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Nuclear Magnetic Resonance Studies on the Solution Conformation of Histone IV Fragments Obtained by Cyanogen Bromide Cleavage†

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ABSTRACT: Two histone IV fragments obtained by cleavage at Met-84 by cyanogen bromide have been examined by proton magnetic resonance (PMR) spectroscopy as a function of temperature, peptide concentration, ionic strength, and pD. Sedimentation and gel electrophoresis studies on these peptides have also been carried out. The 220-MHz PMR spectrum of the N-peptide in both the high- and low-field regions was shown to be almost identical with that calculated for an extended coil N-peptide monomer. The calculated random coil and experimental C-peptide spectra, on the other hand, differ in many respects. Evidence was obtained for the presence of rigid secondary structure in the C-peptide. In addition, the Val, Leu, Ile CH_3 resonance displays a prominent high-field satellite band which shifts downfield with increasing temperature. Sedimentation studies on the N-peptide reveal the formation of extremely large, remarkably homogeneous aggregates at ionic strengths ≥0.01. The C-peptide, on the other hand, does not appear to form aggregates of sufficient size to be detectable in velocity sedimentation studies of a few hours duration. The relative area changes which have previously been noted in the PMR spectrum of histone IV with increasing ionic strength were also observed for the N-peptide but not the C-peptide. Interpretation of these relative area changes has been made in terms of the amino acid sequence of histone IV, and an effort was made to identify that segment of the polypeptide which undergoes secondary structural change with increasing ionic strength.

The histone IV amino acid sequence (DeLange et al., 1969; Ogawa et al., 1969) revealed a high proportion of basic residues within the N-terminal region and a preponderance of nonpolar side chains within the C-terminus. This unexpected amino acid distribution led to early suggestions (anonymous, 1968) that the conformation of this unusually structured protein, both as a free monomer and as a component of native chromatin, might provide important clues to its biological function.

A representative model (Sluyser, 1969) depicted histone IV as essentially a random coil monomer in solution and as a "hairpin" when complexed with DNA. The basic N-terminal region formed a strong electrostatic complex with DNA while the hydrophobic C-terminal region was either folded back along the N-terminal segment or weakly complexed with the same strand of DNA. This model remains attractive to many investigators (DeLange et al., 1972) because it leaves the C-terminal region relatively free to interact with other DNA molecules to form "cross-links" or with regulators of genetic replication and transcription.

The conformational significance of the clustering of the cationic residues of histone IV toward the N-terminal region is the subject of this as well as a previous communication (Pekary et al., 1975). The earlier paper was concerned with the conformational properties and the aggregation behavior of the intact histone IV molecule. In the present work, histone IV was cleaved at Met-84 by cyanogen bromide (CNBr) and the proton magnetic resonance (PMR) spectral characteristics as well as the sedimentation properties of the resultant N- and C-terminal fragments (hereafter referred to as the N- and C-peptides, respectively) have been examined under various conditions of peptide concentration, ionic strength, and pD. The conformational and aggregation properties of these peptides will be discussed in terms of their contributions to the structure and function of the parent histone IV protein molecule.

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Table I: Amino Acid Compositions for the Histone IV CNBr N- and C-Peptides.[a]

	N-peptide	C-peptide
Cysteic acid		
Aspartic acid	4.66 (4)	1.04 (1)
Threonine[b]	5.89 (6)	1.24 (1)
Serine	2.22 (2)	0.20 (0)
Glutamic acid	7.29 (5)	1.21 (1)
Proline	2.10 (1)	
Glycine	10.90 (13)	4.18 (4)
Alanine	7.98 (6)	1.30 (1)
Cysteine		
Valine[b]	6.90 (8)	1.43 (2)[c]
Methionine		
Isoleucine	4.99 (6)	0.17 (0)
Leucine	7.65 (6)	1.94 (2)
Tyrosine[b]	1.76 (2)	1.78 (2)
Phenylalanine	1.17 (1)	0.87 (1)
Histidine	2.25 (2)	
Lysine	9.37 (10)	1.26 (1)
Arginine	9.98 (12)	2.09 (2)

[a] Numbers in parentheses are values calculated from the histone IV amino acid sequence. [b] Values for threonine are corrected by 2.4%, for valine by 9.6%, and for tyrosine by 25% for destruction during 24 hr of hydrolysis. [c] The presence of a Val–Val bond causes this value to be low.

