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Ionic Strength Induced Structure in Histone H4 and Its Fragments†

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ABSTRACT: The salt-induced folding and self-association of histone H4 and its fragments (1–23), (25–67), (69–84), and (69–102) have been studied at the same molar concentration (1 m*M*) by nuclear magnetic resonance (NMR), circular dichroism (CD), and ir spectroscopy. By each of these techniques intact histone H4 exhibited a fast structural change, involving the formation of α_R helix and aggregation, and also a slow change involving β-structure formation. Fragment (25–67) was found to behave in a manner similar to the intact molecule for the fast change, showing both helix formation and aggregation but exhibited no time-dependent effects. All the other fragments were found to be essentially noninteracting. It is concluded that (25–67) contains the region critical for the folding and self-association of histone H4. On the basis of these results a model is proposed for the self-association of histone H4 in which helix is located between residues 49 and 73, while the β structure lies between 74 and the C-terminus.

One of the most striking features of histone H4[1] (F2A1, IV, or GRK) is the very high degree of conservation of its amino acid sequence from species to species in eukaryotes (DeLange and Smith, 1971). Although this near invariance undoubtedly results from an evolutionary need to preserve specific and fundamental interactions in which this histone participates in vivo, physical and biological studies have yet to discover the detailed nature of the uniqueness of these interactions.

The amino acid sequence of histone H4 as determined by DeLange et al. (1969) and Ogawa et al. (1969) displays an asymmetric distribution of basic and apolar residues. Consequently, it has been widely proposed that the most basic portion of histone H4 binds to the DNA in chromatin while the least basic portion (the apolar segment) is free to participate in protein–protein interactions.

Boublik et al. (1970) have shown by nuclear magnetic resonance (NMR) measurements that when the ionic strength of aqueous solutions of histone H4 is increased an aggregation phenomenon occurs involving only a portion of the molecule that corresponds to the apolar segment. Boublik et al. (1970) from optical rotatory dispersion (ORD)

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[1] The new histone nomenclature used here was accepted by the participants at the CIBA Foundation Symposium on the Structure and Function of Chromatin, April 3–5, 1974. This new nomenclature which has been proposed to the appropriate international nomenclature committee is as follows for each histone where the previous names are given in parentheses: H1 (F1, I, KAP), H2A (F2A2, I1b1, ALK), H2B (F2B, I1b2, KSA), H3 (F3, III, ARK), H4 (F2A1, IV, GRK), and H5 (F2C, V, KAS).

and Li et al. (1972) from circular dichroism (CD) measurements also observed ionic strength induced changes in histone H4 which are consistent with the formation of some α_R helix and some β structure.

This paper is concerned with a spectroscopic study of histone H4 and its fragments produced by cleavage at the aspartic acid residue positions 24, 68, and 85. The object of the work is to identify the portion of the chain involved in α_R and β structures and that which takes part in the aggregation phenomenon. A single fragment (25–67) will be shown to behave very similarly to the intact histone with respect to salt-induced secondary structure and aggregation, while other fragments exhibit no such effects. This result, it will be suggested, defines the location of the primary self-interacting site for histone H4 and allows the proposal of a structural model for the self-association of histone H4.

Materials and Methods

Histone H4 was isolated from calf thymus H4/H2A mixture (F2A prepared as described by Johns, 1967) by gel filtration using a Bio-Gel P-10 column (150 × 2.7 cm) eluted with 0.02 *M* HCl. The F2A histone mixture was dissolved in 8 *M* urea + 0.02 *M* HCl and allowed to disaggregate for 8 hr at 4° prior to loading. The histone H4 obtained from the column was free from contamination (<5%) by other histones as judged by disc electrophoresis (Panyim and Chalkley, 1969) and an amino acid analysis comparison with the sequence (DeLange et al., 1969; Ogawa et al., 1969).

Histone H4 was recovered from concentrated column fractions by precipitation with acidified acetone (0.1% HCl). Since this can produce a somewhat aggregated sample, the acidified acetone precipitate was dissolved in 8 *M* urea (50 mg/ml) and allowed to stand for at least 8 hr at 4°. The histone was then precipitated with pure acetone, spun down, and subsequently dried under vacuum. Histone H4 recovered in this way when dissolved in pure water contains markedly less aggregation than histone precipitated by acidified acetone.

Cleavage of histone H4 at the aspartic acid residues was achieved using the method of Partridge and Davis (1950) and Schroeder et al. (1963). Histone H4 dissolved (10 mg/ml) in 0.25 *M* acetic acid was heated for 6 hr at 105° in a sealed flask. The resulting reaction mixture was then applied to a Sephadex G-50 column (150 × 2.7 cm) eluted with 0.02 *M* HCl. The effluent was monitored at 220 nm and the fractions were pooled appropriately. Recovery of the separated fragments was accomplished by first dialyzing the pooled fractions against absolute ethanol which resulted in a tenfold decrease in volume and subsequently adding ten volumes of acidified acetone (0.1% HCl). All of the fragments produced by the aspartic acid cleavage were precipitated by this procedure. The precipitates were then washed twice with dry acetone and dried under vacuum. Typically a fragmentation reaction involved 100 mg of histone H4 from which about 80 mg of the various fragments were recovered by the above procedure. Fragments (1–23) and (69–102) were not separable by gel filtration and so preparative electrophoresis was used as follows (Whitehead et al. (1971)): a Sephadex G-10 column (50 × 1.1 cm) with 0.05 *M* acetic acid as buffer at 500 V (1.4 mA) for 3.5 hr.

Analytical Disc Gel Electrophoresis. Polyacrylamide gels (30%) of the Panyim and Chalkley (1969) type prepared from the following recipe were used to characterize the various fragments: 41.6 g of acrylamide, 0.4 g of *N,N'*-bisacrylamide, 8 ml of glacial acetic acid, 23 g of urea, and 64 ml of water were mixed and degassed. To this mixture was added 0.25 ml of *N,N,N',N'*-tetramethylenediamine and 0.25 g of ammonium persulfate in 0.5 ml of distilled water. The resulting solution was transferred to 32 sealed (10 × 0.7 cm) glass tubes and overlayed with distilled water. The preelectrophoresed gels were run at 200 V for 6 hr and then stained with 0.5% Naphthalene Black 12B in 9.5% acetic acid, 40% ethanol, and 50% water. The gels were destained electrolytically. Double gels were prepared by inserting a small plastic divider down the tube so that two sealed compartments were formed.

Nuclear Magnetic Resonance. All spectra were run on a Bruker 270-MHz NMR Fourier transform spectrometer. D_2O was used as the solvent. The small residual amount of acetone used in the isolation of the peptides was taken as the reference signal at 2.27 ppm with respect to internal sodium dimethylsilapentanesulfonate. Resonance assignments were based on a previous study (Boublik et al., 1970) from this laboratory. Changes in sodium chloride concentration were made by adding small volumes of concentrated salt solutions in D_2O to the liquid sample in the NMR tube, except for the 500 m*M* NaCl solutions which were prepared by adding solid salt; 1 m*M* protein solutions were made up by weight to give an unbuffered pH between 3.0 and 3.5. NMR measurements made at pH 7.7 were on unbuffered protein solutions to which a calculated amount of NaOD/D_2O was added. The pH values are uncorrected meter readings. The number of pulses (60–70°) per spectrum are given in the figure legends. Data collection was for 0.5 sec following each pulse with no delay between the end of data collection and the next pulse. Signal to noise was improved by exponential multiplication of the free induction decay equivalent to line broadening of approximately 2 Hz.

