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Proteins from the Prokaryotic Nucleoid: ^{1}H NMR Study of the Quaternary Structure of *Escherichia coli* DNA Binding Protein NS (HU)†

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ABSTRACT: The quaternary interactions of *Escherichia coli* DNA binding proteins NS1, NS2, and NS (NS1 + NS2) have been studied by ^{1}H NMR spectroscopy at 400 MHz following the reversible spectral changes produced by temperature increases on the resonances (Phe ring and His C-2 protons) whose spectral characteristics reflect the formation and dissociation of either homologous or heterologous interactions. These changes include (a) a progressive intensity decrease of the Phe resonances shifted to high field by stacking interactions, (b) a progressive intensity increase of the resonances due to freely rotating Phe, and (c) splitting of the His C-2 proton resonance. The association constants and thermodynamic parameters for the homologous and heterologous interactions were calculated from the molar fractions of the relevant molecular species by assuming that the above effects are due to the existence of simple association equilibria. It was found that two (out of three) phenylalanine residues of each polypeptide chain are involved in quaternary interactions. Quantitative data concerning the internal mobility and mutual orientations in aggregates of these Phe rings were also obtained. From the calculated association constants, from comparison of these data with recent protein–protein cross-linking results [Losso, M. A., Pawlik, R. T., Canonaco, M. A., & Gualerzi, C. O. (1986) *Eur. J. Biochem. 155*, 27–32], and from other considerations, we suggest that even though stacking of the Phe rings occurs at the interface between monomers, the temperature-dependent alteration of the Phe spectrum monitors shifts of the dimer ⇌ tetramer equilibrium whereas the splitting of the His C-2 proton resonance most likely monitors the equilibrium between tetramers and larger aggregates.

Among the proteins which may play a role in the physical packaging of bacterial chromosome, NS (HU) is the most abundant and the best characterized (Rouvière-Yaniv & Gros, 1975; Berthold & Geider, 1976; Varshavsky et al., 1977; Suryanarayana & Subramanian, 1978; Losso et al., 1982; Miano et al., 1982; Paci et al., 1984; Lammi et al., 1984a,b; Gualerzi et al., 1986). In *Escherichia coli*, NS consists of two 9-kilodalton polypeptide chains, NS1 and NS2, displaying 69% sequence homology (Mende et al., 1978). These proteins have been characterized by ^{1}H NMR spectroscopy (Paci et al., 1984, 1986). The spectra of NS1, NS2, and NS display a large number of high-field-perturbed Phe resonances, shielded and deshielded methyl resonances, and backbone NH protons rather inaccessible to the solvent. These features were attributed to the existence of extensive tertiary and/or quaternary structures which are lost upon heating but that readily re-form upon cooling. It was also shown that, when isolated, NS1 and NS2 undergo self-aggregation but that, when both proteins are mixed, heterologous aggregates are preferentially formed. Furthermore, it has been shown that NS binds to DNA in the aggregated form (Lammi et al., 1984; Paci et al., 1984, 1986) and that the presence of DNA favors its aggregation (Losso et al., 1986).

In this study, we have investigated the quaternary interactions of NS1, NS2, and NS by following the spectral

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changes induced by variation of temperature on the upfield-perturbed Phe resonances and C-2 proton resonance of His-54 of NS2 which are sensitive and characteristic indicators of the quaternary structure of the protein. Here we present data concerning the mutual orientation and mobility of the Phe rings in the aggregates as well as a physicochemical characterization of homologous and heterologous quaternary interactions.

Materials and Methods

Purification of NS1 and NS2 was carried out as described (Losso et al., 1986).