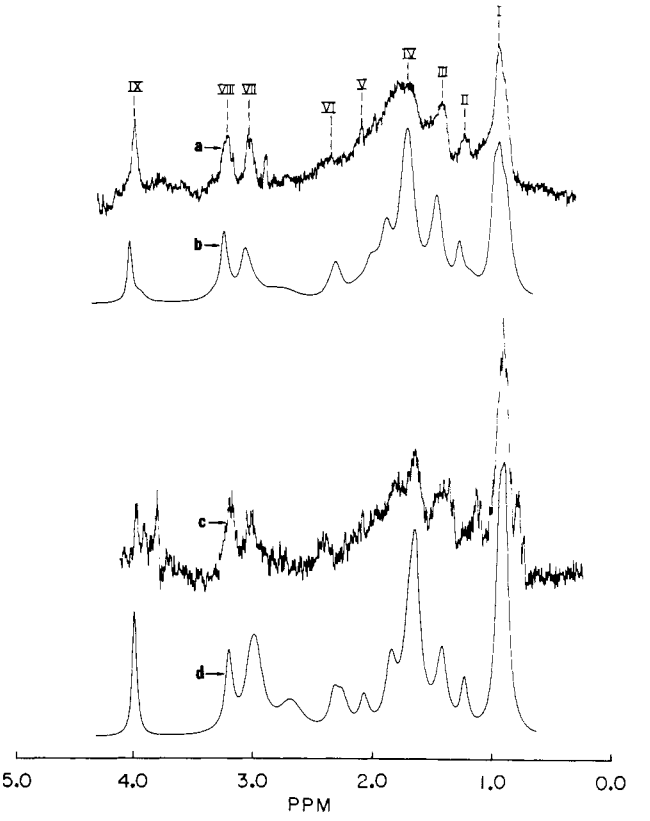

FIGURE 1: The 220-MHz CW PMR spectra in the high-field region. (a) N-peptide; (b) calculated N-peptide spectrum; (c) C-peptide; (d) calculated C-peptide spectrum. pD 3.2; concentrations $\simeq 10^{-3}$ *M*.

Materials and Methods

Histone IV was obtained by previously described methods (Pekary et al., 1975). This protein was cleaved at Met-84 by CNBr using the method of DeLange et al. (1969). The lyophilized product was chromatographed on a 3 × 104 cm G-50 fine Sephadex column eluted with 10% formic acid; 16-ml fractions were collected with a Gilson Model LB1 fraction collector. The elution profile was obtained by measuring the 280 nm ultraviolet (uv) absorbance of each fraction with a Beckman DU spectrophotometer. The homogeneity of the N- and C-peptides was checked by disc electrophoresis in 7.5% acrylamide gels (Bonner et al., 1968). The rates of migration toward the cathode occurred in the following order: histone IV > N-peptide > C-peptide. All bands are stainable with Amido Schwartz 10B but the C-peptide was always lost if cross-electrophoresis rather than diffusion destaining was used. The amino acid composition for the N- and C-peptides (Table I) agreed completely with that calculated from the histone IV sequence (DeLange et al., 1969; Ogawa et al., 1969).

Chromatography of the histone IV CNBr fragments with 10% formic acid resulted in the condensation of formate anions as counterions to the N-peptide. Consequently, a broad, low-field region band, due to the formate CH resonance, was observed in the PMR spectrum of this peptide after it has been lyophilized and then redissolved in pure D_2O. Since this formate resonance obscured the low-field region PMR spectrum of N-peptide, these polycations-condensed formate counterions were exchanged with 99% d_2-formic acid prepared by the thermal decomposition of d_2-oxalic acid (Leonard and Sauers, 1957).

The concentration dependence of the N- and C-peptide 220-MHz high-field region continuous wave (CW) PMR spectrum was obtained by diluting an N- or C-peptide solution (50 mg/ml) in pure D_2O or in 0.05 *M* NaCl (pD 3.7) stepwise with D_2O at pD 3.7 and the corresponding ionic strength. The methods and instrumentation used in these studies have been described previously (Pekary et al., 1975). In the temperature studies, ethylene glycol was used to monitor the probe temperature.

