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pH Dependence of the High-Resolution Proton Nuclear Magnetic Resonance Spectrum of the *lac* Repressor Headpiece†

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ABSTRACT: The pH dependence of the N-terminal 51 amino acid headpiece (HP) of the *lac* repressor has been followed by using ^{1}H NMR spectroscopy to monitor the chemical shifts of resolved aromatic and methyl resonances. The NMR evidence reveals the folded HP domain to be stable from pH 1 to 10 at 23 °C. All observed resonances shift toward their expected random-coil positions above pH 10, which suggests that a general unfolding occurs. The four tyrosine rings reflect a combination of unfolding and titration in the order 7 > 17, 12 > 47. This pH-induced unfolding is completely reversible. In addition, strikingly similar pH behavior for selected tyrosine and methyl resonances at acid pH values suggests that clusters of various tyrosine, methyl, and carboxyl side chains exist in the native structure.

The isolated N-terminal portion of the *lac* repressor protein, termed "headpiece" (HP), has been shown to have a strong affinity for double-helical deoxyribonucleic acid (DNA) (Geisler & Weber, 1977) and to bind to *lac* operator DNA (Ogata & Gilbert, 1978, 1979). The specific binding of whole repressor to *lac* operator DNA controls the lactose genes in *Escherichia coli*. Studies of the structural properties of the HP are therefore of intrinsic interest to questions about genetic control.

No crystals of the HP or repressor suitable for high-resolution X-ray studies are yet available. ^{1}H NMR (Wade-Jardetzky et al., 1979; Buck et al., 1978) and low-angle X-ray studies (Pilz et al., 1980) suggest that the HP forms an independent structural domain whether it is part of the complete repressor or separated from a core domain by proteolytic cleavage. The HP (amino acids 1–51) appears connected to the core (amino acids 60–360) by a flexible hinge localized between residues 50 and 60. The flexible hinge allows the HP DNA-binding domain to have a high degree of motion with respect to the core. Many ^{1}H NMR resonances observable in the native isolated HP are similarly observed in the intact repressor (Wade-Jardetzky et al., 1979).

The 360-MHz ^{1}H NMR spectrum of isolated HP-51 reflects extensive folded structure. The resolution and initial analysis of a considerable number of ^{1}H resonances in the aromatic and aliphatic regions described in previous papers (Ribeiro et al., 1981a,b) allow the use of those resonances as natural probes of the HP structure. Thus, the temperature-induced unfolding of the HP was reported (Wemmer et al., 1981a). Unlike most proteins thus far investigated, this small single-chain polypeptide which possesses no disulfide bridges was found to unfold with weak cooperativity. The thermal denaturation occurs in a gradual and continuous manner in which native and partially unfolded structures are in rapid exchange, and the process appears completely reversible (Wemmer et al., 1981a).

Given the important DNA-binding role of the HP, and its unusual thermal unfolding properties, we have now investigated the effects of pH on the protein structure. In this paper, we consider the phenomena observed in the aromatic and aliphatic

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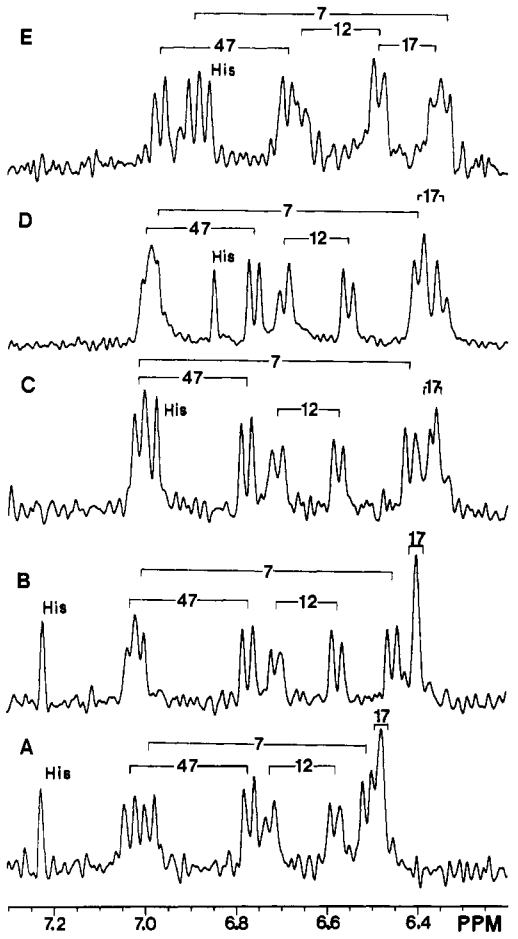

