Histone Redistribution and Conformational Effect on Chromatin Induced by Formaldehyde†

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ABSTRACT: Histone redistributions between endogenous DNA in calf thymus chromatin and exogenous DNA from *Clostridium perfringens* (69% A + T) or from *Micrococcus luteus* (30% A + T) induced by 0.6 M NaCl or by 2% formaldehyde were studied by thermal denaturation. The observed redistribution occurred on histone H1 when the exogenous DNA was (A + T)-richer than the DNA in chromatin, and when the mixture was exposed to 0.6 M NaCl or formaldehyde. When a (G + C)-richer DNA was added as the acceptor for histones, no substantial transfer of histones from chromatin DNA to exogenous DNA was found. Thus the activation energy of histone dissociation from chromatin DNA seems to be substantially lowered by 0.6 M NaCl or formaldehyde such that histones (mostly histone H1) can be dissociated and bind the (A + T)-richer DNA and form a more stable complex. It is suggested that the formaldehyde effect on histones may be due to the loss of positive charges on lysine and arginin residues (probably more on lysine than on arginine) in histones after their rapid reaction with formaldehyde. Formaldehyde treatment of chromatin also distorts the DNA conformation, as revealed by circular dichroism (CD) studies. This structural effect occurs mainly on those base pairs bound by histones other than H1, or within the chromatin subunit. Histone redistribution is treated as a thermodynamic phenomenon of histone binding to DNA. The validity of using formaldehyde to study chromatin structure is discussed.

Histone–DNA interaction has been examined on both native and salt-treated chromatin (Ohlenbusch et al., 1967; Paoletti and Huang, 1969; Li and Bonner, 1971; Smart and Bonner, 1971, Johnson et al., 1972). It is generally assumed that, at certain salt concentration, some histones are dissociated from chromatin and removed by centrifugation, while the rest of the histones remain at their original sites in the partially dehistonized chromatin. Based upon this assumption, the physical properties obtained from partially dehistonized chromatin have been extrapolated to those in native chromatin. Recently Varshavsky and Ilyin (1974) raised a question about this assumption. Using the formaldehyde fixation technique, these authors concluded that histones which remained in chromatin after 0.6 M NaCl treatment were dissociated from the original chromatin and bound to externally added (exogenous) DNA molecules. Using this same technique, Varshavsky et al. (1974) studied distribution of histones in chromatin and suggested clusters of histone H1 (I or f1).

Fixation of histones to DNA by formaldehyde or glutaraldehyde has been utilized for the investigation of histone–DNA interaction in chromatin (Brutlag et al., 1969; Li, 1972), chromatin structure (Olins and Olins, 1974; Griffith, 1975; Oudet et al., 1975; Finch et al., 1975), and histone–histone cross linkage (Hyde and Walker, 1975; Chalkley and Hunter, 1975; Weintraub et al., 1975; Thomas and Kornberg, 1975). Because of the importance of redistribution of histone during preparation of salt-treated chromatin and of the use of formaldehyde fixation technique in the study of chromosomal biology, we have investigated redistribution of histones among DNA molecules in the absence or the presence of 0.6 M NaCl, and with or without formaldehyde fixation using thermal denaturation and circular dichroism (CD[1]). Our results suggest an induction of redistribution of histones among DNA molecules during formaldehyde fixation. Such redistribution is more favored if an (A + T)-rich DNA is added. The same fixation is also shown to induce a substantial alteration in the DNA structure of chromatin subunits.

Materials and Methods

Calf thymus chromatin which was prepared as described by Simpson (1974) was sheared briefly in 0.01 M Tris, pH 8.0. To obtain histone H1-depleted chromatin, the chromatin with A_{260} ca. 10 was dialyzed against 0.6 M NaCl in 0.01 Tris, pH 8.0, at 2 °C for 2 h (Ohlenbusch et al., 1967). The sample was loaded on a Bio-Gel A-50m column (2.5 × 85 cm) equilibrated with 0.6 M NaCl at 2 °C. The column was eluted with 0.6 M NaCl, 0.01 M Tris, pH 8.0. The elution pattern was similar to that of Varshavsky and Ilyin (1974) in that the first peak was histone H1-depleted chromatin, followed by a second peak of dissociated histone H1 and nonhistone proteins. Both chromatin and H1-depleted chromatin were dialyzed against 0.25 mM EDTA, pH 8.0 (EDTA buffer).

DNA from *Cl. perfringens* and *M. luteus* was purchased from Worthington Biochemical Corp. and Miles Laboratories, respectively; each was dissolved and dialyzed against EDTA buffer. A mixture of exogenous DNA and chromatin, or histone H1-depleted chromatin with an input ratio of 0.5 or 1.0 (exogenous DNA/endogenous DNA), was prepared in EDTA buffer. A molar absorption coefficient at 260 nm of 6500 M^{-1} cm^{-1} was used for calf thymus DNA in chromatin, 7400 for *Cl. perfringens* DNA and 7000 for *M. luteus* DNA (Felsenfeld and Hirschman, 1965). An aliquot of the above mixture was dialyzed against 0.6 M NaCl in EDTA buffer at 2 °C for 5 h

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[1] Abbreviations used: CD, circular dichroism; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; tRNA, transfer ribonucleic acid.

