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Interactions of Histone LAK (f2a2) with Histones KAS (f2b) and GRK (f2a1)†

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

ABSTRACT: Measurements of fluorescence anisotropy, relative fluorescence intensity, and circular dichroism (CD) indicate that histones LAK (IIb1; f2a2) and KAS (IIb2; f2b) form a 1:1 complex in solutions of sodium phosphate or sodium chloride, pH 7.0. The order of addition of histone LAK, histone KAS, or salt is not important. The complex is strong and has an association constant of about 10^6 M^{-1}. Upon complexing the number of α-helical residues increases by about 15. The addition of urea reduces complexing. The properties of the LAK–KAS complex are compared with those of KAS–GRK (IIb2–IV) [D'Anna, J. A., Jr., and Isenberg, I. (1973), *Biochemistry 12*, 1035]. Histones LAK and GRK also complex, but the interaction is weaker and comparable to histone self-aggregation. Circular dichroic continuous variation curves indicate an equimolar complex. The interaction of histones LAK and GRK interferes with the slow step of histone GRK.

The treatment of chromatin with varying concentrations of urea (Bartley and Chalkley, 1973; Ilyin *et al.*, 1971) appears to disrupt the compactness of the chromatin and change the circular dichroism (CD) in the 270-nm region to nearly that of free DNA. These results suggest that protein–protein interactions are important to chromatin structure. They are in general agreement with the histone self-aggregation models of Bradbury and Rattle (1972) and Hayashi and Iwai (1971). In those models, parts of histones are bound to DNA and other portions are available for histone–histone interactions.

It has been known for several years that histones aggregate upon addition of salts or at extremes of pH (Cruft *et al.*, 1958; Edwards and Shooter, 1969; Boublik *et al.*, 1970; Barclay and Eason, 1972; Diggle and Peacocke, 1971; Li *et al.*, 1972). Also, some workers have alluded to possible interactions between histones of differing primary structure (Cruft *et al.*, 1958; Laurence, 1966; Shih and Bonner, 1970; Edwards and Shooter, 1970). However, only within the last year has the existence of specific cross-complexes been verified and their characterizations begun (Skandrani *et al.*, 1972; D'Anna and Isenberg, 1973; Kelley 1973).

Skandrani *et al.* (1972) reported that histones LAK[1] and KAS interact during guanidine hydrochloride gradient chromatography on Amberlite resin. From column work and amino acid analyses of the LAK–KAS band, Skandrani *et al.* concluded that the histones form an equimolar complex. Kelley (1973) also reported complex formation based on studies of chromatographic fractions of mixed histones.

We recently reported complex formation between histones KAS and GRK (D'Anna and Isenberg, 1973). CD and fluorescence continuous variation curves imply an equimolar complex. On the basis of a dimer complex, the interaction is strong ($K_A = 10^6$ M^{-1}), and there is an increase of 8 residues of α helix in the complex as compared to the individual histones.

This report describes a number of CD and fluorescence properties of the LAK–KAS complex. We present continuous variation curves and calculate association constants. The effects of urea are examined, and estimates are made of the nature and extent of conformational changes upon complexing. We also examine CD and fluorescence properties of the interaction between histones LAK and GRK. These histones also complex with one another, but much more weakly than LAK–KAS or KAS–GRK.

Experimental Section

Materials and Methods. Calf thymus histone LAK was prepared by the method of Sugano *et al.* (1972) as previously described (D'Anna and Isenberg, 1974). Histone GRK was prepared by exclusion chromatography (Mauritzen *et al.*, 1967), and histone KAS was prepared by the method of Senshu and Iwai (1970). The samples used for fluorescence and CD measurements showed no trace of impurity in electrophoresis (Panyim and Chalkley, 1969), and amino acid analyses agreed with the sequences (Yeoman *et al.*, 1972; Iwai *et al.*, 1970; Ogawa *et al.*, 1969; DeLange *et al.*, 1969).

Fluorescence anisotropy, $r = (E - B)/(E + 2B)$, and CD measurements were obtained as previously described (D'Anna and Isenberg, 1973). CD measurements are reported as $\Delta\epsilon = \epsilon(\text{left}) - \epsilon(\text{right})$ in units of cm^{-1} l./mol of residue or as $\Delta\epsilon' = \epsilon'(\text{left}) - \epsilon'(\text{right})$ in units of cm^{-1} l./mol of histone. The fluorescence anisotropy data as a function of time were

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[1] The nomenclature used in this paper is described in Huberman (1973): GRK = f2a1 = IV; LAK = f2a2 = IIb1; KAS = f2b = IIb2; ARE = f3 = III.

plotted by the use of a computer program written by Dr. E. W. Small.

