High-Resolution Proton Nuclear Magnetic Resonance Studies of Histidine-Binding Proteins J of *Salmonella typhimurium*. An Investigation of Substrate and Membrane Interaction Sites†

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ABSTRACT: The binding of L-histidine to the periplasmic histidine-binding proteins J of *Salmonella typhimurium* strains TA1859, TA301, and TA300 has been studied by high-resolution ^{1}H nuclear magnetic resonance at 250 MHz. The dependence on substrate concentration of the chemical shifts of various resonances of each J protein was used to estimate a range of values for the off-rate constant k_{-1} for the binding of L-histidine to J protein. It was found that J proteins from two of the strains, TA1859 and TA300, have very similar k_{-1} values, while that from TA301 exhibits a much larger k_{-1}. This indicates that the mutation in TA300 has not affected the L-histidine binding, but that the TA301 mutation has altered the conformation of the substrate binding site so that the substrate has a shorter bound lifetime, $\tau_B = 1/k_{-1}$. We have also observed differences between the spectra of the J protein from strain TA1859 (i.e., normal J protein) and the mutant J protein from TA300 or TA301. This shows that there are conformational differences between the native and mutant proteins, which have affected the environment of certain amino acid residues. From these observations we propose certain resonances as possibly arising from amino acid residues at particular regions of the J protein such as the substrate binding site or that part of the J protein molecule at which it interacts with the membrane-bound L-histidine permease or carrier.

The bacterial periplasmic binding proteins are necessary for the transport of certain amino acids, sugars, and ions across the cytoplasmic membranes of gram-negative bacteria. Various models for the translocation process have proposed a direct interaction between the periplasmic binding protein and a membrane-bound carrier or permease. Supposedly, a given binding protein delivers a specific substrate to the carrier, which in turn undergoes a conformational change, allowing the substrate to cross or penetrate the membrane (Oxender, 1972; Singer, 1974; Ames & Spudich, 1976). Conformational changes have been observed in several binding proteins upon attachment of the specific substrates (for example, see Oxender, 1972; Kreishman et al., 1973; Robertson et al., 1977). This structural change could be necessary for the interaction of the two-protein components. Furthermore, if this is the case, then the binding of substrate could alter the structure of that part of the binding protein at which the interaction with the membrane-bound protein occurs.

The high-affinity L-histidine transport system of *Salmonella typhimurium* is one such system. The studies of Ames and her co-workers with this system have shown that it is a two-component system. One component is the histidine-binding protein J (J protein)[1] to which the substrate L-histidine binds as a first step in the transport process (Ames, 1964; Ames & Lever, 1970). Recent nuclear magnetic resonance (NMR) studies have shown that the J protein undergoes an extensive conformational change upon L-histidine binding (Robertson et al., 1977). The second component is the P protein, whose exact function has not yet been determined. However, it has been suggested that the P protein is the membrane-bound carrier of this transport system (Singer, 1974; Ames & Spudich, 1976). If so, a direct interaction between J and P proteins is expected. Evidence for this has been inferred from various genetic studies (Kustu & Ames, 1974; Ames & Spudich, 1976). This interaction is supposed to occur at a specific site on the J protein molecule. The histidine-binding protein J thus has two "functional" sites: one for L-histidine binding and the second for interaction with the P protein (protein–protein interaction site).

Genetic evidence for the existence of these two sites comes from studies of transport in two mutant strains of *S. typhimurium*: TA300 and TA301. Strain TA300 transports L-histidine with a larger K_m than the wild-type strain TA1859. However, the dissociation constant (K_D) for the binding of L-histidine to the J protein is equal to that for TA1859 (Kustu & Ames, 1974). Thus, the mutation in TA300 has not altered the binding properties of the protein. Instead, it has apparently changed some other part of the J protein molecule, so that it cannot properly participate in the translocation step; e.g., the mutation prevents the proper interaction between J and P proteins. Strain TA301 transports almost as well as TA300, but its K_D is approximately 200 times larger (Kustu & Ames, 1974). These results, along with further studies, have led to the suggestion that the J protein from TA300 is altered at the protein–protein interaction site, while the J protein from TA301 is altered at the L-histidine binding site (Ames & Spudich, 1976).

Given the proposed J protein–P protein interaction as discussed above, one would like to seek evidence for a P-interaction site on the J protein molecule and for the effect of substrate binding on the conformation of this site. This may be accomplished by a comparison of the ^{1}H NMR spectra of native J protein from TA1859 and mutant J proteins from TA300 and TA301. The differences among these three J

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[1] Abbreviations used: J protein, histidine-binding protein J which is the product of the *hisJ* gene of *Salmonella typhimurium*; J–His, J protein and L-histidine complex.

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proteins may be further characterized by determination of the off-rate constant k_{-1} for the binding of L-histidine.

We report here the results of a comparison of the 250-MHz ^{1}H NMR spectra of three histidine-binding proteins J from TA300, TA301, and TA1859 of *S. typhimurium*. The off-rate constants may be conveniently obtained from observations of the protein spectra rather than the substrate spectra. In addition, the extent of the conformational and kinetic differences among these three proteins is measured. We are able to suggest some resonances as being at or near each of the two functional sites of the J protein.

Experimental Section

Bacterial Strains. The strains of *S. typhimurium* used in this work were the generous gift of Dr. G. F-L. Ames. TA1859 (*dhuA1*) produces 5–10 times the amount of wild-type histidine-binding protein J (Ames & Lever, 1972) but is considered a "nonmutant" strain for the purposes of this work. Strain TA300, a mutant derived from TA1014 (*dhuA1 hisJ*5625), produces cells which transport L-histidine with a larger K_m ($K_m = 3.7 \times 10^{-8}$ M) than cells containing wild-type J protein ($K_m \approx 0.6 \times 10^{-8}$ M) (Kustu & Ames, 1974). Thus, it transports less efficiently than the native system. However, the binding constant of its J protein for L-histidine is about equal to that of the wild-type J protein ($K_D = 0.8 \times 10^{-7}$ M). Strain TA301 (*dhuA1 hisJ*5626), also containing a mutation in the J protein and derived from TA1014, has a J protein that does not bind L-histidine as well as the native form ($K_D = 167 \times 10^{-7}$ M) and thus does not transport as well either.

