophan ($5 \times 10^{-4}$ M). Fluorotryptophan was added during the lag phase preceding exponential growth. This yielded a maximum incorporation of the tryptophan analogue into J protein (Robertson, 1976). The incorporation of 5-fluorotryptophan into proteins of *S. typhimurium* is similar to that reported by Pratt and Ho (1975) for the incorporation of fluorotryptophans into proteins of *E. coli*.

*Preparation of Osmotic Shock Fluid.* Before harvesting, cells were pretreated by adding 1 M Tris-HCl at pH 7.2 and 1 M NaCl to give final concentrations of 0.03 M. This treatment precludes the time-consuming washing steps as described by Willis et al. (1974). Cells were harvested using a Beckman J-21 refrigerated centrifuge and a Beckman JCF-Z continuous flow rotor. A pumped flow rate of 40 L/h was used. Pretreated wet cell paste (approximately 300 g) was subjected to the osmotic shock procedure of Neu and Heppel (1965). This treatment yielded 6 L of clear shock fluid with an absorbance of approximately 0.5 at 280 nm using a 1-cm path-length cuvette.

*Sodium Dodecyl Sulfate Gel Electrophoresis.* All samples were checked for purity by slab gel electrophoresis in the presence of sodium dodecyl sulfate using the buffer system of Laemmli (1970) and the running gel composition described by Studier (1973) for 12.5% acrylamide. The slab-gel apparatus was constructed using the basic design of Studier (1973) with a gel length of 9 in. Gels were stained with Coomassie brilliant blue in a solution of water–methanol–acetic acid in a volume ratio of 5:5:1.

*Nuclear Magnetic Resonance Studies.* Lyophilized protein samples were dissolved in 10 mM potassium phosphate buffer in $D_2O$ to give final concentrations of approximately 1 mM for both $^1$H and $^{19}$F NMR studies. The samples were placed in standard 5-mm NMR tubes (Wilmad). All spectra were obtained on the MPC-HF 250 NMR spectrometer interfaced with a Sigma-5 computer at an ambient temperature of 27 °C. $^1$H NMR spectra were obtained at 250 MHz. The residual water signal in each sample was used as the internal lock. Regions 1500-Hz downfield and 3000 Hz upfield from this resonance were swept separately. Digital filtering was used to improve sensitivity. Typically, 200–2000 scans with 1.5-s scan times were accumulated using NMR correlation spectroscopy

[1] Abbreviations used are: NMR, nuclear magnetic resonance; J protein, histidine-binding protein J (the product of *hisJ* gene); P protein, the product of *hisP* gene; K protein, the product of *hisK* gene; 5F-Trp J protein, 5-fluorotryptophan histidine-binding protein J; TFA, trifluoroacetic acid; NBS, *N*-bromosuccinimide; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

developed by Dadok and Sprecher (1974) to enhance the signal-to-noise ratio. Proton chemical shifts are referenced with respect to HDO, which appears 4.83 ppm downfield from the proton signal of 4,4-dimethyl-4-silapentane-5-sulfonate at 27 °C. The positive and negative signs of the chemical shifts refer to shifts upfield and downfield from HDO, respectively. The accuracy of $^1H$ chemical shifts is ±0.02 ppm. $^{19}F$ NMR spectra were obtained at 235.2 MHz. Sweep widths of 15 000 Hz were used with sweep times of 0.25 s and with a 0.1-s delay between scans. 1000–25 000 transients were required with 1 mM samples of 5F-Trp J protein to obtain an adequate signal-to-noise ratio. $^{19}F$ chemical shifts are referenced with respect to the $^{19}F$ resonance of trifluoroacetic acid (TFA). The accuracy of $^{19}F$ chemical shifts is ±0.05 ppm. Amino acids were added directly to NMR sample tubes in concentrated form in the presence of 10 mM phosphate buffer at pD 7.0 to avoid large changes in sample concentration. The pD of each NMR sample was measured with a 4.5 mm Ingold Electrode using a Radiometer, Model 26, pH meter. The pD of each sample in $D_2O$ is expressed directly as that obtained from the pH meter reading with no correction for the activity of deuterium ions.

*Tryptophan Analyses.* Analyses of total tryptophan and of tryptophan/5-fluorotryptophan ratios in J protein and 5F-Trp J protein were made using a Beckman, Model 120, amino acid analyzer. The basic procedure of Liu and Chang (1971) was used, except that the short column was eluted with 0.2 M citrate buffer at pH 4.6. This allowed complete separation of L-tryptophan and 5-fluorotryptophan after *p*-toluenesulfonic acid digestion of the protein molecule. This modification also resolved 5-fluorotryptophan and L-lysine which normally elute simultaneously.

*Fluorescence Studies.* Fluorescence measurements were done on a Farrad Mark I spectrofluorometer equipped with a corrected spectral attachment. Sample concentrations of J protein and 5F-Trp J protein were approximately 1 μM in all experiments. Slit widths for excitation and emission were 2.5 nm. Excitation was scanned from 200 to 320 nm while observing emission at 328 nm in the case of J protein. In the case of 5F-Trp J protein, excitation was scanned over the same range while observing emission at 325 nm. Emission spectra were obtained by scanning from 300 to 370 nm while observing emission at 283 nm for J protein and 280 nm for 5F-Trp J protein. All additions to the sample cuvette were made by means of an Eppendorf micropipet (10 μL) using concentrated amino acid solutions. After additions, samples were stirred gently to avoid the formation of air bubbles. Appropriate corrections were made for volume changes inside the cuvette.

