Circular Dichroism and Thermal Denaturation Studies of Nucleohistone IIb2†

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ABSTRACT: Nucleohistone IIb2 (histone IIb2–DNA complex) has been studied by circular dichroism and thermal denaturation. The complex shows strong light scattering which depends upon the input ratio of histone to DNA. Each complex can be separated into two fractions by centrifugation, the pellet and the supernatant. The pellet has much more and the supernatant has much less histone binding than the original complex. The melting profiles of nucleohistone IIb2 can be distinguished into three melting regions, melting band I (about 50°) corresponding to free DNA regions and melting bands II (about 65°) and III (about 88°) to histone-bound regions. Qualitatively they are similar to those of nucleohistone II (IIb1 + IIb2) and chromatin. Quantitatively they are different. The circular dichroism (CD) of nucleohistone IIb2 shows red shifts for λ_{max} of the positive band near 275 nm and λ_c of the crossover near 255 nm and a reduction of the amplitude of the 275-nm band which resemble the CD changes in chromatin.

Histone–DNA interaction is important because of its relation to chromosome structure and gene regulation in higher organisms. Circular dichroism (CD) (Olins, 1969; Fasman *et al.*, 1970, 1971; Shih and Fasman, 1970, 1971, 1972; Shih and Lake, 1972: Simpson and Sober, 1970; Permogorov *et al.*, 1970; Li *et al.*, 1971; Wagner and Vandegrift, 1972; Johnson *et al.*, 1972; Sponar and Fric, 1972) and thermal denaturation (Olins, 1969; Shih and Bonner, 1970; Li and Bonner, 1971; Ansevin and Brown, 1971; Ansevin *et al.*, 1971; Li, 1972, 1973: Li *et al.*, 1972, 1973) have been used for studying this interaction.

In native chromatin, histone binding results in changes of the CD of DNA such as red shifts of both the positive band near 275 nm and the crossover near 255 nm. The amplitude of the positive band is also reduced (Shih and Fasman, 1970; Simpson and Sober, 1970; Permogorov *et al.*, 1970). Thermal denaturation of chromatin shows two melting bands corresponding to histone binding (Li and Bonner, 1971; Ansevin *et al.*, 1971; Li, 1972; Li *et al.*, 1973). This phenomenon has also been observed for reconstituted nucleohistone II (Li and Bonner, 1971).

This communication presents the results of both thermal denaturation and CD studies of reconstituted nucleohistone IIb2. To some extent the binding of this histone to DNA is cooperative and the complexes show strong light scattering. Qualitatively the CD changes of DNA resulting from the binding of histone IIb2 are similar to those in chromatin. Thermal denaturation of reconstituted nucleohistone IIb2 shows some similarity to that of chromatin or reconstituted nucleohistone II (IIb1 + IIb2) (Li and Bonner, 1971; Li *et al.*, 1973). However, there are significant differences which will be discussed later.

Materials and Methods

Whole histone was extracted from calf thymus chromatin as previously described (Shih and Bonner, 1969). Crude histone II was obtained from the whole histone by Amberlite CG-50 chromatography as described by Bonner *et al.* (1968). Histone IIb2 was further purified from crude histone II by CM-cellulose chromatography as described by Senshu and Iwai (1970). It was electrophoretically pure and had the same electrophoretic mobility as the major band of histone IIb (Bonner *et al.*, 1968).

In order to test the possibility of formyl ester formation on serine and threonine with the hydroxy group on the side chains, unfractionated histone IIb (6 mM amino acid residue) was incubated with 2.6 M formic acid, 0.1 M sodium formate, and 50% ethanol at room temperature for 65 hr. The condition was the same as that of CM-cellulose chromatography. Formic acid was labeled by adding a tiny amount of highly ^{14}C-labeled sodium formate (35 Ci/mol Mallinckrodt) to a final specific radioactivity of 4.8×10^4 cpm/μmol of formic acid. After incubation the mixture was dialyzed against water and lyophilized. It was then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (0.6×10 cm of 15% gel) in 1% sodium dodecyl sulfate, 0.04 M Tris, 0.02 M acetate, 2 mM EDTA (pH 7.4). The gel was sliced into 20 equal fractions and digested with 1.5 ml of alkaline Protosol (New England Nuclear) to release the radioactivity from the gel. The radioactivity was counted in a toluene base medium with a Beckman liquid scintillation counter.