All bacteria were grown according to the procedure of Willis et al. (1974), with the salt concentrations slightly increased to produce a more stable pH during the long growth period. Salt concentrations used were Na_2HPO_4, 18 g/L; KH_2PO_4, 9 g/L; NH_4Cl, 5 g/L; Na_2SO_4, 1.1 g/L; and $MgSO_4$, 0.1 g/L. The carbon source was 3% glycerol. Bacteria were grown 15–20 h with vigorous aeration in 5-gal. carboys each containing 15 L or on a shaker in 20 4-liter flasks each containing 1 L and at 37 °C. Cells were grown to the stationary phase. When the maximum optical density (OD 8–10 at 550 nm) had been reached, harvesting was carried out by centrifugation at 8000 rpm in a Sorval GS-3 rotor.

Shock fluid was prepared according to Willis et al. (1974). Treatment of shock fluid to isolate J protein followed the procedure of this laboratory (Robertson et al., 1977). Typically 60 L of strain TA1859 at an OD of 10 at $\lambda = 550$ nm yields 80–100 mg of histidine-binding protein J.

NMR Samples. Lyophilized protein was dissolved in 10 mM sodium phosphate buffer in D_2O at pH 6.5 to give a final concentration of ~1 mM, for each strain. Concentrations were determined using an extinction coefficient of 0.71 for a 0.1% protein solution in a 1-cm cuvette at 280 nm (Lever, 1972). Protein solutions were then transferred to a standard 5-mm NMR sample tube, and the pH was adjusted to ~6.2 directly in the tube with a 4.5-mm Ingold electrode (Model 6030-04). pH measurements were made on a Radiometer Model 26 pH meter and are accurate to ±0.02 units. pH is given as the direct pH meter reading uncorrected for D_2O. When a titration of protein with L-histidine was required, aliquots of an L-histidine solution (~8 mM, 15.5 mg/12.5 mL of buffer) were added to the protein solution in the NMR sample tube. After the combined solution was carefully mixed, the ^{1}H NMR spectra were recorded.

Separate spectra of the aliphatic and aromatic resonances of J protein were obtained on the MPC-HF 250 NMR spectrometer. This instrument operates at 250 MHz for ^{1}H resonances at a temperature of 27 °C. An internal lock was provided by the residual HDO in each sample. The signal to noise ratio was improved by NMR correlation spectroscopy (Dadok & Sprecher, 1974). Usually, 500–600 scans were taken of each region of each sample. The sweep width was 1500 Hz, with a sweep time of 2 s/scan. Reported ^{1}H chemical shifts are parts per million (ppm) from HDO, which resonates 4.83 ppm downfield from the proton resonance of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). A negative sign indicates that a resonance is downfield from HDO. Accuracy of chemical shift measurements is ±0.01 ppm.

Amino Acid Analysis. Amino acid analyses were performed on a Durrum Model D-500 automated amino acid analyzer equipped with a Mark I integration system. Three procedures were used for the analyses. Firstly, for the determination of all amino acids except cysteine and tryptophan, the sample was hydrolyzed in 6 N HCl at 110 °C in vacuo for 24, 48, and 72 h. Threonine, serine, and tyrosine values were corrected for decomposition by extrapolation to zero time. Secondly, cysteine was converted quantitatively to cysteic acid by hydrolysis with 6 N HCl in the presence of dimethyl sulfoxide (Spencer & Wold, 1969). Thirdly, the hydrolysis of the sample with 3 N *p*-toluenesulfonic acid (Liu & Chang, 1971) permitted the quantitative recovery of tryptophan.

Carbohydrate Analysis. Analysis for sugars was performed on 5-mg samples of J protein from each strain (TA1859, TA301, and TA300) using gas–liquid chromatography (Clamp et al., 1971).

Theoretical Section

Theory for Chemical Exchange. Each J protein molecule binds one substrate molecule (Lever, 1972; Kustu & Ames, 1974). The binding will then be represented as follows

$$J + His \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} J\text{–}His \qquad (1)$$

where J is the uncomplexed or free binding protein J, His is the substrate (L-histidine), J–His is the J protein with one molecule of substrate bound, k_1 is the on-rate constant, and k_{-1} is the off-rate constant for the binding of L-histidine to J protein. The J protein resonances in this situation undergo chemical exchange between two environments, free and complexed.

The proton resonances of free J protein appear at certain characteristic positions. In the presence of fast or intermediate exchange, some of these resonances will appear at new positions governed by the proportions of free and complexed J protein (Pople et al., 1959; Sykes & Scott, 1972). Only one resonance will be seen for each proton undergoing fast exchange, and it can be expressed as

$$\delta_{obsd} = \delta_F + P_B\Delta \qquad (2)$$

where δ_{obsd} is the observed chemical shift, δ_F is the chemical shift in the absence of substrate, Δ is the difference between bound and free chemical shifts ($\Delta = \delta_B - \delta_F$), and P_B is the fraction of J protein molecules which have substrate bound. This is given by (Sykes & Scott, 1972)

$$P_B = \frac{[J\text{–}His]}{[J]_t} \qquad (3)$$

where [J–His] is the concentration of J protein with histidine bound and $[J]_t$ is the total concentration of J protein. The concentration of bound protein, [J–His], is calculated by standard methods from the definition of the equilibrium dissociation constant:

$$2[J\text{–}His] = ([J]_t + [His]_t + K_D) - [([J]_t + [His]_t + K_D)^2 - 4[J]_t[His]_t]^{1/2} \qquad (4)$$

Table I: Amino Acid Analyses of Histidine-Binding Proteins J of *S. typhimurium*

residue	no. of residues/J protein[a]			
	TA1859	TA300	TA301	TA1014[b]
aspartic acid	26.4	26.7	25.8	24.0
threonine	13.2	13.3	13.0	11.9
serine	10.3	11.1[c]	10.8	12.6
glutamic acid	30.4	30.1	30.2	27.4
proline	8.8	8.3	8.8	8.2
glycine	20.4	20.6	20.9	19.8
alanine	23.8	24.3	24.3	21.8
cysteic acid	1.8	2.7[c]	2.0	1.7
valine	12.0	13.2[c]	11.8	11.4
methionine	3.1	3.0	3.0	2.0
isoleucine	13.8	12.9[c]	14.5	12.8
leucine	18.0	18.2	18.5	13.3
tyrosine	7.5	7.6	6.8[c]	6.7
phenylalanine	11.6	10.6[c]	11.9	10.4
histidine	1.1	1.3	1.9[c]	3.3
lysine	23.0	23.1	22.4	29.7
tryptophan	1.3	1.4	1.4	3.7
arginine	8.7	7.6[c]	8.1	12.2

[a] Based on a molecular weight of 26 000 for J protein. [b] Data taken from Lever (1972). [c] Indicates compositional differences between the mutant and native J proteins.