FIGURE 1: Resolution-enhanced 360-MHz ^1H NMR spectra over the aromatic region of the *lac* repressor headpiece. The FID's were obtained at a sweep width of ±2000 Hz, 16K points for a digital resolution of 0.49 Hz/point, and resolution enhanced before Fourier transformation. Each spectrum was obtained on 6.7×10^{-5} M to 1×10^{-4} M samples in high salt-low phosphate buffer in ^2H_2O at the given p^2H and is the sum of 2000 scans at 23 °C. (A) p^2H 1.04, (B) p^2H 4.08, (C) p^2H 7.26, (D) p^2H 9.87, and (E) p^2H 10.71. The coupling relationships of the four tyrosines are indicated at each pH. The shift of the His-29 C4 singlet serves as a convenient gauge of the pH in solution.

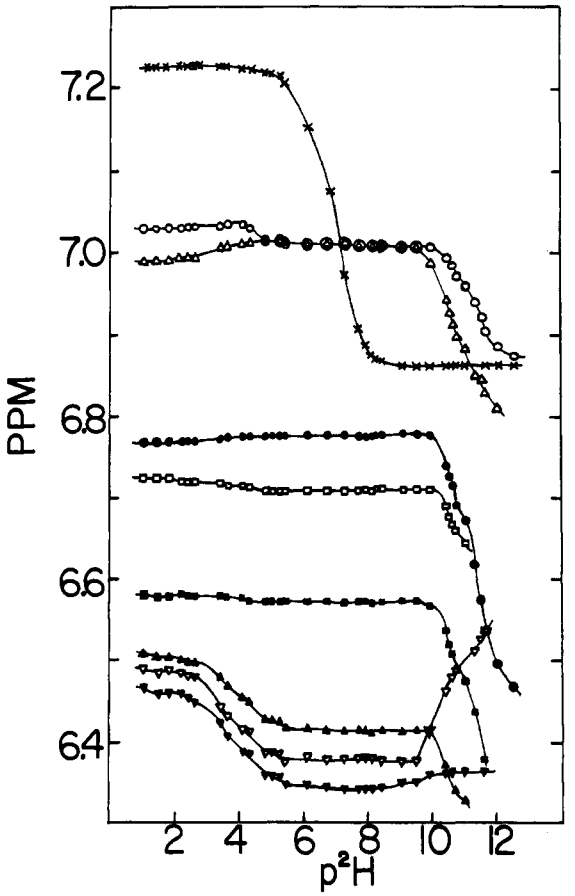

FIGURE 2: Chemical shift as a function of the p^2H meter reading for the His-29 C4 and the 2,6 and 3,5 proton resonances of Tyr-7, Tyr-12, Tyr-17, and Tyr-47 in the *lac* repressor headpiece (HP-51). Peaks are represented as follows: His-29 C4 (×); 2,6 proton resonances of Tyr-7 (△), Tyr-12 (□), Tyr-17 (▽), and Tyr-47 (○); 3,5 proton resonances of Tyr-7 (▲), Tyr-12 (■), Tyr-17 (▼), and Tyr-47 (●).

methyl regions of the ^1H NMR spectrum. In the accompanying paper (Wemmer et al., 1981b), we discuss the pH and kinetic behavior of the exchangeable protons of the HP.