Circular Dichroism. The CD spectra were measured on a Cary 61 CD spectrometer. The histone solutions measured were the same ones used for the above NMR measurements. The path length of the cells used is indicated in the figure legends. All spectra were recorded at the ambient temperature (≈22°). Salt concentrations were varied as in the NMR section.

Infrared Spectroscopy. Infrared (ir) spectra were measured on a Grubb-Parsons ir spectromaster. The histone solutions measured were the same ones used in the NMR and CD measurements. The cell had barium fluoride windows and the path lengths are indicated in the figure legends. A similar reference cell containing D_2O was used to cancel the water contribution to the spectrum. All spectra were recorded at the ambient temperature.

Results

Aspartic Acid Cleavage of Histone H4. The amino acid sequence of bovine thymus histone H4 (DeLange et al., 1969; Ogawa et al., 1969) in which the three aspartic acid residues at positions 24, 68, and 85 are indicated by arrows is shown in Figure 1. Although the dilute acid hydrolysis of proteins is quite specific for aspartic acid under the conditions used (Schultz, 1967), the following minor complications occur. The hydrolysis of the aspartic acid backbone can take place at either the amino or carboxyl end (Light, 1967) and therefore for the reaction time used here (6 hr) it can be expected that the fragments will be composed of molecules with and without a C- or N-terminal aspartic acid residue. Also histone H4 as isolated from calf thymus contains an acetylated N-terminal serine residue and in

Ac-Ser-Gly-Arg-Gly-Lys-Gly-Gly-Lys-Gly-Leu^{10}-Gly-
Lys-Gly-Gly-Ala-Lys^{AC}-Arg-His-Arg-mLys^{20}-Val-Leu-
Arg-Asp*-Asn-Ile-Gln-Gly-Ile-Thr^{30}-Lys-Pro-Ala-Ile-
Arg-Arg-Leu-Ala-Arg-Arg^{40}-Gly-Gly-Val-Lys-Arg-Ile-
Ser-Gly-Leu-Ile^{50}-Tyr-Glu-Glu-Thr-Arg-Gly-Val-Leu-
Lys-Val^{60}-Phe-Leu-Glu-Asn-Val-Ile-Arg-Asp*-Ala-Val^{70}-
Thr-Tyr-Thr-Glu-His-Ala-Lys-Arg-Lys-Thr^{80}-Val-Thr-
Ala-Met-Asp*-Val-Val-Tyr-Ala-Leu^{90}-Lys-Arg-Gln-Gly-
Arg-Thr-Leu-Tyr-Gly-Phe^{100}-Gly-Gly

FIGURE 1: The amino acid sequence of histone H4 as determined by DeLange et al. (1969) and Ogawa et al. (1969). The three aspartic acid residues at positions 24, 68, and 85 are indicated by asterisks.

50% of the molecules an acetylated lysine at position 16 both of which probably slowly hydrolyze under the reaction conditions used. Finally, the residues asparagine (positions 25 and 64) and glutamine (positions 27 and 93) might also be subject to slow acid hydrolysis to aspartic acid and glutamic acid, respectively. Therefore it can be expected that some of the peptides will exhibit microheterogeneity in analytical electrophoretic gels (Panyim and Chalkley, 1969) due to the presence of a C- or N-terminal aspartic acid as well as to the variations in the net peptide charge from the presence or absence of the 1- or 16-acetyl groups. A list of the nine major possible fragments is given in Table I.

The Sephadex G-50 column separation of the 6-hr aspartic acid cleaved histone H4 reaction mixture is shown in Figure 2. The magnitude of the chromatographic peaks 0, 1, 2, 3, and one of the two components of peak 5 was found to depend on reaction time such that beyond 6-hr these peaks *decreased* in intensity reaching zero by 36 hr. It will be shown that the peptides in these peaks contain one or two uncleaved nonterminal aspartic acid residues.

Table I: Histone H4 and Its Aspartic Acid Cleaved Fragments.

Species[a]	Molecular Weight[b]	Net Charge[c] at pH 3
Histone H4	11294	27, 26
25–102, 24–102	8814, 8929	17
1–84, 1–85	9310, 9425	24, 23, 25
25–84, 25–85, 24–84, 24–85	6814, 6929, 7044	13
1–67, 1–68	7177, 7292	20, 19, 21
25–67, 24–67, 25–68, 24–68	4696, 4811, 4926	10
69–102, 68–102	4003, 4117	7
86–102, 85–102	1886, 2001	3
69–84, 68–84, 69–85, 68–85	2003, 2118, 2233	4
1–23, 1–24	2366, 2481	10, 9, 11

[a] All peptide species listed are consistent with either or both a C or N terminal cleavage of aspartic acid at positions 24, 68, and 85. [b] Based on all ionizable size chains in their neutral form. [c] The sum of all the histidine, lysine, and arginine residues in the peptide. Variations are due to only 50% of histone H4 molecules being acetylated at position 16 and the possibility of acid hydrolysis of the N terminal serine residue.

Double disc electrophoretic gels of each of the Sephadex G-50 peaks 1–7 as well as uncleaved histone H4 (peak 0) vs. the 6-hr reaction mixture are shown in Figure 3 on the right and left sides of each gel, respectively. The bands (or groups of bands) have been labeled a–j. The double gel of histone H4 vs. the 6-hr reaction mixture establishes band a as that due to histone H4 (peak 0). Since the order of elution from a gel filtration column is directly related to the peptide molecular weight, the second component of peak 1 is most likely fragment (1–84) corresponding to band c.

