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Conformational Changes of Histone ARE (F3, III)†

Joseph A. D'Anna, Jr., and Irvin Isenberg*

ABSTRACT: The conformational changes of histone ARE are a function of the phosphate concentration added to the solution. The conformational changes are monitored by intrinsic tyrosine fluorescence anisotropy, circular dichroism (CD), and light scattering. Histone ARE is shown to be similar to histone GRK [H. J. Li, R. R. Wickett, A. M. Craig, and I. Isenberg (1972), *Biopolymers 11,* 375] in that salt induces both fast and slow conformational changes. However, it is like histone KAS [J. A. D'Anna, Jr., and I. Isenberg (1974), *Biochemistry 13,* 2093], in that the fast step of ARE is very sensitive to changes in phosphate concentration. The fast conformational changes are characterized by increased tyrosine rigidity and α-helix formation of about 19 residues at 10^{-5} M histone. There are no slow changes below 1.5 mM phosphate. Above this critical phosphate concentration, there are time-dependent increases in light scattering, anisotropy, and CD. During this slow change, histone ARE aggregates. At the end of the aggregation there are about 11 residues of α helix and about 33 residues of β sheet per histone molecule. As with GRK, the slow changes are sensitive to temperature and histone concentration. The conformations of ARE, LAK, KAS, and GRK are summarized and compared.

This laboratory recently reported studies of conformational changes of histone GRK[1] (Li *et al.,* 1972; Wickett *et al.,* 1972; Wickett and Isenberg, 1972: Li and Isenberg, 1972; Small *et al.,* 1973; Smerdon and Isenberg, 1973, 1974), histone KAS (D'Anna and Isenberg, 1972), and histone LAK (D'Anna and Isenberg, 1974a). These studies indicated that at least part of each histone molecule has a definite folded configuration in salt solutions. The required salt concentration is, in each case, in the physiological range.

Histones GRK and KAS complex strongly with one another (D'Anna and Isenberg, 1973) as do LAK and KAS (Skandrani *et al.,* 1972; D'Anna and Isenberg, 1974b; Kelley, 1973). Upon complexing, there is a marked conformational change in one or both partner molecules (D'Anna and Isenberg, 1973, 1974b).

In the present paper, these studies are extended to an investigation of histone ARE, and a study of the complexes of ARE with other histones appears in the accompanying paper (D'Anna and Isenberg, 1974c).

A number of properties of histone ARE are known. The

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[1] The nomenclature used in this paper is described in Huberman (1973): GRK = IV = F2a1, KAS = IIb2 = F2b, LAK = IIb1 = F2a2, ARE = III = F3.