The samples for NMR spectroscopy were concentrated by batch elution from small phosphocellulose columns to yield protein concentrations of 4–8 mg/mL (about 0.6 mM). The samples were exhaustively dialyzed against 10 mM potassium phosphate buffer, pH 7.5 (meter reading), containing 100 mM KCl (Merck Suprapur) in 99% deuterium oxide (Merck). ^1H NMR spectra were recorded on a Bruker WM-400 spectrometer operating at 400 MHz with a 4-μs (30°) pulse, a 6000-Hz spectral width, and a 2.0-s relaxation delay. Magnetization decays obtained in the quadrature detection mode were accumulated on 16K of memory, and a sensitivity enhancement of 0.5 Hz was applied before the Fourier transform (FT). Samples were observed in standard 5-mm tubes. HDO was partially suppressed by using an inverse gated pulsed irradiation technique for a duration of 1.5 s. The typical number of scans ranged from 1000 to 4000. Spectra were recorded at the temperature (±1 °C) indicated in the figures. (Trimethylsilyl)propionate sodium salt (TSP) was used as an internal reference for the chemical shift scale. To obtain quantitative data, integration of the resonances was performed either in a direct way or, in the crowded spectral regions, by comparison between simulated and experimental spectra.

The simulation was obtained with a trial and error program which computes spectra as a sum of Lorentzian-shaped bands by using the number, the chemical shifts, the half-height line width, and the integrated intensity of the resonances as adjustable parameters.

Assuming simple association equilibria, the dependence of the apparent equilibrium constants on temperature allowed us to obtain van't Hoff plots and thereby to evaluate the molar thermodynamic quantities ΔG, ΔH, and ΔS. The activation energies for the hindered internal rotations of the Phe rings were calculated from the frequency of the passage across the energy barrier at different temperatures evaluated from the chemical shift differences between the resonance bands corresponding to the individual Phe ring protons in different environments (Gutowsky & Holm, 1956). The $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ values for the energy barrier of the hindered motion were calculated by direct application of the Eyring equations.

Results

The spectra of NS1, NS2, and NS differ in the position and intensity of the upfield-perturbed Phe resonances between 6.95 and 6.10 ppm (Figure 1) as well as in the presence, only in NS2 and NS, of the C-2 and C-4 proton resonances of NS2 His-54. Closer examination of the spectra also reveals that the chemical shift of the C-2 proton of His-54 of NS2 is not identical in the sample of NS2 alone and in that containing a small amount of NS2 in the presence of a large excess of NS1 (i.e., under conditions where all NS2 is presumably aggregated with NS1). This difference is due to the existence of two alternative chemical environments for the His C-2 proton in the homologous (NS2–NS2) and in the heterologous (NS2–NS1) interaction. This premise is confirmed by the finding that, in the spectrum of NS, the His C-2 proton resonance is composed of two bands, the major one having the same chemical shift as NS2 in the presence of an excess of NS1, and a shoulder with the same chemical shift as in NS2 alone. This finding indicates that the His C-2 proton resonance of NS2 can be influenced by the quaternary interactions of this protein. Furthermore, since it is clear that the spectrum of NS does not correspond either qualitatively or quantitatively to the sum of the NS1 and NS2 spectra (Figure 1), it is obvious that the patterns of perturbed Phe resonances are also due to quaternary interactions. Thus, we decided to use the spectral signal of the C-2 proton of NS2 as well as those of the upfield-perturbed Phe resonances to monitor the equilibria of both homologous and heterologous quaternary interactions.

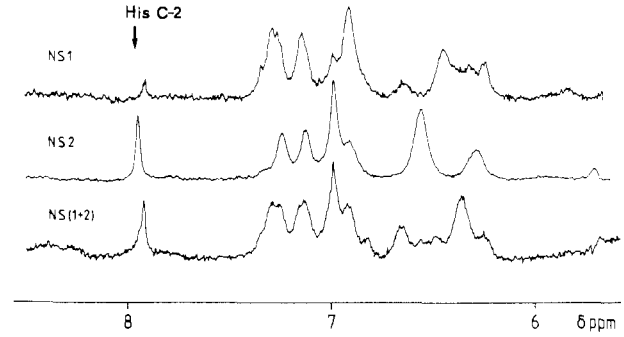

FIGURE 1: Comparison of the enlarged aromatic region of the spectra of NS1, NS2, and NS. The 8.5–5.5 ppm spectra of NS1, NS2, and NS (NS1 + NS2) are juxtaposed to emphasize the differences. The peaks at 7.94 and 7.0 ppm are assigned to the His C-2 and C-4 proton resonances. The bands downfield from the latter are attributed to freely rotating Phe rings and the upfield ones to Phe ring protons perturbed from their original position by magnetic shielding effects induced by stacking interactions.