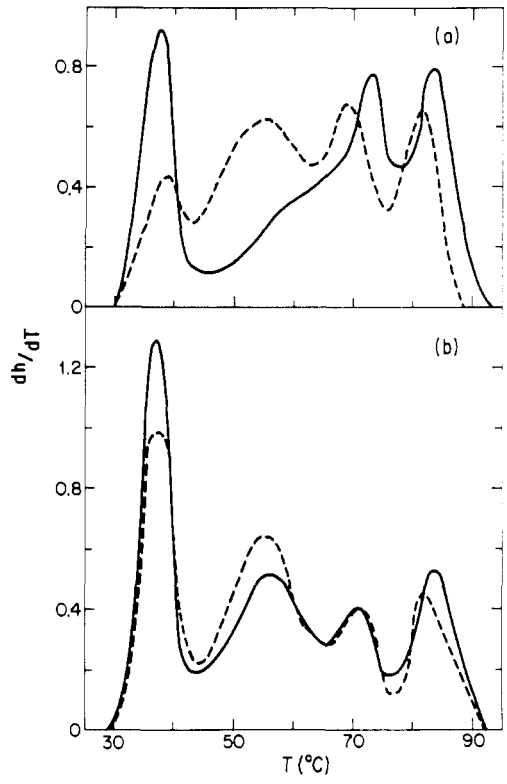

FIGURE 1: Derivative melting profiles of a mixture of *Cl. perfringens* DNA and calf thymus chromatin (a) or H1-depleted chromatin (b). Mixed and melted in EDTA buffer (—); mixed in EDTA, dialyzed to 0.6 M NaCl for 5 h and back to EDTA (- - -). The input ratio of *Cl. perfringens* DNA to chromatin DNA is 1.0.

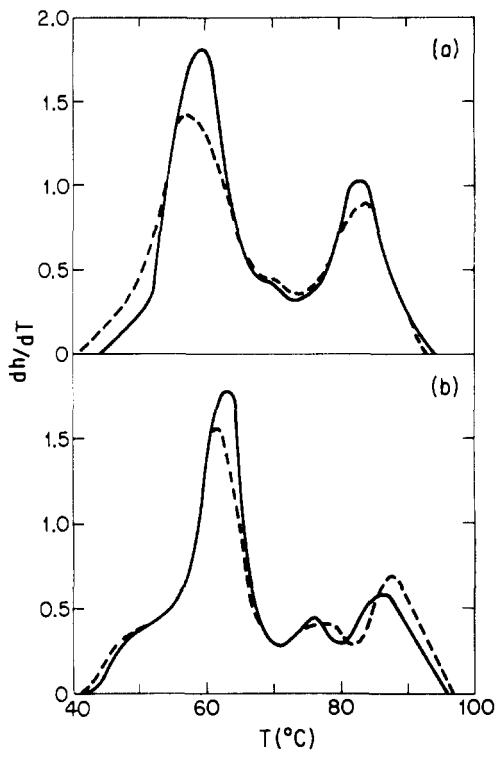

FIGURE 2: Derivative melting profiles of a mixture of *M. luteus* DNA and calf thymus chromatin (a) or H1-depleted chromatin (b). Mixed and melted in EDTA buffer (—); mixed in EDTA, dialyzed to 0.6 M NaCl for 5 h and back to EDTA (- - -). The input ratio of *M. luteus* DNA to chromatin DNA is 1.0.

and then extensively dialyzed back to EDTA buffer without salt. Another aliquot of the above mixture was dialyzed against 5 mM triethanolamine, pH 7.8, and fixed with formaldehyde (Fischer Scientific Co.) according to the method of Varshavsky and Ilyin (1974). These formaldehyde fixed samples were removed after 1 or 24 h of reaction and dialyzed against EDTA buffer.

Thermal denaturation of the samples was measured on a Gilford spectrophotometer Model 2400-S. The percent increase in hyperchromicity (h) was measured on a spectropolarimeter Model J-20 at room temperature. The CD results are reported as $\Delta\epsilon = \epsilon_l - \epsilon_R$ where ϵ_L and ϵ_R are, respectively, the molar absorption coefficients for the left- and the right-handed circularly polarized light. The units of $\Delta\epsilon$ are M^{-1} cm^{-1}, where M is moles/liter in nucleotide. Both melting and CD measurements were made in 0.25 mM EDTA, pH 8.0.