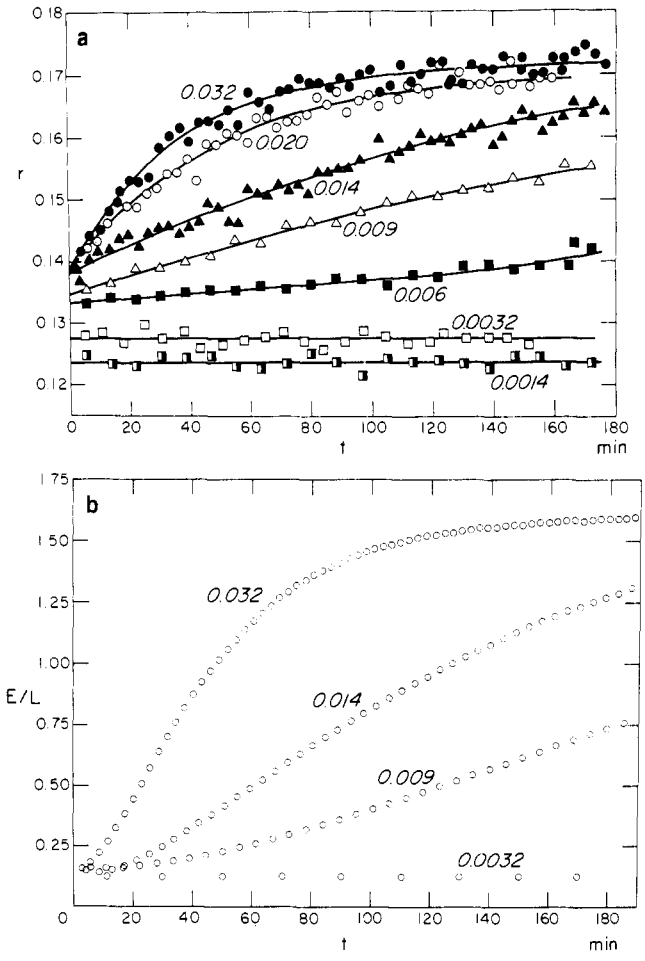

FIGURE 1: Fluorescence anisotropy and relative intensity of scattered light as functions of time at several different phosphate concentrations: (a) anisotropy, (b) scattered light. Histone ARE is 1.0×10^{-5} M, and the phosphate concentration is given on the curves.

proteins from different species have been sequenced (DeLange *et al.*, 1972; Brandt and Von Holt, 1972; Hooper *et al.*, 1973) and the amino acid sequence of ARE, like GRK (DeLange *et al.*, 1969), is highly conserved. ARE has cysteine; lower organisms have one and higher organisms have two (Panyim *et al.*, 1971).

Sedimentation and osmotic pressure measurements indicate that histone ARE forms large aggregates in salt solution (Diggle and Peacocke, 1971; Edwards and Shooter, 1969), and Laurence (1966) has observed time-dependent increases of the fluorescence intensity of 8-anilino-1-naphthalenesulfonic acid in salt solutions of histone ARE. Studies of GRK (Li *et al.*, 1972; Wickett *et al.*, 1972) have shown that there are both fast and slow conformational changes and that, in the slow change, aggregation occurs. This suggests that the aggregation observed by Diggle and Peacocke and by Edwards and Shooter might be associated with the slow changes reported by Laurence (1966), and, indeed, this is what we have found. In addition, we have found that there is also a fast conformational change analogous to that observed for GRK, and we shall describe a number of properties of the fast and slow steps.

Experimental Section

Calf thymus histone ARE was purified by affinity chromatography (Ruiz-Carrillo and Allfrey, 1973). The protein was electrophoretically pure (Panyim and Chalkley, 1969), and the amino acid analysis agreed with the sequence (DeLange *et al.*, 1972). Histone ARE was stored until used as an acetone dried powder, in desiccators, at −15°.

Prior to physical measurements, the histone was reduced and chromatographed on Sephadex G-25. To reduce the histone we used a scaled-down version of the procedure of Ruiz-Carrillo and Allfrey (1973); 8–10 mg of histone ARE was dissolved in 0.33 ml of glass-distilled water containing 6 mg of Sigma dithiothreitol (DTT),[2] and 0.49 ml of 9.3 M urea in 0.010 M Tris (pH 9.0) was added. The tube was covered with parafilm under argon, and the sample was then incubated in a 40° water bath for 1 hr.

After incubation, the solution was desalted on a 1.2 × 26 cm column of G-25 Sephadex equilibrated with 0.01 M HCl and 0.001 M DTT. The 0.01 M HCl was chosen because a low pH retards the oxidation of sulfhydryls (Hirs, 1967; White, 1967). Fractions of 1.1 ml were collected and assayed by their absorbance at 275 nm. The major fractions were pooled to make a stock solution of about 1.4 mg/ml; this solution was stored at 4°. Electrophoresis of the stock protein showed it was reduced by DTT treatment and, when kept at 4°, remained so for at least a week. We note that absorbance measurements of 1.0×10^{-3} M DTT in 0.01 M HCl demonstrate that negligible oxidation of DTT occurs at room temperature for at least 8 hr. However, at 0.01 M phosphate, pH 7.0, there is about a 7% oxidation of DTT within 2 hr, and after 24 hr, the DTT is nearly completely oxidized.

Measurements of fluorescence anisotropy of ARE in phosphate buffer were the same at 10^{-3} M DTT or 10^{-4} M DTT. Circular dichroism (CD) spectra were measured at the lower DTT concentration so that we could avoid excessive absorption by the solvent. Based upon the DTT absorbance measurements, there is probably 10–15% oxidation of the DTT in our samples after a 3-hr measuring period. As we shall see later, possible sulfhydryl oxidation has no bearing on the aggregation.