*N-Bromosuccinimide Reaction.* Determination of the tryptophan reactivity to *N*-bromosuccinimide (NBS) was made using the procedure of Spande and Witkop (1967).

*Protein Assays.* Protein concentrations were determined either by the procedure of Lowry et al. (1951) or spectrophotometrically using the reported absorbance of 0.71 for a 0.1% J protein solution at 280 nm (Lever, 1972). All spectrophotometric measurements were made on a Zeiss PMQ II spectrophotometer.

## Results

*Isolation and Purification of J Protein and 5F-Trp J Protein.* Isolation of J protein from crude osmotic shock fluid essentially followed the procedure of Willis et al. (1974). This procedure consists of an acid precipitation, a CM-23 column purification, and an ammonium sulfate (65% saturation)

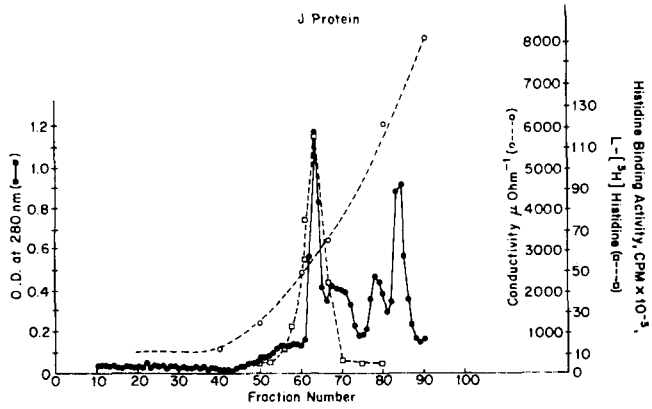


FIGURE 1: The elution pattern of the 65% ammonium sulfate supernatant on Whatman DE-52 cellulose. A 1.5 × 30 cm column was equilibrated with 5 mM Tris-HCl at pH 8.35 and loaded with 800 mL of protein solution from the $(NH_4)_2SO_4$ supernatant dialyzed extensively against 5 mM Tris-HCl at pH 8.35. After loading, the column was eluted with a concave gradient from 0 to 0.2 M NaCl. 12.5-mL fractions were collected.

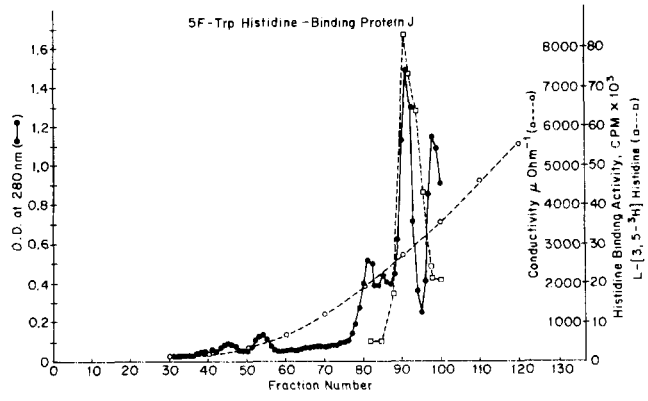


FIGURE 2: The elution pattern of the 65% ammonium sulfate supernatant on Whatman DE-52 cellulose of a preparation from 5-fluorotryptophan grown *S. typhimurium.* Conditions for the cellulose column are identical to those in Figure 1. Ten milliliter fractions were collected.

precipitation step, followed by chromatography on a DE-52 column. The DE-52 elution profile of a J protein preparation from the TA1859 strain of *S. typhimurium* is illustrated in Figure 1. The L-histidine binding activity is confined to one peak, which normally elutes at a conductivity of 3000 $\mu ohm^{-1}$. Protein from fractions 59 through 66 was pooled and was judged pure by sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis. A total of 80 mg of J protein was recovered from 6 L of shock fluid.

5F-Trp J protein was isolated in a similar manner from strain TA1010. Its DE-52 elution pattern is shown in Figure 2. Although this pattern differs somewhat from that in Figure 1, the L-histidine binding activity is again eluted at a conductivity of 3000 $\mu ohm^{-1}$. A total of 50 mg of 5F-Trp J protein was recovered from 6 L of shock fluid. The protein was judged pure by sodium dodecyl sulfate gel electrophoresis.

Molar extinction coefficients at 280 nm were estimated for both J protein and for 5F-Trp J protein. Using a molecular weight of 26 000 for J protein and 5F-Trp J protein, the extinction coefficients were estimated to be 17 000 $M^{-1}$ for J protein (in agreement with the value reported by Lever (1972)) and 25 000 $M^{-1}$ for 5F-Trp J protein (Robertson (1976)).