Results

NaCl (0.6 M) Induced Histone Redistribution. The mixture of *Cl. perfringens* DNA and calf thymus chromatin in EDTA buffer shows three major melting bands (Figure 1a): 38 °C for *Cl. perfringens* DNA, and 73 and 83 °C for histone-bound DNA in calf thymus chromatin (Li et al., 1973). There is only about 30% of the melting area under the band at 38 °C despite a 1:1 ratio of *Cl. perfringens* DNA to chromatin DNA. This could possibly be due to a lower hyperchromicity of the former (20–25%) than the latter (about 33%). If this mixture was exposed to 0.6 M NaCl for 5 h before it was dialyzed back to EDTA buffer, the melting pattern was greatly changed (Figure 1a). The melting band of 38 °C is reduced with a new enhanced band at 55 °C. The melting bands at 73 and 83 °C are both shifted to lower temperature by 2 to 4 °C. These results could be explained as a shift of a substantial amount of histone in chromatin from endogenous DNA to exogenous DNA: (a) a removal of some histone from endogenous DNA would increase the amount of free regions in calf thymus chromatin and an increase of its melting band around 50 °C; (b) a binding of histones to exogenous DNA would reduce its free DNA regions and melting band at 38 °C; (c) the binding of histones to (A + T)-richer DNA is expected to show a reduced melting temperature in histone-bound regions such as those around 70 and 80 °C.

The transfer of histones from chromatin to exogenous DNA is greatly reduced if 0.6 M NaCl-treated chromatin, from which histone H1 already has been removed (Ohlenbusch, 1967), is used (Figure 1b), indicating that the histones which are shifted from chromatin to exogenous DNA at 0.6 M NaCl, (Figure 1a) are mainly histone H1. This is not unexpected since this histone is dissociated from chromatin at this salt concentration. The transfer of additional histones to exogenous DNA after histone H1 removal (Figure 1b) may suggest the release of a small portion of other histones (probably slightly lysine-rich histones H2A and H2B) from endogenous DNA as more exogenous free DNA becomes available for binding during a 5-h period. Considerations of both kinetic and equilibrium binding of histone to DNA are in favor of this suggestion.

Although the competition for histone binding at 0.6 M NaCl between endogenous DNA from calf thymus (58% A + T) and exogenous DNA from *Cl. perfringens* (69% A + T) favors the latter, this trend becomes less apparent if the exogenous DNA is a (G + C)-richer DNA, such as that from *M. luteus* (30% A + T). Figures 2a and 2b show results of such a competition. When native chromatin is used, some free DNA regions in calf thymus chromatin are generated, as shown by a slight increase

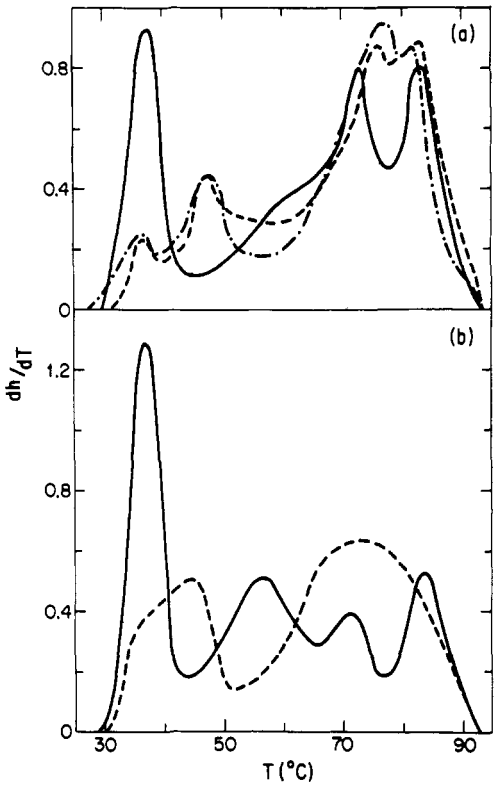

FIGURE 3: Derivative melting profiles of a mixture of *Cl. perfringens* DNA and calf thymus chromatin (a) or H1-depleted chromatin (b) treated by formaldehyde. Control (without formaldehyde treatment) (—), treated by formaldehyde for 1 h (— · —) or 24 h (- - -). The input ratio of *Cl. perfringens* DNA to chromatin DNA is 1.0.

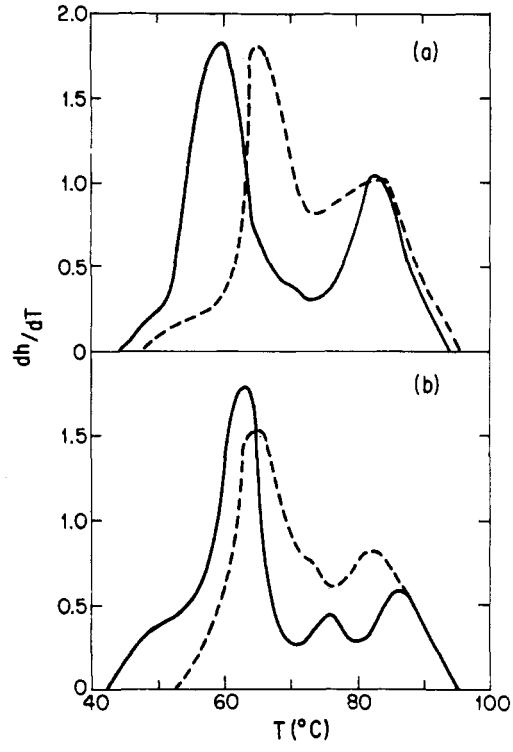

FIGURE 4: Derivative melting profiles of a mixture of *M. luteus* DNA and calf thymus chromatin (a) or H1-depleted chromatin (b) treated by formaldehyde. Control (without formaldehyde treatment) (—), treated by formaldehyde for 24 h (- - -). The input ratio of *Cl. perfringens* DNA to chromatin DNA is 1.0.