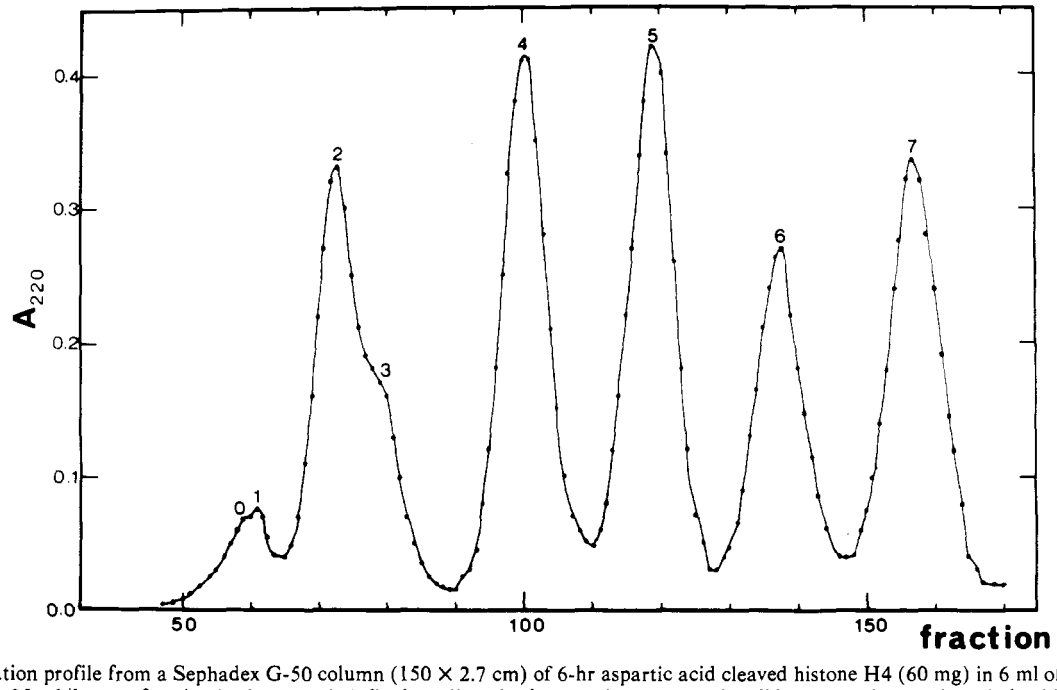

FIGURE 2: Elution profile from a Sephadex G-50 column (150 × 2.7 cm) of 6-hr aspartic acid cleaved histone H4 (60 mg) in 6 ml of 0.25 *M* acetic acid. Flow rate, 35 ml/hr; one fraction is about 4 ml. A final small peak after number seven which did not contain protein as judged by Cl_3CCOOH precipitation is not shown. It is presumed that this peak contains free aspartic acid and the initial acetic acid in which the sample was dissolved.

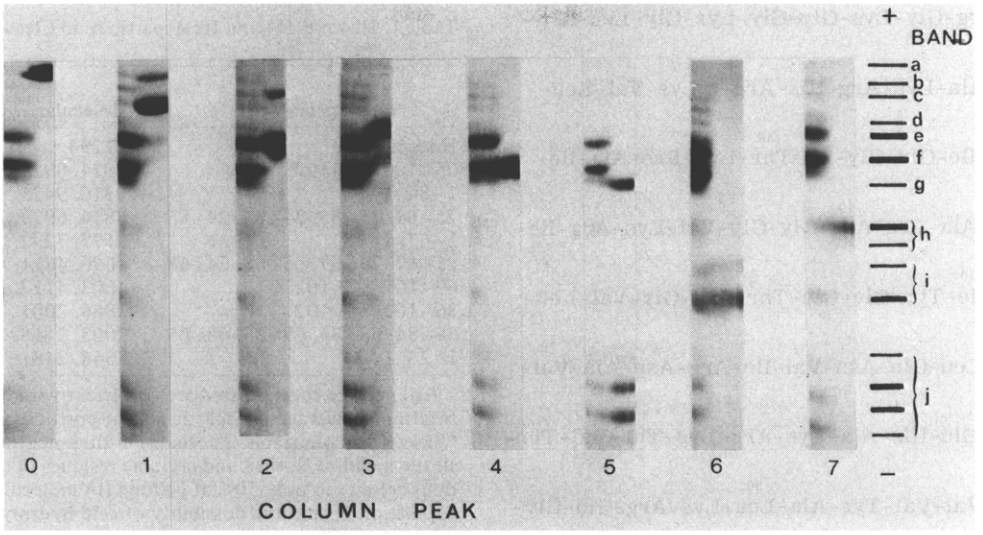

FIGURE 3: Double electrophoretic disc polyacrylamide (30%) gels of the Sephadex G-50 column peaks 0 through 7, respectively, on the right vs. the total 6-hr reaction mixture on the left. A labeled schematic representation of the observed bands is shown on the extreme right.

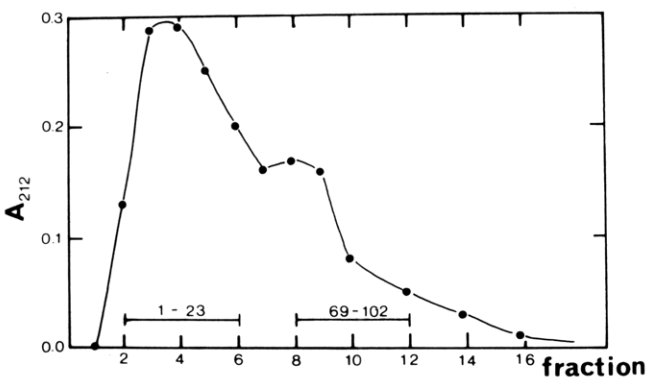

FIGURE 4: Elution profile from a Sephadex G-10 preparative electrophoresis column (50 × 1 cm). The sample was 3 mg of peptide obtained from the Sephadex G-50 column peak number 5 dissolved in 0.3 ml of 0.05 *M* acetic acid. The column was electrophoresed for 3.5 hr at 500 V and then eluted with 0.05 *M* acetic acid at 6 ml/hr. The fractions of 1 ml each for (1–23) and (69–102) which were pooled are indicated by bars (|—|).

Similarly peak 2 has two components, bands b and e which are probably peptides (25–102) and (1–67). Both on the basis of size and charge the faster moving band e is probably (1–67) (67 residues, +20 charge) and band b (25–102) (78 residues +17 charge). Peak 3 consisting of only band d is most likely the next smallest peptide (25–84) (60 residues) while peak 4, also consisting of a single band f, is probably the next smallest peptide 25–67 (43 residues). Peak 5 consists of two groups of widely spaced bands, g and j. The multiplicity of the bands in group j suggests fragment (1–23), which contains 50% acetylated lysine at position 16. By elimination band g would correspond to peptide (69–102) (34 residues). This assignment is substantiated by the observation that band g decreases to zero intensity by 36-hr reaction time, consistent with the residual internal aspartic acid 85, while band j increases in intensity. Peaks 6 and 7 probably correspond to fragments (69–84) (16 residues) and (86–102) (17 residues). Amino acid analyses confirm that the two bands labeled h (peak 7) are indeed peptide (86–102) while the bands labeled i (peak 6) are peptide (69–84). Presumably the presence and absence of a terminal aspartic acid residue give rise to the band doubling. Column peak 5 containing peptides (1–23) and (69–102) was fractionated further by preparative electrophoresis. The profile of the column effluent is shown in Figure 4. Clearly column peaks 1, 2, and 3 of Figure 3 require further fractionation before the component peptides can be considered further. This publication will be concerned with only the following five fragments which have been obtained in pure condition: (1–23), (25–67), (69–84), (86–102), and (69–102). An amino acid analysis of each of these fragments as well as the calculated composition based on the sequence is presented in Table II. Work is in progress with purifying the larger fragments (1–84), (1–67), (25–102), and (25–84) by ion exchange chromatography.