The concentration of histone was determined from the absorbance of histone in water at 275 or 230 nm. Respective absorption coefficients of 4.05×10^3, 5.4×10^3, and 6.7×10^3 cm^{-1} l./mol of histone were used at 275 nm for histones LAK, GRK, and KAS; coefficients of 4.3×10^4, 4.2×10^4, and 5.4×10^4 were used at 230 nm.

Reagent grade urea was deionized on a mixed resin of Dowex 50-X4 and Dowex 2-8X. Other chemicals and salts were reagent grade.

Series of solutions for continuous variation curves were prepared from separate stock solutions of histones in water. Mixed solutions were prepared, by pipetting, such that the sum of the two concentrations, after dilution with buffer, equaled C_0.

Equations. Previously (D'Anna and Isenberg, 1973), we presented continuous variation equations relating the concentration of a complexed species AB_n to the measured fluorescence and CD properties. In the development of those equations, it was assumed that the independent species A and B interacted to form a complex, AB_n. Although effects of histone self-dimerization were not considered in the derivation of the equation, estimates indicate that such a dimerization will result in at most only a 5% change in the continuous variation plots for the LAK–KAS complex. In a continuous variation plot, the value of n is obtained from the maximum deviation between measured values and values computed for noninteracting solutions by $n = (1 - X_A^M)/X_A^M$ where X_A^M is the mole fraction of component A at which the maximum deviation occurs. However, the formation of a complex of the type $A_{n_1}B_{n_2}$ will also give a maximum at the same place if $n_2/n_1 = n$. Our previous equations may easily be generalized to consider this possibility. For CD, the concentration of the complex, $A_{n_1}B_{n_2}$, is related to our observables by

$$\Delta\epsilon' - \Delta\epsilon_I' = \frac{\Delta\epsilon_{An_1Bn_2}' - n_1\Delta\epsilon_A' - n_2\Delta\epsilon_B'}{C_0}[A_{n_1}B_{n_2}] \quad (1)$$

We previously presented (D'Anna and Isenberg, 1972) continuous variation equations for anisotropy data. These equations ignored the variation of the absorbance of the exciting light as a function of the mole fraction of histones. For the concentration of histones used, this was an approximation that resulted in a neligible change in the continuous variation curve. In the present work, because of the much weaker interaction of LAK and GRK, higher concentrations of histones were used. If we now take into account variations of light absorbed with changes in mole fraction, the equations read

$$(F - F_I)/\theta = k[\epsilon_{An_1Bn_2}q_{An_1Bn_2} - n_1\epsilon_Aq_A - n_2\epsilon_Bq_B][A_{n_1}B_{n_2}] \quad (2)$$

$$(Fr - F_Ir_I)/\theta = k[\epsilon_{An_1Bn_2}q_{An_1Bn_2}r_{An_1Bn_2} - n_1\epsilon_Aq_Ar_A - n_2\epsilon_Bq_Br_B][A_{n_1}B_{n_2}] \quad (3)$$

$$F_I = k\epsilon([A]\epsilon_Aq_A + [B]\epsilon_Bq_B) \quad (4)$$

ϵ_i and q_i are the molar extinction coefficient and quantum yield of the ith species; θ is defined as $(1 - 10^{-A})/(A \cdot \ln 10)$ in which A is the absorbance of the sample and k is a constant. The subscript I refers to values for noninteracting molecules. These equations were used in the present paper, but we found that even at the higher protein concentrations used here, the use of a nonconstant value of θ made no difference in the results.

The concentrated solutions of urea used in this work were found to have a residual fluorescence, undoubtedly due to an impurity, of low anisotropy, $r = 0.015$. The fluorescence anisotropy of concentrated urea–histone solutions was therefore corrected for this solvent fluorescence by use of eq 5 in

$$r_{cor} = [1/(I_M - I_u)][I_Mr_M - I_ur_u] \quad (5)$$

which r_{cor} is the corrected value of r, r_m is the measured value, and r_u is the anisotropy of the urea solvent. The subscripts of I have the same meaning as those for r. The values r_{cor} and $I_{cor} = I_M - I_u$ were used in eq 3 for the continuous variation curves.

Results

Histone LAK–Histone KAS Interaction. Histones LAK and KAS have been found to interact in both sodium phosphate and sodium chloride solutions. Addition of these salts leads to instantaneous changes, by our techniques, which exceed those calculated for noninteracting mixtures of the histones. We note that there is no slow change such as that observed in histone GRK (Li *et al.*, 1972; Wickett *et al.*, 1972).

We have found that our results do not depend on the order of addition of histone LAK, histone KAS, and salt. Furthermore, we find interactions only in the presence of salt. Therefore, we prepare mixtures of the histones in water and then add salt.

