Conformational Changes in Histone GRK (f_2a_1)†

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ABSTRACT: The fast and slow conformational changes of histone GRK have been investigated at different temperatures by measuring the fluorescence anisotropy of the tyrosine residues, circular dichroism of the peptide bond, and light scattering. At low temperatures the slow change does not occur and the fast change can be decomposed into two different processes. The first is a rapid conformational change which appears similar to the renaturation of a denatured globular protein. The second varies slowly with time at low temperatures and is shown to be the dimerization of histone GRK monomers. Aggregation occurs only during the room temperature slow change. This is a complex kinetic process that does not satisfy simple kinetics.

Past studies have shown that the addition of salts to aqueous solutions of histone GRK results in conformational changes in the protein (Li *et al.*, 1972; Boublik *et al.*, 1970; Bradbury *et al.*, 1967; Tuan and Bonner, 1969). The overall process appears complex, but it may be decomposed into more elementary steps (Li *et al.*, 1972).

Upon the addition of salt there is a rapid conformational change that has been named the *fast step* (Li *et al.*, 1972). If the salt concentration is below a critical value, which depends on the histone concentration and the type of salt, *only* the fast step occurs (Wickett *et al.*, 1972). Above the critical concentration, the fast step is followed by a considerably slower change, referred to as the *slow step*. At room temperature, the fast step is over by the time a circular dichroism (CD) or anisotropy measurement can be made, whereas the slow step may take minutes to hours, depending on the histone and salt concentrations.

Recently, we reported that the slow step could be drastically reduced by lowering the temperature of the solution (Smerdon and Isenberg, 1973). During these studies we discovered that, at low temperatures, the fast step itself could be decomposed into two events, one of which was still instantaneous when monitored by CD or fluorescence anisotropy measurements, while the other process, which we detected by light scattering measurements, took many minutes at 2°, although it was instantaneous at room temperature. Li *et al.* (1972) found that the amount of fast step product was a function of the histone concentration, and that this concentration dependency satisfied a dimer equilibrium equation. In this paper we shall show that the process which is instantaneous at room temperature, and slow at 2°, is the dimerization that was predicted in Li *et al.* In addition, we shall show that the slow step is not a simple process. It evolves by complex kinetics which change as a function of temperature. for didactic purposes we discuss the slow step first.

Materials and Methods

Histone GRK, prepared by the method of Ogawa *et al.* (1969), was electrophoretically pure and its amino acid composition agreed with the published sequence (Delange *et al.*, 1969; Ogawa *et al.*, 1969). Its concentration was determined spectrophotometrically using a molar extinction coefficient of 5.4×10^3 cm^{-1} per mol of histone per liter at 275 nm (D'Anna and Isenberg, 1973).

Stock solutions of phosphate were used as their own buffers and adjusted to pH 7.4 by the addition of NaOH. Prior to each experiment, phosphate solutions, and histone GRK in water, were brought to the temperature at which they were to be studied. At this temperature, aliquots of each were rapidly mixed. The time of mixing is called time zero. In all cases the phosphate concentration after mixing was 0.007 M.

The fluorescence anisotropy [$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$] and light scattering were measured on a computer controlled polarization spectrometer constructed in our laboratory (Ayers *et al.*, 1974).

For the fluorescence anisotropy measurements, the samples were excited at 279 nm and the emissions were measured at 325 nm. The light scattering measurements were made at 90° using the method described in Smerdon and Isenberg (1973). To account for lamp fluctuations, the scattered intensity was divided by a quantity proportional to the incident light intensity. In this paper, the ratio is called I_E/L.

Circular dichroism (CD) measurements were made on a Jasco Model J-10 CD recorder. The data obtained are reported as $\Delta mgrke = \epsilon$ (left) $- \epsilon$(right) in units of cm^{-1} liter per mole of residue.

Temperatures of the samples were monitored with a thermistor probe and a YSI telethermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio). For the anisotropy and light scattering measurements, the thermistor probe was imbedded in the sample jacket. Control measurements showed less than 0.25° between sample and jacket temperatures. For CD measurements, a jacketted cell was used, and the thermistor probe was immersed in the solution through a hole in the Teflon cap.

For all experiments at low temperatures, condensation of moisture was prevented by circulating dry nitrogen through the sample chamber.