*Proton NMR Studies of J Protein and of 5F-Trp J Protein.* The aliphatic and aromatic regions of the $^1H$ NMR spectra of J protein are shown in the upper panels of Figures 3 and 4,

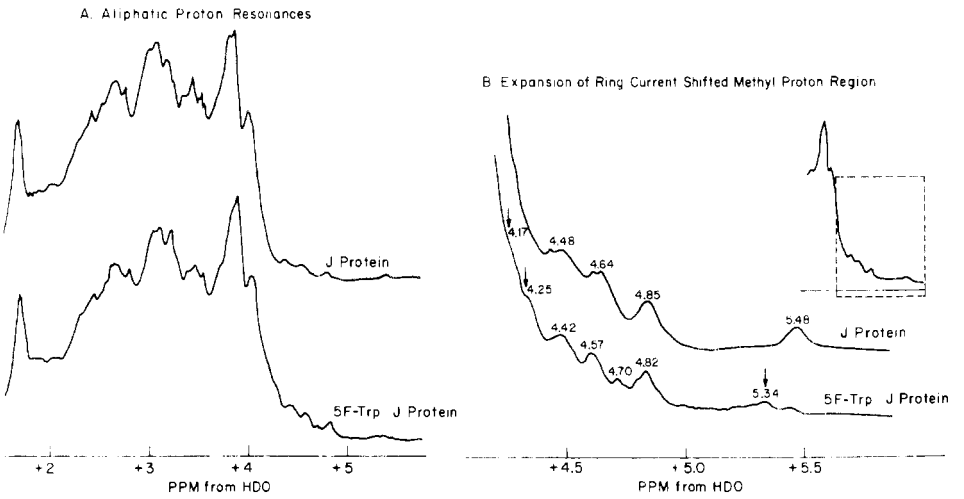


FIGURE 3: 250 MHz $^1$H NMR spectra of the aliphatic and ring-current shifted resonances of J protein and 5F-Trp J protein in 10 mM potassium phosphate at pD 6.8 and 27 °C. Arrows indicate differences in the ring-current shifted methyl region of the $^1$H NMR spectra between native J protein and the 5F-Trp J protein.

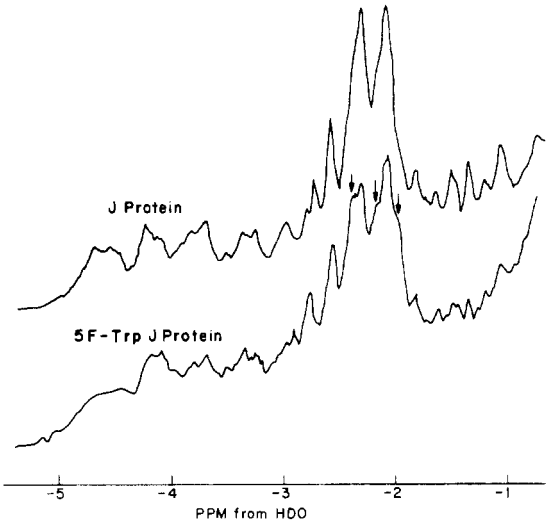


FIGURE 4: A comparison of the 250 MHz $^1$H NMR spectra of the downfield proton resonance region of J protein and 5F-Trp J protein. Differences are indicated by arrows. Experimental conditions are the same as those given in Figure 3.

respectively. The secondary and tertiary structures of J protein are responsible for many features not evident in denatured samples (Robertson, 1976). The aliphatic region of the spectrum is composed primarily of methylene and methyl resonances. While most of the methyl resonances occur near +4.0 ppm from HDO, a number of ring-current shifted methyl resonances appear between +4.2 and +5.5 ppm. The region between −0.5 and −2.6 ppm from HDO is composed principally of aromatic resonances whose line positions and widths are determined by their environment in the folded protein molecule. A number of exchangeable amide proton resonances also appear in the aromatic proton resonance region, between −2.6 and −5.0 ppm. The bulk (but not all) of these protons can be exchanged for deuterons by heating J protein to 55 °C in $D_2O$ (Robertson, 1976).

A single titratable resonance was observed in the region characteristic of histidine C2 proton resonances. Its p$K$ was found to be 6.5 from a titration curve of chemical shifts vs. pD (Robertson, 1976). This is characteristic of protein histidine residues that are exposed to the solvent (Markley, 1975). Its rather narrow line width (~10 Hz) also suggests that at least one of the three histidine residues (Lever, 1972) is quite mobile.

The aliphatic and aromatic proton resonance regions of the 5F-Trp J protein spectra are shown in the lower panels of Figures 3 and 4, respectively. The similarity of these spectra to those of J protein suggests that 5-fluorotryptophan incorporation has no major conformational consequences. This observation is strongly supported by the similarity in isolation properties and binding constants of the J and 5F-Trp J proteins (Figures 1 and 2; Robertson, 1976). Nevertheless, some spectral differences are both expected and observed, particularly in the aromatic and ring-current shifted methyl regions. Differences in the aromatic region can be traced primarily to spectral differences between tryptophan and 5-fluorotryptophan, while differences in the ring-current shifted methyl region can be attributed to differences in ring-current intensity between tryptophan and 5-fluorotryptophan. A comparison of the ring-current shifted methyl regions shows that several resonances, which are absent from the J protein spectrum, appear in the 5F-Trp J protein spectrum, for example those at +4.25, +4.70, and at +5.34 ppm from HDO. At the same time, the resonance at +5.48 ppm loses much of its intensity in the 5F-Trp J protein spectrum. We surmise that this resonance corresponds to a methyl group shielded by residual nonfluorinated tryptophan, while the resonance at +5.34 ppm corresponds to the same methyl group shielded by 5-fluorotryptophan. This is supported by the fact that the ratio of the +5.34 and +5.48 ppm intensities corresponds roughly to the 5-fluorotryptophan/tryptophan ratios found from amino acid analyses of 5F-Trp J protein preparations (for this preparation the ratio was 80:20). A comparison of the aromatic regions of J and 5F-Trp J proteins (Figure 4) shows that, as expected, some differences exist in the region characteristic of the indole resonances. These differences are indicated in the figure by arrows. The rest of the aromatic $^1$H NMR spectra are very similar however.