All measurements were made in 1.0×10^{-5} M histone, pH 7.0, unless otherwise stated. Solutions were prepared by adding buffer to approximately 1.0×10^{-4} M stock histone. Because the stock solution contained 0.01 M HCl, the pH of the buffers was adjusted so that the final pH was 7.0. Before mixing the buffer and the stock histone, both solutions were adjusted to the temperature at which measurements were to be made. Fluorescence anisotropy and circular dichroism were measured as previously described (D'Anna and Isenberg, 1973; Smerdon and Isenberg, 1973). Right angle light scattering was measured at 365 nm as described by Smerdon and Isenberg (1973). Fluorescence and light scattering data as a function of time were plotted using a computer program written by E. W. Small.

Results

Effects of Phosphate Concentration on Conformation of Histone ARE. Phosphate induces fast and slow changes in histone ARE; in this respect, ARE resembles histone GRK (Li *et al.*, 1972; Wickett *et al.*, 1972). Figure 1 shows anisotropy and light scattering results. It may be seen from the fluorescence data that there are instantaneous changes upon the addition of phosphate, and, at higher phosphate concentrations, there are also slow changes. Just as with GRK conformational changes, the slow step requires a criti-

[2] Abbreviation used is: DDT, dithiothreitol.

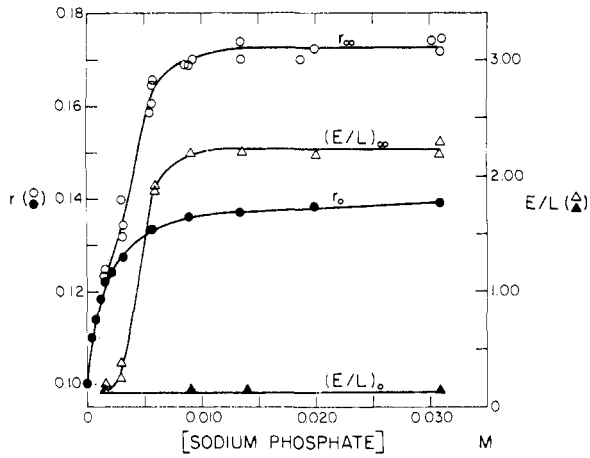

FIGURE 2: Fast step and final slow change values of anisotropy and relative scattered light as functions of phosphate: fast step anisotropy, r_0; fast step light scatter, $(E/L)_0$; final slow step anisotropy r_∞; and final slow step light scatter $(E/L)_\infty$. Histone is 1.0×10^{-5} M. No correction is made for the cell, but the scatter is similar for all of the phosphate buffers and is about 0.05 unit.

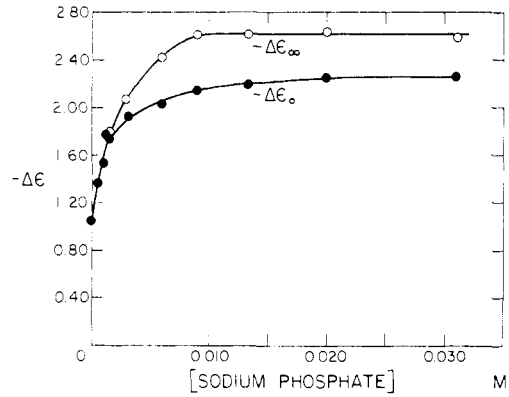

FIGURE 3: CD fast step, $-\Delta\epsilon_0$, and final slow change value, $-\Delta\epsilon_\infty$, as functions of phosphate concentration. Histone is 1.0×10^{-5} M.

cal salt concentration below which no slow change occurs.

In Figure 2, the anisotropy immediately after phosphate addition, r_0, and the anisotropy after 24 hr, r_∞, are plotted as functions of phosphate concentration. Analogous light scattering results are also given in Figure 2. Both show that there is no slow change below 1.5 mM phosphate. Above this critical concentration, the amount of slow change increases rapidly with salt. It may also be noted that the plateau region in the light scattering curve occurs at the plateau region of r_∞. This correspondence between the 24-hr light scattering and the r_∞ values shows that histone aggregation accompanies slow step formation. Figure 3 shows that changes in secondary structure accompany the fast and slow change. The same critical salt concentration is apparent in the CD data.