As P_B increases from 0, the observed resonance will shift upfield or downfield by an amount depending on the magnitude of Δ, i.e., depending on the difference in the environment of the resonance in free and complexed J protein.

If the exchange of a J protein resonance between its two environments is slow, its chemical shifts will be unaffected. However, two resonances will, in theory, be apparent, one each from the proton when in the free or complexed J protein environment. The relative intensity of these resonances will depend on the value of P_B. As more L-histidine becomes bound J protein, the resonance(s) from free J protein will disappear.

Results

Amino Acid Analyses of J Proteins

The results of the amino acid analyses of the J proteins from each of the three strains are shown in Table I along with the amino acid composition of strain TA1014 (*dhuA1*) found by Lever (1972). The wild-type strains for J protein are TA1014 and TA1859. The results show that J protein from TA300 differs from the wild-type J protein in having different numbers of, at most, six amino acid residues. It has one less of isoleucine, phenylalanine, and arginine but one additional each of cysteine, serine, and valine. Ames has found from peptide mapping that J protein from TA300 has one arginine replaced by cysteine (G. F-L. Ames, personal communication). Our results are not incompatible with her finding. J protein from TA301 has one less tyrosine and (possibly) arginine and one more histidine than the native protein. Our results show that there is no significant difference in the molecular weight among these three J proteins.

Each of the three proteins has 1.3–1.4 tryptophans. It is quite possible that tryptophan is partially destroyed during the hydrolysis by *p*-toluenesulfonic acid, and so we believe that the actual number of tryptophans is two per J protein. This is one-half the number found by Lever (1972) (see Table I). Our results do, however, agree with those of Robertson (1976) in which J protein from TA1859 was found to contain 1.2 mol of tryptophan. Our results disagree with those of Lever (1972) in other respects also. Lever has found several more of the residues arginine and lysine but fewer of aspartic acid, glutamic acid, alanine, and leucine. Lever's results also show two more

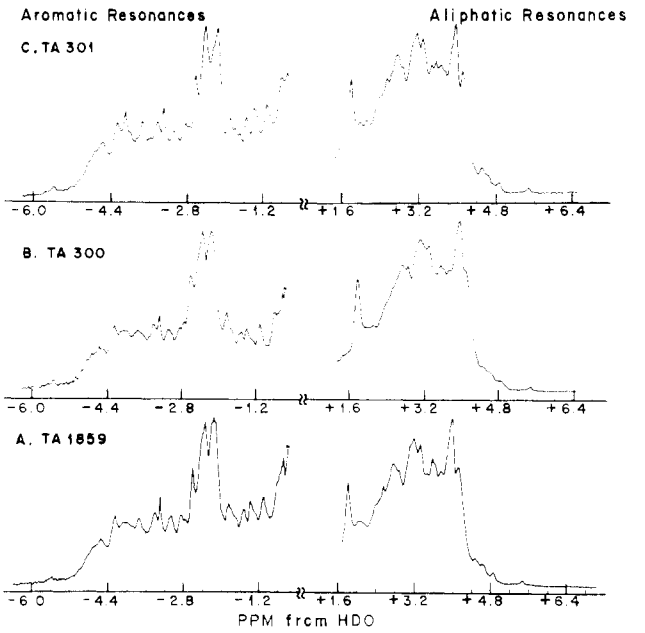

FIGURE 1: 250-MHz ^{1}H NMR spectra of the aliphatic and aromatic resonances of histidine-binding proteins J from strain (A) TA1859, (B) TA300, and (C) TA301. Substrate (L-histidine) has not been added.

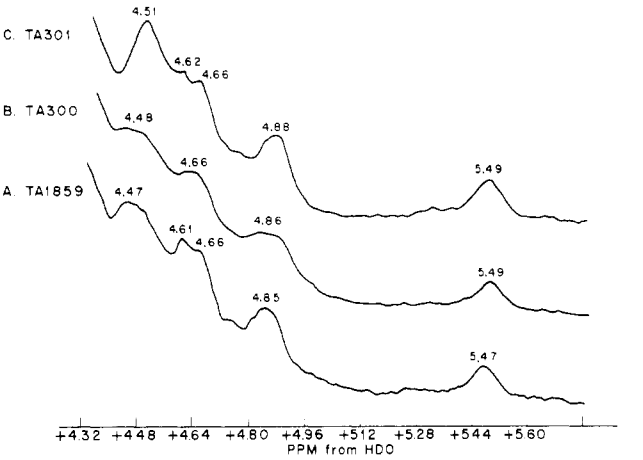

FIGURE 2: 250-MHz ^{1}H NMR spectra of the ring-current-shifted resonances of histidine-binding proteins J from strains (A) TA1859, (B) TA300, and (C) TA301. Substrate (L-histidine) has not been added.

of histidine (3.3 vs. 1.1) and two more serines (12.6 vs. 10.3) than do our results.

Nevertheless it can be seen that the differences between the wild-type J protein and each mutant are few, while the functional differences are substantial. Therefore, the NMR spectra should reflect changes at specific parts of the molecule which are functionally important.

Carbohydrate Analyses of J Proteins

These analyses showed that J protein from neither TA1859 nor TA300 contained sugars. We have found, however, that TA301 has approximately 1 mol of glucose/mol of protein (0.75 mol/mol of J protein).