Results

1. Slow Step. The anisotropy and CD of histone GRK in water, with no added salt, are functions of the temperature (Figure 1). In studying the changes induced by salt, it is therefore more appropriate to examine $r(t) - r_w$ and $\Delta\epsilon(t) - \Delta\epsilon_w$ rather than $r(t)$ and $\Delta\epsilon(t)$.

Lowering the temperature markedly decreases slow step formation as may be seen from the anisotropy data (Figure 2) and CD data (Figure 3). Each technique permits a calculation of the fraction of molecules undergoing slow step formation.

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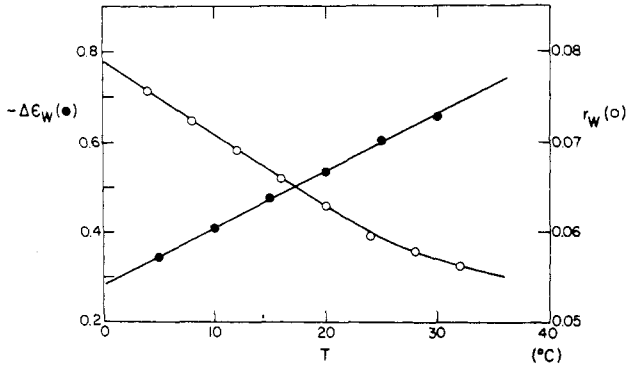

FIGURE 1: Fluorescence anisotropy, r_w, and CD at 220 nm, $\Delta\epsilon_w$, of 0.8 × 10^{-5} M histone GRK in water as a function of temperature. The subscript w means that the solvent was water.

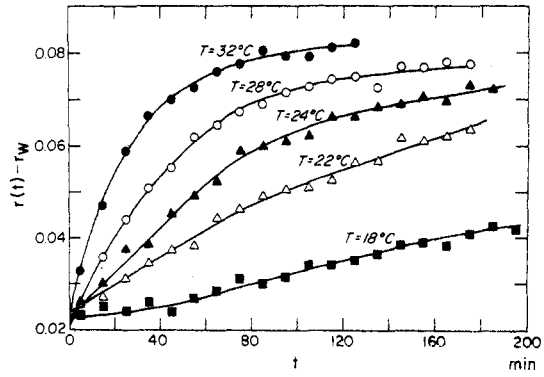

FIGURE 2: Fluorescence anisotropy of 0.8 × 10^{-5} M histone GRK as a function of time at various temperatures. Phosphate was added at time zero to give a final concentration of 0.007 M, pH 7.4.

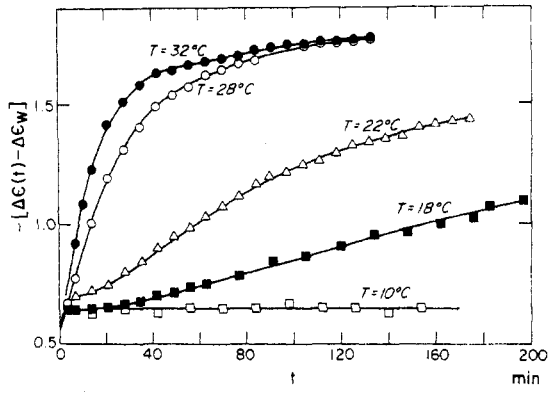

FIGURE 3: CD at 220 nm of 1.2 × 10^{-5} M histone GRK as a function of time at various temperatures. Solution conditions were the same as those for Figure 2.

Let f be the fraction *not* undergoing change. Then, for anisotropy measurements (Evett and Isenberg, 1969)

$$f = \frac{I(t)}{I(0)}\left[\frac{r(\infty) - r(t)}{r(\infty) - r(0)}\right]$$

where $r(t)$ and $I(t)$ are the fluorescence anisotropy and fluorescence intensity at time t, respectively. For CD measurements

$$f = \frac{\Delta\epsilon(\infty, 220) - \Delta\epsilon(t, 220)}{\Delta\epsilon(\infty, 220) - \Delta\epsilon(0, 220)}$$

where $\Delta\epsilon(t, 220)$ is the CD value at 220 nm at time t.

The data obtained were analyzed to see if first-order kinetics could be satisfied. In Figure 4, $\ln f$ is plotted against t. It may be seen that the data do not satisfy first-order kinetics. At about 23° it appears to do so, but this appearance is misleading.[1] For second-order kinetics, $(1/f) - 1$ *vs.* t should be a straight line. Figure 5 shows that anisotropy data do not satisfy second-order kinetics. CD data, not shown here, also do not satisfy second-order kinetics.