LAK–KAS Fluorescence Continuous Variation Curves. Mixtures of histones LAK and KAS in solutions of sodium phosphate or of sodium chloride show an increased anisotropy in excess of the values expected for noninteracting histones. However, the characteristics of the fluorescence intensity differ for the two salts. When sodium chloride is used, there is a considerable increase in fluorescence intensity over that for noninteracting species; but when sodium phosphate is used, the increase is small. It may be seen in Figure 1 that the plot for sodium chloride fluorescence shows marked curvature, while that for phosphate is almost linear.

Maxima in the continuous variation curves of Figure 1 occur at $X_{LAK} = 0.45$ for 0.0031 M phosphate, and at 0.40 for 0.016 M phosphate. The curves from the sodium chloride solutions have maxima at 0.47 for anisotropy measurements (eq 3) and at 0.50 for intensity (eq 4).

LAK–KAS CD Spectra and CD Continuous Variation Curves. Continuous variation curves, monitored at 220 nm, are given for a number of salt concentrations in Figure 2. Maxima in the CD curves occur at 0.48 for 0.003 M phosphate, 0.42 for 0.016 M phosphate, and 0.48 for 0.20 M NaCl–0.006 M cacodylate.

CD spectra of the mixed histones at a 1:1 molar ratio are given for three different solvents in Figure 3. The difference spectra between the salt solutions and the 1.0×10^{-4} M HCl solution are also shown. The 1.0×10^{-4} M HCl solvent was selected to ensure the random coil conformation of histone LAK (D'Anna and Isenberg, 1974). The CD spectra and difference spectra are very similar to those obtained for the KAS–GRK complex (D'Anna and Isenberg, 1973) or for the individual histones (Li *et al.*, 1972; Wickett *et al.*, 1972; D'Anna and Isenberg, 1972, 1974). Interpretation of the difference spectra by the method of Li *et al.* (1972) and comparison with standard curves (D'Anna and Isenberg, 1972) suggest that the changes in secondary structure involves a number of residues going into α-helical conformation.

Effects of Urea on the LAK–KAS Complex. We have explored the effect of urea on the LAK–KAS complex. In these