Proton Magnetic Resonance of Histone H4. Histone H4 in the presence of salt undergoes conformational changes and can be divided into two overlapping phases, one fast the other slow (Li et al., 1972). The fast change is essentially instantaneous for 1 m*M* histone H4 solutions while the salt concentration dependent slow change for NaCl concentrations less than 60 m*M* is about 30% complete after 1 hr. At 100 and 200 m*M* NaCl the slow change is more than 50% complete in 1 hr. For this reason the results of the NMR measurements of histone H4 solutions with increasing salt concentration at pH 3 and pH 7.7 with no special regard to time dependence will be given first. These results therefore contain an admixture of the fast and slow changes but nevertheless illustrate the differential resonance broadening effect. Subsequently the results of a time dependent NMR study will be described.

(i) Salt Induced Changes. The high and low field spectra of 1 m*M* histone H4, pH 3, with increasing NaCl concentration are shown in Figures 5a and b, respectively. The spectra shown constitute a series made with the same histone sample at 30-min intervals by adding after each run an aliquot of a concentrated NaCl solution in D_2O. The most striking feature of these spectra is that certain resonances such as the Leu, Ile, and Val CH_3 peak (0.93 ppm) appear to decrease in intensity at a much greater rate with increasing salt concentration than, say, the glycine CH_2 resonance (3.99 ppm). This effect, reported previously for this histone by Boublik et al. (1970), is due to the formation of molecular associates in which resonances from protons within the associates experience dipolar broadening due to reduced

Table II: Observed and Calculated Amino Acid Compositions of Histone H4 Fragments.

	(1–23)		(25–67)		(69–84)		(86–102)		(69–102)	
Amino Acid	Obsd	Calcd	Obsd	Calcd	Obsd	Calcd	Obsd	Calcd	Obsd	Calcd
Lysine	4.4	5	3.3	3	2.0	2	1.1	1	3.4	3
Histidine	0.9	1	0.1	0	0.9	1	0.0	0	0.9	1
Arginine	3.6	4	6.9	7	1.0	1	1.9	2	3.0	3
Aspartic Acid	0.7	0	1.6	2	0.4	0	0.7	0	0.9	1
Threonine	0.2	0	2.0	2	3.3	4	0.9	1	4.0	5
Serine	1.5	1	1.1	1	0.2	0	0.0	0	0.8	0
Glutamic Acid	0.4	0	4.2	4	1.1	1	1.1	1	2.0	2
Proline	0.0	0	1.2	1	0.0	0	0.0	0	0.0	0
Glycine	7.0	8	5.3	5	0.4	0	3.6	4	4.3	4
Alanine	1.4	1	2.4	2	2.8	3	1.0	1	5.2	4
Valine	0.9	1	3.4	4	1.9	2	1.6	2	3.2	4
Methionine	0.0	0	0.0	0	0.9	1	0.0	0	0.8	1
Isoleucine	0.2	0	5.2	6	0.1	0	0.1	0	0.5	0
Leucine	1.8	2	4.4	4	0.1	0	2.0	2	2.1	2
Tyrosine	0.1	0	1.0	1	0.9	1	1.8	2	2.2	3
Phenylalanine	0.0	0	0.7	1	0.0	0	0.9	1	0.8	1

mobility while those from protons in the unassociated parts of the same molecule remain comparatively unconstrained and maintain narrow line widths. From a knowledge of the sequence of this histone it has been possible (Bradbury and Rattle, 1972) to determine by computer simulation what portion of the protein is constrained. The results of that study for histone H4 indicate that residues 33–102 are markedly immobilized by 200 m*M* NaCl at pH 3.

(ii) pH Dependence. A series of spectra (not shown) similar to those shown in Figure 5 was obtained at pH 7.7. It was found that the differential broadening effect was identical with that found at pH 3, the only difference being that about 30 m*M* less salt concentration was necessary to produce the same changes. This result is expected if the salt acts by screening the side chain charges since the net charge on the histone H4 molecule at pH 3 is about +27 while at pH 7.7 the net charge is about +18.

(iii) Time Dependence. The spectra shown in Figure 5 were run sequentially at 30-min intervals so that the 200 m*M* NaCl sample was completed at the end of 4 hr (when the slow step was essentially complete). In order to investigate the time dependence of the resonance broadening a histone H4 sample was dissolved in D_2O (1 m*M*, pH 3) and salt was added to bring the concentration to 60 m*M* NaCl and the sample run directly. Spectra were taken at 3 min, 15 min, 1 hr, 2 hr, 3 hr, and 54 hr on the same sample. It was found that the two 60 m*M* NaCl spectra run within 15 min were very similar to the 50 m*M* NaCl spectra shown in Figure 5 while the 2- and 3-hr spectra were intermediate between the 50 and 100 m*M* NaCl spectra. The 54-hr spectrum was very similar to the 200 m*M* NaCl spectrum shown in Figure 5. These results confirm the existence of a fast and slow step (Li et al., 1972) and show that under the present conditions (1 m*M* histone) *both* steps are accompanied by resonance broadening. The half-widths of the resonances in the time-dependent spectra (not shown) and also those in the spectra of Figure 5 change very little as salt is added. This result means that certain resonances experience a very considerable increase in width as the ionic strength rises and effectively disappear from the spectrum. A gradual increase in line width is *not* observed. Since globular proteins with molecular weights up to 50,000 have well-defined NMR spectra, such marked line broadening demonstrates the formation of aggregates of considerable size in both the fast and slow steps. The absence of lines of intermediate width indicates the essential absence under these conditions of states of intermediate molecular weight such as dimers or tetramers. Molecular weight studies (Diggle and Peacocke, 1971) have demonstrated the very high degree of aggregation induced in H4 by salt.

(iv) Slowly Exchanging Amide Protons. The existence of hard to exchange amide protons in histone H4 when quickly dissolved in D_2O can be seen from Figure 5b, the first spectrum of which was run within 30 min after dissolving the solid histone into the D_2O. Since the half-life of peptide hydrogens in D_2O at pH 3 is about 7 min for a fully random structure (Hvidt and Nielsen, 1966) this result shows that histone H4 possesses some nonrandom structure in water. The detailed structure visible in the residual amide spectrum indicates that the more slowly exchanging protons are at specific sites and not randomly distributed throughout the molecule.

(v) Histone H4 Preparation. The method of final recovery of histone H4 influences the conformational properties of the protein when dissolved in water. Histone H4 is usually recovered by adding acidified acetone (0.1% HCl) to an aqueous solution of the histone. This procedure yields a product whose NMR spectrum in pure D_2O (1 m*M* histone) has already considerable resonance broadening. This was verified by obtaining the spectrum of the same sample in 8 *M* urea: the apolar and aromatic resonances were found to be considerably sharper and more intense. However, if the histone H4 prepared as above was redissolved in 8 *M* urea, precipitated with *pure* (not acidified) acetone at 4°, spun down, washed with acetone, and then dried under vacuum, the resulting NMR spectrum of 1 m*M* protein in D_2O was indistinguishable from the spectrum in 8 *M* urea. Presumably the addition of the 0.1% HCl (≈25 m*M* in Cl^- ion) to the acetone induces H4 aggregation which is preserved in the precipitate.