We previously reported that, at sufficiently low temperatures, the slow step is effectively blocked (Smerdon and Isenberg, 1973); at 2° both CD and anisotropy data show this. The ability to block the slow step is of critical importance in understanding the nature of the fast step.

II. Fast Step. Although the anisotropy and CD are constant at 2°, the light scattering intensity varies with time (Figure 6). This is surprising in view of the fact that the slow step is completely blocked at 2°. From this fact, however, it follows that lowering the temperature to 2° slows down some part of the fast process. We asked ourselves if the event showing this time dependency could be the dimerization of histone GRK, predicted in Li *et al.* (1972). To this end we tested the data to see if they satisfied the equation for dimerization (see Appendix).

$$\frac{1}{I(t) - I(0)} = \frac{\alpha}{t} + \beta$$

Figure 7 shows that the data obey this relationship. It should be noted, furthermore, that the data cover the very long time interval of 150 min. In addition, the fit was found to be good at two different wavelengths—365 and 435 nm. There can be little doubt, therefore, that the scattering data do represent dimer formation.

It is usually difficult to obtain good light scattering data on protein solutions when the proteins are relatively small and concentrations are low. Dust and other adventitious scatterers interfere with measurements of absolute scatter. The method that we use here avoids such problems. We were able, therefore, to establish the existence of dimers of low molecular weight proteins.

Finally, we note that there is only a little change, and that only at short wavelengths, in the CD spectrum during the dimerization process (Figure 8).

Discussion

The addition of salt induces an immediate conformational change in histone GRK at all temperatures, and this change is followed by dimer formation. At room temperature both appear to occur together; however, at low temperatures, the events may be separated since, in this case, dimerization takes a long time to occur.

Previous work (Li *et al.* 1972) had suggested that dimers formed, because the anisotropy had a functional dependence on the histone concentration which agreed with dimer equilibrium equations. The present study demonstrates that the dimerization of histone GRK does indeed take place.

We note that, at low temperatures, the anisotropy and CD remain constant during the dimerization process. Therefore, at least at low temperatures, there is very little change in α-helical content or local rigidity of the tyrosine residues during the formation of dimers. We do not know, of course, if this still holds at room temperature.

Combining our new information with previous results (Li *et al.*, 1972; Wickett *et al.*, 1972) we can now summarize the fast step as follows. (1) Upon the addition of salt, histone GRK mo-

[1] The work in Li *et al.* (1972) reported that the slow process could be represented by a single exponential. It may now be seen that this was due to taking all data in that paper at room temperature.

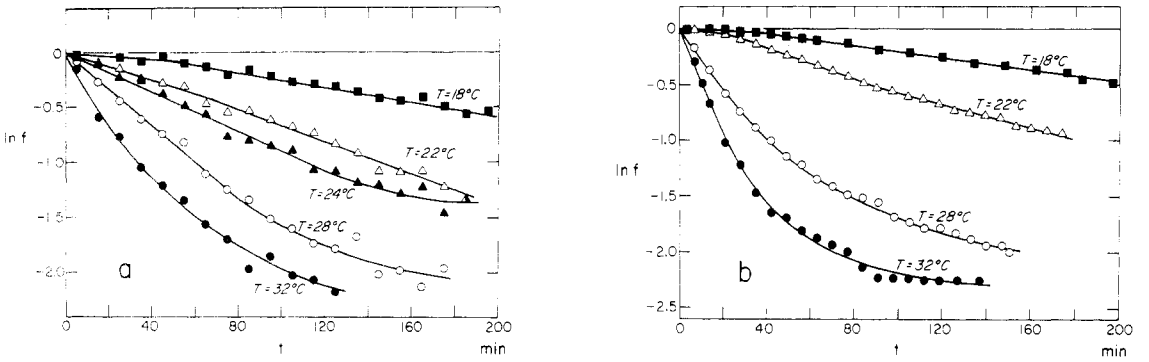

FIGURE 3: First-order plots, *viz.*, $\ln f$ *vs.* t, of (a) fluorescence anisotropy data and (b) CD data. It may be noted that at low temperature the plots are concave downward, and at high temperatures, concave upwards. The changeover occurs at around room temperature.