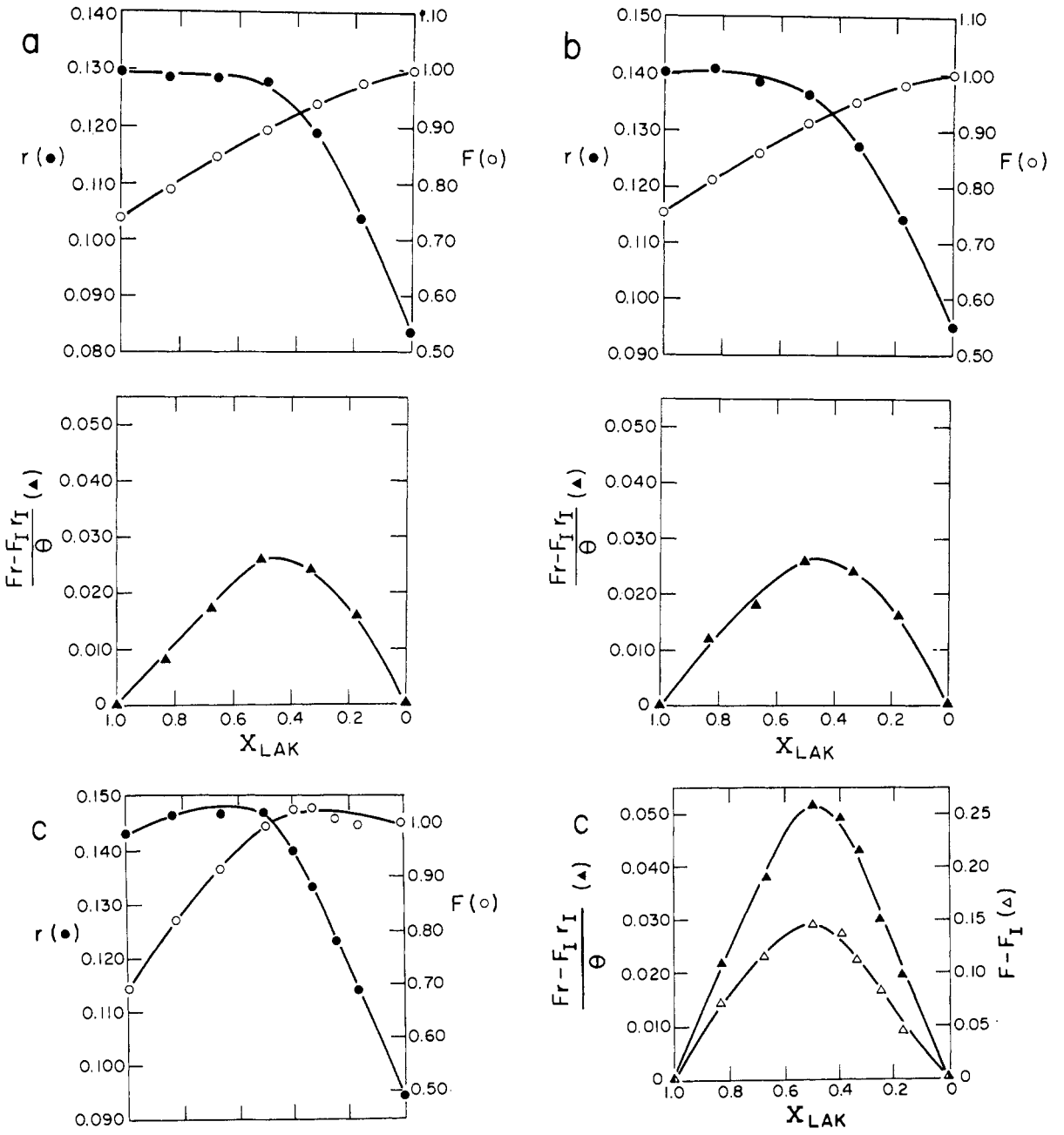

FIGURE 1: Fluorescence continuous variation curves of histone LAK plus histone KAS: anisotropy, r (●); relative fluorescence intensity, F(○); $(Fr - F_I r_I)/\theta$, (▲); or $F - F_I$ (△) *vs.* X_{LAK} in (a) 0.0032 M phosphate, (b) 0.016 M phosphate, and (c) 0.20 M NaCl–0.006 M cacodylate. For all curves, $C_0 = 1.0 \times 10^{-5}$ M and pH is 7.0.

experiments solutions containing salt, buffer, and urea were added to concentrated aqueous solutions of the mixed histones. The resultant deviations from ideality of the continuous variation curves are shown in Figure 4. At 2.0 M urea complexing still occurs, but the degree of association has dropped to 40% of its initial value; at 4.0 M urea the interaction is eliminated.

Stoichiometry of the LAK–KAS Complex and Association Constants. The CD and fluorescence continuous variation curves exhibit values of $X_{LAK}{}^{M}$, between 0.50 and 0.40. The average value is 0.46. This suggests a 1 : 1 complex but, of course, experimental errors make more complicated stoichiometries possible.

Recently, Kelley has studied the chromatography of a mixture of histones KAP, LAK, and KAS from salt eluted BioRex 70 columns. He observed a fraction that contained histones LAK and KAS. An equilibrium ultracentrifuge study at 15,000 rpm indicated a complex of LAK and KAS of molecular weight 28,500 ± 800.

We have made equilibrium runs under Kelley's conditions, using a 1 : 1 molar mixture of histones LAK and KAS, to which salt was added. Log plots of concentration *vs.* the radius squared were linear for most of the 3.0-mm column length, and one gets a molecular weight of about the dimer value. However, we have also observed that about half of the protein sedimented to the bottom of the sectored cell. These ultracentrifuge data were taken at initial concentrations about ten times higher than those used for CD and fluorescence studies. Under such conditions, there is not only the complex between the two histones, but there is a higher order aggregation as well.

Assuming now that there is a complex containing a single molecule of each histone, we can calculate association con-