of melting around 50 °C and a slight decrease at 60 °C. Such a shift is much smaller in Figure 2a than in Figure 1a. Similar results were observed if 0.6 M NaCl-treated chromatin was used (Figure 2b). Consequently the problem can be treated in terms of thermodynamics as described below:

$$D_{ex} + D_{en}\text{-}H \rightleftarrows D_{ex}\text{-}H + D_{en} \qquad (1)$$

where D_{ex} and D_{en} represent, respectively, the exogenous and endogenous DNA, while H is the histone involved in the transfer. The above equilibrium will be shifted to the right by (a) increasing the NaCl concentration, (b) increasing the concentration of exogenous DNA, and (c) increasing its A + T content.

Formaldehyde-Induced Histone Redistribution. If a mixture of *Cl. perfringens* DNA and chromatin is exposed to formaldehyde either for 1 or 24 h, the melting curve is very different from that of the control (Figure 3a). The 38 °C band is reduced by 70–80%, while at the same time, a new, distinct band appears at 48 °C. Concurrently, the resolution of the two melting bands at high temperature is reduced, which is in agreement with the earlier report (Li, 1972). These results indicate the induction of a shift of histone from calf thymus DNA in chromatin to *Cl. perfringens* DNA in the presence of formaldehyde for 24 h or even for as short a time as 1 h.

If histone H1 is selectively removed first by 0.6 M NaCl and then treated with formaldehyde, the melting curve can be differentiated into two main regions, a lower one at 35–45 °C and a higher one at 60–90 °C. The lower melting regions presumably represent the *Cl. perfringens* DNA not bound by histones after formaldehyde treatment and some free regions in calf thymus chromatin. Calf thymus DNA alone, after formaldehyde treatment, has a melting band at 50 °C, while *Cl. perfringens* DNA alone has a peak at 38 °C and a shoulder at 45 °C. The results in Figure 3 are parallel to those of Figure 1. They suggest that histone H1 can be dissociated from chromatin DNA by 0.6 M NaCl or by formaldehyde, when an (A + T)-richer DNA (*Cl. perfringens*) is used as the competitor for binding. Under similar conditions other histones (probably slightly lysine-rich histones) can also be transferred but to a much lesser extent.

Figure 4 shows results of experiments similar to those of Figure 3, except that *M. luteus* DNA with 30% A + T was used. When mixed with either chromatin or histone H1-depleted chromatin from calf thymus, formaldehyde treatment shifts the lower melting band to higher temperature, from 60–63 to 65 °C. After formaldehyde treatment, *M. luteus* alone also shows a melting band at 65 °C. Figure 4 shows no melting at 50 °C comparable to that of free calf thymus DNA. Consequently, one can conclude from Figure 4 that, although additional free DNA from *M. luteus* is added to chromatin solution for histone binding, the lower A + T content of this exogenous DNA prevents it from offering competition to the endogenous DNA of the chromatin. Figure 4b shows a shift of melting of the 50 °C shoulder in H1-depleted to higher temperature after formaldehyde fixation. A similar shift was observed when H1-depleted chromatin was fixed by formaldehyde in the absence of exogenous DNA (Li, 1972).

Circular Dichroism of Formaldehyde-Treated Chromatin. Figure 5a shows CD spectrum of native chromatin and that of chromatin treated with formaldehyde either for 1 or 24 h. Formaldehyde treatment causes a permanent decrease and red shift of the positive CD band near 275 nm and a slight decrease in the negative CD band near 220 nm, changes which occur mostly during the first hour of fixation. These results confirm the original observations of Senior and Olins (1975). If $\Delta\epsilon_b$ is