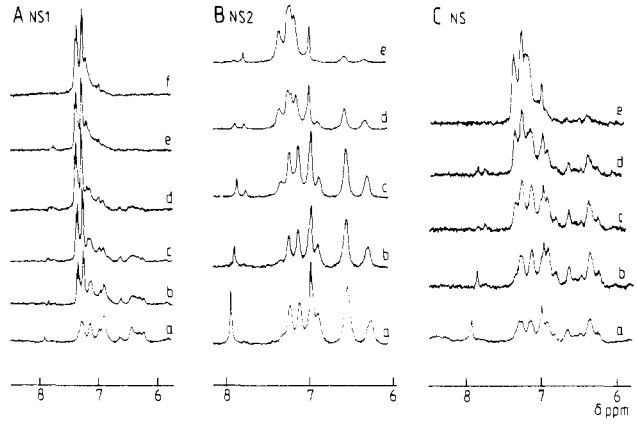

FIGURE 2: Aromatic region of the NS1, NS2, and NS spectra at various temperatures. The temperature of the samples was increased by 2–5 °C increments and kept at each temperature for approximately 60 min (an equilibration time of 20 min and an accumulation time of 40 min). Only some representative spectra are reported. The temperatures at which the spectra were recorded are for NS1, (a) 19, (b) 29, (c) 31, (d) 35, (e) 45, and (f) 50 °C; for NS2, (a) 23, (b) 34, (c) 41, (d) 45, and (e) 55 °C; and for NS, (a) 23, (b) 40, (c) 48, (d) 53, and (e) 58 °C.

The temperature-induced variations in the aromatic region of the spectra of NS1, NS2, and NS are shown in Figure 2. Increasing the temperature (from the bottom toward the top spectra) induces a progressive disappearance of the upfield-shifted resonances with a concomitant increase of intensity of the peaks characteristic of freely rotating Phe. In addition, at temperatures above 30 °C, the His C-2 proton resonance splits, giving rise to a "new" high-field resonance whose intensity increases at the expense of the original low-field one until, above 55 °C, only the high-field resonance remains

Table I: Evaluation of Thermodynamic Quantities for Equilibria Monitored by His-54 C-2 and Phe-Perturbed Proton Resonances[a]

protein	resonance monitored	K_a (M^{-1})	$\Delta G°$ (kcal M^{-1})	$\Delta H°$ (kcal M^{-1})	$\Delta S°$ (cal K^{-1} M^{-1})
NS2	His C-2	1.6×10^5	−5.6	−32.4	−90
NS (NS1 + NS2)	His C-2	4.4×10^5	−6.6	−32.8	−88
NS1	Phe ring	1.3×10^6	−7.6	−93.4	−288
NS2	Phe ring	4.8×10^5	−6.7	−22.4	−52.8
NS (NS1 + NS2)	Phe ring	3.9×10^6	−8.8	−42.8	−114

[a] The listed values were evaluated at 298 K from the van't Hoff plots shown in Figure 3A (for the His-54 C-2 resonances) and Figure 3B (for the Phe-perturbed resonances).