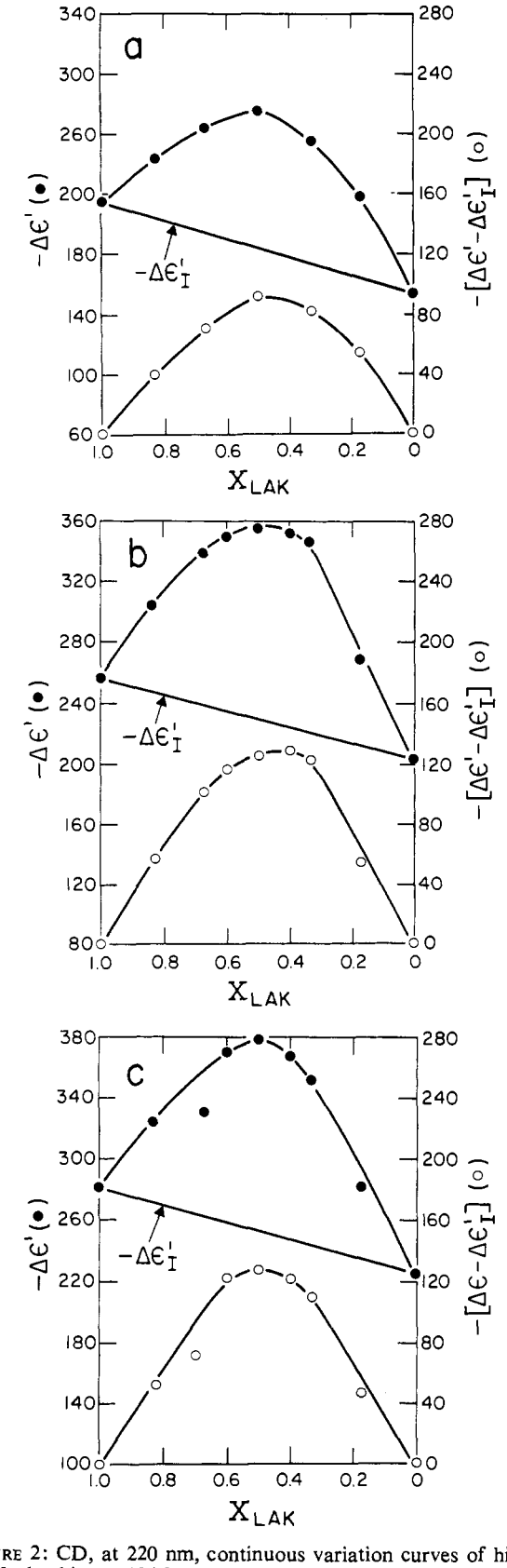

FIGURE 2: CD, at 220 nm, continuous variation curves of histone LAK plus histone KAS: $-\Delta\epsilon'$ *vs.* X_{LAK} (●) and $-[\Delta\epsilon' - \Delta\epsilon_I']$ *vs.* X_{LAK} (○) at (a) 0.003 M phosphate, (b) 0.016 M phosphate, and (c) 0.20 M NaCl–0.006 M cacodylate. For all curves $C_0 = 0.5 \times 10^{-5}$ M and the pH is 7.0.

stants. These are given in Table I. The values were obtained from extrapolation of the initial slopes of the continuous variation curves (Schaeppi and Treadwell, 1948). The ends of

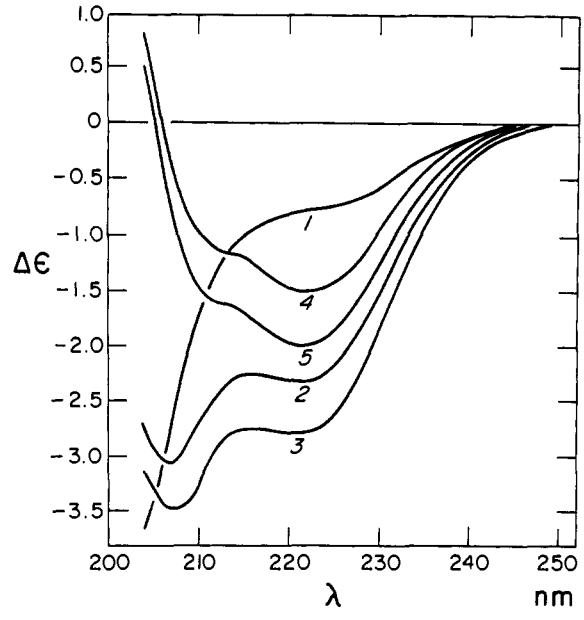

FIGURE 3: CD spectra of 0.375×10^{-5} M histone LAK plus 0.375×10^{-5} M histone KAS in 1.0×10^{-4} M HCl (1), 0.003 M phosphate (pH 7.0) (2), and 0.20 M NaCl–0.006 M cacodylate (pH 7.0) (3). CD difference spectra were computed by subraction of curves 2 and 1 (4) and curves 3 and 1 (5).

these curves are flattened because of histone self-aggregation. The calculated equilibrium constants therefore have a relatively large error. Nevertheless, it is clear that the cross-interaction is quite strong. K has an order of magnitude of 10^6 M^{-1} which, we note, is the order of magnitude of the association constant of the KAS–GRK complex (D'Anna and Isenberg 1973).

Effects of LAK–KAS Complexing on Secondary Structure. The CD at 220 nm has been measured as a function of phosphate concentration for the following solutions (Figure 5): LAK alone at $c = 0.5 \times 10^{-5}$ M, KAS alone at $c = 0.5 \times 10^{-5}$ M, and LAK plus KAS, 0.25×10^{-5} M in each. All of the samples were prepared from the same stock solutions of LAK, KAS, and phosphate. At all phosphate concentrations used, the measured CD exceeds the value expected for a noninteracting mixture. The CD spectral changes are similar to those induced in the parent histones and are typical of α-helix formation. These results parallel those for the KAS–GRK complex, and we shall employ the same arguments to estimate the structural increase accompanying complexing of histones LAK and KAS (D'Anna and Isenberg, 1973).