Calf thymus DNA was purchased from Sigma Chemical Co. and was purified by phenol extraction. Nucleohistone IIb2 was prepared by continuous salt gradient dialysis (2.0 M to about 0.1 M NaCl) in the presence of 5 M urea–0.02 M Tris (pH 8.0) over a period of 24 hr or longer. Urea was then continuously dialyzed out in the presence of 0.015 M NaCl–0.01 M Tris (pH 8.0) and the mixture was finally dialyzed against 2.5×10^{-4} M EDTA (pH 8.0) for CD and thermal denaturation measurements. The purpose for using this buffer with low ionic strength is that melting bands of free and histone-bound base pairs in nucleohistone can be well separated (Shih and Bonner, 1970; Li and Bonner, 1971).

A portion of each complex was centrifuged at 10,000 rpm in a Sorvall SS-34 rotor at 4° for 30 min. The supernatant was collected and the pellet was dissolved in EDTA buffer. Glass centrifuge tubes were used.

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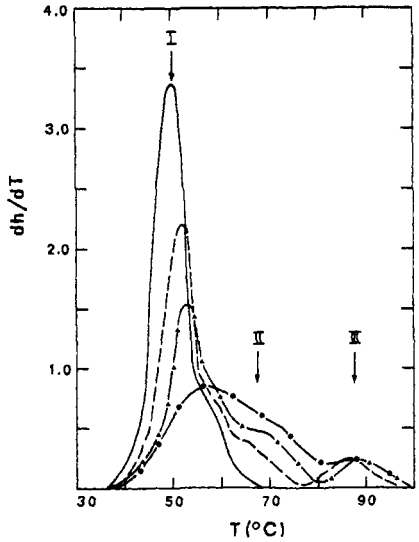

FIGURE 1: Derivative melting profiles of nucleohistone IIb2. B = 0(—), 1.0(- - -), 1.5(—▲—), and 3.0(—●—).

A molar extinction coefficient of 4.7×10^2 M^{-1} cm^{-1} in amino acid residue at 230 nm is used for histone in water (Ohlenbusch *et al.*, 1967) which is close to 4.3×10^2 M^{-1} cm^{-1} used by D'Anna and Isenberg (1972). After the concentration is determined in water it is diluted into 2.0 M NaCl–5 M urea–0.01 M Tris (pH 8.0) for complex formation. Molar extinction coefficient of 6500 M^{-1} cm^{-1} at 260 nm is used for DNA. Difficulty was found for determining DNA concentration in the complexes because of strong light scattering. We tried several methods such as making up the complexes into 2.0 M NaCl–5 M urea in EDTA buffer, boiling the complexes in 0.5 N $HClO_4$ (Shih and Fasman, 1971), or using 0.1% sodium dodecyl sulfate and 0.15 M NaCl (Shih and Lake, 1972). In each case the ratio of A_{320}/A_{260} of the complex has been reduced but not to a negligible level. Correction of light scattering was still needed. The complexes were therefore dissolved in 0.1% sodium dodecyl sulfate and 0.15 M NaCl and the contribution of light scattering to the absorbance at 260 nm was corrected according to Leach and Scheraga (1960).

Thermal denaturation measurements were made on a Gilford spectrophotometer, Model 2400-S. Hyperchromicity, h, is defined as per cent increase in absorbance at 260 nm. h_{max} is the maximum hyperchromicity when the melting is finished at about 100°. The derivative plots, dh/dT, of the melting curves are reported as we did before (Shih and Bonner, 1970; Li and Bonner, 1971). CD measurements were made on a Durrum-Jasco spectropolarimeter, Model J-20, at room temperature. $\Delta\epsilon = \epsilon_L - \epsilon_R$ is reported where ϵ_L and ϵ_R are respectively molar extinction coefficients of the left- and the right-handed circularly polarized light. Cells with 1 cm path length were used and A_{260} of the samples were always below 1.0.