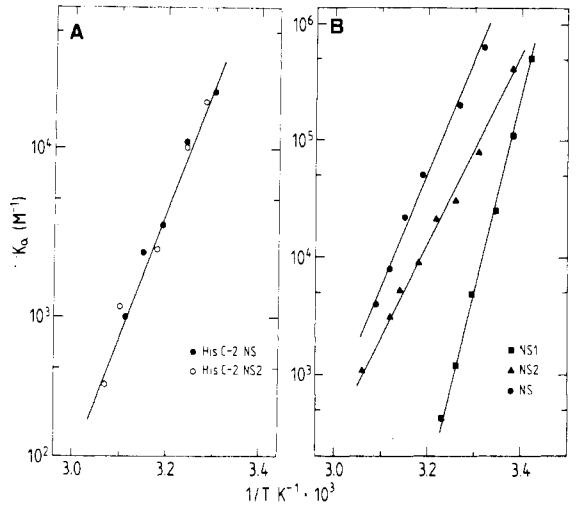

FIGURE 3: van't Hoff plots for the association equilibria monitored by the His-54 C-2 and by the perturbed Phe ring proton resonances. (A) ln K_a vs. $1/T$ plot for His C-2 in NS2 alone (○) or in the presence of a stoichiometric amount of NS1 (●). (B) ln K_a vs. $1/T$ plots for the reshuffling of the perturbed Phe ring proton resonances in NS1 (■), NS2 (▲), and NS (●).

visible. All these effects are fully reversible upon cooling (Paci et al., 1984; data not shown), indicating that the chemical environment responsible for the original spectral features of these proteins is attained again at lower temperatures. The splitting of the His C-2 and the reshuffling of the Phe resonances indicate the existence of dynamic equilibria between forms of the proteins characterized by the presence of the His and Phe residues in different chemical environments. Temperature increases induce shifts of these equilibria toward the formation of the molecular species characterized by the higher field His C-2 and by the absence of perturbed Phe rings.

To follow quantitatively these equilibria, the resonance peaks were integrated either in a direct manner, in the case of the His C-2 proton, or with the aid of a computer simulation of the spectra, in the case of the Phe resonances. From the molar fractions of the alternative spectral forms of His and Phe, we calculated the apparent equilibrium constants of these equilibria at the different temperatures. The plot of ln K_a vs. $1/T$ for the His C-2 proton in NS2 alone and in the presence of a stoichiometrically equivalent amount of NS1 is presented in Figure 3A. The thermodynamic quantities calculated from the van't Hoff plot are reported in Table I. The simulated spectra of NS1, NS2, and NS must contain, based on the known primary structures of these proteins (Mende et al., 1978), 15, 16, and 15.5 protons, respectively. Thus, from the integral values of the resonances, one can calculate the number of perturbed and freely rotating phenylalanines at the various temperatures. In the temperature range examined, these numbers span from approximately 1.1 to 3.0 and from 1.9 to 0 for the freely rotating and perturbed phenylalanines, respectively. These noninteger numbers suggest that the increase of temperature induces a shift of an intermolecular equilibrium, thereby altering the molar fractions of the various types of Phe

Table II: Chemical Shift Difference between Proton Resonances of Freely Rotating Phenylalanines and the Corresponding Perturbed Phe Resonances in Homologous and Heterologous Interactions[a]

assigned ring protons[b]	type of interaction: NS1–NS1 (ppm)	NS2–NS2 (ppm)	NS1–NS2 (ppm)
o	0.3	0.3	0.4
o′	0.8	0.7	0.9
m	0.2	0.6	0.6
m′	0.9	0.9	0.9
p	~0.4	~0.6	~0.9

[a] Data were obtained as described under Materials and Methods and in the text comparing the experimental and the simulated spectra reported in Figure 4. [b] o, o′ = ortho; m, m′ = meta; p = para protons.

Table III: Activation Energy for Rotation of Perturbed Phenylalanine Rings around the C_β–C_γ Bond[a]

type of interaction	$\Delta G^\ddagger$ (kcal M^{-1})	$\Delta H^\ddagger$ (kcal M^{-1})	$\Delta S^\ddagger$ (cal K^{-1} M^{-1})
NS1–NS1	16	4	−38
NS2–NS2	15	2	−44
NS1–NS2	15	10	−18

[a] Values obtained from the chemical shift differences between the resonances of Figure 4 as a function of temperature. The interconversion rate was obtained as described (Gutowsky & Holm, 1956), and the activation energy was estimated by using the Eyring equation.

protons, and it can be calculated that two (out of three) phenylalanines are involved in the quaternary interaction. The apparent association constants of the aggregate and the related thermodynamic quantities were evaluated from the molar fraction of the freely rotating vs. perturbed Phe protons at various temperatures. These data are presented in Figure 3B and in Table I. From comparison of the aromatic spectra of NS1, NS2, and NS and from their simulation spectra, information concerning the mutual orientation and mobility of the phenylalanine rings in the quaternary interactions can also be obtained. The spectral shapes of the perturbed Phe resonances in the homologous and heterologous contacts as well as the relative assignments of the various types of ring protons are presented in Figure 4.