Since salt is necessary for formation of the complex, we estimate the properties of a solution, in which a maximum

TABLE I: Apparent Association Constants of Histone LAK–Histone KAS Interaction from Continuous Variation Plots.

Medium, pH 7.0 (M)	C_0 (M)	Technique	$K \times 10^{-6}$ (M^{-1})
Phosphate (0.0030)	0.5×10^{-5}	CD	0.7
Phosphate (0.0032)	1.0×10^{-5}	CD	1.6
Phosphate (0.016)	0.5×10^{-5}	CD	1.2
Phosphate (0.016)	1.0×10^{-5}	Fluor anis.	0.4
NaCl (0.20)	0.5×10^{-5}	CD	3.4
NaCl (0.20)	1.0×10^{-5}	Fluor anis.	2.9
NaCl (0.20)	1.0×10^{-5}	Fluor intensity	1.2

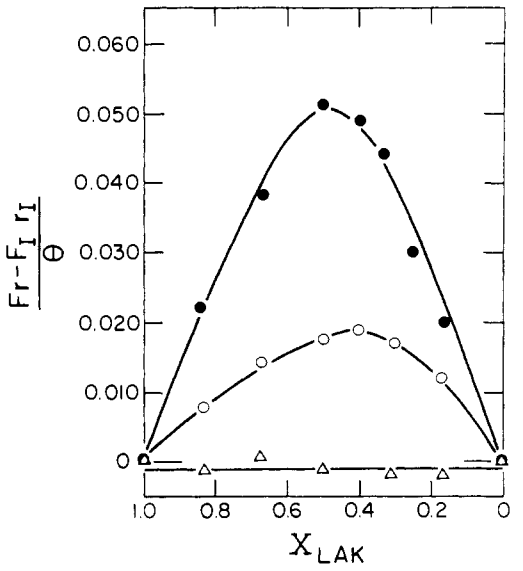

FIGURE 4: Fluorescence continuous variation curves of histone LAK plus KAS, $(Fr - F_I r_I)/\theta$ *vs.* X_{LAK} in 0.20 M NaCl–0.005 M cacodylate and urea concentrations of 0.0 M (●), 2.0 M (○), and 4.0 M (△); $C_0 = 1.0 \times 10^{-5}$ M.

degree of complexing occurs, by extrapolating to infinite salt concentration. Similar extrapolations may be used for the individual histones. Subtraction of extrapolated values of the noninteracting mixture and the real mixture then gives the minimum structural increase due to complex formation. The estimate is a minimum value, because we do not know if all of the histones are actually complexed in the extrapolated state. Our values are given in Table II and are interpreted in terms of α-helical content. In the dimer, there is then a minimal increase of about 15 residues of α helix accompanying complex formation in phosphate buffer, pH 7.0.

LAK–GRK Interaction. The interaction between LAK and GRK is much weaker than that between histones LAK and KAS or between histones KAS and GRK. Circular dichroic continuous variation curves at C_0 values up to 3.0×10^{-5} M show only small variations from noninteracting mixtures (Figure 6). The deviation of $\Delta\epsilon'$ at its maximum is only about 8% greater than the ideal value. However, Figure 6 does indicate a 1:1 molar ratio for the complex. In contrast, we have found that any deviation in the fluorescence continuous

TABLE II: α-Helical Content of the Individual Histones and of the LAK–KAS Complex in the Presence of Phosphate, pH 7.0.

Sample	$-[\Delta\epsilon(\infty) - \Delta\epsilon(0)]$ $(cm^{-1}\ M^{-1})^a$	% α Helix	Helix Content
LAK	2.12–0.85[b]	12.7	16.4 (of 129)
KAS	2.06–0.80[b]	12.6	15.8 (of 125)
LAK + KAS (measd)	2.69–0.83	18.6	47.2 (of 254)
LAK + KAS (ideal)	2.10–0.83	12.7	32.2 (of 254)

[a] Obtained from extrapolation of $\Delta\epsilon$ *vs.* the reciprocal of phosphate concentration. [b] The coil form from spectra at 1.0×10^{-4} M HCl.

variation curves are below experimental error (Figure 7)—an apparent anomaly. Because histone GRK, itself, shows time-dependent changes, the data for the LAK–GRK complex were obtained by extrapolating the observables to zero time (D'Anna and Isenberg, 1973).