The CD spectrum of histone ARE in 1.0×10^{-3} M HCl (Figure 4a) resembles those of unordered polypeptides (Tiffany and Krimm, 1969) and is similar to the unordered forms of other histones (Li *et al.*, 1972; Shih and Fasman, 1971; D'Anna and Isenberg, 1972, 1974a). We have interpreted the CD spectra using a difference spectrum method (Li *et al.*, 1972) and standard curves (D'Anna and Isenberg, 1972). The fast change is accompanied by α-helix formation. During the slow change β sheet appears, and there is a loss of some α helix (Figures 4 and 5). At 1.4 mM phosphate, which is below the critical concentration, the CD

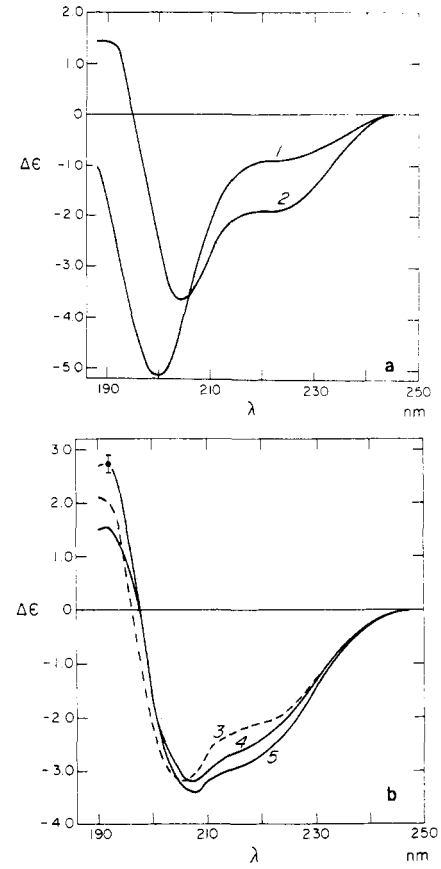

FIGURE 4: (a) CD spectra of histone III (1×10^{-5} M) at 1.0×10^{-3} M HCl, curve 1, and at 0.0016 M phosphate, curve 2. (b) CD spectra of histone III after 24 hr at 0.0032 M phosphate, curve 3, 0.0059 M phosphate, curve 4, and at 0.020 M phosphate curve 5. Spectra were measured in 1.0- and 2.0-mm cells.

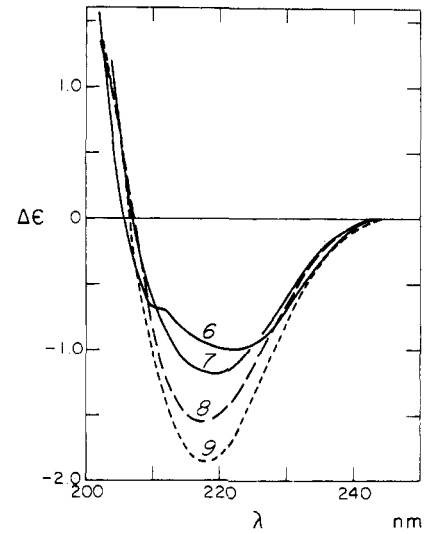

FIGURE 5: CD difference spectra computed from the CD spectra of Figure 4: curve 6, from curves 2 and 1; curve 7, from curves 3 and 1; curve 8, from curves 4 and 1; and curve 9, from curves 5 and 1.

spectrum does not change with time. The shape of the difference spectrum indicates only the presence of α helix and random coil. In the plateau region of Figure 3, that is above 9 mM phosphate, the CD spectra after 24 hr are independent of salt. They indicate the presence of β sheet, in addition to α helix and random coil. At 3.4 and 6.0 mM phosphate concentrations, the spectra are intermediate in character, containing α helix, random coil, and an amount of β

TABLE I: Classification of CD Changes and the Secondary Structure of Histone III at Different Times and at Different Phosphate Concentrations.