*Effects of Substrates on J Protein and 5F-Trp J Protein.* At a substrate/protein molar ratio of 2:5, specific changes begin to appear in the $^1$H NMR spectrum of J protein. The spectral change is essentially complete at a ratio of 4 mol of substrate to 5 mol of protein, indicating an approximate stoichiometry of binding of 1:1 in agreement with Lever (1972). An expansion of the aliphatic region of the $^1$H NMR spectrum before and after L-histidine addition is shown in Figure 5. In

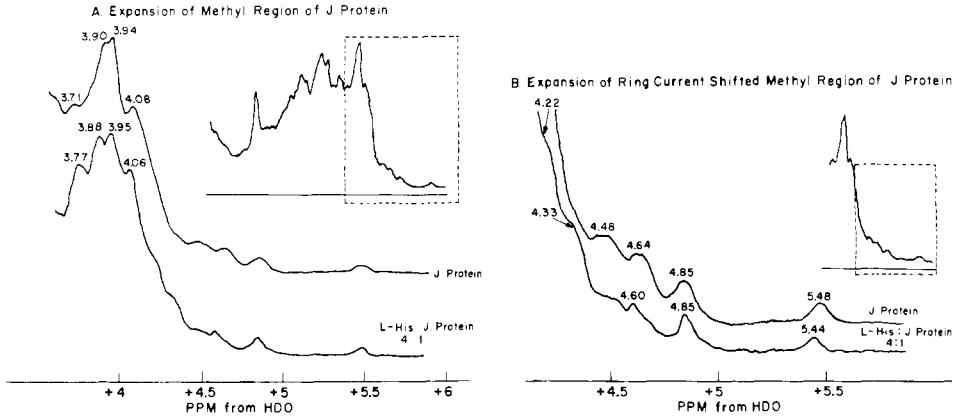


FIGURE 5: Expansion of the 250 MHz $^1$H NMR spectra of the methyl and ring-current shifted methyl regions of J protein before and after L-histidine addition. Spectra were obtained in 10 mM phosphate at pD 6.1 and 27 °C. J protein concentration was 0.9 mM. Chemical shifts from HDO are indicated for each resonance. L-histidine was added to a threefold molar excess.

the methyl region it is apparent that a considerable number of protons have changed environment within the J protein molecule. This general trend is evidenced by a shift of the +3.71 ppm line to +3.77 ppm as well as the slight downfield shift of the resonance at +4.08 ppm from HDO. A greater separation of the two resonances at +3.90 and +3.94 ppm is also apparent. Of great interest is the area of the ring-current shifted methyl protons shown in Figure 5B. Since the methyl residues which give rise to these peaks are either above or below aromatic rings, movement of these groups relative to each other will result in a chemical shift change. It is apparent from Figure 5B that a number of such changes have occurred upon substrate binding. New resonances appear at +4.22 and +4.33 ppm from HDO, while the resonance which appeared at +4.48 ppm from HDO in the absence of L-histidine disappears in the presence of L-histidine. The peak at +4.64 ppm shifts downfield to +4.60, while the line at +4.85 ppm appears to sharpen considerably. Since each of these chemical shift changes reflects a movement of a methyl group relative to nearby aromatic rings, the binding of L-histidine appears to induce a conformational change which encompasses a considerable number of amino acid residues. Earlier we suggested that the +5.48 ppm resonance corresponds to a methyl group shielded by a tryptophan residue. Its chemical shift change in the presence of L-histidine (0.04 ppm) therefore indicates that at least one of the tryptophans is involved in the conformational change.

Figure 6 is an expansion of the aromatic proton resonance region under conditions identical to those given in the preceding figure. Again, significant spectral changes are observed, most notably in the intense aromatic proton resonances. The peak at −2.33 ppm shifts about 0.07 ppm downfield and the −2.14 ppm line loses considerable intensity, while its line width is reduced by about 20 Hz. The addition of L-histidine also causes a complete disappearance of the resonance at −1.36 ppm. The gradual disappearance of this line with incremental L-histidine addition is quite apparent from Figure 4. General spectral features in the region between −1.25 and −0.77 ppm are also drastically influenced by the addition of L-histidine. These spectral changes again provide evidence for the existence of an L-histidine induced conformational change which involves a substantial number of amino acid residues.

We have also studied the effect of L-glutamate, L-arginine, and D-histidine on the $^1$H NMR spectrum of J protein. Both L-arginine and D-histidine are low-affinity substrates for J protein ($K_D \sim 10$ and 500 $\mu$M, respectively). L-Glutamate has

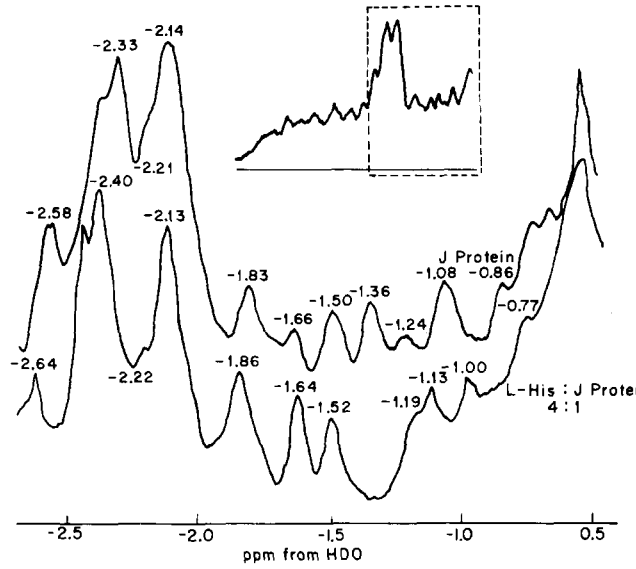


FIGURE 6: Expansion of the downfield resonance region of the 250 MHz $^1$H NMR spectra of J protein before and after addition of a threefold molar excess of L-histidine. Spectra of a 0.9 mM J protein sample were obtained in 10 mM phosphate at pD 6.1 and at 27 °C. Chemical shifts from HDO are indicated for each resonance.