The interaction between histones KAS and GRK causes a reduction in the rate of the histone GRK slow step formation (Figure 8). In the strong KAS–GRK complex (D'Anna and Isenberg, 1973) we observed that complexing blocked the slow step of GRK. If we now assume that in the LAK–GRK mixture the uncomplexed GRK molecules go through a slow change and the complex does not, we can then make an estimate of the concentration of uncomplexed GRK. Let ΔA be the absorbance(left) − absorbance(right). We then compute the initial rate of change of ΔA with time, $(d(\Delta A)/dt)_{t=0}$, for the mixed histones and for GRK alone. An equilibrium constant for dimer formation of about $4 \times 10^4\ M^{-1}$ at 0.0067 M phosphate is then obtained. It is difficult to measure these derivatives accurately. Consequently, the value of $4 \times 10^4\ M^{-1}$ must be considered an order of magnitude estimate only. However, it is of interest that Li *et al.* (1972) gave an equilibrium constant of 150 l. (mole of residue)$^{-1}$ for histone GRK which, in protein concentration units, is $1.5 \times 10^4\ M^{-1}$. The calculated association constant for the cross dimer is thus of the same order of magnitude as the association constant for histone GRK self-dimerization. This is consistent with the experimental evidence that the cross-complexing

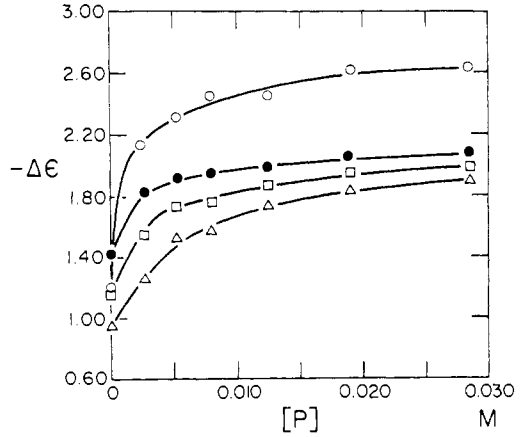

FIGURE 5: CD, at 220 nm, of histone LAK, histone KAS, and histones LAK plus KAS as functions of phosphate concentration, pH 7.0: 0.50×10^{-5} M LAK (●), 0.50×10^{-5} M KAS (△), 0.25×10^{-5} M LAK plus 0.25×10^{-5} M KAS as measured (○) and as calculated (□).

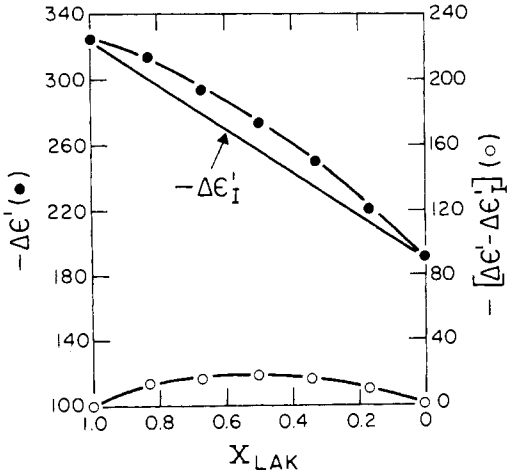

FIGURE 6: CD continuous variation curves of histones LAK and GRK, $C_0 = 3.0 \times 10^{-5}$ M at 0.0067 M phosphate: $-\Delta\epsilon'$ (●) and $[\Delta\epsilon' - \Delta\epsilon_I']$ (○) *vs.* X_{LAK}.

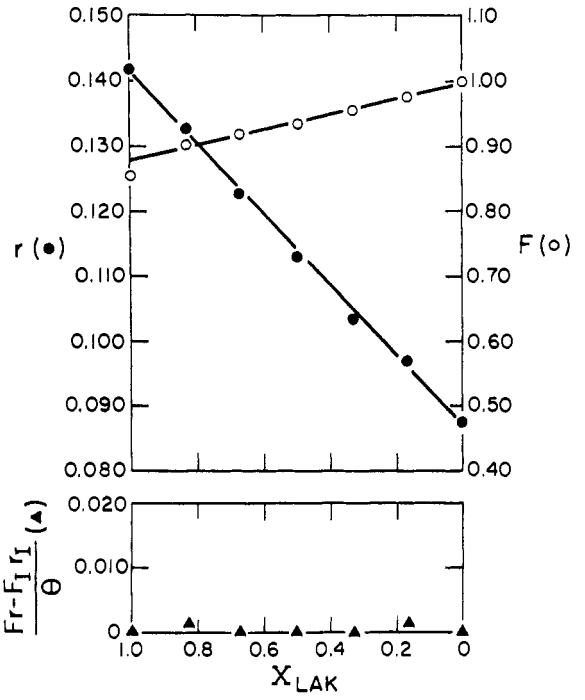

FIGURE 7: Fluorescence continuous variation curves of histones LAK and GRK, $C_0 = 3.0 \times 10^{-5}$ M at 0.0067 M phosphate: anisotropy, r (●); relative fluorescence intensity, F (○), and $(Fr - F_1r_1)/\theta$ (▲) *vs.* X_{LAK}.