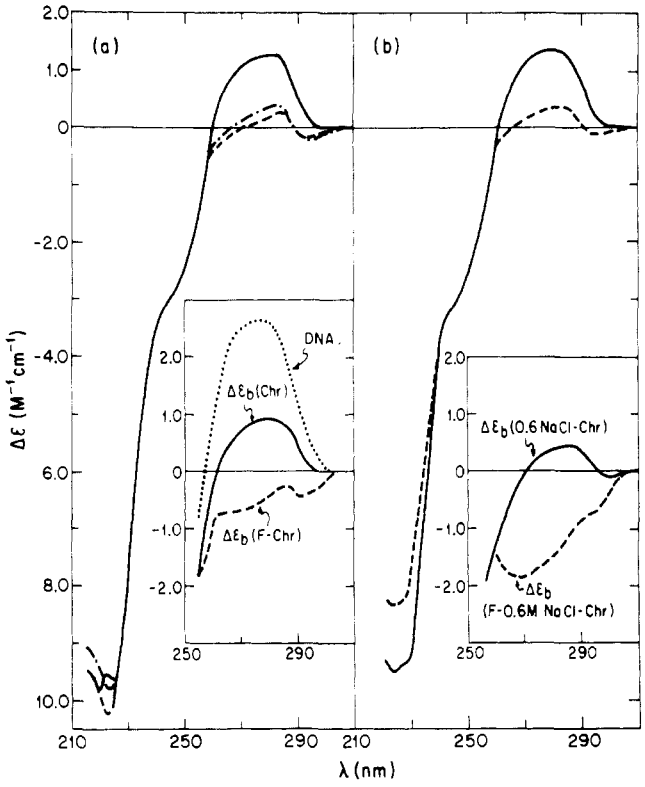

FIGURE 5: Circular dichroism spectra of chromatin (a) and H1-depleted chromatin (b) fixed by formaldehyde. Control (without fixation) (—); treated by formaldehyde for 1 hr (— · —) or for 24 h (- - -). Calculated CD ($\Delta\epsilon_b$) of histone-bound DNA without fixation (—) or after 24 h of fixation (- - -) (inserted).

considered to be the average CD of DNA bound by all histones (including H1), the following equation can be used for the calculation of $\Delta\epsilon_b$ above 250 nm in chromatin and formaldehyde-treated chromatin (Chang et al., 1973):

$$\Delta\epsilon = (1 - F)\Delta\epsilon_f + F\Delta\epsilon_b \quad (2)$$

where F is the fraction of DNA base pairs bound by all histones and $\Delta\epsilon_f$ is the CD of histone-free DNA base pairs, based upon the assumption that $\Delta\epsilon_f$ is the same as that of pure DNA in both formaldehyde-treated or untreated chromatin. This assumption is not unreasonable, because pure DNA does not show changes in its CD spectrum after formaldehyde treatment (Senior and Olins, 1975). Since it was estimated that, as an average, about 80% of DNA base pairs in calf thymus chromatin are bound by histones (Li et al., 1973), $F \approx 0.8$ was used for the calculation. As calculated, $\Delta\epsilon_b$ for untreated chromatin shows a positive band at 280 nm (Figure 5a), while that of treated chromatin shows negative bands around 270 and 290 nm.

Figure 5b shows the CD spectrum of 0.6 M NaCl-treated chromatin before and after formaldehyde fixation. The results are nearly the same as for native chromatin, shown in Figure 5a. It is noted that in 0.6 M NaCl-treated chromatin, histone H1 has already been removed resulting in more histone-free base pairs. The similarity in the CD spectra of these two chromatins before fixation indicates that histone H1 makes little contribution to changes in the DNA CD near 280 nm (Simpson and Sober, 1970; Chang and Li, 1974; Hjelm and Huang, 1974; Wilhelm et al., 1974; Hanlon et al., 1974), and protein CD near 220 nm (Li et al., 1975). Similarity in the CD spectra of these two chromatins after formaldehyde fixation implies two points: (a) the fixation of histone H1 to DNA in chromatin has little effect on DNA conformation and (b) the main conformational effect of formaldehyde fixation occurs in DNA regions bound by whole histones minus H1 or within the chromatin subunit.

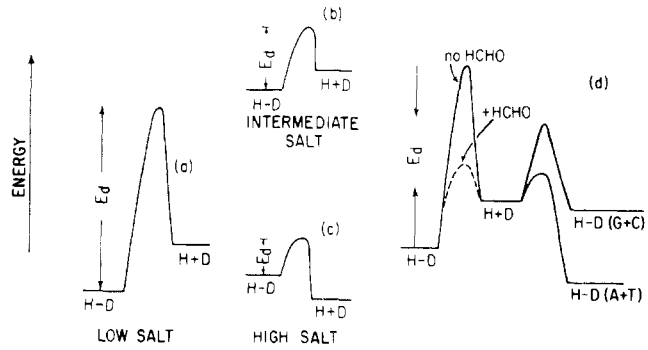

FIGURE 6: Schematic energy diagrams of histone-DNA complexes under various conditions. H is free histone, D free DNA, and H-D associated complex. E_d is the activation energy required for dissociation.

Since the melting curve of 0.6 M NaCl-treated chromatin shows 50–60% DNA base pairs still bound by histones (Li et al., 1973), $F = 0.55$ was used in eq 2 for the calculation of $\Delta\epsilon_b$. For H1-depleted chromatin without formaldehyde fixation, there is a small positive band ($\Delta\epsilon_b = 0.4$) at 285 nm and a small negative band near 300 nm. It should be pointed out that the calculated CD, $\Delta\epsilon_b$, is sensitive to the exact F value chosen and that such F value can only be determined with an accuracy of about 5 to 10%. Our previous calculation of $\Delta\epsilon_b$ for partially dehistonized chromatin showed a variation in amplitude of 0 ± 0.5 between 260 and 290 nm (Li et al., 1975). When a similar equation was used for calculating $\Delta\epsilon_b$ in formaldehyde-fixed H1-depleted chromatin, a negative CD with a peak at about 270 nm and a shoulder at about 290 nm was obtained (Figure 5b). The results in Figures 5a and 5b indicate that, although the CD of formaldehyde-fixed chromatin or H1-depleted chromatin is similar to that of C-type DNA in high salt (Tunis-Schneider and Maestre, 1970), the calculated CD of histone-bound DNA itself is negative. Within chromatin subunits without histone H1, it becomes more negative than when H1-bound regions are also included in the averaging process.