no effect on the $^1$H NMR spectrum of J protein. However, if either L-arginine or D-histidine is added to a sample of J protein, several spectral changes are observed. These changes are characteristic of the added amino acid, and differ from those observed in the presence of L-histidine. Thus, while the presence of L-histidine results in a greater resolution of the methyl resonances at +3.90 and +3.94 ppm (Figure 5), these resonances are completely unresolved in the presence of L-arginine or D-histidine. The +5.48 ppm resonance which shifts downfield in the presence of L-histidine (Figure 5) is shifted similarly by L-arginine, but is unaffected by D-histidine. More changes occur in the aromatic region, as shown in Figure 7. In the presence of L-arginine or D-histidine, the −1.36 ppm line disappears, as it does in the presence of L-histidine. The behavior of the remainder of the −2.2 to −1 ppm region, however, is different for each of the added substrates. L-Arginine, for example, causes considerable sharpening of the −1.5 and −2.13 ppm lines, as well as an intensity loss in the resonance at −1.86 ppm. In contrast, the latter resonance becomes sharper in the presence of L-histidine. The effects of D-histidine in this region are similar to those of L-arginine, except that the

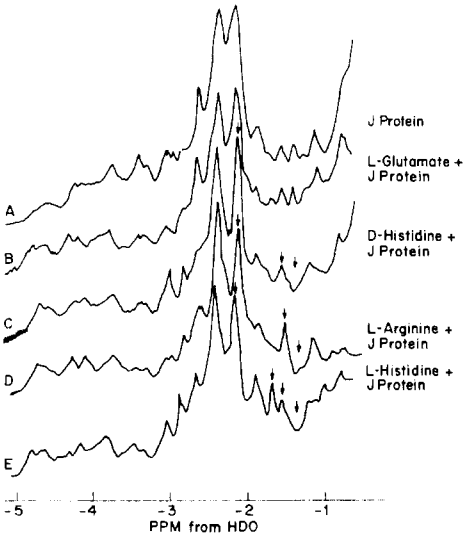


FIGURE 7: 250 MHz $^1$H NMR spectra of the downfield regions of 1.2 mM samples of J protein and J protein plus threefold excess of either L-glutamate, D-histidine, L-arginine, or L-histidine. The spectra were obtained in 10 mM phosphate at pD 6.8 and at 27 °C. Spectral differences are indicated by arrows for each sample.

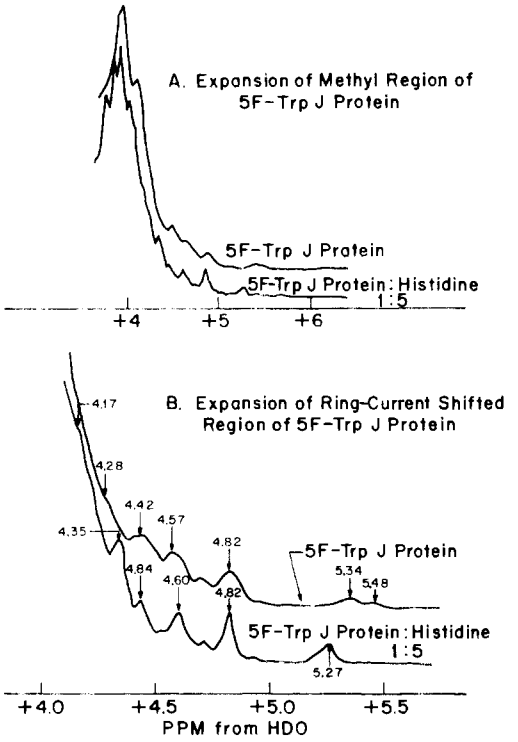


FIGURE 8: 250 MHz $^1$H NMR spectra of the methyl and ring-current shifted methyl regions of 5F-Trp J protein before and after addition of 1 molar excess of L-histidine. Shifts are indicated in ppm from HDO. Spectra were obtained in 10 mM phosphate at pD 6.8 and at 27 °C.

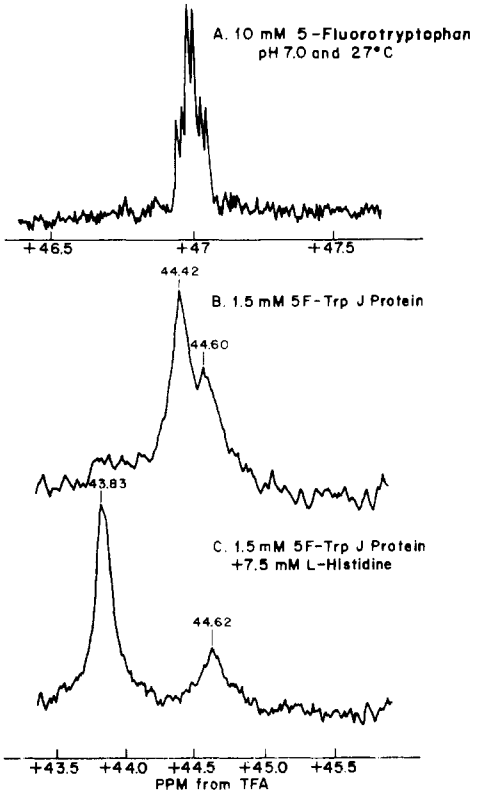


FIGURE 9: 235.2 MHz $^{19}$F NMR spectra of 5-fluorotryptophan and 5F-Trp J protein in 10 mM phosphate plus 10 mM NaCl at pD 6.8 and at 27 °C. Indicated shifts are referenced to the position of the $^{19}$F resonance of trifluoroacetic acid (TFA).