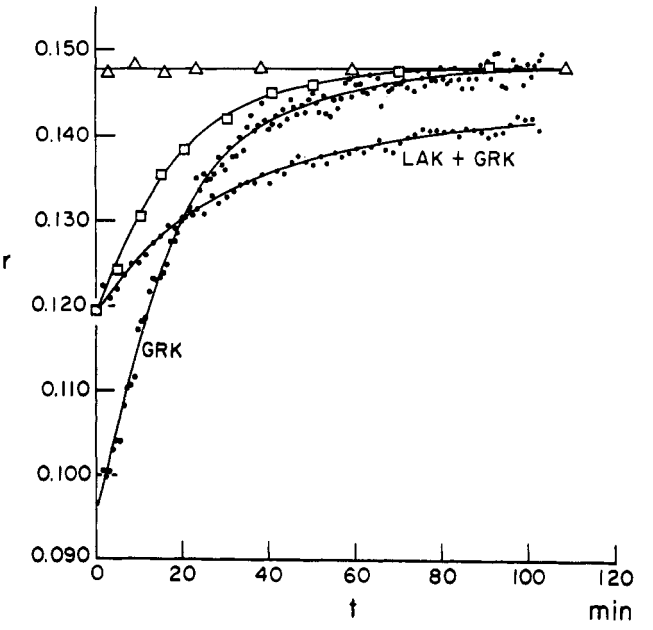

FIGURE 8: Fluorescence anisotropy curves as functions of time for histone LAK, 6.0×10^{-5} M (*Z*), histone GRK, 6.0×10^{-5} M (as indicated), measured LAJ plus GRK, 6.0×10^{-5} M in each (as indicated), and the calculated curve for noninteracting LAK plus GRK (□).

occurs but is not strong. The self-complex and the cross-complex are competing processes.

Discussion

The LAK–KAS complex has many characteristics in common with that of KAS–GRK. Both are formed only in the presence of salt, they are strong ($K_A = 10^6$ M^{-1}), and they are characterized by increases in CD, fluorescence intensity, and anisotropy as compared to noninteracting solutions. Upon complex formation, there are increases in the α-helical content. For a dimer, there are increases of $\geq$15 residues in LAK–KAS and increases of $\geq$8 residues in KAS–GRK. These results are in general agreement with the equilibrium scheme proposed for histones KAS and GRK (D'Anna and Isenberg, 1973).

On the other hand, the LAK–GRK complex is about two orders of magnitude weaker than that of LAK–KAS or KAS–GRK. Nevertheless, even this weak interaction interferes with the slow changes of histone GRK. We have also seen that the fluorescence continuous variation curves of LAK and GRK do not deviate from ideality, although the CD and kinetic data indicate complexing. This implies that the rotational relaxation and the fluorescence intensity of the tyrosine residues in the KAS–GRK complex are, essentially, the same as those in KAS or GRK alone at C_0.

It is of some interest that our analyses of the α-helical content of the three histones, GRK, KAS, and LAK, all show about the same number of residues in the α-helical state. Furthermore, in each case, we have found that a Wu–Kabat (1971) analysis of the amino acid sequence, followed by a Schiffer–Edmundson (1967) helical wheel analysis, gives a number of residues in an α helix not far different from the measured value.

The Schiffer–Edmundson concept rests upon finding presumed α-helical regions which have a relatively large hydrophobic area on one side of the α helix. For stability, the tertiary structure must have such regions inside rather than outside of the molecule. This may be done in several ways. There may be an intramolecular folding of a monomer that achieves such a conformation; there may be a dimer in which the hydrophobic regions are internal, or there may be cross-complexes in which the hydrophobic groups are internal. In cross-complexing, the number of α-helical residues may change and, of course, we find this to be the case. With our present state of knowledge, it appears fruitless to attempt a detailed structural model. However, the broad picture that we suggest here is that each of the three histones has a similar secondary and, perhaps, a similar tertiary structure which facilitates their interaction.

We believe that histone cross-complexing could be important in determining the structure of chromatin. Recent studies suggest that histones bind with some degree of base specificity (Clark and Felsenfeld, 1972; Combard and Vendrely, 1970; Sponar and Sormova, 1972). Varshavsky and Georgiev (1972) have reported that histones ARE and GRK bind to DNA in blocks. While such studies and our own cross-complexing experiments are incomplete, the trends prompt us to suggest the following scheme: histones bind to specific regions of DNA. These regions may be determined by the relative affinity of the histones for the base sequence, or perhaps, by histone modification as suggested by Louie *et al.* (1972), or perhaps by a specific interaction between histones and nonhistone proteins which, in turn, interact with specific DNA sequences. These regions then bind specifically to other histone bound regions *via* histone cross-complexing. Such interactions could effect much of the ordering and packaging of chromatin and changes in morphology during the cell cycle.

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