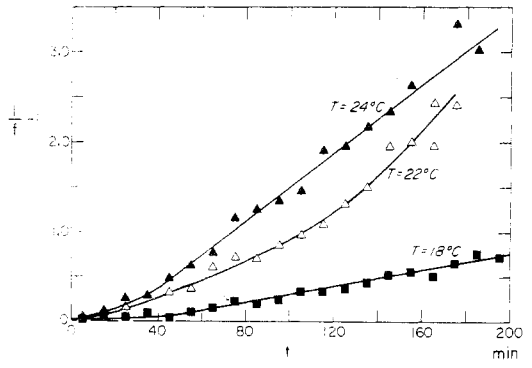

FIGURE 5: Second-order plot, *viz.*, $(1/f) - 1$ *vs.* t, of fluorescence anisotropy data.

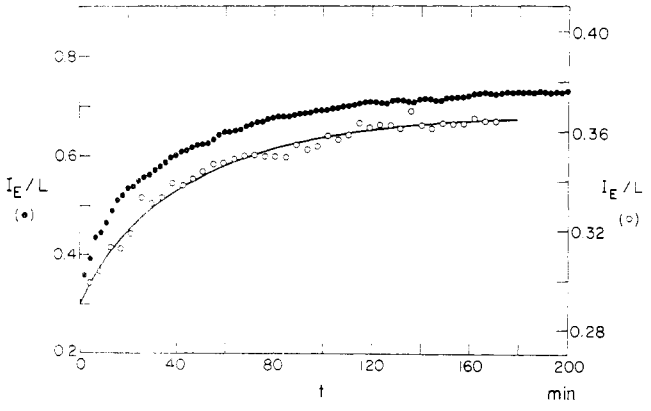

FIGURE 6: Light scattering intensity at 365 nm (●) and 435 nm (○) of 0.8×10^{-5} M histone GRK as a function of time after adding phosphate. Temperature was 2°, pH was 7.4, and final phosphate concentration was 0.007 M.

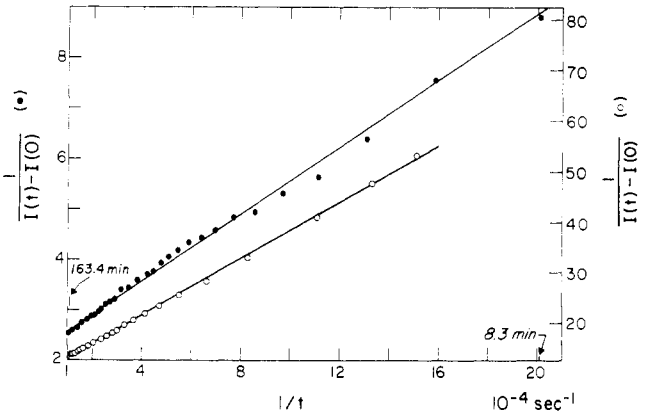

FIGURE 7: Dimerization test plot. The data are from Figure 6: 365 nm (●), 435 nm (○). The 435-nm data are from the fit shown in Figure 6.

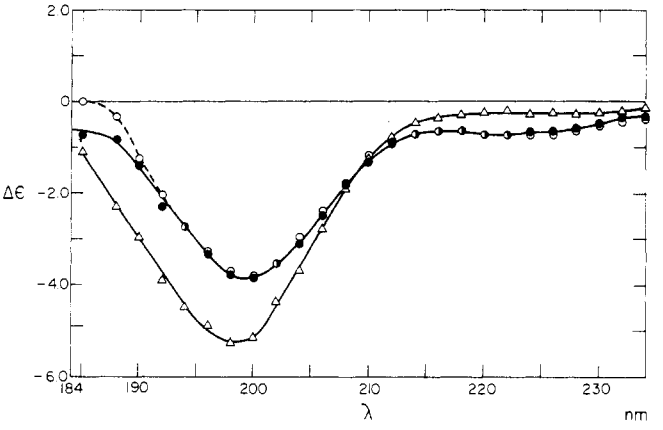

FIGURE 8: CD spectra of 0.8×10^{-5} M histone GRK, at 2°, in water (△) and in 0.007 M phosphate, pH 7.4. The scans of histone GRK in phosphate were from t = 5 to 20 min (●) and t = 180 to 195 min (○). The optical path length was 1 mm.

nomers rapidly go from a random coil to a folded configuration (Li *et al.*, 1972). (2) In this form there are 17–22 α-helical residues (Li *et al.*, 1972).[2] (3) This folding has the properties of the renaturation of a denatured globular protein (Li *et al.*, 1972; Wickett *et al.*, 1972). The folding is not appreciably affected by lowering the temperature. (4) After the folding, the molecules form dimers. (5) The dimerization becomes slower as the temperature is lowered. (6) At least at low temperatures, the dimerization does not affect the rigidity of the tyrosine residues or secondary structure of the molecules.