^{1}H NMR Studies of J Proteins

A. Comparison of J Proteins in the Absence of L-*Histidine.* Figure 1 shows the aromatic and aliphatic regions of the three J proteins from TA300, TA301, and TA1859 in the absence of L-histidine. Figures 2, 3, and 4 show expanded regions of the aromatic and aliphatic resonances. It is obvious that the

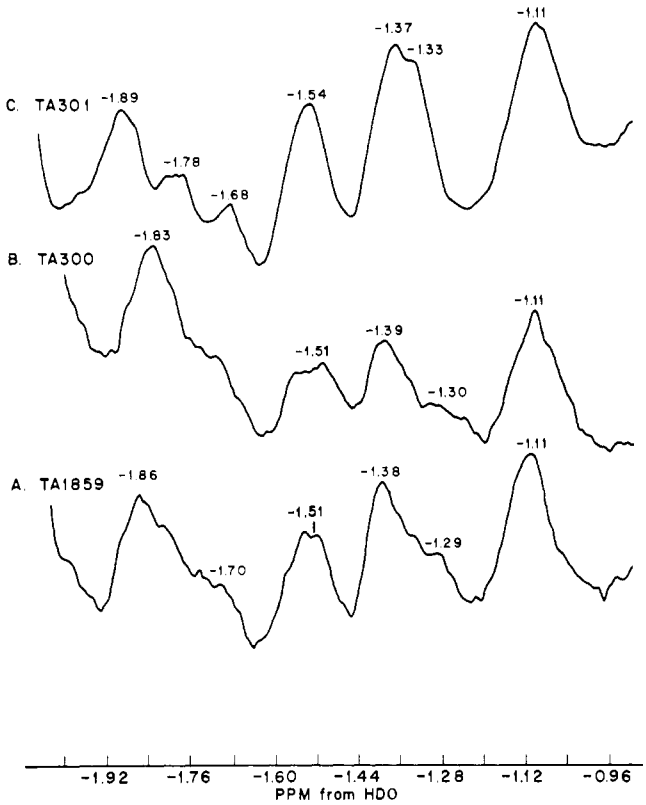

FIGURE 3: 250-MHz ^{1}H NMR spectra of the aromatic resonances of histidine-binding proteins J from strains (A) TA1859, (B) TA300, and (C) TA301. Substrate (L-histidine) has not been added.

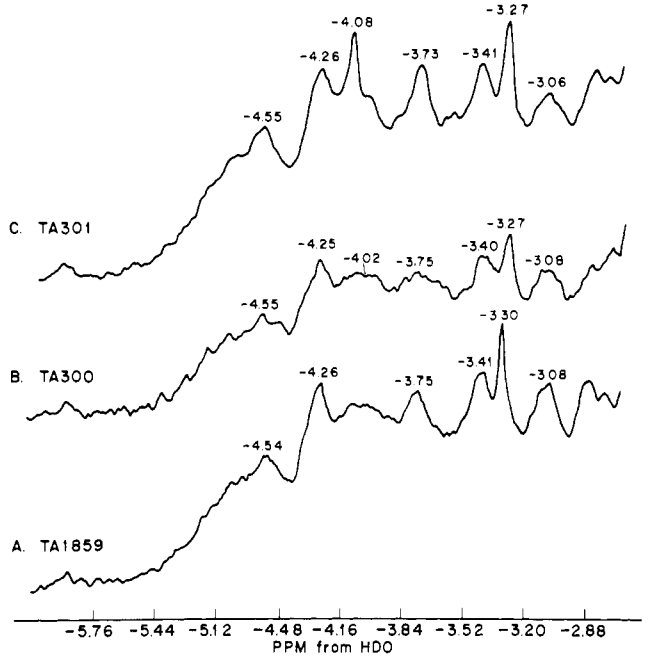

FIGURE 4: 250-MHz ^{1}H NMR spectra of the aromatic and exchangeable resonances of the histidine-binding proteins J from strains (A) TA1859, (B) TA300, and (C) TA301. Substrate (L-histidine) has not been added.

spectra of J proteins from TA300 and TA1859 are more similar than those from TA301. Yet there are definite differences among the spectra of all three proteins.

Consider Figure 2 which shows the ring-current-shifted proton resonances. The general appearance is quite similar for all three J proteins—each having four or five peaks in this region. However, TA300 has one peak less than the others, in that there is no resonance at 4.61 ppm from HDO. Also, in the spectrum of TA301 the peak at 4.51 ppm is much more intense than its counterparts in TA300 and TA1859. The most upfield peak is in a slightly different position in TA1859 (5.47 ppm) than in TA300 or TA301 (5.49 ppm).

The expansion of the −1- to −2-ppm aromatic region is shown in Figure 3. J proteins from TA300 and TA1859 yield very similar spectra, but there is one difference in that the resonance at −1.51 ppm is much broader in the TA300 spectrum. It appears that several peaks could be overlapping here. TA301 has a spectrum which contains two extra peaks, at −1.78 and −1.68 ppm. In addition, there are two overlapping peaks at −1.37 and −1.33 ppm, whereas in the spectra of TA300 and TA1859 only one peak appears at this position.

Figure 4 shows the aromatic and exchangeable resonances between −2.5 and −5.8 ppm from HDO. The native J protein spectrum exhibits several relatively broad resonances and one sharp one at −3.30 ppm. J protein from TA300 is similar to the TA1859 spectrum except that the peaks at −4.55, −4.25, and −3.75 ppm are less intense in TA300. The spectrum of TA301 has much the same features as that for TA1859 with the addition of an extra peak at −4.08 ppm. This peak could arise from the extra histidine, found by amino acid analysis to be part of the J protein from TA301 (Table I). Also, the sharp resonance at −3.30 ppm is shifted slightly upfield in TA300 and TA301 (−3.27 ppm).

These comparisons show that in the absence of L-histidine the mutation at the L-histidine binding site in TA301 has a large effect on its NMR spectrum when compared to that of the native J protein. The mutation in TA300 does not produce as much change in its spectrum.