Materials and Methods

Preparations of HP-51 (M_r ~6000) were described previously (Ribeiro et al., 1981a). Samples (0.4–0.6 mg) of HP-51 dissolved in 500-μL aliquots of 1 M KCl in H_2O were lyophilized to remove the H_2O and then dissolved in 400-μL volumes of buffer in ^2H_2O. These gave solutions of the HP at 6×10^{-5} to 1×10^{-4} M in buffer with 0.015 M potassium phosphate, 1.26 M KCl, 5×10^{-6} M EDTA, and 5×10^{-6} M dithiothreitol and samples at ionic strength of 1.3 M. Experiments in previous papers were at HP concentrations of $(2.5–8.3) \times 10^{-4}$ M. No concentration dependence of the HP NMR spectra is detected over this concentration range. The p^2H of solutions was determined before and after NMR measurements. The term p^2H is used to indicate a glass electrode meter reading uncorrected for the deuterium isotope effect on the glass electrode. The conversion factor is p^2H = pH + 0.4 (Glasoe & Long, 1960).

NMR experiments were performed on the 360-MHz spectrometer at the Stanford Magnetic Resonance Laboratory. Chemical shifts are referenced to a sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS) solution in an external glass capillary. Spectral resolution was improved with a Lorentzian-Gaussian double-exponential transform (Ferrige & Lindon, 1978). For resonances reflecting titration shifts, we have plotted log $[(\delta_A - \delta)/(\delta - \delta_B)]$ vs. pH and equated the pH at which the log term = 0 with the pK_a (Brown & Bradbury, 1975). For groups which monitor simple one-proton titration behavior, straight lines with slopes near 1 are obtained. For groups with more complex titration behavior, this graphical method gives an apparent pK (pK_{app}) corresponding to the p^2H at which the observed resonance has moved to a halfway position between the fully protonated (δ_A) and fully deprotonated (δ_B) positions.

Results

Four tyrosine residues at positions 7, 12, 17, and 47 and a single histidine at position 29 contribute to the aromatic region, and 23 amino acids—7 Ala, 3 Thr, 8 Val, 1 Ile, 2 Leu, and 2 Met—give rise to 34 methyl resonances in the aliphatic region of the ^1H NMR spectrum of the HP. Their resonance identification and/or assignments were reported earlier (Ribeiro et al., 1981a,b).

^1H NMR spectra of tyrosine and histidine C4 protons as a function of p^2H are shown in Figure 1. The His-29 C2 proton gives rise to a singlet at 8.58 ppm at p^2H 1.04. There is nothing unusual about the His-29 residue. Both the His C4 and C2 singlet resonances shift upfield with increasing p^2H and yield "normal" titration curves (Figure 2). The graphical procedure of Brown and Bradbury leads to a pK_a of ~7.0, with a slope near 1 as expected for a one-proton titration process.

The four tyrosine groups on the other hand give rise to eight nonequivalent tyrosine doublets whose relative chemical shifts and consequent line shapes are sensitive to pH. At each p^2H, the 2,6 protons of each tyrosine group are low field of its 3,5 protons (Wemmer et al., 1981a). Figure 1A reveals that at p^2H 1.0 tyrosines-7, -12, and -47 give rise to well-resolved doublets, whereas the Tyr-17 protons give rise to an unusual line shape. A two-dimensional NMR experiment previously demonstrated that the 2,6 and 3,5 protons of Tyr-17 are nearly chemical-shift equivalent (Ribeiro et al., 1981a). The line shape in Figure 1A is thus identified as that of a strongly coupled AB quartet.