[Phos], M	Type of CD Changes[b]	% α Helix	% β Sheet	Residues[a] α Helix	Residues[a] β Sheet
0.0014, 24 hr	R → α	10	0	14	0
0.0032, 24 hr	R → 50% α + 50% β	8.6	8.6	12 ± 2	12 ∓ 3
0.0061, 24 hr	R → 25% α + 75% β	6.8	20.5	9 ± 3	28 ∓ 6
0.020, fast scan	R → α	13.5	0	18	0
0.020, 24 hr	R → 25% α + 75% β	8.2	24.6	11 ± 4	33 ∓ 8

[a] The error estimation allows for a 10% error in the assignment of the CD changes. [b] See D'Anna and Isenberg, 1972.

TABLE II: Secondary Structure of the Fast and Slow Steps in Phosphate Buffer.

Conformation	$-[\Delta\epsilon_{220}(\infty) - \Delta\epsilon_{220}'(R)]$[a]	% α Helix	% β Sheet	Residues α Helix	Residues β Sheet
Fast step	2.33 − 0.92 (220 nm)	14.1	0	19	0
Slow step	2.82 − 0.98 (218 nm)	8.2	24.6	11 ± 4	33 ∓ 8

[a] The solution for the random coil is 1.0×10^{-3} M in HCl and 1.0×10^{-4} M in DTT.

sheet which increases with salt. The analysis of these spectra are given in Table I.

At each salt concentration, one has an equilibrium between different states of the histone (Li *et al.*, 1972), and the number of molecules undergoing the transition increases with phosphate concentration. We therefore calculate the number of α-helical residues in each molecule by extrapolating the fast step data to infinite phosphate concentration where we assume that all of the molecules have undergone the transition. This extrapolation is made in a simple fashion by a reciprocal plot and the extrapolated Δε value and the calculated α-helical content are given in Table II.

In the plateau region of Figure 3, where the slow change has been completed, we estimate that there are 11 residues of α helix and about 33 residues of β sheet per histone molecule (Table II).

Two-State Analysis of the Fast Changes. The fast conformational changes observed in histone GRK may be described by a simple two-state equilibrium process in which it is assumed that salt binding induces the changes (Li *et al.*, 1972). We also note that histone KAS satisfies the same scheme (D'Anna and Isenberg, 1972), but LAK does not (D'Anna and Isenberg, 1974a).

Two-state equilibrium is governed by

$$K = f/(1 - f)[P]$$

or

$$1/f = 1 + (1/K[P])$$

where f is the fraction of molecules which have changed and [P] is the phosphate concentration. K is an effective binding constant for inducing a conformational change.

Plots of $1/f$ *vs.* $1/[P]$ are given in Figure 6. Both the CD and fluorescence data satisfy linear plots; therefore, the data are consistent with the two state assumptions. The CD data yield $K = 6.6 \times 10^2$ M^{-1}, while the fluorescence data yield $K = 7.1 \times 10^2$ M^{-1}. These values are close enough so that they may be considered the same. This implies, there-

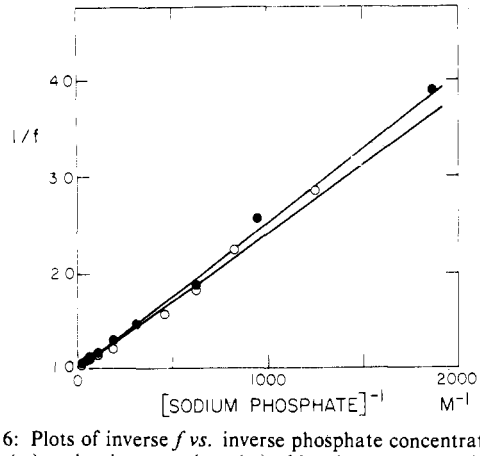

FIGURE 6: Plots of inverse f *vs.* inverse phosphate concentration from CD data (●) and anisotropy data (○). f has been calculated from the data of Figures 2 and 3 using eq 3 and 9 of D'Anna and Isenberg (1972).