intensity of the −1.5 ppm line remains relatively unchanged. Finally, if L-histidine is added in fourfold excess to a solution of J protein together with a fourfold excess of one of the lower affinity substrates, the spectral characteristics are the same as those seen in the presence of L-histidine alone. This clearly indicates that each of the substrates binds to the same site.

We have also studied the effect of L-histidine on the $^1$H NMR spectrum of 5F-Trp J protein. The methyl and ring-current shifted methyl regions for 5F-Trp J protein in the presence and absence of L-histidine are shown in Figure 8. Of interest is the downfield (0.07 ppm) shift observed for the +5.34 ppm resonance. We suggested earlier that this resonance may be assigned to a methyl group shielded by 5-fluorotryptophan. Both the +5.34 ppm and the corresponding J protein resonance undergo downfield shifts upon histidine binding, although the latter shift is smaller. That the shifts are different in magnitude is not surprising in view of the expected differences in ring-current intensities. We also point out that the +5.48 ppm resonance disappears in the presence of L-histidine. In view of our assignment of this resonance to a methyl group shielded by a residual L-tryptophan, we expected this resonance to shift downfield. There are a number of explanations for this behavior which have not been resolved at present. Finally, the changes observed in the aromatic region are similar to those observed for J protein (Robertson, 1976).

*$^{19}$F NMR Studies of 5F-Trp J Protein.* Earlier we concluded that at least one of the tryptophans is involved in the substrate-induced conformational change in J protein. We have studied this involvement in more detail from the effect of L-histidine on the 5-fluorotryptophan $^{19}$F resonances of 5F-Trp J protein. 235.2 MHz $^{19}$F NMR spectra of 5-fluorotryptophan and 5F-Trp J protein in the absence and presence of L-histidine are shown in Figure 9. No proton decoupling was used for these experiments because of the possible existence of a negative nuclear Overhauser effect (Sykes et al., 1974). As a result, each of the line widths contain a 30 Hz contribution from fluorine–proton spin–spin coupling. In the absence of L-histidine, the protein spectrum consists of two partially overlapping resonances with an intensity ratio of about 2:1. These resonances appear approximately 2.5 ppm downfield from that of 5-fluorotryptophan. The addition of L-histidine results in a large downfield shift (136 Hz or 0.59 ppm) of the more intense

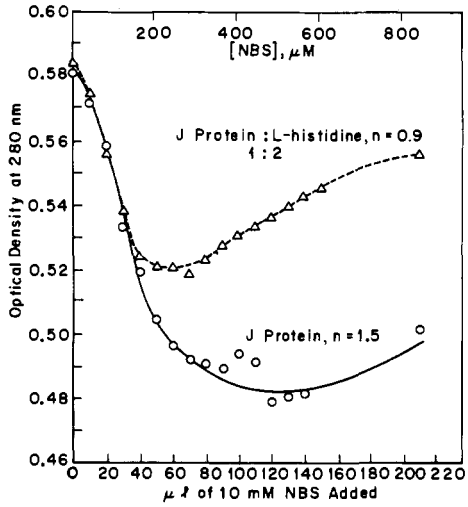


FIGURE 10: Titrations of J protein and J protein plus L-histidine with *N*-bromosuccinimide. These experiments were performed in 0.1 M acetate buffer at pH 3.6 and at 23 °C. The decrease in optical density at 280 nm corresponds to the formation of oxindole from tryptophan. In the figure, *n* represents the number of moles of oxindole formed per mole of protein, as calculated from the expression of Spande and Witkop (1967).

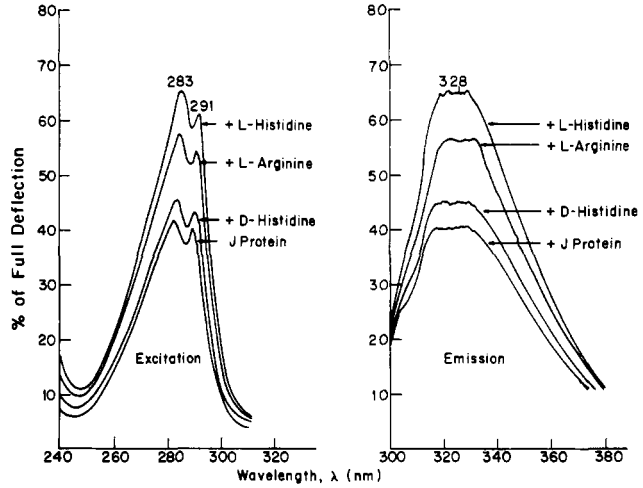


FIGURE 11: Effects of ligands on the excitation and fluorescence emission spectra of histidine-binding protein J. Emission spectra were obtained by excitation at 283 nm. Protein concentrations in each case were 1 μM and ligands were added in 19-fold molar excess. All spectra were taken of solutions in 10 mM phosphate at pD 6.8 and at 23 °C.