B. Comparison of the Substrate Titrations of J Proteins. To examine differences among the proteins in detail and also to obtain kinetic data, it is necessary to obtain spectra at several points at which L-histidine is less than saturating. Titrations with L-histidine have been performed for all three proteins. To indicate the relative amounts of added L-histidine, a "fraction bound" (P_B) has been calculated (see eq 3 and 4) for each sample and is given in Figures 5–7. Spectra were obtained at additional values of P_B but are not shown in these figures.

−2.8- to −5.6-ppm Region. In the native J protein (TA1859), many changes are apparent for these resonances (Figure 5A). Changes begin to occur at the lowest value of $P_B = 0.097$ (results not shown). One of the more interesting changes here is the very apparent disappearance of the resonance at −3.30 ppm ($P_B = 0$) (compare $P_B = 0$ to $P_B = 0.29$, 0.48, and 0.87 in Figure 5A). Concomitant with this change is the appearance of another resonance at −3.09 ppm ($P_B = 0.48$). The second peak at −3.09 ppm is presumably the same proton, at a different chemical shift because this proton is now in a different environment in the presence of L-histidine. As P_B increases, the second peak increases in intensity while that from "free" J protein decreases until it is all but invisible ($P_B = 0.87$). Thus we see evidence for the existence of an equilibrium between two forms of J protein: free J (i.e., no L-histidine bound) and bound J (J–His), with one L-histidine bound. At $P_B = 0.48$ it is especially obvious that the two forms are coexisting.

J protein from TA300 (P protein interaction site mutant) behaves quite similarly to that from TA1859 in many respects (Figure 5B). The peak at −3.27 ppm ($P_B = 0$) behaves as does its counterpart in the native J protein. Again, we believe this is the result of "slow" exchange of some proton between two different environments (see below). The major difference between J proteins from TA1859 and TA301 in this region is the behavior of the peak at −3.27 ppm (Figure 5C). In the

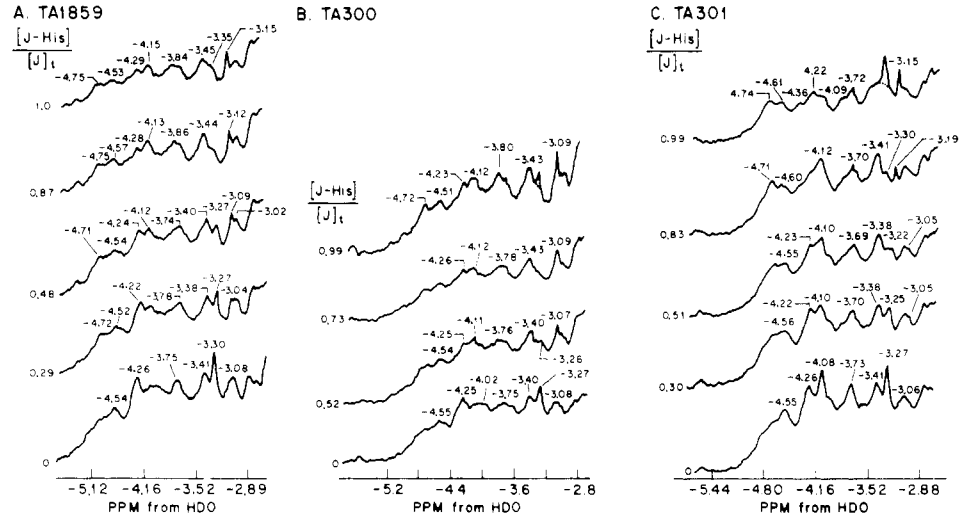

FIGURE 5: Effect of L-histidine concentration on the 250-MHz ^{1}H NMR spectra of the aromatic and exchangeable resonances of histidine-binding proteins J from strains (A) TA1859, (B) TA300, and (C) TA301. Spectra at selected values of P_B are shown.

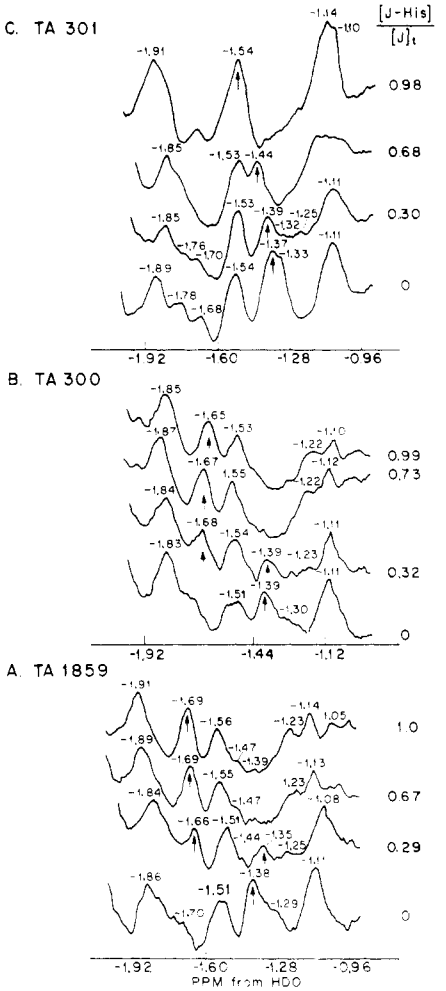

FIGURE 6: Effect of L-histidine concentration on the 250-MHz ^{1}H NMR spectra of the aromatic resonances of histidine-binding proteins J from strains (A) TA1859, (B) TA300, and (C) TA301. Spectra at selected values of P_B are shown.