If the salt concentration exceeds a critical value, different for each anion (Wickett *et al.*, 1972), the fast change is followed by a slow one (Li *et al.*, 1972). It is here that aggregation occurs.

Historically, the aggregation of certain histones has evolved as one of the more intensely studied characteristics of these proteins (Boublik *et al.*, 1970; Cruft *et al.*, 1957, 1958; Davison and Shooter, 1956; Diggle and Peacocke, 1971; Edwards and Shooter, 1969; Johns, 1971; Mauritzen and Stedman, 1959). The pioneering work by Cruft *et al.* (1958) on the aggregation

[2] Of course, the actual number of residues is somewhat uncertain because the calculation depends on the CD standard that is assumed. Our value is based on taking long chain length poly L-lysine as a standard, but, if it is ever found that this standard is inappropriate, the estimate of the number of α-helical residues will need revision.

of "β-histone" (histones GRK and ARE) showed that their ability to aggregate was dependent on such things as salt concentration, pH, and temperature.

Our laboratory has shown that the onset of histone GRK aggregation is associated with the slow step (Smerdon and Isenberg, 1973). We have also shown that the final products of the aggregation process are very large units, comprised of many monomers (Small *et al.*, 1973). From these studies, the ones by Li *et al.* (1972), Wickett *et al.* (1972), Li and Isenberg (1972), and the present communication, we may now list the following basic physical properties which allow us to visualize this aggregation phenomenon in a much more precise way. (1) For a given histone GRK concentration, a critical salt concentration is needed for slow step formation (Wickett *et al.*, 1972). (2) Urea shifts the critical salt concentration to higher values (Li and Isenberg, 1972). (3) During the slow step, the onset of aggregation occurs (Smerdon and Isenberg, 1973). (4) The aggregation is slow, indicating a large activation energy, probably involving unfolding of previously existing structures. (5) The kinetic processes associated with the slow step are not simple and the rate-limiting step involves complex intermolecular interactions. (6) A large amount of β-sheet formation occurs during the slow step (Li *et al.*, 1972). (7) The final products that are formed are large units, containing many monomers (Small *et al.*, 1973). (8) The aggregation process is highly sensitive to temperature and can be stopped at sufficiently low temperatures.

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Appendix

For dimerization

$$2H \underset{k'}{\overset{k}{\rightleftharpoons}} H_2$$

with the dissociation constant $K = k'/k$.

If n is the number of moles of monomer per unit volume at time t and n_0 is the total number, then

$$\frac{dn}{dt} = -kn^2 + \frac{kK}{2}(n_0 - n)$$

This integrates to

$$n = n_0\left(\frac{(K/4) + a\coth kat}{n_0 + (K/4) + a\coth kat}\right) \qquad (1)$$

where $a^2 = (K/4)[2n_0 + (K/4)]$.

From scattering theory

$$I(t) = A'\sum_i n_i M_i^2$$

where $I(t)$ is the scattering intensity at time t, M_i is the molecular weight of the ith component, and A' is a constant for the solution.

Therefore, for dimer formation,

$$I(t) = A(2n_0 - n)$$

where $A = A' \times$ (molecular weight of the monomer), or

$$I(t) - I(0) = A(n_0 - n)$$

From eq 1, after algebraic manipulation, we find that

$$\frac{1}{I(t) - I(0)} = \left(\frac{\alpha}{t} + \beta\right)[1 + \delta(K, t)]$$

where $\alpha = 1/kAn_0^2$, $\beta = 1/An_0$, and

$$\delta(K, t) = \frac{kt(K/4 + a\coth kat) - 1}{1 + kn_0 t}$$

For short times

$$\delta \longrightarrow kKt/4$$

δ is monotonic in t and, for large times, approaches its maximum value

$$\delta_{max} = \frac{1}{n_0}\left(\frac{K}{4} + a\right)$$

For strong binding, $K \ll n_0$, and

$$\delta_{max} = \sqrt{\frac{K}{2n_0}}$$

Therefore, for all times $\delta(K,t) \ll 1$.

Thus, for a dimerization process, with $K \ll n_0$

$$\frac{1}{I(t) - I(0)} = \frac{\alpha}{t} + \beta$$

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