Discussion

Thermodynamic Consideration of Histone Binding to DNA in Chromatin. Although histones are considered to bind irreversibly to DNA in chromatin at physiological and low ionic strength, strictly speaking, this binding is actually reversible but has a high energy barrier which prevents dissociation (Figure 6a). Thermodynamically histone association with DNA in chromatin can be described by

$$H\text{-}D \rightleftarrows H + D \quad (3)$$

where H-D is the complex, and H and D represent dissociated histone and DNA, respectively. It should be borne in mind that differences in binding affinity do exist among the various species of histones and base sequences along the DNA and, though our discussion is a general one, it can be applied to each individual case.

At low salt (Figure 6a), the activation energy for dissociation (E_d) could be so high that the probability for a histone to dissociate from the DNA is extremely low. Therefore, practically, one can consider the binding to be irreversible. However, an increase of ionic strength is expected to weaken the binding

strength and reduce the activation energy E_d; under these circumstances, some histone molecules (lysine-rich histone H1, for example) can climb over the energy barrier and be dissociated (Figure 6b). At high salt, the dissociated state (H + D) is more stable than the associated state (H-D) so that histones are dissociated from DNA (Figure 6c).

Previously, it was shown that lysine-rich histone H5 favors (A + T)-rich DNA for binding (Hwan et al., 1975). The results in this report seem to indicate that histone H1 exhibits the same preference and probably also the slightly lysine-rich histones H2A and H2B. For these histones, Figure 6d shows an energy diagram for describing competition among DNAs for histone binding. In a complexed state, (A + T)-rich H-D is more stable than endogenous H-D, which in turn is more stable than (G + C)-rich H-D. At very low salt, the transfer of histones from endogenous DNA to a more favorable exogenous (A + T)-rich DNA may not proceed readily because of its high energy barrier E_d. If this energy barrier can be lowered by some means, such as formaldehyde or NaCl, these dissociated histones will bind the available (A + T)-rich DNA and form a more stable complex. If, on the other hand, the exogenous DNA is richer in G + C, compared with the endogenous DNA, the dissociated histones will reassociate more favorably with the endogenous DNA. Thus the results in Figures 1 and 2 can be described as a thermodynamic phenomenon.

The results in Figure 1b indicate a transfer of some histones other than H1 to the exogenous (A + T)-rich DNA in 0.6 M NaCl. The amount of these transferred histones, however, must be greater than what there might have been during preparation of NaCl-treated, partially dehistonized chromatins because of the following reasons: (a) the exogenous DNA in Figure 1 is (A + T)-richer than the endogenous DNA in calf thymus chromatin; it can force more histones (slightly lysine-rich histones, for example) to dissociate from their original binding sites than can the free DNA regions in calf thymus chromatin (see Figure 6 and the above discussion); (b) the ratio of free DNA (or free binding sites) to histone-bound DNA (or occupied binding sites) in the experiments of Figure 1 is a few times greater than that in an ordinary preparation of NaCl-treated chromatin due to the addition of extra free DNA in Figure 1; thermodynamically these additional free binding sites will cause more dissociation. Therefore, it is unlikely that histones, which are supposed to remain in 0.6 M NaCl-treated, H1-depleted chromatin, are dissociated during preparation. Nevertheless, our experiments still cannot exclude the possibility of sliding of histones from the original binding sites to the neighboring free sites.

Histone Redistribution Induced by Formaldehyde. Formaldehyde has been used extensively in the study of nucleic acids (see McGhee and von Hippel, 1975, and references therein) and protein-nucleic acid complexes such as chromatin. In the latter case, Brutlag et al. (1969) first reported covalently linked histone-DNA complexs in chromatin which could not be dissociated by acid or high concentration of CsCl following formaldehyde fixation. This fixation has been applied by many laboratories in the studies of chromatin (see below). Although the report of Brutlag et al. (1969) showed fixation of histones to DNA, these authors did not show that the fixed histones remained at the same locations as those in the native state without formaldehyde treatment. It is necessary to question this assumption since it is critical for the validity of conclusions drawn on formaldehyde-fixed chromatin and it has not been tested by those who applied this technique.

The results in Figures 3 and 4 show that, in fact, relocation of histones is induced in the presence of formaldehyde provided that the exogenous DNA is (A + T)-richer than the endogenous DNA. Such relocation can occur at low salt only if the formaldehyde reaction lowers the energy barrier, E_d, substantially such that, within as little as 1 h of reaction time, some histones (mostly histone H1 and probably some histones H2A and H2B), climb over the barrier, dissociate themselves from endogenous DNA, and bind the exogenous DNA to form a more stable complex. Theoretically, this is very plausible by considering the chemistry involved in formaldehyde fixation.