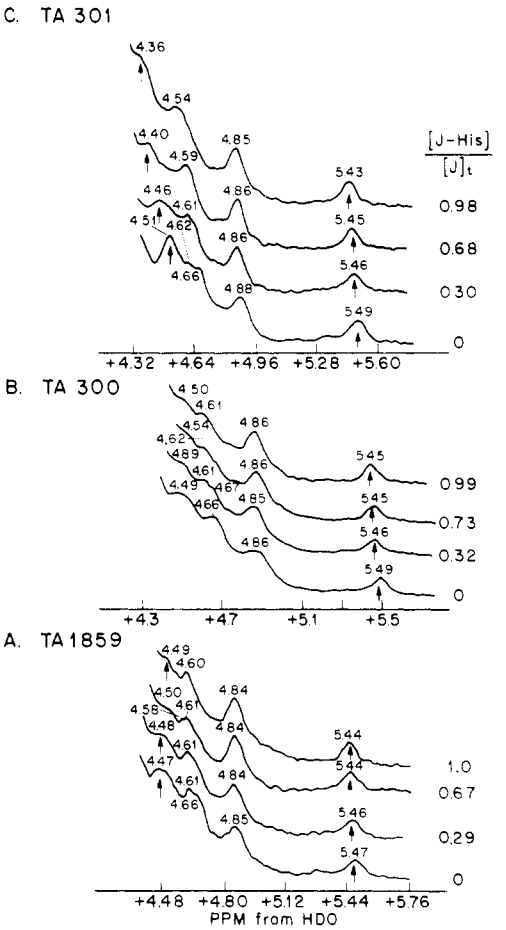

FIGURE 7: Effect of L-histidine concentration on the 250-MHz ^{1}H NMR spectra of the ring-current-shifted resonances of histidine-binding proteins J from strains (A) TA1859, (B) TA300, and (C) TA301. Spectra at selected values of P_B are shown. The arrows indicate changes discussed in the text.

normal J protein spectra, its intensity appears to decrease, while the second peak at −3.09 ppm increases in intensity. In the L-histidine binding site mutant, TA301, this peak decreases in intensity (P_B = 0.30 and 0.51) and shifts continuously upfield. At P_B = 0.68, its intensity begins to increase again while continuing to shift upfield. This behavior is believed to result from intermediate or fast exchange (see below).

Aromatic Region: −1 to −2 ppm. This region also exhibits large changes in resonance positions and intensities (Figure 6). In native J protein, as L-histidine is added, the peak at −1.86 ppm sharpens and shifts downfield to −1.91 ppm (this type of behavior is manifested in the two mutants also). The peak at −1.51 ppm shifts downfield by a total of 0.05 ppm. A peak at ~−1.69 ppm first appears at P_B = 0.097 (not shown) and continues to grow as P_B increases. Along with

this increase, there is a decrease in the intensity of the peak at −1.38 ppm, beginning at $P_B = 0.097$. It is completely gone by $P_B = 0.87$. It appears that the larger the peak at −1.69 ppm the smaller is the peak at −1.38 ppm. Thus, it could be that these two peaks arise from the same proton, except in two different environments. This is evidence of a slow chemical exchange.

There are some differences between the spectra of J proteins from TA300 and TA1859 in this region at $P_B = 0$ (see above). However, the changes in the resonances of TA300 follow very closely those of TA1859. For example, as the intensity builds up at ~−1.68 ppm ($P_B = 0.32$), the resonance at −1.39 ppm shrinks. Again we may be seeing slow exchange. The peak at −1.51 ppm sharpens up considerably as P_B is increased. This could be a true sharpening or, possibly, two overlapping peaks at low P_B become nonoverlapping as P_B increases.

The TA301 spectra show greater differences from TA1859. The main change is that the peak at −1.37 ppm decreases only slightly in intensity and that it shifts downfield to about −1.54 ppm at $P_B = 0.98$ (in TA300 or TA1859 no shift was observed). Moreover, there is no growing peak at −1.68 ppm as in the other two protein titrations. This indicates that in J protein from TA301 this residue (corresponding to −1.37 ppm at $P_B = 0$) undergoes fast rather than slow exchange. The absence of a resonance at −1.68 ppm also makes it more likely that this resonance, seen in TA1859 and TA300 spectra, is from the same residue as the resonance at ~−1.38 ppm.

Ring-Current-Shifted Aliphatic Resonances: 4.3–5.5 ppm. We observe fast exchange behavior in the resonance at 4.51 ppm in J protein from TA301 not observed in either TA300 or TA1859. The other ring-current-shifted resonances behave essentially identically in all three proteins (Figure 7).

In the spectra of native J protein, the two peaks at 4.61 and 4.66 ppm appear to merge at $P_B = 0.29$ into one peak at 4.61 ppm. At 4.47 ppm, the peak gradually disappears, without any apparent change in the chemical shift. The most upfield peak of the entire aliphatic region (5.47 ppm) shifts downfield by ~0.03 ppm as P_B increases.

The P interaction site mutant (TA300) differs from TA1859 only in that there is no resonance at 4.61 ppm when L-histidine is absent. The peak at 4.66 ppm shifts to 4.61 ppm at P_B = 0.32 where it remains. The shift downfield of the peak at 5.49 ppm is slightly greater in this mutant than in TA1859.

J protein from TA301 is different in two ways. Firstly, the ring-current-shifted resonance at 4.51 ppm loses intensity as L-histidine is added and shifts downfield to 4.36 ppm. Secondly, the peaks at 4.62/4.66 merge ($P_B = 0.3$) as in TA1859 but then shift downfield to 4.54 ppm. This downfield shift is greater than in either of the other proteins. Either or both of these two resonances is undergoing a fast exchange not observed for normal J protein or J protein from TA300. The change in chemical shift of the last peak (5.49 ppm) is the greatest observed in all three proteins.

C. Estimates of the Off-Rate Constant k_{-1}. Several resonances of these three J proteins exhibit changes in chemical shift as the concentration of substrate present increases. The dependence of these changes on fraction bound (P_B) yields estimates of the range of possible k_{-1} values.