Results

Thermal Denaturation of Nucleohistone IIb2. Figure 1 shows the derivative plots of the melting profiles of nucleohistone IIb2 of varied B values, the input ratios of histone to DNA reported as amino acid residues per nucleotide. Three melting regions are assigned. Melting band I (about 50°) corresponds to the melting of free DNA regions while melting band II (about 65°) and III (about 88°) to that of histone-bound regions on DNA. The separation of melting bands I and II is not large and the latter does not appear as a distinct peak. However, the increase of melting amplitude near 65° at higher ratios of histone to DNA allows us to assign it as a separate band.

TABLE I: Hyperchromicities and Turbidities of Nucleohistone IIb2.[b]

Complexes	B (Amino Acid/ Nucleotide)	h_{max} (%)	A_{320}/A_{260} 30°	A_{320}/A_{260} 100°
Originals	0	36.5	0.027	0.017
	0.5	35.7	0.058	0.041
	1.0	31.0	0.140	0.114
	1.5	29.0	0.208	0.191
	2.0	24.2	0.290	0.270
	3.0	24.4	0.368	0.372
Pellets[a]	0.5	–	–	–
	1.0	19.7	0.400	0.370
	1.5	18.7	0.413	0.400
	2.0	22.2	0.400	0.405
	3.0	22.4	0.430	0.440
Supernatants[a]	0.5	35.6	0.036	0.028
	1.0	31.5	0.093	0.070
	1.5	31.0	0.107	0.097
	2.0	27.4	0.141	0.120
	3.0	23.5	0.160	0.145

[a] B values for the pellets or the supernatants are not the real values. They are the B values of the original complexes from which they are obtained after centrifugation (see Materials and Methods). [b] The average values are obtained from three preparations.

As more histone is added to DNA, the amplitude of melting band I decreases and its melting point ($T_{m,I}$) shifts slightly to higher temperatures, which is a common phenomenon in the melting of nucleoprotein (Olins *et al.*, 1967; Olins, 1969; Shih and Bonner, 1970; Li and Bonner, 1971; Ansevin *et al.*, 1971; Li *et al.*, 1973). Differences are clear if the results are carefully examined. The increase of amplitude of melting band II is approximately proportional to the input ratio of histone to DNA while that of melting band III is not. Further, the complexes in Figure 1 have strong light scattering as shown in Table I. For the complex of 3.0 amino acid residues per nucleotide the A_{320}/A_{260} ratio is as high as 0.40 which is comparable with that of reconstituted polylysine–DNA complexes (Olins *et al.*, 1967). In nucleohistone II this ratio is always as low as 0.04 (H. J. Li and J. Bonner, unpublished results). When strong light scattering occurs it is often thought that perhaps the distribution of protein on DNA molecules is heterogeneous that some molecules are highly bound while the others are not, as is the case in reconstituted polylysine–DNA complexes (Olins *et al.*, 1967). Therefore, the complexes were separated into two fractions, the supernatant and the pellet, by centrifugation (see Materials and Methods).

Thermal denaturation results of a typical complex, its supernatant and pellet are shown in Figure 2. Compared with the original complex, the supernatant has more melting at $T_{m,I}$ and less melting at $T_{m,II}$ and $T_{m,III}$ which reflects a complex of more free DNA regions and less histone-bound regions. On the other hand, the pellet has less melting at $T_{m,I}$ and more melting at $T_{m,II}$ and $T_{m,III}$.

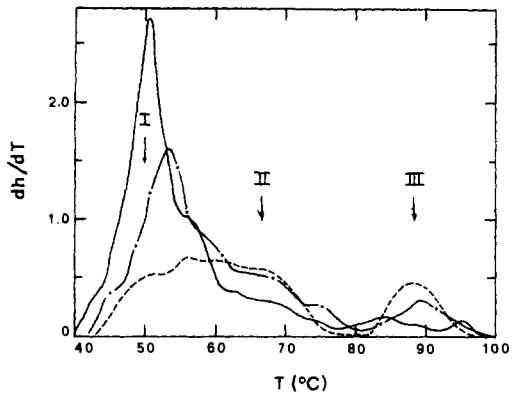

FIGURE 2: Derivative melting profiles of nucleohistone IIb2 (B = 1.5): original complex (—·—), the pellet (- - -), and the supernatant (—).