Low-field region Fourier transform (FT) PMR spectra of histone IV, N-peptide, and C-peptide were obtained with a Varian HA-100 spectrometer interfaced with a Varian V-4356 Fourier transform accessory. A trifluoroacetic acid containing capillary was used for frequency locking. Samples were measured at the probe temperature of 30°.

Velocity sedimentation studies on 1×10^{-4} *M* N-peptide in distilled H_2O (pH 3.3; pH 0.04) and 0.1 *M* NaCl (pH 3.3) and 1×10^{-4} *M* C-peptide in 0.1 *M* NaCl (pH 3.3) were performed with a Hermes Model E analytical ultracentrifuge. Absorption scans of the 12-mm optical path double sector cells at 268 nm were taken every 4 min at 44000 rpm with the temperature maintained at 20° and distilled water used as the reference solution.

Results

The 220-MHz CW PMR spectra for the histone IV N- and C-peptide in the high-field region are shown in Figure 1 (1a and c, respectively). For comparison, calculated spectra for the monomeric random coil N- and C-peptides are also presented (Figure 1b and d). The chemical shifts and half-widths used in the computer simulation were based on corresponding shifts and half-widths observed for random coil proteins (McDonald and Phillips, 1969). Except for some broadening of peak III (Lys γ-CH_2, Ala-CH_3, Ile-CH_2), peak IV (Lys δ-CH_2 + β-CH_2, Arg γ-CH_2, Leu β-CH_2 + γ-CH), and peak VI (Glu γ-CH_2, Gln γ-CH_2, Val β-CH),

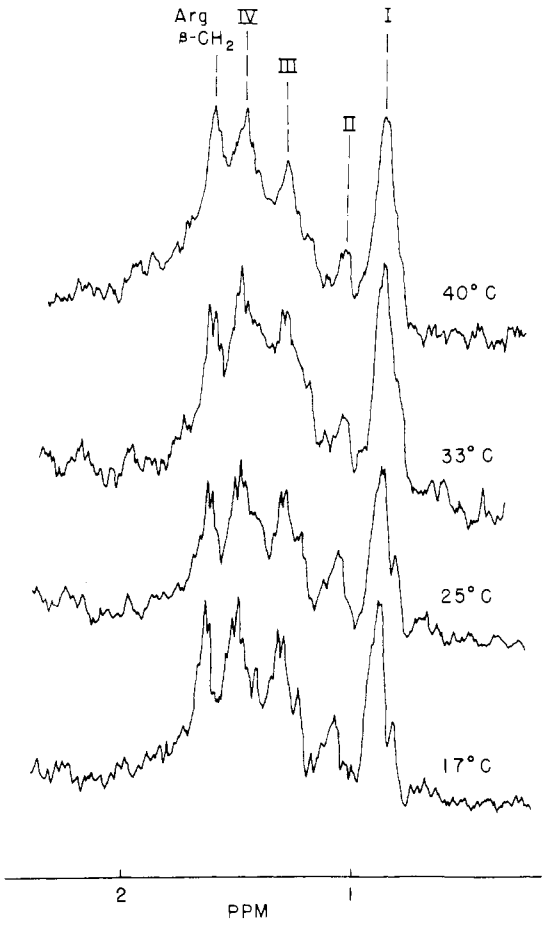

FIGURE 2: Effect of temperature on the 220-MHz CW PMR spectrum of 1.5×10^{-3} *M* C-peptide in the high-field region. pD 3.2.

the experimental and calculated N-peptide spectra correspond in almost every respect. The observed C-peptide spectrum, on the other hand, contains several features which do not appear in the calculated spectrum, including a small satellite peak on the high-field side of peak I, an upfield-shifted peak II (Thr CH_3), and multiple resonances within peak IX (Gly CH_2). The bandwidths observed for peaks III, VII, and VIII of the C-peptide are also noticeably broader than the corresponding widths in the simulated spectra.