From the chemical shift differences between freely rotating and perturbed Phe protons in the various types of interactions (Table II), the mutual orientation of the stacked Phe rings can be estimated (Johnson & Bovey, 1959) by assuming that no other center of magnetic anisotropy exists in the neighborhood of the phenylalanine residues. These results are sketched in Figure 5 which shows a view perpendicular to the ring plane as well as an edge-on view of the phenyl rings in the various interactions.

Within the temperature range examined, small variations of the relative position of the resonances of the perturbed phenylalanines have been recorded. These variations can be attributed to a modification of the interconversion rate between the relative positions of the aromatic ring and thus allow the evaluation of their interconversion rate (i.e., of their rotation) (Gutowsky & Holm, 1956). This rate depends on the acti-

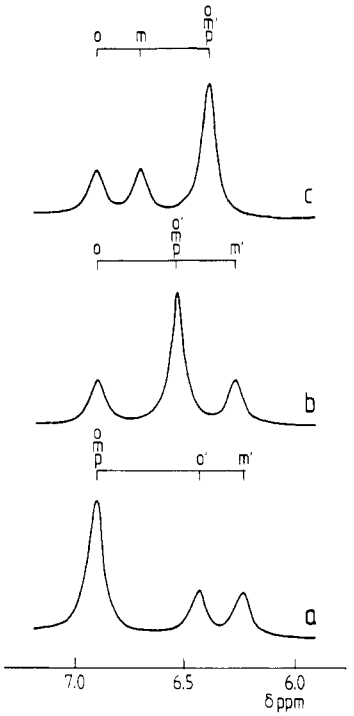

FIGURE 4: Spectral shape of the perturbed phenylalanine resonances in the homologous and heterologous interactions. Simulated spectral shapes of the resonances due to the perturbed Phe in homologous interactions in NS1 (a) and in NS2 (b) and to heterologous interactions in NS (c). The simulations were obtained as described under Materials and Methods, omitting the resonances of the freely rotating phenylalanines and, when appropriate, that of the C-4 proton of His. In the case of NS, simulated NS1 and NS2 spectra of normalized intensity were subtracted from the simulated NS spectrum to yield a simulated spectrum which reflects the resonances of the Phe of NS1 and NS2 engaged in heterologous interactions. The relative assignments of the various peaks are also reported: o, o′ = ortho; m, m′ = meta; p = para protons.

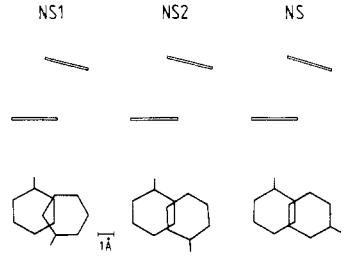

FIGURE 5: Mutual orientation of the Phe rings in the homologous and heterologous interactions of NS1, NS2, and NS. The relative positions and orientations of the Phe rings of NS1 and NS2 in the homologous and heterologous interactions have been obtained from the chemical shift differences between the freely rotating and the perturbed Phe resonances reported in Table II. The upper scheme refers to an edge-on view and the lower one to a view perpendicular to the ring plane.

vation energy barrier required to overcome all the steric hindrances to the rotation of the ring. From the Eyring equation, the energy barrier for the hindered internal rotation of the perturbed Phe rings around the C_β–C_γ bond was calculated. The results are reported in Table III.

Discussion

Previous ^{1}H NMR studies on *E. coli* histone-like proteins NS1, NS2, and NS have shown that these proteins undergo extensive homologous aggregation when isolated and that they preferentially form heterologous aggregates when present together (Paci et al., 1984). It has also been shown that the quaternary structure of these proteins depends on hydrophobic interactions, involves the stacking of some phenylalanine rings (Paci et al., 1984), and remains unaffected by their interaction with DNA [i.e., these proteins bind in the aggregated form to the nucleic acid (Lammi et al., 1984)].