TABLE I: A Compilation of Data for Absorbance and Fluorescence Increases of 1 μM J Protein Samples with Ligands Added in 19-Fold Molar Excess.

| Added Ligand | % Increase[a] at 284 nm | 291 nm | 328 nm |
|---|---|---|---|
| D-Histidine | 11.6 | 11.4 | 11.0 |
| L-Arginine | 42.0 | 50.0 | 41.0 |
| L-Histidine | 62.0 | 59.0 | 61.0 |

[a] % increase refers to the increase in full scale absorbance or emission on the Farrad Mark I spectrofluorometer.

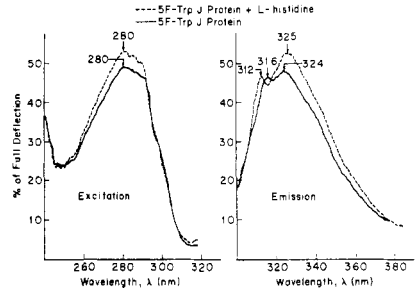


FIGURE 12: The effect of L-histidine on the excitation and emission spectra of 5F-Trp J protein. Emission spectra were obtained by excitation at 280 nm of 0.8 μM samples in 10 mM phosphate at pD 6.8 and at 23 °C. L-Histidine was added in 19-fold molar excess.

resonance (at +44.42 ppm from TFA), while its line width remains virtually unchanged. The less intense resonance (at +44.60 ppm from TFA) undergoes no change under these conditions. Similar observations were made for L-arginine, although the shift observed in this case is considerably smaller (0.22 ppm). That these shifts are a result of substrate binding is evident from the lack of any discernible spectral changes in the presence of either a fourfold molar excess of L-glutamate or L-leucine. Since there appear to be a total of approximately three tryptophans per protein molecule (Lever, 1972), the data appear to suggest that two tryptophan residues are affected to the same extent by L-histidine binding, while one of the residues is unaffected. An interesting feature is that two tryptophans should have such similar environments both in the presence and absence of L-histidine. In view of recent studies which show that the $^{19}F$ resonance of 5-fluoroindole moves downfield with decreasing solvent polarity (Robertson, 1976), we conclude that the environment of at least one of the tryptophans becomes more hydrophobic in the presence of L-histidine. Judging from the similarity in line widths (55 Hz) the tryptophan mobility appears to be unaffected.

*N-Bromosuccinimide Reactivity of the Tryptophan Residues.* The $^{19}F$ NMR data suggest that at least one of the tryptophan residues moves to a more hydrophobic environment in the presence of L-histidine. In an attempt to determine whether this change in environment is accompanied by a change in solvent exposure, we have studied the reactivity of the protein tryptophan residues toward NBS, in the presence and absence of L-histidine. In these experiments, the absorbances at both 280 and 261 nm were observed to monitor the indole to oxindole reaction (Spande and Witkop, 1967). The results at pH 3.6 are shown in Figure 10. At this pH, the tryptophan residues appear to be most reactive. The figure depicts the drop in optical density at 280 nm (the tryptophan absorption maximum) during the titration with NBS. The data clearly show that fewer tryptophans are available for the NBS reaction in the presence of L-histidine. We infer from this that at least one of the tryptophans is less exposed to the solvent in the presence of L-histidine than in its absence.

*Fluorescence Studies of J Protein.* Both the position of the emission band and quantum yield associated with tryptophan fluorescence are sensitive to changes in the tryptophan environment. We have therefore measured the tryptophan fluorescence of both J and 5F-Trp J protein solutions in the presence and absence of substrate. The results of these experiments are shown in Figures 11 and 12. Figure 11 shows that J protein exhibits absorbance maxima at 283 and 291 nm. Excitation at 283 nm results in a broad fluorescence emission band between 318 and 334 nm. Neither L-glutamate nor L-leucine has any effect on these spectra. However, addition of L-histidine, D-histidine, or L-arginine causes striking changes in both the absorption and emission spectra. These results are summarized in Figure 11 and in Table I. D-Histidine, which has a reported dissociation constant of 500 μM, increases the quantum yield

at 328 nm by 11%, while L-arginine, whose dissociation constant is 10 $\mu$M, increases the quantum yield by 41%. The most striking change occurs upon addition of L-histidine, which has the highest affinity for J protein. In this case, a 61% increase was observed at 328 nm. Fluorescence spectra were also obtained for 5F-Trp J protein with and without L-histidine. The results for this experiment are shown in Figure 12. 5F-Trp J protein has a broad absorption band with a maximum at 280 nm. Excitation at this wavelength results in a fluorescence emission with maxima at 316 and 324 nm. The addition of a 19-fold molar excess of L-histidine leaves the position of the 324-nm band unchanged while the 316 nm band shifts toward shorter wavelength by 4 nm. This shift is accompanied by an 11% increase in the absorption and emission intensities.

## Discussion

The high-affinity transport system for L-histidine in *S. typhimurium* requires the presence of both the histidine-binding protein J and a second protein, the P protein (Ames and Lever, 1970; Ames, 1972). It has been suggested that the P protein is membrane bound (Ames and Lever, 1970). Although no detailed molecular model exists for the J-P transport system, recent genetic evidence has implicated a direct and specific interaction between the J and P proteins as an essential condition for the active transport of L-histidine across the cytoplasmic membrane of *S. typhimurium* (Ames and Spudich, 1976). One of the proposed models for this interaction envisions an L-histidine-induced conformational change of the J protein molecule, which precedes the J-P interaction. In this study, we have undertaken a physical-chemical investigation of the J protein conformation in aqueous solution. An investigation of this aspect of J protein and a characterization of the structural changes upon binding of L-histidine are essential for a study of the J-P interaction and/or J protein-lipid interaction in the energy-coupled translocation of L-histidine.