These spectral results indicate that the N-peptide behaves as a random coil in pure D_2O, whereas some secondary structure is present in the C-peptide. This latter result might be anticipated on the basis of the amino acid composition of the C-peptide. Other than one Asp acid and three basic residues (2 Arg and 1 Lys), the C-peptide contains 14 other residues which are primarily hydrophobic in character, and this amino acid composition should confer on the C-peptide a compact conformation in aqueous solution. The magnetic nonequivalence of the four glycyl residues (peak IX) is most likely the consequence of this secondary structure, as is the upfield shift observed for the resonance due to the only Thr in the C-peptide. Similarly, the small satellite peak which appears to the high-field side of peak I can be attributed to secondary structure. This peak was found to be temperature dependent. We have followed the position of this signal over the temperature range between 17 and 40° and have noted that it gradually shifts downfield with increasing temperature (Figure 2), at least until 40° whereupon it merges with the much stronger peak I envelope (Val, Leu CH_3).

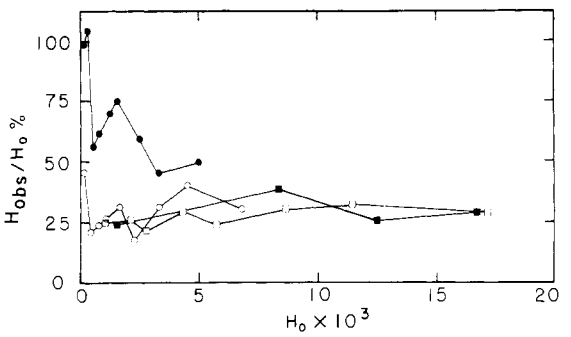

FIGURE 3: Ratio of the PMR visible peptide concentration to the stoichiometric concentration, $[H_{obsd}]/[H_0]$, for the N-peptide in pure D_2O (●) in 0.05 *M* NaCl (○), the C-peptide in pure D_2O (■) and in 0.05 *M* NaCl (□). Concentrations are expressed in moles of peptide/liter.

As in the case of the parent histone IV, the PMR estimated concentration, $[H_{obsd}]$, of the N-peptide in 0.05 *M* NaCl and of the C-peptide in both pure D_2O and 0.05 *M* NaCl has been found to be invariably less than the stoichiometric peptide concentration, $[H_0]$. In addition, these PMR estimated concentrations were found to be essentially invariant over the concentration range 2×10^{-2}–10^{-4} *M* (Figure 3). On the other hand, for the N-peptide in pure D_2O, the percentage of the peptide contributing to the PMR spectrum increases from 50 to 100% as $[H_0]$ is decreased from 10^{-3} to 10^{-4} *M*. The relatively uniform loss in the PMR band areas for the N-peptide in 0.05 *M* NaCl may be attributed to partial aggregation. A solution of the N-peptide in 0.1 *M* NaCl (pH 3.3) and 1×10^{-4} *M* in concentration was found to exist predominantly in the form of relatively homogeneous aggregates with $s_{20,w} = 14.5$ S. In pure D_2O, apparently the N-peptide becomes totally dissociated at sufficiently low peptide concentrations to give essentially a random coil. This complete dissociation of the N-peptide upon dilution may be the result of increased electrostatic repulsion between the many cationic residues as the counterion concentration is reduced with sample dilution (Tanford, 1961).

The observed intensity loss associated with the PMR spectrum of the C-peptide most probably arises from intermolecular aggregation. Considering the size of the C-peptide, its rigid secondary structure (A. E. Pekary and S. I. Chan, 1975) does not appear to be a likely source of the intensity loss. The observed lack of concentration dependence may be attributed to an extremely slow dissociation rate for the C-peptide aggregates (Cruft et al., 1958). There is also no apparent effect of ionic strength on the PMR spectra. Within the experimental error of our PMR area measurements, there is no detectable change in the peak I area for a 1×10^{-4} *M* solution when the NaCl concentration was varied from 0 to 0.8 *M*. Since the CD spectrum of the C-peptide (A. E. Pekary and S. I. Chan, 1975) was also found to be independent of the ionic strength as well as pD, it is apparent that these variables do not affect the secondary structure or the aggregation properties of the C-peptide.