More recently, the X-ray crystal structure of a *Bacillus stearothermophilus* DNA binding protein homologous to NS has been obtained, confirming the localization of the phenylalanines at the contact surface between subunits in a position suitable for stacking interactions (Tanaka et al., 1984).

In this paper, we have examined quantitatively the effect of temperature on the aromatic ^{1}H NMR spectra of NS1, NS2, and the reconstituted complex NS and thereby characterized, from a physicochemical point of view, the interaction between these proteins. Our results show that two of the three phenylalanines present in each polypeptide chain are involved in intermolecular stacking interactions with the approximate orientation shown in Figure 5. The mobility of these rings in the aggregate was found to be strongly impaired (interconversion rate $\simeq 25$ s^{-1}) with respect to a freely rotating ring (10^3–10^5 s^{-1}) (Jardetzky & Roberts, 1981). The activation energy barriers for ring rotation were also calculated and found to be very similar in NS1, NS2, and NS and comparable to those reported for aromatic rings with reduced internal mobility due to steric hindrances. The third phenylalanine of each molecule, on the other hand, was found to remain unstacked and substantially free to rotate.

Temperature changes drastically affect the phenylalanine proton resonance pattern. Increasing the temperature produces an overall simplification of the aromatic spectra with a progressive decrease in intensity of those peaks due to phenylalanines which are shifted high field by centers of magnetic anisotropy (i.e., perturbed phenylalanines) and with a concomitant increase in intensity of those resonances having chemical shifts characteristic of freely rotating phenylalanines. In addition to these large effects, the temperature increase induces the splitting of the His C-2 proton resonance; this effect can be attributed to a deprotonation of the imidazole ring. The behavior of both Phe and His resonances reflects that expected for a temperature-influenced chemical equilibrium. Thus, assuming simple association equilibria for the transitions monitored by the spectral changes of the Phe and His proton resonances, we have calculated the pertinent thermodynamic quantities and found that all interactions are characterized by negative ΔG, ΔH, and ΔS values and that the heterologous interactions are characterized by more negative free energy (i.e., they are the most favored interactions).

In a recent study (Losso et al., 1986), we have shown that, at 20 °C, dimers and tetramers prevail at low NS concentration ($<10^{-5}$ M) while at higher protein concentrations, comparable to the presumed intracellular ones and to those used in NMR studies, interactions between tetramers, yielding larger aggregates, may become prominent. The precise nature of the quaternary interactions monitored in this study cannot be determined directly, but our data suggest that the association equilibria monitored by the Phe and His residues might be different. From comparison of the association constants determined here with those previously estimated for the formation of dimer ($K \geq 10^9$ M^{-1}), tetramer ($K \simeq 10^6$ M^{-1}), and larger aggregates ($K \simeq 10^4$ M^{-1}), it seems reasonable to rule out that the effects studied here reflect the monomer $\rightleftharpoons$ dimer equilibrium and to assume instead that the perturbation of the Phe resonances reflects the dimer $\rightleftharpoons$ tetramer equilibrium. The splitting of the His resonance, on the other hand, probably reflects the dissociation of protein aggregates larger than tetramers. The above interpretation is consistent with the

finding that the association constants as well as the other thermodynamic quantities calculated for the dimer ⇌ tetramer and tetramer ⇌ larger aggregate interactions of NS are strikingly similar to those determined calorimetrically for the equilibria H2A–H2B + $(H3–H4)_2$ ⇌ hexamer and hexamer + H2A–H2B ⇌ octamer, leading to the assembly of nucleosomal octamers (Benedict et al., 1984). Finally, since the Phe residues of NS1 and NS2 are located on protein surfaces which are presumably involved in the major dimeric interaction (Tanaka et al., 1984), if our interpretation of the nature of the association equilibria is correct, it must be concluded that a stacking interaction between Phe rings of different protein subunits at the dimeric interface does not take place (or at least not to the extent observed by NMR) until a tetrameric structure is produced by an interaction between two dimers.

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