Proton Magnetic Resonance of Histone H4 Fragments. (i) Fragments (1–23), (69–84), (86–102), and (69–102). The NMR spectra (not shown here) of these fragments at 1 m*M* protein concentration have been obtained over a range of NaCl molarities up to 500 m*M* and showed little change over this ionic strength range. These results mean that these peptides do not participate in the formation of observable aggregation and that no detectable globular structures occur. It is of considerable practical interest that the spectra allow a positive identification of the fragment and an as-

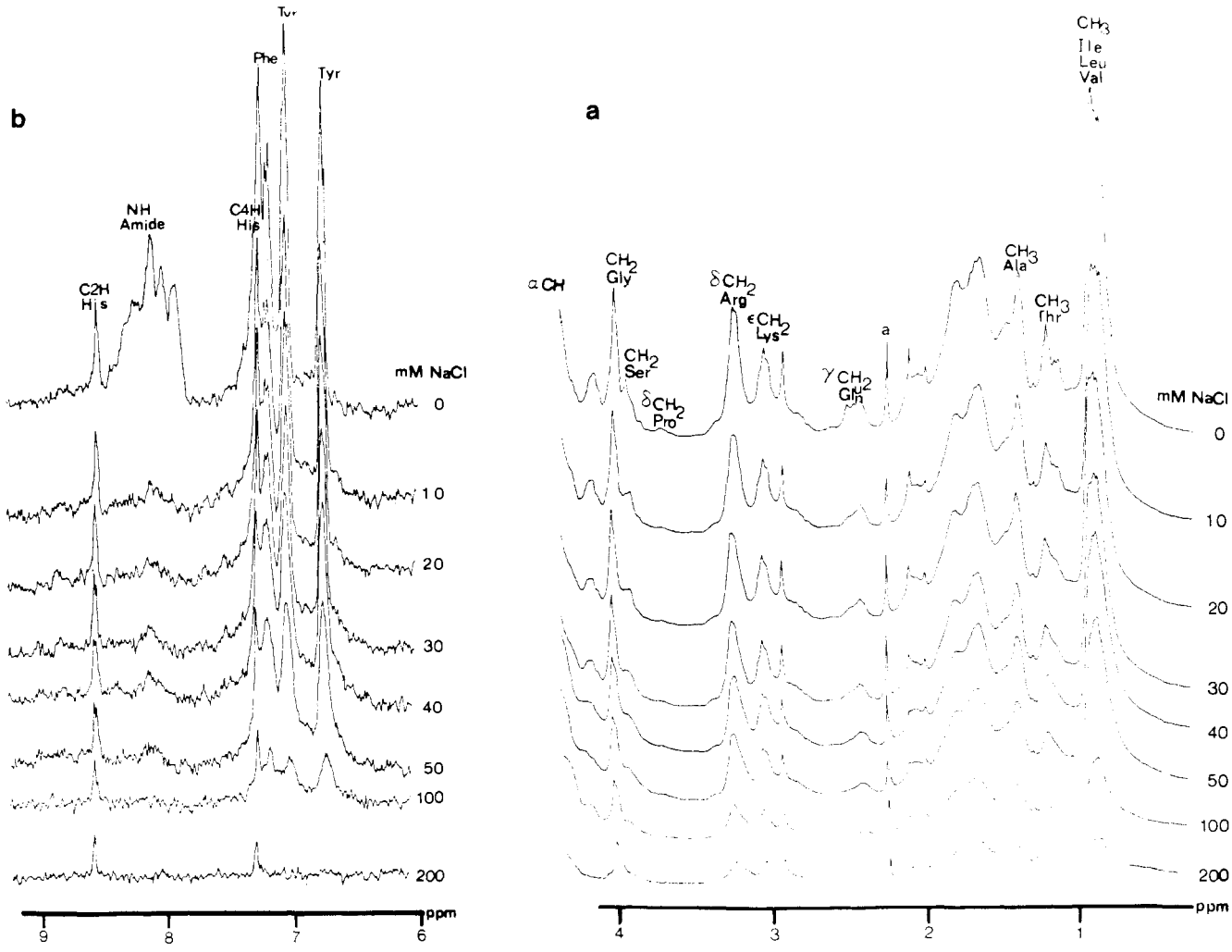

FIGURE 5: (a) Upfield and (b) downfield 270-MHz NMR spectra of 1 m*M* histone H4 sample initially dissolved in D_2O (pH 3.0) to which appropriate aliquots of concentrated NaCl in D_2O were added at 30-min intervals to give the indicated salt concentrations. Each spectrum was obtained after 1250 pulses (approximately 15 min). The final histone H4 concentration was 0.7 m*M* after the salt additions. The proton resonance at 2.27 ppm labeled a is from residual acetone used in the preparation of the protein.

sessment of purity, largely because of their aromatic residue distribution.

(ii) Fragment (25–67). The upfield and downfield NMR spectra of fragment (25–67) obtained in the same manner as the intact histone are shown in Figure 6a and b. It can be seen that these spectra are strikingly similar to the spectra of intact H4 shown in Figure 5, and therefore demonstrate the formation of large aggregates with this fragment. However, no time-dependent changes were observed for this fragment (in contrast to the intact molecule). From a rough analysis of peak height changes it is possible to deduce that the region 35–67 is involved in the interacting segment at 200 m*M* NaCl (not shown) a result that compares favorably with the interacting segment 33–102 deduced by Bradbury and Rattle (1972) for the intact molecule at this salt concentration. At 500 m*M* NaCl the whole of the fragment is included in the aggregate.

The aromatic spectrum of 25–67 involves a single Phe (61) and a single Tyr (51) resonance. On salt addition both these resonances lose area without any increase in line width and maintain their relative areas of 5 (Phe) to 4 (Tyr) (Figure 6b). One can conclude that both residues are equally involved in the associated state and that this state is large enough for a high resolution spectrum to be unobservable. Furthermore, any exchange of molecules between the aggregated state and random coil at intermediate ionic strengths must be slow on the NMR time scale (<30 sec^{-1}). If large aggregates are formed then slow exchange is to be expected. We have discussed general models of histone self-aggregation elsewhere (Bradbury et al., 1975).

Similar NMR measurements on fragment (25–67) were made at pH 7.7. It was found that identical resonance broadening to that found at pH 3.5 occurred but that about 30 m*M* less NaCl was required to effect the same change. This was the same result as found for the intact histone. The fragment (25–67) has some specific hard to exchange amide protons (Figure 6b), suggesting some nonrandom structure in pure water.