The effects of pD on the intensities of peak I (Val, Leu CH_3) for both the N- and C-peptide are summarized in Figure 4 at a peptide concentration of 1×10^{-4} *M*. These intensities are expressed as the percentage expected based on the stoichiometric peptide concentration. Between pD 2 and 9 in distilled water, peak I of the N-peptide was found to contain essentially the expected intensity within a line width corresponding to the random coil. Thus there is no ev-

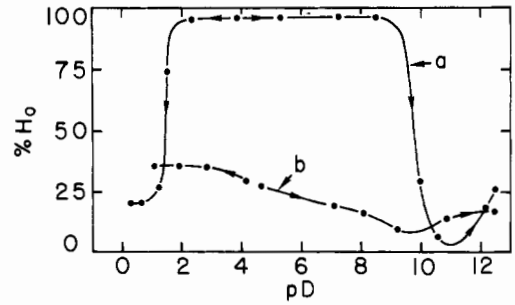

FIGURE 4: Variation of the peak I intensity with pD for the N-peptide (a) and the C-peptide (b). The arrows indicate the direction of titration.

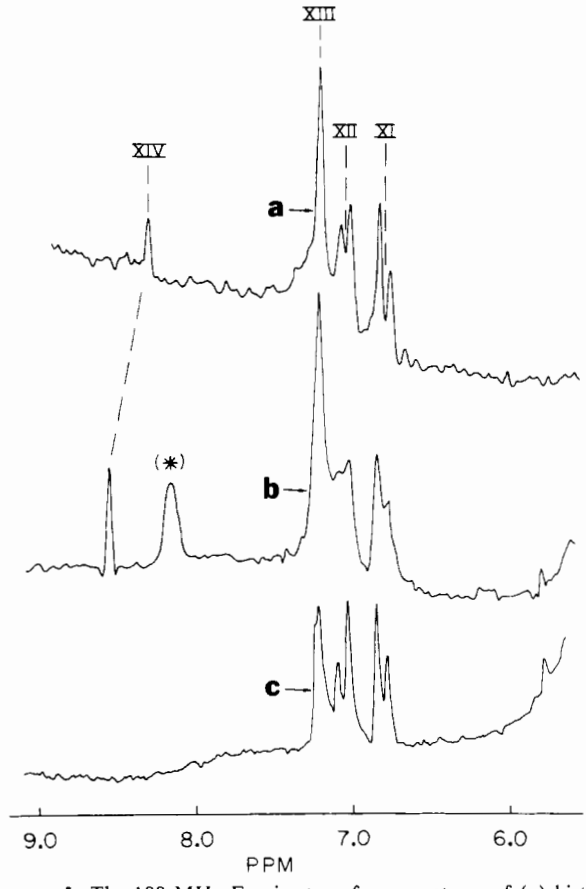

FIGURE 5: The 100-MHz Fourier transform spectrum of (a) histone IV; (b) N-peptide; and (c) C-peptide, in the low-field region. Acquisition time: 0.4 sec; pD 3.2; concentrations $\simeq 10^{-3}$ *M*. (*) denotes residual formate CH resonance.

idence for gross aggregation of the N-peptide at a concentration of 1×10^{-4} *M* over this total pH range. Sedimentation velocity measurements of the N-peptide at a pH of 3.3 substantiate this conclusion. The sedimentation velocity of the N-peptide at the concentration of 1×10^{-4} *M* was observed to be less than 0.1 S. Since the sedimentation velocity will be retarded by a factor of $1/(1 + n)$, with n being the number of counterions (Bowen, 1970), $s_{20,w}$, with this primary charge effect removed, would be less than 3 S. By contrast, at a pH of 0.04 in HCl solution, 1×10^{-4} *M* N-peptide forms large aggregates of 28.8 S, presumably as a result of Cl^- condensation and the resultant electrostatic shielding of the cationic Arg and Lys residues. As expected, at pD >9, the N-peptide precipitates, as the isoelectric point (p*I* $\approx$ 11) is approached.