Our $^1$H NMR results of J protein and 5F-Trp J protein indicate that there is indeed a substrate-induced conformational change in this protein molecule. This is clearly reflected by spectral changes of J protein in the aromatic, aliphatic, and ring-current shifted methyl proton resonance regions. Judging from the extent of the spectral changes, a considerable number of amino acid residues are involved in the substrate-induced conformational change. This strengthens the argument that there is a general conformational change in the J protein molecule, that is, one consisting of structural changes in different regions of the protein molecule rather than simply a conformational change in the substrate-binding site. L-Arginine and D-histidine which bind to J protein with much lower affinity than L-histidine ($K_D \sim 10$ $\mu$M and $K_D \sim 500$ $\mu$M, for L-arginine and D-histidine, respectively, vs. $\sim$0.15 $\mu$M for L-histidine (Ames and Lever, 1972)) also alter the conformation of J protein. However, this change differs from that induced by L-histidine. This is evidenced by subtle, but observable, differences in their $^1$H NMR spectra. Each of these three amino acids appears to bind to the same site in J protein. It seems, therefore, that there may be differences in the stereochemical details of their binding. It is conceivable that these differences could affect the J-P interaction.

$^{19}$F NMR spectroscopy is a valuable technique to investigate the structure-function relationship in a protein molecule if an appropriate $^{19}$F label can be incorporated biosynthetically or can be attached (covalently or noncovalently) to certain groups in a protein molecule (for example, see Sykes et al., 1974; Pratt and Ho, 1975; Huestis and Raftery, 1972). $^{19}$F NMR studies of 5F-Trp J protein have provided a unique insight into the involvement of the tryptophan residues in the substrate-induced conformational change. The fact that only one of the two $^{19}$F resonances shifts in the presence of L-histidine implies that at least one (and possibly two) of the three to four tryptophan residues in J protein undergoes a change in environment. Thus, while the $^1$H NMR data suggest that a considerable number of amino acid residues are involved in the substrate-induced conformational change, the $^{19}$F NMR results show that only one or at most two of the total number of tryptophan residues change their environment. Furthermore, the fact that a much smaller shift is observed in the presence of L-arginine (0.22 ppm) than in the presence of L-histidine (0.59 ppm) supports the notion that there are appreciable differences in the stereochemistry of binding between these two amino acids.

Hull (1975) has postulated that there is a relationship between the $^{19}$F chemical shift of *m*-fluorotyrosine in fluorotyrosine-labeled alkaline phosphatase and its environment; as the $^{19}$F resonance shifts downfield, the environment becomes more hydrophobic. The $^{19}$F chemical-shift dependence of 5-fluoroindole in solvents of differing polarity supports this relationship (Robertson, 1976). From this, we infer that the downfield shift observed for 5F-Trp J protein upon substrate binding indicates a change in environment of at least one of the 5-fluorotryptophan residues to more hydrophobic surroundings. In view of the fact that there is no line-width change, the rotational freedom of the 5-fluorotryptophan residue(s) appears to be unaffected (assuming a constant intermolecular contribution to the line width).

Our tryptophan fluorescence data confirm the conclusion drawn above. The increased quantum yields observed upon addition of substrates indicate that at least some of the tryptophan residues move to a more hydrophobic environment (Wada and Ueno, 1964; Konev, 1967; Borenboim et al., 1969). The increase in quantum yields is related to the substrate affinity which increases in the order D-histidine < L-arginine < L-histidine. A maximum gain in fluorescence intensity (61%) is observed for L-histidine binding. We surmise that this trend reflects the highly stereospecific nature of the substrate binding site.

Similar fluorescence experiments have been performed for the galactose-binding protein of *E. coli* in the presence and absence of substrates (Silhavy et al., 1974) and for the sulfate-binding protein of *S. typhimurium* in the presence and absence of sulfate (Langridge et al., 1970). In both of these cases, the environment of at least some of the tryptophan residues became more hydrophobic in the presence of substrate.

The change in the environment of the tryptophan residue(s) is accompanied by a change in solvent exposure. This is clearly shown by the fact that fewer tryptophan residues are available for attack by *N*-bromosuccinimide in the presence of L-histidine. This is also another indication that there is a substrate-induced conformational change in J protein. In any case, the data indicate that in the presence of substrate the environment of one or more of the tryptophan residues becomes more hydrophobic in nature and less exposed to the aqueous solvent, while the remaining tryptophan is essentially unaffected.

In conclusion, our results have clearly shown the existence of a substrate-induced conformational change in histidine-binding protein J. We have also shown that the environment of at least one of the tryptophan residues becomes more hydrophobic and less accessible to the solvent upon binding of L-histidine. The existence of a conformational change upon L-histidine binding provides strong support for those models

which envision such a conformational change as a necessary step for the interaction of the J protein with the P protein in the active transport of L-histidine across the cytoplasmic membrane of *S. typhimurium*. Nevertheless, the relationship between the conformational changes observed and that deemed essential for the J–P interaction remains uncertain. We are at present attempting to clarify this relationship.

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