As the p^2H increases, selective shifts of tyrosine resonances occur. The Tyr-47 and -7 2,6 proton doublets that are resolved at p^2H 1 merge at p^2H 4.0, are essentially chemical-shift equivalent at p^2H 7.3, diverge from each other at p^2H 9.9, and are again well resolved above p^2H 10.0 (Figure 1A–E). Double-resonance experiments irradiating their corresponding 3,5 proton doublets at acid, neutral, and alkaline pH show that the Tyr-7 and Tyr-47 2,6 proton resonances do not cross each other. They appear to merely merge and then diverge. The Tyr-47 3,5 doublet and the Tyr-12 resonances remain resolved and show small chemical-shift changes up to p^2H 9.9 (Figure 1A–D). These resonances then shift upfield above p^2H 10.0 (Figure 1E).

The strongly coupled AB quartet of Tyr-17 partially overlaps the Tyr-7 3,5 doublet at p^2H 1.0. When the p^2H is raised to 4.0, the components of the quartet are resolved from the Tyr-7 doublet. At p^2H 7.3, the 2,6 protons of the AB quartet shift notably away from the 3,5 protons of this quartet. This chemical-shift difference increases further with pH so that by p^2H 9.9 the Tyr-17 2,6 proton doublet has shifted onto the Tyr-7 3,5 proton doublet, giving the line shape seen in Figure 1D. By p^2H 10.7, the Tyr-17 2,6 proton resonances shift downfield so that they overlap the Tyr-12 3,5 proton doublet (Figure 1E). Concomitantly, the Tyr-17 3,5 proton doublet has crossed to low field of the Tyr-7 3,5 proton doublet.

As the pH is further increased, the tyrosines continue to shift. Individual peaks eventually broaden and become lost from the spectrum, suggesting that the titration of the tyrosine hydroxyl protons in alkaline medium is accompanied by protein unfolding effects. At p^2H 12.5, the aromatic region shows two tyrosine envelopes centering at ~6.9 and 6.5 ppm, corresponding to the random-coil form of the HP.

The tyrosine pH curves in the alkaline region (Figure 2), therefore, do not represent simple deprotonation of ring hydroxyl protons in the case of the HP. Instead, they reflect a complex combination of hydroxyl titration and/or protein unfolding effects. The approximate midpoints of these processes as represented by the term "apparent pK_a" are for Tyr-7 p^2H 10.8 and for Tyr-47 p^2H 11.4. The midpoints of the curves for Tyr-12 and Tyr-17 are more difficult to quantitate due to disappearance of these resonances at ~p^2H 12.0. A reasonable guess from Figure 2 gives an apparent pK_a of ~11.0.

In addition to the shifts observed above p^2H 10.0, all eight tyrosine resonances are sensitive to p^2H in the range 2–6. The p^2H range corresponds to titration of acidic groups and suggests that the tyrosine rings are in the proximity of side-chain carboxyl groups that titrate with pH. These acidic titration effects of the tyrosine resonances differ in magnitude and direction for the various tyrosine protons. The Tyr-17 protons shift upfield by over 0.1 ppm (~36 Hz at 360 MHz). The Tyr-7 3,5 protons similarly shift upfield by ~0.1 ppm; the Tyr-7 2,6 protons however shift *downfield* by ~0.03 ppm. The

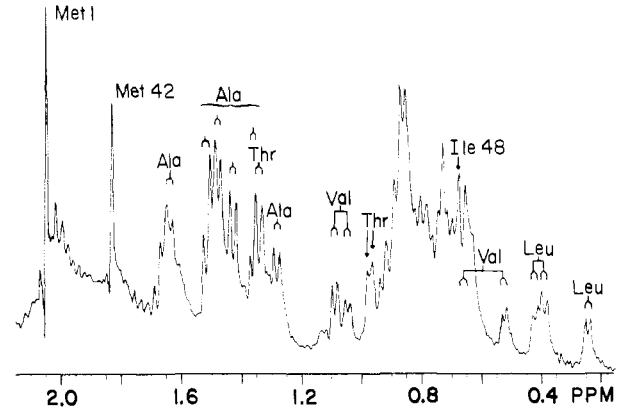

FIGURE 3: Resolution-enhanced 360-MHz ^1H NMR spectrum between 0.15 and 2.15 ppm showing the identified and/or assigned methyl resonances of the *lac* repressor headpiece. Of the 34 possible methyl resonances arising from 23 methyl-possessing amino acids, 33 have been identified. Only the resolved resonances are labeled here; difference spectra allow the observation of selected peaks hidden under the 0.6–1.0-ppm envelope.