FT PMR spectra at 100 MHz of (a) histone IV, (b) N-peptide, and (c) C-peptide in the low-field region are exhibited in Figure 5. The resonance position for the peak XI (Tyr, ortho protons), peak XII (Tyr, meta protons), and peak XIII (Phe, aromatic protons) correspond in every case with the chemical shift data previously obtained with proteins in the random coil state (McDonald and Phillips, 1969). In Figures 6 and 7 we compare the effects of concentration on the 220-MHz PMR spectrum of histone IV and the N-peptide in the low-field region. These studies revealed a concentration dependence splitting of peak XIV (His C-2 proton) and a conspicuous decrease in the intensities of peak XI (Tyr, ortho protons) and peak XII (Tyr, meta protons) relative to peak XIII with increasing concentration for both the parent protein (Figure 6) and the N-peptide (Figure 7). The splitting of the His C-2 proton is most likely due to nonequivalence of the chemical shifts experienced by His-18 and His-75. As we shall later argue, the observed intensity variations of the resonance peaks arise from changes in the secondary structure of the polypeptide. Surprisingly, the spin–spin splitting associated with peaks XI and XII of the histone IV protein, so clearly evident in Figure 5 at 100 MHz, is only barely discernible in the CW spectrum at 220 MHz (Figure 6). This apparent discrepancy is due in part to the absence of saturation broadening effects and hence the somewhat greater inherent resolution in FT spectroscopy (Ernst and Anderson, 1966). The fact that the appearance of these Tyr resonances is not changed significantly when the temperature of the sample is increased from 18 to 30° (the ambient HA-100 probe temperature) would seem

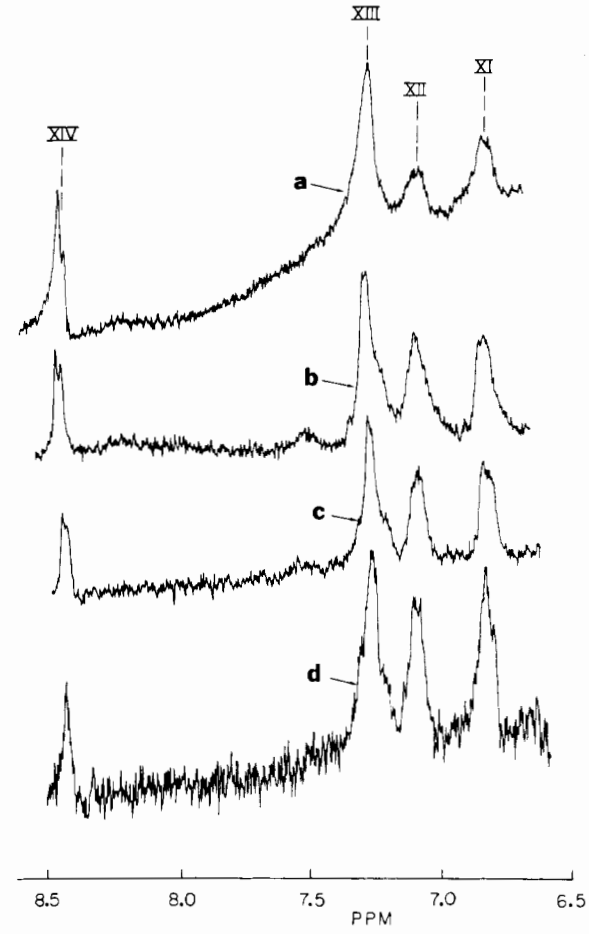

FIGURE 6: The 220-MHz spectra of histone IV in the low-field region at various concentrations: (a) 7.25×10^{-3} *M*; (b) 3.62×10^{-3} *M*; (c) 1.81×10^{-3} *M*; (d) 9.07×10^{-4} *M*. pD 3.2.