1. J Protein from TA1859. The resonance at 5.47 ppm ($P_B = 0$) undergoes fast exchange between two environments. The chemical shift of this peak has been plotted as a function of P_B according to eq 2 (Figure 8). [In calculating P_B from eq 3 and 4, the value of K_D was 0.8×10^{-7} M for J proteins from TA1859 and TA300 or 167×10^{-7} M for J protein from TA301 (Kustu & Ames, 1974).] The slope of the plot is equal

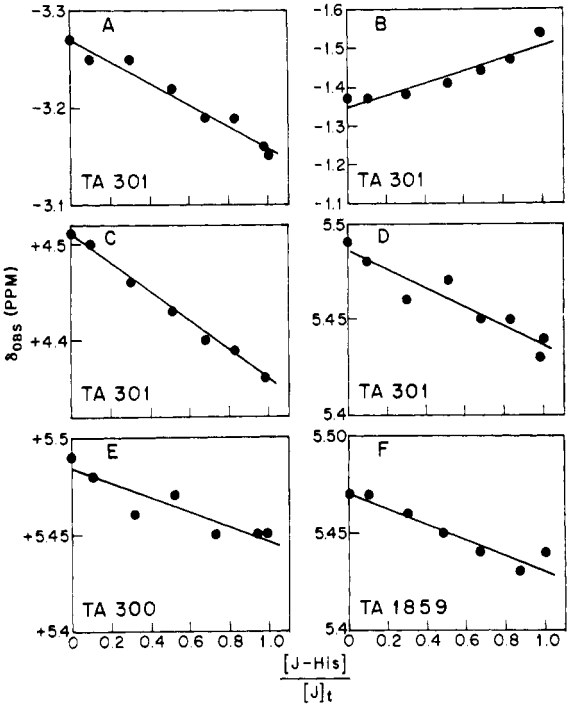

FIGURE 8: Plots of δ_{obsd} vs. $[J\text{–}His]/[J]_t$ for several resonances of the J proteins. From these data, values of $\Delta = \delta_B - \delta_F$ were calculated (see text): (A) peak at −3.27 ppm for J protein from TA301; (B) peak at −1.37 ppm for J protein from TA301; (C) peak at 4.51 ppm for J protein from TA301; (D) peak at 5.49 ppm for J protein from TA301; (E) peak at 5.49 ppm for J protein from TA300; and (F) peak at 5.47 ppm for J protein from TA1859.

Table II: Chemical Shifts and Δ Values for Several Resonances of J Proteins

J protein	chemical shift[a] (ppm from HDO)	Δ (ppm)
TA1859	5.47	−0.039
	−3.30	0.18
	−1.38	−0.31
TA300	5.49	−0.038
	−3.27	0.18
	−1.39	−0.26
TA301	5.49	−0.05
	4.50	−0.152
	−3.27	0.113
	−1.37	−0.16

[a] In the absence of L-histidine.

to Δ ($\equiv \delta_B - \delta_F$). Slopes and intercepts were calculated from a linear least-squares fit to the data (Table II). For fast exchange the relationship between k_{-1} and Δ is given by $k_{-1}^2 \gg \Delta^2$ (Smallcombe et al., 1972), where Δ is in radians. Thus we find a lower limit for k_{-1} of $2\pi \times 9.75 = 61$ s^{-1}.

The resonance at −3.30 ppm ($P_B = 0$) is subject to slow exchange. The peak that appears at −3.09 ppm comes from the bound state of this residue. The difference in chemical shift ($\delta_B - \delta_F$) of bound and free states (at $P_B = 0.48$) gives an estimate of $\Delta = -3.09 - (-3.27) = 0.18$ ppm or 45 Hz. The condition for slow exchange is given by $k_{-1}^2 \ll \Delta^2$ (Smallcombe et al., 1972) and so the upper limit for k_{-1} is 283 s^{-1}. Thus, we obtain an estimate for k_{-1} as being somewhere between 61 and 283 s^{-1}.

The resonance at −1.38 ppm is also probably involved in slow exchange. The resonance from the bound residue is at −1.69 ppm (see above). The difference between its free and bound shifts is $\Delta = -0.31$ ppm. This would give an upper limit of 487 s^{-1} for k_{-1}. This value is compatible with, but does not

alter, the range of k_{-1} given above.

2. J Protein from TA300. The resonances at 5.49 and −3.27 ppm behave in the same manner as those at 5.47 and −3.30 ppm in TA1859, respectively. The same type of plot and calculations for these two peaks yield a lower and upper limit for k_{-1}. From the shift behavior of the peak at 5.49 ppm, a value of $\Delta = 9.00$ Hz was obtained. The slow exchange of the peak at −3.27 ppm gave $\Delta = 45$ Hz. Thus the range of the rate constant is $57 < k_{-1} < 283$ s^{-1}, essentially equal to that found for TA1859.

The slow exchange behavior of the resonance at −1.39 ppm gives an estimate of the upper limit for k_{-1} ($k_{-1} < 408$ s^{-1}). This value is close to that found for TA1859 and does not alter the range of k_{-1} values presented here.

3. J protein from TA301. Several of the peaks in the spectrum of J protein from TA301 undergo fast exchange while their counterparts in TA1859 and TA300 spectra undergo slow exchange. These are the resonances at −1.37 and −3.27 ppm from HDO. A ring-current-shifted resonance at 4.51 ppm also exhibits fast-exchange properties. And, as in the other two proteins, the most upfield peak, at 5.49 ppm, undergoes fast exchange. However, we were not able to observe any resonances in the slow-exchange limit. Therefore, only a lower limit for k_{-1} can be found.

The observed shifts of each of these four resonances were plotted vs. fraction bound, P_B. Values of Δ were obtained for each peak and are given in Table II. Each value of Δ (in rad/s) is then used to find a lower limit for k_{-1}. These lower limits are 251, 177, 235, and 79 s^{-1} for the −1.37-, −3.27-, 4.50-, and 5.49-ppm peaks. It is not possible for the binding of L-histidine to J protein to have four different values of k_{-1}. Therefore, in order for one k_{-1} to govern the exchange process of all four resonances, the lower limit must be equal to the largest of all: i.e., 251 s^{-1}. Thus, we estimate $k_{-1} > 251$ s^{-1}.

Discussion

Observations of changes in the chemical shift or intensity upon increasing P_B indicate that fast or slow exchange occurs for several resonances in each protein. The observed shift behavior for any resonance can be explained by a two-state model; i.e., J protein residues exist only in free or bound environments. Since one process controls the equilibrium between these states, the off-rate constant k_{-1} (see eq 1) must be the same for each resonance, and thus whether the exchange is fast or slow is determined by that resonance's Δ value. For example, Δ of the −3.27-ppm peak is larger than for the 5.48-ppm resonance (Table II), so that the chemical shift dependence on P_B indicates slow exchange for the former and fast exchange for the latter.