Tyr-47 2,6 resonances shift upfield by ~0.03 ppm while its 3,5 resonances shift downfield by ~0.01 ppm. Both Tyr-12 resonances shift by ~0.02 ppm (~7 Hz). The shifts for Tyr-12 and Tyr-47 may initially be thought to be near the limits of error in chemical-shift differences. However, the peak positions in the 360-MHz spectra were determined to $<\pm 1$ Hz. The centers of the tyrosine doublets were also followed on expanded spectra and found to fluctuate by no more than 2 Hz in the pH 6–8 region. Given this situation, both the magnitude and direction of the shifts observed for the Tyr-47 and Tyr-12 resonances at pH 2–6 are above experimental error.

The aliphatic methyl region of the ^1H NMR spectrum of the HP is shown in Figure 3. Selective chemical modification and extensive double-resonance experiments have resulted in the assignment of the Met-1 and Met-42 SCH_3 singlets and the Ile-48 δ-methyl triplet, and the identification of six of the seven Ala methyl doublets, the 16 doublets of the eight Val residues, the three Thr methyl doublets, and the four leucyl methyl resonances arising from Leu-6 and Leu-45 in HP-51 (Ribeiro et al., 1981a). These identifications allow us to assess the effects of pH on methyl groups located at many disparate positions in the HP structure. The pH dependence of proton methyl resonances of proteins has been discussed only recently in a few cases (Lauterwein et al., 1978; Brown et al., 1978; Moore & Williams, 1980).

The pH dependence of the chemical shifts of various of the HP methyl resonances is shown in Figure 4. Like the aromatic region, the methyl resonances have distinct chemical shifts from pH 1 to 10, evidencing the presence of the folded structure. Above pH 10, the resolved leucyl and valyl doublets from 0.2 to 0.5 ppm shift into the methyl envelope between 0.6 and 1.0 ppm. Much of the fine structure in the envelope region is lost. Alanyl doublets resolved from ~1.4 to ~1.8 ppm merge into a widespread envelope centering at 1.4 ppm. The Met-42 SCH_3 singlet, distinctly upfield at the 1.8-ppm region at lower pH, shifts to the 2.0-ppm region. All of these phenomena are consistent with unfolding of the HP structure.

In addition to the general effects on many resonances above pH 10, individual methyl resonances show selective behavior below pH 10.0. The N-terminal Met-1 SCH_3 singlet, for example, is constant in chemical shift at 2.06 ppm from p^2H 1 to 8.0 and shifts upfield in the p^2H 8–9 region with an apparent pK_a of about 8.0. This resonance presumably reflects the titration of the N-terminal amino group in the HP and