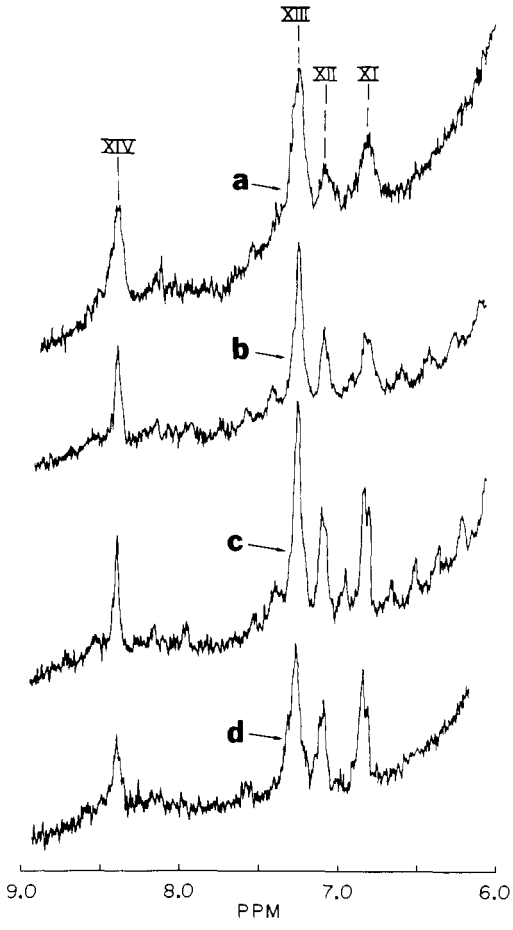

FIGURE 7: The 220-MHz spectra of the N-peptide in the low-field region at various concentrations. (a) 8.36×10^{-3} *M*; (b) 4.18×10^{-3} *M*; (c) 2.09×10^{-3} *M*; (d) 1.05×10^{-3} *M*. pD 3.2.

to rule out any broadening effects arising from chemical exchange.

Discussion

In our previous paper on histone IV (Pekary et al., 1975) the PMR spectra and their variation with concentration and ionic strength were used to deduce the conformational properties as well as the aggregation tendencies of the protein in aqueous solution. In this analysis it was assumed that only the histone IV monomer contributes to the high resolution features of the spectra and that aggregate species of the protein possess spectra which are so broad that they contribute only to the base line. However, even for the monomeric histone IV molecule, only 70–80% of the residues are PMR visible. We attributed the missing signals to rigid secondary structure in the polypeptide, particularly in the C-terminus. This conclusion is now substantiated in the present work, as removal of 18 residues from the C-terminus of the histone IV protein leaves an N-peptide which is fully observable in pure D_2O in the monomeric state. Like the parent protein, the N-peptide is also very susceptible to gross aggregation with increasing concentration, particularly in the presence of NaCl, yielding species which are also PMR invisible. We attribute the NaCl induced aggregation to Cl^- counterion shielding of the repulsive cationic residues.

The above conclusions are at variance with those previously reported by Boublik et al. (1970) and Bradbury and Rattle (1972). Although these workers have also interpreted their nuclear magnetic resonance data in terms of secondary structure and intermolecular aggregation of the histone IV protein, they concluded that any changes in the secondary structure are intermolecularly induced. Specifically, it was assumed that the intermolecular association leads to immobilization of only those regions of the polypeptide which are involved in the intermolecular interactions, and those parts which are not are thought to be always sufficiently mobile to yield high resolution PMR spectra. By contrast, in our model, immobilization of the entire polypeptide is assumed upon histone IV association. An important source of divergence between the results from the two groups is that Boublik et al. (1970) and Bradbury and Rattle (1972) interpreted relative area losses in their spectral data only, whereas we took full account of the absolute areas actually observed as well. It is essential that absolute areas also be used in this analysis. Relative area changes alone can be misleading if a significant percentage of the intensity for each individual resonance peak is not accounted for. For example, both groups of workers noted the relatively greater area loss observed for peak I relative to peaks II–V with changes in the solution concentration and ionic strength. On the basis of these relative area losses, Boublik et al. (1970) and Bradbury and Rattle (1972) concluded that intermolecular interactions occur principally between the hydrophobic C-terminal regions exclusively and the N-terminal part of the histone IV protein remains free in solution even in the aggregated state. First, we point out that although band I of histone IV decreases about 25% in intensity relative to peaks II–V when the histone concentration is increased from 3×10^{-4} to 4×10^{-3} *M*, in terms of the total area expected should the histone IV protein be totally in the random coil form, the measured peak I and peaks II–V areas actually vary from 65 to 21% and 67 to 32%, respectively. The actual difference between the area losses of peak I and the aggregate intensity of peaks II–V is thus only 11%.

Secondly, we have evidence to suggest an alternate explanation for the relative area losses observed upon the addition of NaCl. To be sure, part of the additional area losses observed with increasing ionic strength is due to further aggregation of the histone IV, but in part they also arise from further changes in the secondary structure of the polypeptide, particularly in the N-terminus of the protein. If we compare (Table II) the peak I to peaks III–V ratios for the N-peptide, we find that these ratios are decreased upon the addition of NaCl. On the other hand, the C-peptide peak I

Table II: Peak I to Peaks III–V Intensity Ratio for N- and C-Peptide.