Infrared Spectroscopy of Histone 4 and Fragment (25–67). The amide I band of 1 m*M* histone H4 solutions in D_2O, pH 3.0 with increasing concentrations of NaCl, are shown in Figure 7A. Each spectrum was run within 3 min of adding the concentrated salt solution to the aqueous H4 sample. As the salt concentration is raised beyond 60 m*M* NaCl the peak at 1638 cm^{-1}, characteristic of the α_R helix and random conformations, decreases while a peak at 1610 cm^{-1} characteristic of β structure appears (Miyazawa and Blout, 1961; Timasheff et al., 1967; Elliott, 1969). In Figure 7B spectra are shown as a function of time for a 1 m*M* histone H4 solution in 60 m*M* NaCl. It can be seen that by

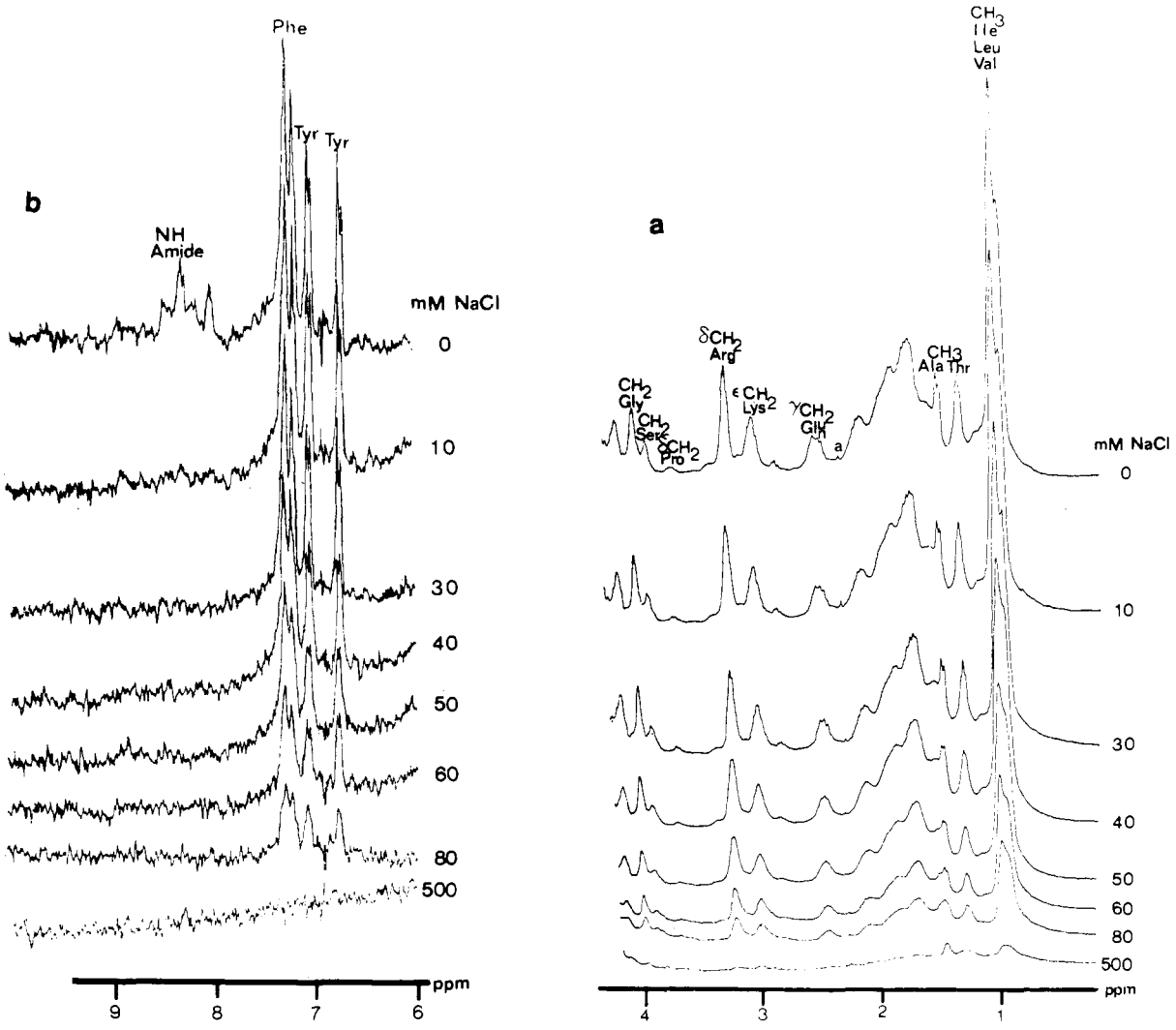

FIGURE 6: (a) Upfield and (b) downfield 270-MHz NMR spectra of 1 m*M* fragment 25–67 sample initially dissolved in D_2O (pH 3.5) to which appropriate aliquots of concentrated NaCl in D_2O were added to give the indicated salt concentrations, 1500 pulses/spectrum. The final peptide concentration was 0.7 m*M* after the salt additions.

20 hr a limiting amount of β structure has formed and furthermore it was found that the same final spectrum was obtained using 500 m*M* instead of 60 m*M* NaCl and transition was complete in about 2 hr. The 500 m*M* spectrum in Figure 7A shows less than the limiting amount of β structure since it was obtained in substantially less than 2 hr after salt addition. Using the spectral data and band separation procedures of Chirgadze et al. (1973) and Chirgadze and Brazhnikov (1974), the limiting amount of β structure in H4 (when the slow step is complete, 20- and 60-hr spectra, Figure 7B) is ~30% (~30 residues).

The ir spectra for fragment (25–67), 1 m*M*, pH 3.5, are shown in Figure 7C. Just as for the intact molecule a β peak at 1610 cm^{-1} increases in intensity on salt addition to a limiting value that is lower than for the intact H4 molecule. Above 180 m*M* NaCl the proportion of β structure is estimated at ~20% (9 residues). As in the NMR measurements no time dependence of these spectra was detected.

Circular Dichroism of Histone H4 and Its Fragments. The salt-induced changes in the CD spectra of histone H4 and its fragments (1 m*M* in protein) at pH 3.5 are shown in Figure 8. The spectra for histone H4, run within 30 min of sample preparation, show that for salt concentrations less than 50 m*M* NaCl the changes are consistent with the formation of α_R helix as seen by the onset of a trough at 222 nm. At higher salt concentrations a component of β structure is evident from the disappearance of the strong shoulder at 222 nm and the appearance of an ellipticity minimum at lower wavelengths. These ellipticities are in good agreement with the results of Isenberg and coworkers (Li et al., 1972; Wickett et al., 1972; D'Anna and Isenberg, 1973).

Although it is possible to analyze the H4 CD data directly in terms of α_R, random, and β structure contributions we have preferred to take the β content as determined from the ir spectrum (30%). When this is subtracted from the total CD curve the remainder is divided into random plus helix. The ellipticities of β and α_R are taken from Chen et al. (1974) (−3360 and −30,000°, respectively at 222 nm). The random coil value used (−1000° at 222 nm) is that which gives the best fit for histones (see Bradbury et al., 1975). This method of analysis leads to an α_R content of ~25% (25 residues) for H4 in 200 m*M* NaCl.