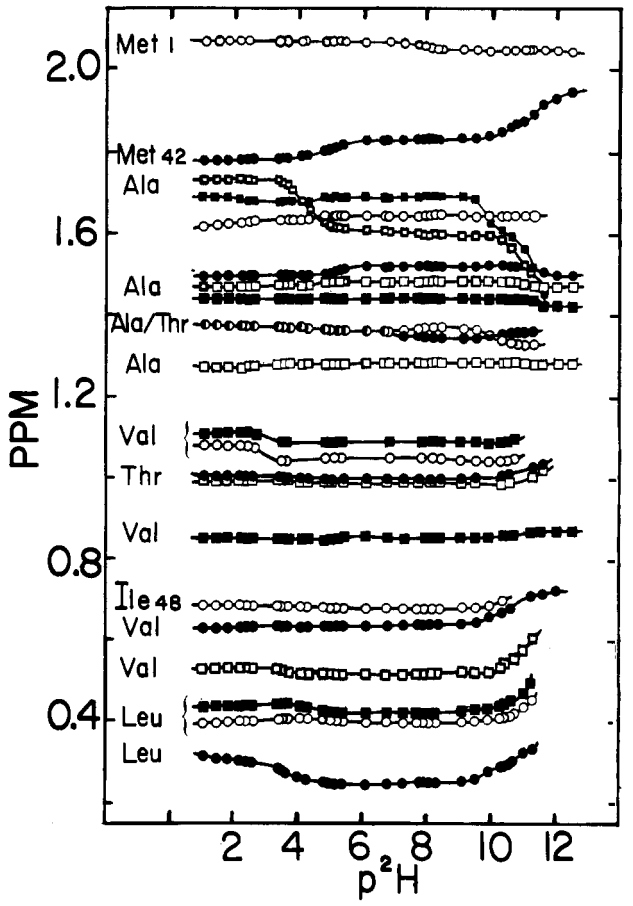

FIGURE 4: Chemical shift as a function of the p²H meter reading for resolved methyl resonances of the *lac* repressor headpiece.

hence gives us an indirect estimate of the intrinsic pK_a of the N-terminal amino group. This is consistent with Met-1 being a surface residue, exposed to solvent. The Met-42 SCH_3 group on the other hand is known to be buried since it is insensitive to chemical modification and is chemically shifted to an unusual position at ~1.8 ppm (Ribeiro et al., 1981b). When the pH is increased, this group shifts downfield between p²H 4 and 6 and levels off in shift between p²H 6 and 10.

In the alanyl methyl region of the HP spectrum (1.3–1.7 ppm), the various resonances show differential behavior. For example, the 1.42-ppm Ala doublet is constant in shift over the entire p²H range from 1 to 10, and shifts only above p²H 10, reflecting the protein unfolding. The 1.73-, 1.70-, 1.47-, and 1.49-ppm resonances on the other hand show distinct shifts of varying magnitude and direction between p²H 4 and 6. The 1.49-ppm alanyl resonance behaves remarkably similarly to the Met-42 SCH_3 singlet. A threonine methyl resonance overlaps an alanyl doublet at ~1.28 ppm at acid pH. These resonances diverge at neutral pH and surprisingly cross each other at alkaline pH.

The bulk of the methyl resonances in the envelope region between 0.5 and 1.0 ppm shows little dependence on p²H between 1 and 10. The overlap of lines in the envelope prevents a reliable, detailed analysis. However, the valyl doublet at 0.63 ppm, the δ-methyl of Ile-48, and the resolved Thr resonances at 1.0 ppm can reliably be followed, and these groups show little change in shift until above p²H 10.

The resolved valyl methyl doublets at 1.08 and 1.11 ppm at p²H 1.0 arise from the same valyl residue (Ribeiro et al., 1981b). These doublets shift upfield between p²H 2.4 and 4 and are then constant in shift until p²H 10.0. The resolved high-field leucine doublet resonance at 0.31 ppm behaves in a similar manner. The direction and magnitude of the acidic transitions for these valyl and leucyl groups are highly similar to those observed for the Tyr-7 and Tyr-17 resonances (Figure 2).