The dependence of the chemical shift on P_B has allowed us to determine Δ for several peaks and from these values to estimate k_{-1} for binding of substrate to each protein as described above (see Results). It has been found that histidine-binding proteins J from TA300 and TA1859 have essentially identical ranges for k_{-1} ($60 < k_{-1} < 280$ s^{-1}). This is consistent with the proposal of Ames and co-workers (Kustu & Ames, 1974; Ames & Spudich, 1976) that the mutation in J protein from TA300 has acted at the P interaction site. In addition it implies that the mutation has not affected the substrate binding site; that is, a certain interaction site conformation is not required for proper L-histidine binding. The value of k_{-1} for histidine-binding protein J from TA301 appears larger than that of histidine-binding protein J from either TA300 or TA1859, since its lower limit ($\sim$251 s^{-1}) is about equal to their upper limits. This is consistent with the TA301 mutation acting at the L-histidine binding site. The mutation has produced a conformational change at this site, which decreases the bound lifetime ($\tau_B = 1/k_{-1}$) of the substrate.

Our results also show that k_{-1} can be obtained easily without observation of the ^{1}H NMR spectrum of the substrate itself. Typically, rate constants for substrate binding have been obtained from observations of line broadening in the substrate resonances brought about by exchange with the protein (Sykes & Scott, 1972). However, because of the very small K_D ($\sim 10^{-7}$ M) for L-histidine binding to J protein from TA1859, it is unlikely that such exchange broadening could be observed, even at low ratios of substrate to protein (i.e., high P_B). Moreover, the spectrum of the substrate itself can often be obscured by the protein spectrum at these high values of P_B. Thus, other than isotopic substitution of the substrate (i.e., incorporation of a ^{19}F label) or deuteration of the protein, the observation of the protein spectrum is a very practical way to obtain k_{-1}.

In addition to permitting estimation of k_{-1}, the Δ values provide information on which resonances may come from residues affected by the TA301 mutation and also information on the magnitude of the conformational change affecting certain resonances. The Δ values for the peaks of J protein from TA301 do not exhibit as wide a range as those of J protein from TA300 or TA1859. Δ of the −1.37-ppm resonance is approximately one-half that of the same peak in either of the other two proteins. The value of Δ for the −3.27 ppm peak is about 30% smaller than that found for this resonance in J protein from TA1859. The resonance at 5.49 ppm undergoes a larger downfield shift (0.05 vs. 0.039 ppm) in J protein from TA301. These comparisons to the native J protein indicate that the peaks at −1.37, −3.30, and 5.49 ppm have been affected by the TA301 mutation and therefore could be at or near the L-histidine binding site.

Histidine-binding proteins J from TA300 and TA1859 have essentially identical values of Δ for all three peaks. This is expected if the TA300 mutation acts away from the substrate binding site and if these three resonances are from amino acid residues close to the L-histidine binding site. Therefore, from the data for J protein from TA300, it appears that none of the peaks at −3.27, −1.37, or 5.49 ppm are near the protein–protein interaction site. This agrees with the J protein data from strain TA301 discussed above.

As yet, there are no clear indications of which resonance, if any, might be near the protein–protein interaction site. A direct comparison of the ^{1}H NMR spectra of the histidine-binding proteins J from TA300, TA301, and TA1859 sheds some light on this problem. Figures 2, 3, and 4 show that the genetic mutations have brought about different and subtle conformational alterations for J proteins from TA300 and TA301; i.e., different parts of the J protein molecule have been changed by each mutation. The strain TA300 was believed altered at the protein–protein interaction site, and so the spectral differences between TA300 and TA1859 are likely to arise from changes in the environment of amino acid residues at or near this site. Such altered resonances could be taken as arising from residues near the P interaction site. Similarly, the resonances which have different chemical shifts or intensities in the spectra of J protein from TA301 as compared to those of TA1859 could come from amino acid residues near the L-histidine binding site.

From a comparison of the ring-current-shifted resonances of J proteins from TA300 and TA1859 (Figure 2), it appears that the peak at 4.61 ppm, missing in the spectrum of J protein from TA300, is a possible monitor of the "second" site. Other

probes of this region are more difficult to find. The −1.51-ppm resonance (Figure 3) and that at −3.75 ppm (Figure 4) are two other possibilities obtained by comparing spectra of J proteins from TA300 and TA1859. Other resonances can be proposed as coming from the L-histidine binding site residues. In Figure 2, it can be seen that the resonance at 4.51 ppm is much sharper in the spectrum of J protein from TA301 than in that of J protein from TA1859. This, along with comparisons of Δ in J proteins from TA301 and TA1859 (see above), shows that the peaks at ~4.50, −1.37, and 5.47 ppm are monitors of the L-histidine binding site conformation.

The addition of substrate to the J protein from TA1859 causes a large shift in the position of the peaks appearing at 5.47 and 4.66 ppm, and the intensities of the −1.38-, −3.30-, and 4.47-ppm resonances decrease. This shows that L-histidine binding produces a conformational change in the histidine-binding protein J molecule, as originally observed in this laboratory (Robertson et al., 1977). It can be said that our observations suggest a subtle conformational change at the L-histidine binding site, because four of these resonances (−1.38, −3.30, 4.47, and 5.47 ppm) are probably from amino acid residues at or near this site. The peak at 4.61 ppm, a possible protein–protein interaction site probe, is not substantially affected by addition of L-histidine. This suggests that the binding of L-histidine does not affect the conformation of this protein–protein interaction site. On the other hand, the resonance at −1.51 ppm, also a possible probe, does sharpen and shift downfield upon adding substrate. This implies a change in the interaction site conformation, subsequent to L-histidine binding. However, it is possible that both resonances are from residues affected by a conformational change, but only one (−1.51 ppm) is from a residue sensitive enough to its microenvironment to reflect this conformational change as an alteration of the spectrum. In this case we would conclude that L-histidine binding produces a structural change at the P interaction site. Firmer conclusions in this regard await further information on the three-dimensional structure, identification of resonances with specific amino acid residues, or incorporation of labeled (e.g., ^{2}H, ^{13}C, and/or ^{19}F) amino acids into the histidine-binding protein J.

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