Fragment (25–67) exhibits CD changes similar to those of intact H4 (see Figure 8) in that largely α_R is formed at the lower ionic strengths but considerable amounts of β occur at 200 m*M* NaCl. Analysis of the 200 m*M* NaCl curve by the above procedure (β content taken as 20%)

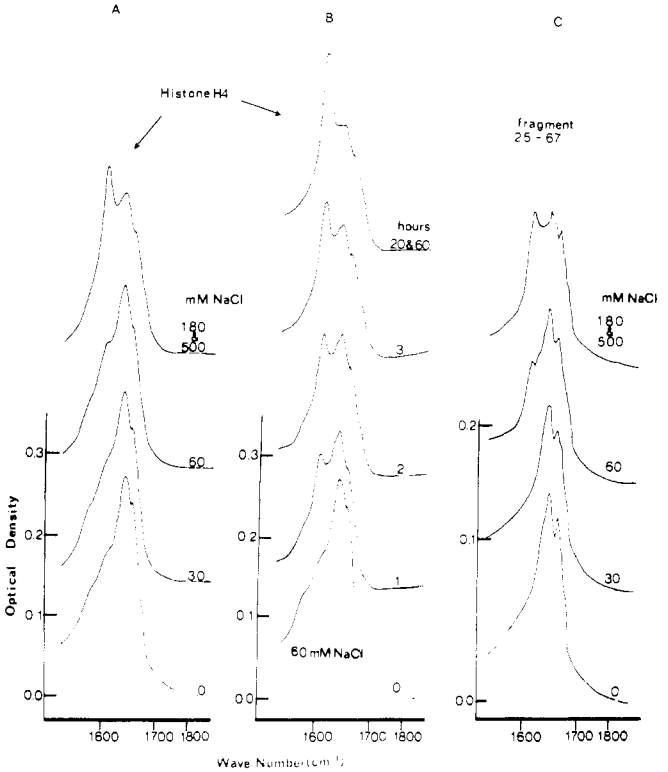

FIGURE 7: (A) Ir spectra of 1 m*M* histone H4 in D_2O (pH 3.0) to which a solution of NaCl in D_2O was added. Each spectrum was made from histone H4 in D_2O plus an appropriate amount of saline solution and run in less than 3 min. The cell path length was 0.075 mm. (B) Ir spectra of 1 m*M* histone H4 in D_2O (pH 3.0) plus 60 m*M* NaCl as a function of increasing time. (C) Ir spectra of 1 m*M* fragment 25-67 in D_2O (pH 3.5) to which aliquots of NaCl in D_2O were added. The cell path length was 0.075 mm. In each of these figures the vertical scale applies to the bottom spectrum only. The scale must be raised for the other spectra.

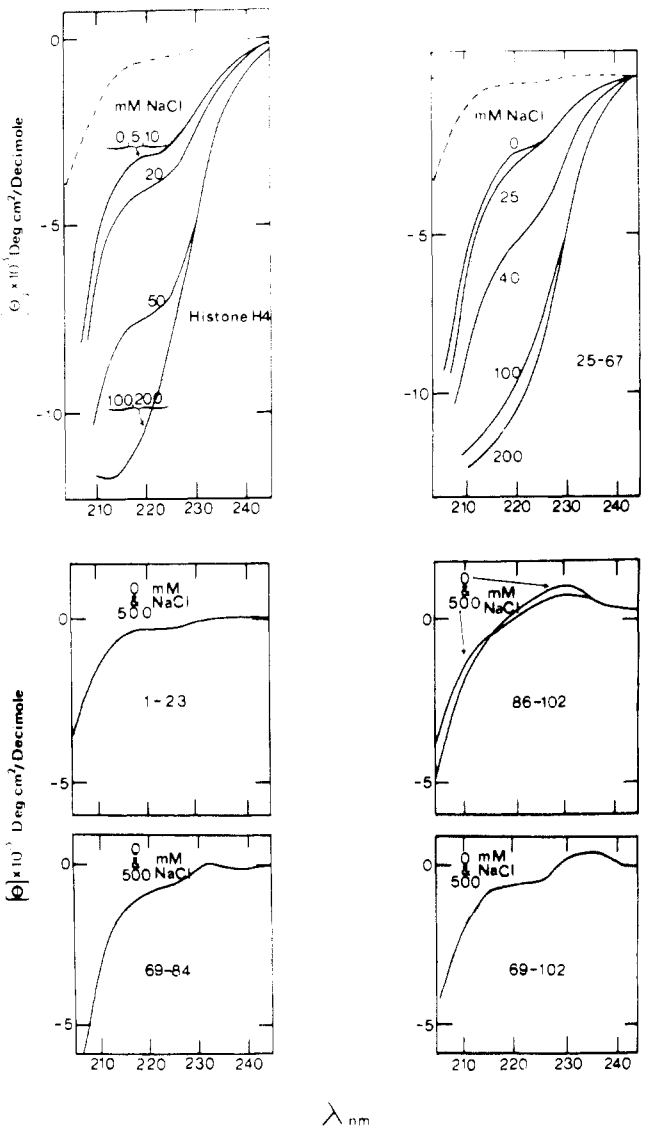

FIGURE 8: CD spectra for 1 m*M* D_2O solutions of histone H4 and fragments (25-67), (1-23), (69-84), (86-102), and (69-102) as labeled in the figure. Each spectrum was run within 20 min of adding the salt aliquots. The dashed spectrum in the upper two boxes is the spectrum of fragment (1-23). The cell path lengths were 0.1 and 0.2 mm for histone H4 and fragment (25-67), respectively. A 1-mm pathlength cell was used for the other peptides

leads to an α_R estimate of 29% (12 residues).

The CD spectra for fragments (1-23), (69-84), (86-102), and (69-102) (Figure 8) are essentially unaffected by salt addition and are clearly all characteristic of a fully random conformation. It is worthy of note that the CD of both H4 and (25-67) in water differ markedly from that of these four structureless peptides and it is therefore concluded that H4 and (25-67) both contain residual structure in water. This confirms the NMR observations on this point. The residual structure could be α_R helix since a small negative trough at 222 nm is observed (−3300° for H4). However, the ellipticity at this wavelength is very susceptible to all conformational restrictions and this residual structure cannot therefore be defined with certainty.

Discussion

On the basis of the present results and the earlier data on the whole molecule it is possible to derive a model for the structural changes taking place in H4 on salt addition. In the region of ionic strength up to about 0.06 *M* there is a gradual increase in the helix content of H4 accompanied by a rise in the degree of H4 association. This increase in molecular weight is presumably due to the interaction of folded chains that include a high proportion of helix. Where then in the chain is the helix located? The predictions of Lewis and Bradbury (1974) at high ionic strength indicate the region from 49 to 95 (47 residues or almost twice the observed amount) but there is a dip in the helicity profile for low ionic strength at about residue 78. The rules of Chou and Fasman (1974) predict 49-71 as helical (23 residues) while Vorobev and Birshtein (1971) and Vorobev et al. (1972) favor the region between about 50 and 75. The latest methods of Ptitsyn et al. (1974) predict two helical regions: 52-69 (18 residues) and 83-93 (11 residues). The H4 sequence is such that helix before residue 49 is very unlikely but the predictions do indicate some helix potential between about residues 80 and 90. All authors are agreed on high helix potential near the center of the molecule in the 50-70 region and since ~25 helical residues are observed by CD we have incorporated the section 49-73 as helical in our model. This is consistent with the finding that 25-67 has considerable helicity and that no helix can be induced in the peptide (69-102). The association of molecules having helix between 49 and 73 is the proposed first (fast) step (see Figure 9).