The formaldehyde reaction in the presence of an amino group can be described by the following two steps (Feldman, 1973)

$$R{-}NH_2 + HCHO \rightleftarrows R{-}\overset{H}{N}{-}CH_2OH \qquad (4)$$

$$R{-}\overset{H}{N}{-}CH_2OH + R'NH_2 \longrightarrow R{-}\overset{H}{N}{-}CH_2{-}\overset{H}{N}{-}R' + H_2O \qquad (5)$$

The first step is rapid and reversible while the second step (methylene bridge formation) is slow and irreversible. Immediately after the chromatin is exposed to formaldehyde, a rapid reaction will occur between the formaldehyde and the amino groups of the histones, which could cause a substantial loss of positive charges in the latter and reduce their binding affinity to DNA. Consequently, a lowering of energy barrier in Figure 6d encourages dissociation of histones as well as other perturbations, so that relocation of histones becomes a possibility before the fixation or methylene bridge formation (a slow step) occurs. The fixation might still proceed when formaldehyde was removed by the dialysis (24-48 h, for example) since the decomposition of methylol derivative is very slow (half time about 60 h for adenine derivative, McGhee and von Hippel, 1975). Therefore, the redistribution of histones H2A, H2B, H3, and H4 between endogenous and exogenous DNA at 0.6 M NaCl and in 2% formaldehyde reported by Varshavsky and Ilyin (1974) could probably be caused by a lowering of energy barrier in the interaction between these histones and endogenous DNA by both ionic strength and formaldehyde.

Distribution of Histone H1 in Chromatin. Both electron microscopy (Bonner and Griffith, 1969) and thermal denaturation (Li, 1972; Li et al., 1974) of histone H1-depleted chromatin suggest a dispersed binding of histone H1 in chromatin. On the other hand, histone H1 clusters in chromatin have been implied by the findings of long stretches of free DNA in histone H1-depleted chromatin after formaldehyde fixation (Varshavsky et al., 1974; Doenecke and McCarthy, 1975a,b), and the formation of polymers of histone H1 by glutaraldehyde fixation of chromatin (Chalkley and Hunter, 1975).

H1-depleted chromatins used by Varshavsky et al. (1974) and Doenecke and McCarthy (1975a,b) were prepared by adding tRNA to chromatin. According to Varshavsky et al. (1974), chromatin treated by tRNA (their DNP_{F1}) lacks histone H1 and most nonhistone proteins. Treatment of these H1-depleted chromatins by formaldehyde fixation might induce relocation of histones, through the sliding of separated histone subunits into clusters (cooperative binding, for example). If such sliding occurs, the long stretches would be an artifact of formaldehyde treatment. If, however, such sliding does not occur, these long stretches still could represent the E regions of chromatin (Li, 1975) which are the DNA stretches bound by nonhistone proteins alone or by nonhistone proteins plus histone H1. They do not necessarily represent the DNA segments bound by clusters of histone H1.

Using purified DNA from *Drosophila* to compete for histone binding with *Drosophila* chromatin in the presence of formaldehyde, Doenecke and McCarthy (1975b) showed a release to exogenous DNA of only about 1% of the total chromosomal proteins from chromatin when the input ratio of DNA to chromatin was 1:10, and no detectable transfer when the input ratio was increased to 1:1. If 5% is considered to be the upper limit of the undetectable shift and if histone H1 is considered to be the protein causing the shift, our calculations suggest a transfer of not more than 25% of histone H1, if the transfer does occur at all. Their results do not disagree with those of this report because, in their experiments, the added DNA had the same A + T content as that in chromatin, which have the same binding affinity for histones. In our experiments, the additional binding sites offered [exogenous (A + T)-rich DNA] have a greater affinity than the endogenous DNA for histone binding. Therefore, consideration of the thermodynamics of histone binding to DNA does help us explain many apparent differences in experimental results.

The formation of polymers of H1 by treating chromatin with glutaraldehyde (Chalkley and Hunter, 1975) could possibly be explained by the preferential dissociation of histone H1 from chromatin DNA when formaldehyde was used. Their results and the observations reported here are compatible with the earlier report of Brutlag et al. (1969) that histone H1 was selectively fixed to chromatin DNA at very low formaldehyde concentration.

Based upon thermal denaturation, Li et al. (1973) concluded that one histone H1 molecule would bind about 30 base pairs which are not bound by other histones. Li et al. (1975) further suggested that histone H1 might not protect DNA against nuclease digestion as well as do other histones. By incorporating the octamer hypothesis of Kornberg (1974), Li (1975) proposed specifically that one histone H1 binds 30–40 base pairs of DNA next to each chromatin subunit with 130–150 base pairs bound by 8 molecules of other histones. These suggestions have been supported by experiments reported recently.

Sollner-Webb and Felsenfeld (1975) and Simpson and Whitlock (1976) reported that, after a brief digestion of chromatin by nuclease, DNA fragments of 185–205 base pairs were obtained which were precursors of other fragments of 140–160 base pairs after limit digestion. Shaw et al. (1976) and Varshavsky et al. (1976) further reported two types of monomers of nuclease-resistant fragments of chromatin: the larger monomers contained 180–200 base pairs of DNA and all histones, while the smaller ones contained 140–170 base pairs with all histones minus H1 or minus H1 + H5. Apparently one histone H1 binds 30–40 base pairs adjacent to the 140–170 base pairs bound by an octamer of other histones.