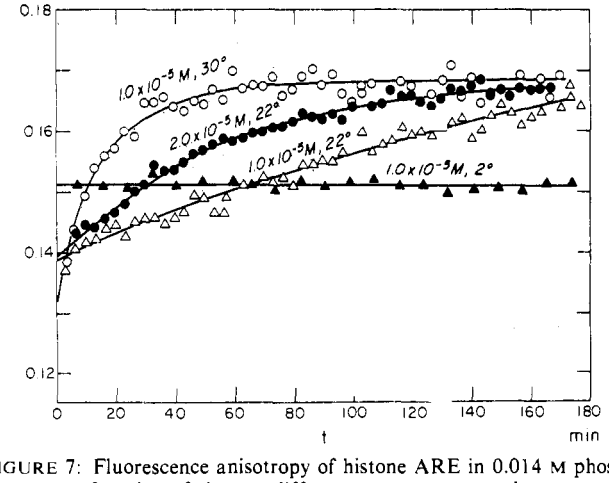

FIGURE 7: Fluorescence anisotropy of histone ARE in 0.014 M phosphate as a function of time at different temperatures and concentrations. The histone concentration and sample temperature are given for each curve.

TABLE III: A Tabulation of the Conformational Changes of Histones ARE, GRK, LAK, and KAS.

Histone	K (M^{-1})	Phosphate Concentration for $f = 0.50$	Fast Step[d] α Helix	Slow Step?	Slow Step[d] α Helix	Slow Step[d] β Sheet
LAK[a] f2a2	Nonlinear	0.0019	16–18	No		
KAS[b] f2b	220	0.0045	17	No		
ARE f3	700	0.0016	19	Yes	11	33
GRK[c] f2a1	80	0.012	15	Yes	15	33

[a] D'Anna and Isenberg, 1974a. [b] D'Anna and Isenberg, 1972. [c] Wickett *et al.*, 1972. [d] In phosphate buffer.

fore, that both techniques really examine different aspects of one overall protein folding. We note that this effective association constant is about eight times larger than that of GRK (Wickett *et al.*, 1972) and about three times larger than that of KAS (D'Anna and Isenberg, 1972). ARE is, therefore, very sensitive to salt and its sensitivity is comparable to that of histone LAK (D'Anna and Isenberg, 1974a).

Effects of Temperature and Concentration of Protein upon the Aggregation. The rate of slow change of GRK is very sensitive to both temperature and the histone concentration (Smerdon and Isenberg, 1973; Li *et al.*, 1972). We, therefore, looked for such sensitivities in histone ARE (Figure 7). The aggregation is indeed more rapid at 2×10^{-5} M than at 1×10^{-5} M, as might have been expected. In addition, lowering the temperature slows the aggregation and, at 2°, the slow change is still below detectable limits at 24 hr. Similar temperature effects have been observed in CD and light scattering data. The CD spectra of solutions at 2° are indicative of only random coil and α helix, and do not change for at least 24 hr.

Discussion

Histone ARE conformation changes are very similar to those of histone GRK (Li *et al.*, 1972; Wickett *et al.*, 1972; Smerdon and Isenberg, 1973) in that there are both fast and slow changes. In the fast change of ARE there is a folding of the protein in which 19 residues go into α-helical conformation. In the slow change there is aggregation and β-sheet formation. The aggregation occurs only above a critical salt concentration. When the aggregates form there is a loss of about eight residues of α helix and a formation of about 33 residues of β sheet per molecule. Like GRK, the aggregation of ARE is sensitive to temperature changes and can be effectively stopped for very long periods of time at 2°.

It is important to note that the effective equilibrium constant for inducing fast CD changes is equal to the effective constant for inducing fluorescence changes. This indicates that upon the addition of salt, the molecules fold in a definite, cooperative manner so that a two-state theory becomes applicable. Measurements of the CD of the peptide linkage and measurements of the fluorescence anistropy of the tyrosines are then probes of one basic event—the overall folding of the molecule. This does not mean, of course, that the entire polypeptide chain assumes a folded structure, but it does imply that the part that folds acts in a manner similar to the renaturation of a denatured globular protein.

In the Experimental Section we observed that only about 10–15% of the dithiothreitol oxidizes in phosphate buffer in 3 hr. The data of Figure 1 show that at the higher phosphate concentrations the slow step is completed in 3 hr when the dithiothreitol is still in excess. Therefore very little, and perhaps none, of the aggregation that occurs in the slow step is due to disulfide bridge formation. Furthermore, if there were to be disulfide bridges formed within 1–3 hr during the slow step, we would expect much more oxidation at 24 hr. Yet, at 1.4 mM phosphate, which is below the critical concentration, there is no aggregation at 24 hr.