Table I: Apparent pK_a and Chemical-Shift Effects Observed for the Titrating Aromatic and Aliphatic Methyl Resonances in the *lac* Repressor Headpiece[a]

resonance	chemical shift (ppm) (at p²H 1.0)	app pK_a	direction (±)/magnitude (ppm)	app pK_a	direction (±)/magnitude (ppm)
His-29 C2	8.58	7.0	+0.992		
His-29 C4	7.23	7.0	+0.361		
Tyr-47 2,6	7.03	4.7	+0.024	~11.4	+0.133
Tyr-47 3,5	6.77	broad	−0.012	~11.4	+0.306
Tyr-7 2,6	6.98	3.4	−0.027	10.8	+0.200
Tyr-7 3,5	6.51	3.8	+0.090	~10.8	>+0.094
Tyr-12 2,6	6.72	broad	+0.022		>+0.067
Tyr-12 3,5	6.58	broad	+0.012	~11.0	>+0.196
Tyr-17 2,6	6.49	3.6	+0.114	11.0	>−0.165
Tyr-17 3,5	6.47	3.7	+0.118		>−0.027
Met-1	2.06	8.0	+0.027		
Met-42	1.78	5.4	−0.050	>11.0	>−0.110
Ala	1.72	4.2	+0.15		>+0.15
Ala	1.49	5.3	−0.46		+0.03
Ala	1.47	4.8	−0.022		+0.02
Val[b]	1.11	2.8	+0.039		
Val[b]	1.08	2.8	+0.040		
Val	0.53	4.0	+0.020		
Leu[b]	0.44	5.0	+0.025		
Leu[b]	0.40	5.0	+0.022		
Leu	0.31	3.7	+0.072		

[a] pH shifts measured at 23 °C. + = upfield shift; − = downfield shift. Only identified resonances which shift by more than 0.01 ppm are included in the table. Other resonances in the methyl envelope between 0.6 and 1.0 ppm may also shift with pH, but their shifts are small and difficult to measure exactly in the forest of peaks in the envelope. Values of pK_a are in p²H units uncorrected for the deuterium isotope effect. [b] These methyl resonances arise from the same individual amino acid residue.

Smaller shifts are observed for the 0.40- and 0.44-ppm leucyl methyl pair between p²H 4 and 5. The pH behavior and the apparent pK_a of this leucyl group appear similar to those of Tyr-47. The apparent pK_a's of the resonances shifting in the aromatic and aliphatic regions have been obtained by a graphical method (Brown & Bradbury, 1975) where possible and are summarized in Table I.

Discussion

The X-ray crystal structures of the HP and whole repressor are not yet available. The present NMR studies continue our efforts to characterize a protein of unknown structure in solution. In papers 1 and 2 of this series we built a data base of ^1H NMR resonance assignments and identifications and followed the temperature dependence of the HP structure.

The NMR studies presented here demonstrate that the folded HP structure is intact and stable over the pH range 1–10. The large shifts of many methyl resonances and the eight tyrosine resonances above pH 10 are interpreted to reflect a general unfolding of the protein structure which may be concomitant with titration of the tyrosine rings and other groups. Spectra at pH 12.5 show only two tyrosine resonances at ~6.9 and ~6.5 ppm, complete loss of the four high-field methyl doublets between 0.2 and 0.5 ppm, and broad methyl envelopes at 0.7, 0.85, and 1.4 ppm. These approximate the random-coil form of the HP. This pH denaturation is completely reversible. Lowering of pH from the alkaline region on several denatured HP samples yielded spectra with all the features of the folded HP structure.

Since the tyrosine shifts in the alkaline region reflect both the ring hydroxyl titration and the protein unfolding, it is not possible to quantitate with certainty the pK_a for the intrinsic titration of the individual tyrosine rings. The alkaline shift changes for Tyr-47 however notably lag behind the changes of the other tyrosine residues.