Effect of Formaldehyde Fixation on Chromatin Structure. Since formaldehyde can cause cross-linking to occur between histones and DNA and between histones themselves, a treatment of chromatin by formaldehyde is expected to effect two modifications in chromatin: induction of permanent binding of histones to DNA and condensation or packing of DNA–histone subunits into a smaller space, in addition to the possible relocation of histones which was discussed earlier.

The beaded structure in chromatin (Olins and Olins, 1974; Griffith, 1975; Oudet et al., 1975; Finch et al., 1975) very likely could be stabilized or even induced by formaldehyde treatment. Oudet et al. (1975) showed a 25% reduction in the diameter of beads when the chromatin was treated by formaldehyde. Carlson et al. (1975) showed that the disappearance of ν bodies observed in 5 M urea did not occur if chromatin was fixed in formaldehyde before adding the 5 M urea. These results support the argument (Li et al., 1975) that the beaded (or condensed) structure of chromatin subunits might be induced by such treatment. Figure 5 shows that the main CD effect occurs in fixation of histones other than H1 to DNA in chromatin subunits. The calculated CD of histone-bound DNA in H1-depleted chromatin is greatly distorted by formaldehyde fixation (Figure 5b). Therefore, the accumulated data seem to support the viewpoint that the condensed bead is likely to be induced by formaldehyde. Nevertheless, due to the great stimulation given to research on chromatin structure by the report of ν bodies (Olins and Olins, 1974), it was suggested (Li, 1976) that the following experiments be undertaken to clarify the issue of beaded structure in chromatin: (a) preparation of chromatin *without any fixation*; (b) examination of a large number of chromatin molecules (several hundred, for example) under the electron microscope; (c) measurement of the total length of DNA (in terms of base pairs) in the regions showing regular beads and those without; and (d) calculation of the probability of observing regular beads along the DNA molecule in a chromatin. Since about 80% of total DNA base pairs are covered by histones (Li et al., 1973), one might expect to obtain a probability as high as 0.8 if the beaded structure is indeed the one in a native chromatin.

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Steady State Kinetic Analysis of the Mechanism of Guanosine Triphosphate Hydrolysis Catalyzed by *Escherichia coli* Elongation Factor G and the Ribosome†

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ABSTRACT: The mechanism of guanosine triphosphate (GTP) hydrolysis catalyzed by elongation factor G and the ribosome in the absence of other participants in protein synthesis was examined by steady-state kinetic analysis. Optimal hydrolytic conditions were determined to be approximately pH 8.0, 20 mM Mg^{2+}, and 80 mM NH_4^+. Kinetic analyses were performed under these conditions at constant elongation factor G concentrations and variable ribosome and GTP concentrations. The resulting double-reciprocal plots in conjunction with the inhibition patterns obtained with GDP indicated that the reaction occurs by an ordered mechanism in which GTP is the leading obligatory substrate. Dissociation constants for GTP and guanosine diphosphate (GDP), as well as limiting Michaelis constants for GTP and ribosomes, were calculated from the double-reciprocal plots. These values are: K_s^{GTP} = 37.0 μM, K_s^{GDP} = 16.5 μM, K_M^{GTP} = 8.0 μM, K_M^R = 0.22 μM. Inhibition was also observed at high ribosomal concentrations and suggests that inhibition was due both to the decreased breakdown of the tertiary elongation factor G·GDP·ribosome posthydrolytic complex and to the formation of a nonproductive elongation factor G·ribosome complex. A sequential mechanism with a dead-end elongation factor G·ribosome complex has been constructed to describe the hydrolysis of GTP catalyzed by elongation factor G and the ribosome.

Since its discovery (Nishizuka and Lipmann, 1966), the uncoupled GTPase[1] reaction catalyzed by elongation factor G (EF-G) and the ribosome has served as a convenient model system for the examination of the mechanism of EF-G action. The failure to detect any binary complexes or the postulated tertiary EF-G·ribosome·GTP Michaelis complex has confined the majority of the investigations of this mechanism to events occurring after the formation of the tertiary complex. These studies have shown that a relatively stable posthydrolytic EF-G·ribosome·GDP complex is formed, indicating that release of P_i precedes the release of GDP (Brot et al., 1969; Parmeggiani and Gottschalk, 1969). It has also been shown that the hydrolytic step in the mechanism is irreversible and that cleavage occurs between the γ-phosphorus atom and the oxygen bridging the β- and γ-phosphorus atoms (Rohrbach et al., 1974).

Early kinetic analyses of the reaction employed a single fixed

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[1] Abbreviations used are: EF-G, elongation factor G; GTPase, guanosine triphosphatase; GTP, GDP, guanosine tri- and diphosphates; P_i, inorganic phosphate; in denoting equilibrium constants, the superscript R refers to ribosome.