With the present work and previous papers (Li *et al.*, 1972; Wickett *et al.*, 1972; Wickett and Isenberg, 1972; Smerdon and Isenberg, 1973; D'Anna and Isenberg, 1972, 1974a), we are now in a position to summarize and compare the conformational properties of histones GRK, LAK, KAS, and ARE (Table III). The major points are these. (1) All four histones undergo a rapid, salt induced, conformational change. (2) This change satisfies a simple two-state model for three of the histones—GRK, KAS, and ARE, but not for the fourth, LAK. The folding of LAK is more complex. (3) It is striking that all four histones, in their folded state, have about the same α-helical content. The absolute numbers of α-helical residues is somewhat uncertain, primarily because it is necessary to pick definite standards and basis spectra when estimating α-helical and β-sheet content. We have chosen L-poly(lysine) α helix and β-sheet spectra for standards (Greenfield and Fasman, 1969), and histones in 10^{-3} M HCl for a random coil basis spectrum. However, if at some time it is found that the standards that we have chosen are inappropriate, the absolute value of the α-helical and β-sheet content will have to be altered but, in all probability, there will still be about equal numbers of α-helical residues in each of the four histones. We have suggested (D'Anna and Isenberg, 1974b) that the similarity in the secondary structure of different histones may underlie histone–histone interactions, both self-dimerization (Li *et al.*, 1972; Smerdon and Isenberg, 1974) and cross-complexing (D'Anna and Isenberg, 1973, 1974b,c; Skandrani *et al.*, 1972; Kelley, 1973). (4) The conformations of LAK and ARE are much more sensitive to salt changes than KAS and GRK. (5) GRK and ARE show a slow change in addition to the fast one. In the slow change the protein aggregates. At the low protein concentrations that were used, KAS and LAK do not have a slow change, at least over the time period in which they were studied. (6) For the onset of the slow aggregation, a critical salt concentration is needed. (7) The concentrations of salt needed to induce conformational changes in each of the four histones are all in the physiological range. (8) The fast change is a folding like that which occurs when a denatured globular protein renatures. However, only part of the histone molecule may fold.

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A Histone Cross-Complexing Pattern†

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ABSTRACT: The interactions of histone ARE with histones LAK, KAS, and GRK have been studied by fluorescence anisotropy, circular dichroism (CD), and light scattering. ARE and GRK form a very strong 1:1 complex. We verify the result of R. D. Kornberg and J. O. Thomas [(1974), *Science 184,* 865] that the complex is a tetramer. In 8.5 mM phosphate (pH 7.0) the complex has a binding constant of 0.7×10^{21} M^{-3}. Upon complexing, one or both histones undergo a conformational change, and about nine additional residues per tetramer go into an α-helical conformation. Complexing inhibits the slow aggregation of ARE and GRK. Histones ARE and LAK also interact in a 1:1 molar ratio, but there is no increased α helicity upon complexing, and slow aggregation and β-sheet formation occur. ARE and KAS also interact, but, upon complexing, there is no change in the tyrosine rigidity or in the circular dichroism. A pattern of the interactions between LAK, KAS, ARE, and GRK is presented.

We have reported (D'Anna and Isenberg, 1973, 1974a) that histones LAK and KAS, and histones KAS, and GRK, form strong complexes with a 1:1 molar ratio. Histones LAK and GRK also interact to form a 1:1 complex, but more weakly (D'Anna and Isenberg, 1974a). The complexes require salt for stability and, in fact, they were formed by adding salt to histone mixtures in water. One or both of the partner histones in the strong complexes, KAS-GRK and LAK-KAS, show marked conformational changes upon complexing, and the complex shows considerably more α-helical content than the uncomplexed molecules.

Skandrani *et al.* (1972) and Kelley (1973) have also reported a 1:1 complex of histones LAK and KAS, which was isolated by chromatographic fractionation of mixed his-

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[1] The nomenclature used in this paper is described in Huberman (1973): GRK = IV = F2a1, KAS = IIb2 = F2b, LAK = IIb1 = F2a2, ARE = III = F3.