Peptide	Concn (*M*)	NaCl (*M*)	Peak I/ Peaks III–V
N	5.00×10^{-3}	0.00	0.41
N	6.77×10^{-3}	0.05	0.34
N	1.56×10^{-4}	0.00	0.39
N	2.12×10^{-4}	0.05	0.20
C	2.50×10^{-2}	0.00	0.48
C	1.72×10^{-2}	0.05	0.60
C	1.56×10^{-3}	0.00	0.29
C	1.07×10^{-3}	0.05	0.33

to peaks III–V ratios are slightly *increased* upon the presence of NaCl. In the case of the intact histone IV protein, these peak I/peaks III–V ratios were found to decrease with added salt, which is what one would expect if ionic strength induces similar secondary structural change in the N-terminal region of the histone IV protein as in the N-peptide fragment.

Comparative CD studies of the N- and C-peptide as well as the histone IV protein, the results of which we shall report in a separate communication (A. E. Pekary and S. I. Chan, 1975), demonstrate that all the structural-*promoting* effects of added salt are indeed confined to the N-terminus of the histone IV. Thus although the present work indicates that the C-peptide as well as the C-terminus of the histone IV protein contain rigid secondary structure, it appears that this secondary structure is not affected significantly by substantial changes in the ionic strength or pD of the solution. This result can also be inferred from detailed analysis of the relative area changes. In fact the observed "concentration induced" variations in the relative areas of the various resonances in the low-field spectra observed at 220 MHz for the intact histone IV molecule (Figure 6) and the N-peptide (Figure 7) may be used to ascertain those regions of the histone IV N-terminus which acquire salt-induced secondary structure. Although only the peptide concentration is varied in these experiments, due to the large number of counterions required to maintain the overall neutrality of these histone IV and N-peptide solutions, the ionic strength of these solutions actually also varies from about 0.02 to 0.2, or over a range of about 10, and the observed *relative* area changes are more likely due to changes in the ionic strength rather than the concentration of polypeptide per se. Figure 6 reveals two distinct His C-2 resonances (peak XIV), the areas of which change significantly with respect to each other as the histone concentration increases. There are two His's in histone IV, one at position 75, near the C-terminus, and one at position 18, near the N-terminal region. One or the other of these respective regions must be undergoing a conformational change with ionic strength increase sufficient to limit the PMR visibility of its component His. As we have already noted, this change occurs simultaneously with a substantial decrease in the areas of peaks XI (Tyr, ortho protons) and XII (Tyr, meta protons) relative to peak XIII (Phe, aromatic protons). Similar relative PMR band area changes also occur within the low-field region PMR spectrum of the N-peptide (Figure 7) over a concentration range (and therefore ionic strength range) similar to that used for corresponding measurements on histone IV (Figure 6). Since (1) any conformational transition which would limit the PMR "visibility" of His-75 very likely would also affect the observability of Tyr-72 due to their proximity within the histone IV sequence, and (2) Tyr-72 and His-75 occur within a region of the histone IV sequence which is "permissive" to α-helix formation (Wu and Kabat, 1971; Chou and Fasman, 1974), while (3) the His-18 is located within a region of high linear charge density (Manning, 1969) which will tend to oppose the formation of stable secondary structure in the absence of gross aggregation, these observations combined with a consideration of the histone IV sequence (DeLange et al., 1969; Ogawa et al., 1969) lead us to conclude that it is the histone IV sequence just preceding Met-84 (the residue destroyed during CNBr cleavage) which undergoes substantial conformational change with ionic strength increase.

Conclusions

CNBr cleavage of histone IV at Met-84 produces two fragments with distinctive structural and physical properties. The 83 residue N-terminal fragments (N-peptides) at very low ionic strengths are completely extended coils which form large, relatively homogeneous aggregates as the Cl^- concentration exceeds 0.01 *M* (NaCl) or pD <2 (DCl). This behavior is characteristic of cationic polymers. The 18 residue C-terminal fragments (C-peptides), on the other hand, possess extensive secondary structure as evidenced by certain PMR spectral anomalies. This secondary conformation is not perturbed by large variations in the peptide concentration and ionic strength of pD, but is somewhat affected by temperature. Relative area changes within the PMR spectrum of histone IV, which occur with ionic strength increase, are attributed to conformational modifications within the N-terminal segment, since the only CNBr fragment of histone IV which undergoes a corresponding variation is the N-peptide.

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