Of greater significance are the observations that all four tyrosines and several aliphatic methyl groups shift selectively at acidic pH, i.e., pH 2–6. Unlike the intrinsic titrations of the histidine at neutral pH and the tyrosine hydroxyls at alkaline pH, the acidic shifts must arise from through-space, conformation-dependent interactions between different groups of the folded HP polypeptide chain. Interactions in proteins may be direct interactions, e.g., through-space shift effects due to local shielding of nearby side chains by aromatic rings or anisotropic carboxyl groups or even direct hydrogen bonding between two groups (Bundi & Wüthrich, 1979). They may also be indirect interactions transmitted by changes of conformation arising as a result of the deprotonation of one or several ionizable groups. Since relatively few resonances have been individually assigned and located in protein structures and the pK_a values of ionizable groups in the interior of protein resonances are not generally well established, it is not possible to distinguish these two mechanisms for causing indirect shifts at the present time. However, given the primary sequence of the HP (Geisler & Weber, 1977) and the observation that the single His-29 is still fully protonated at p^2H 5.0 (Figure 2), only six residues in the HP are likely to possess ionizable groups in the acid region. These are the carboxyl side chains of Asp-8, Glu-11, Glu-36, Glu-39, and Glu-44 and the Arg-51 carboxyl C terminus.

There appear to be at least four distinct clusters of resonances which show similar pH behavior in the folded HP. It is reasonable to believe that these similarities are more than coincidence and that the pH titrations reflect clusters of side chains that are interacting with a common ionizable group (or groups) in the native structure. The most striking of the clusters consists of the Tyr-7, Tyr-17, the 0.31-ppm leucyl, and the 1.0-ppm valyl groups. These residues show *large* shifts, suggesting strong interactions with a mutual set of ionizable carboxyl side chains.

Reciprocal intramolecular nuclear Overhauser effects have also been noted between the Tyr-7 ring and the 0.31-ppm leucyl methyl resonances (Ribeiro et al., 1981b). The pH and NOE evidence thus establish a triangular relationship in which the Tyr-7 ring, the 0.31-ppm leucyl methyl group, and at least one ionizable group mutually interact in the folded HP structure.

These findings are particularly important since the Tyr-7 and Tyr-17 rings have been directly implicated to be part of the contact region in the in vitro binding of HP to double-helical nonoperator DNA (Buck et al., 1980).

A second subset of similar pH behavior appears in the Tyr-47 and the 0.40- and 0.44-ppm leucyl groups. The markedly higher acidic pK_a of their transitions, however, suggests they are near a different ionizable carboxyl group than those experienced by Tyr-7 and Tyr-17. Since the Tyr-47 ring and the 0.40- and 0.44-ppm leucyl methyls also show reciprocal nuclear Overhauser effects (Ribeiro et al., 1981b), a second triangular relationship appears in which the Tyr-47, the 0.40- and 0.44-ppm Leu, and a carboxyl group interact in the folded HP structure.

A third subset appears in the pH behavior of Tyr-12. The shift changes for Tyr-12 are less dramatic than those for the other tyrosines, suggesting weaker interactions. However, the broad transitions observed over p^2H 2–6 for Tyr-12 suggest it interacts with at least two ionizable carboxyl groups. Finally, the similarity of the shifts and pK_{app} for the Met-42 SCH_3 singlet and the 1.49-ppm Ala doublet suggests these groups reflect at least one other ionizable group.

The distribution of the six ionizable carboxyl groups into these four clusters cannot be given at this time. The interactions observed could arise from short-range interactions between neighboring residues in the amino acid sequence or from long-range interactions, i.e., interactions between polypeptide segments widely disparate in the primary structure. The deprotonation of the ionizable groups, however, clearly induces effects at four clusters of resonances thus far identified.

In conclusion, the data presented here show that the folded HP structure is retained at p^2H 1–10 and that unfolding occurs in the alkaline region. This pH-induced unfolding is completely reversible. In the folded HP structure, the four tyrosine groups and several methyl resonances interact with ionizable carboxyl groups. Three triangular relationships involving (i) Tyr-7, one Leu, and ionizable group(s), (ii) Tyr-47, another Leu, and an ionizable group, and (iii) Met-42, one Ala, and an ionizable group are inherent features of the folded HP structure. When firmer assignments of the HP resonances and distances between groups are established, a detailed model of the HP structure will be possible.

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