The addition of more salt induces *slow* β formation in the intact H4 molecule (~30 residues) but *immediate* β forma-

tion (~9 residues) in the peptide (25–67): it can be seen from Figure 7 that at 0.06 *M* NaCl H4 shows no β structure when run immediately although (25–67) does show a small amount. This kinetic difference in β formation implies that the β structure in (25–67) is not the same as that which occurs in intact H4 and is a specific feature of this peptide. It is therefore probably located close to the points of cleavage, i.e., in the N- or C-terminal regions of (25–67). Now the NMR spectra of (25–67) show that the *whole* peptide is included in the aggregates at salt molarities about 500 m*M* while the NMR spectra of intact H4 show (Bradbury and Rattle 1972) that residues 1–32 remain free under these conditions. We therefore conclude that it is the N-terminal residues that become included in β structures when the peptide 25–67 aggregates. Residues 25–34 are predicted to have β potential by the procedure of Chou and Fasman (1974). In the model of H4 self-interaction (Figure 9) this β structure is *not* of course included.

What then is the location of the β structure (~30 residues) of intact H4? At 0.1–0.2 *M* NaCl the region of H4 from about residue 33 up to the C terminal end is involved in the intermolecular associates. Thus the β structure is either on the C terminal side of the helix (between 74 and 102) or on the N-terminal side (between 33 and 48). If it were between 33 and 48 then it should be strongly evident in (25–67), bearing in mind that this peptide both forms helix and aggregates in like manner to intact H4. Considerable quantities of slowly forming β structure are not, however, observed in (25–67) and the β structure must therefore lie between 74 and 102. Since this β formation is slow and follows helix formation and aggregation it must be intermolecular, which under these circumstances means between already formed aggregates. An anti-parallel interleaving of the C-terminal regions is therefore incorporated in the model. The anti-parallel chain β arrangement is supported by the fact that a careful study of the amide I region of the H4 infrared spectrum in 0.2 *M* NaCl (Shestopalov and Chirgadze 1974) shows the presence of the $\nu(0,\Pi)$ vibration at ~1680 cm^{-1} characteristic of the anti-parallel β chain arrangement. The β structure is assumed in the model to cover the whole of the 74–102 region (29 residues) since the observed number of residues is 30. In this context it is particularly noteworthy that Ziccardi and Schumaker (1973) have observed that the peptide H4 (1–84) does not have the potential to form high molecular aggregates, which is clearly a property of the C-terminal end.

Another feature of the model requiring comment is the parallel association of H4 molecules proposed for the fast step. This arrangment is preferred since we have observed that an H4 dimer (formed by oxidation of sea urchin H4 that contains a single cysteine at position 73 (P. Sautiere, private communication)) shows very similar NMR changes to those of the thymus monomer shown in Figure 5. This indicates a similar pattern of aggregation in the monomer and in the residue 73-linked dimer, suggesting that in the fast step aggregate residues 73 of adjacent chains are close together, i.e., there is a parallel chain arrangement. Furthermore, the parallel arrangement provides a mechanism whereby the N-terminal 33 residues remain free despite the formation of large aggregates.

The data presented here appear to conflict with that of Smerdon and Isenberg (1973) which show that in 7 m*M* phosphate (pH 7.4) H4 initially associates to a dimer. The difference between their results and ours is due to the difference in protein concentration and this can be illustrated as follows. At an H4 concentration of 1 m*M* the present results (at room temperature) show that the addition of about 60 m*M* NaCl *immediately* induces a helicity of greater than 20% ($\theta_{222} \simeq -8000°$) and drives at least half of the H4 into large aggregates (as seen from the area changes in Figure 5) that contain little or no β structure (see Figure 7). Subsequently the maximum amount of β structure (~30%) forms. Wickett et al. (1972), on the other hand, show that at 0.8×10^{-5} *M* H4 concentration (room temperature also), 50 m*M* NaCl induces only a little helix ($\theta_{222} \simeq -3800°$) and that no slow change follows. It is clear therefore that in the present measurements we have passed rapidly through the dimerization stage to higher orders of aggregation.

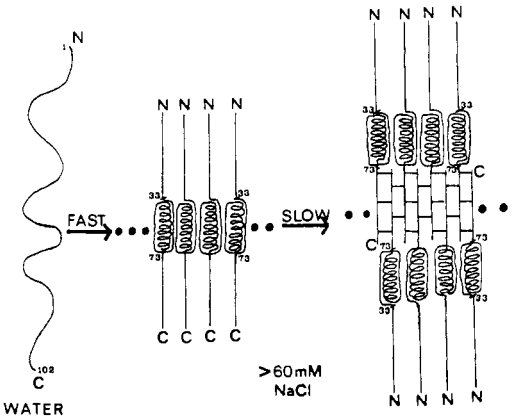

FIGURE 9: Schematic representation of structural changes induced by salt (>60 m*M* NaCl) in a 1 m*M* solution of H4. No residual structure is shown in water due to uncertainty as to its nature. Both the fast and slow step products are shown for clarity in a very extended form: they may, however, have a more compact tertiary structure.

The CD data of Li et al. (1972) and Smerdon and Isenberg (1973) demonstrate that under conditions for which dimer forms and no "slow step" takes place (7 m*M* phosphate (pH 7.4), 2°) the helicity of H4 is low ($\theta_{222} \simeq -2600°$). In fact is does not appear possible to obtain a dimer state of H4 in which the helicity is greater than about 5%. We therefore suggest that the first stage of H4 aggregation is a dimer of low helicity which then associates further with the formation of more helix (to a maximum of 25%) yielding the fast step product of Figure 9. These aggregates then associate further with β-structure formation.

It has recently been shown that H4 can cross-interact both with H3 (Kornberg and Thomas, 1974; D'Anna and Isenberg, 1974) and with H2B (D'Anna and Isenberg, 1973) to form strong 1:1 complexes containing about 25% helix but *no* β structure. The interactions in these complexes may bear a close resemblance to those occurring in chromatin and the helicity of the complexes is indeed similar to the sum of that inducible in the pure histone components. However, the self-association of H4 involves the slow formation of ~30 residues of β structure and it follows that the C terminal portion of H4 has a different conformation in the mixed H4/H3 and H4/H2B systems from that in pure H4. We therefore conclude that residues 73–102 of H4 is a site for cross-interaction with H3 and H2B.

Acknowledgments

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