Melting curves of pellets of complexes with varied input ratios of histone to DNA are shown in Figure 3. These pellets are almost identical with one another as far as thermal denaturation is concerned.

The melting results of the supernatants are shown in Figure 4. Quite different from those results in the pellets (Figure 3), the melting curves of the supernatants depend upon the input ratio of histone to DNA (B). As B is increased, melting band I is decreased, band III is almost constant, while band II is slightly increased. Though the apparent greater light scattering of the supernatant with higher B values (see Table I) may lead to a lower hyperchromicity and smaller melting band, the fraction of the area of melting bands (II + III) is still increased with higher B value. It indicates that more DNA base pairs are bound by histone IIb2 in the supernatant of the complex with higher B value.

Table I summarizes the ratio of A_{320}/A_{260} of these complexes before and after melting and their h_{max}. For the original complexes, there is more light scattering at higher ratios of histone to DNA. On the other hand, all the pellets have nearly the same light scattering irrespective of the input ratios of histone to DNA in the original complexes. The supernatants in general have lower light scattering which is still significantly high. The lower h_{max} in the complex than that in pure DNA is due to light scattering. Light scattering increases the

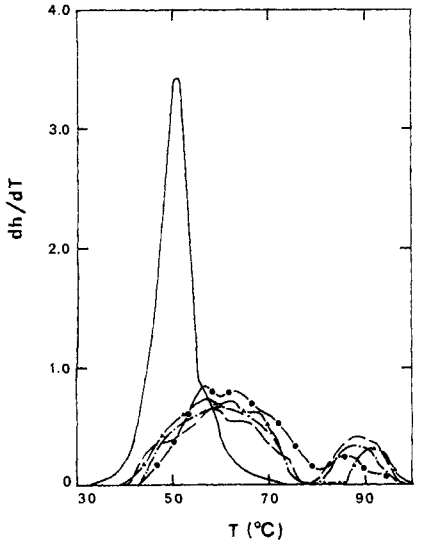

FIGURE 3: Derivative melting profiles of the pellets of nucleohistone IIb2. B values of the original complexes are respectively 1.0 (- -), 1.5 (—·—), 2.0 (—▲—), and 3.0 (—●—); DNA (—).

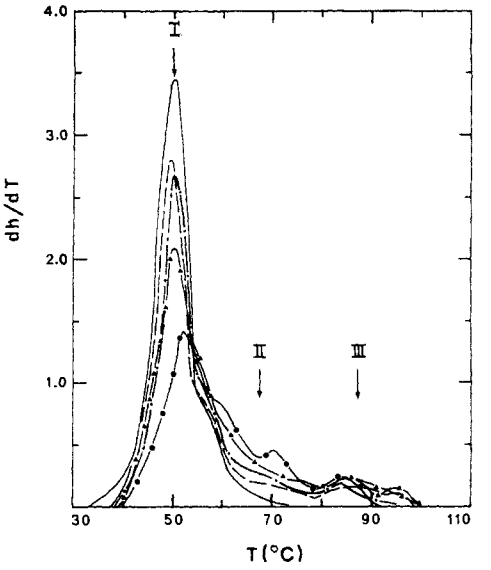

FIGURE 4: Derivative melting profiles of the supernatants of nucleohistone IIb2. B values of the original complexes are respectively 1.0 (- -), 1.5 (—·—), 2.0 (—▲—), and 3.0 (—●—); DNA (—).

apparent A_{260} before melting and therefore reduces the measured h_{max}. It is also interesting to notice from Table I that A_{320}/A_{260} ratios of the complexes before and after melting are almost identical. It implies that aggregation simply increases apparent A_{260} and reduces its h_{max}. The fraction of area in each melting band can still be used for an estimation of fraction of base pairs in nucleohistone which melts at that particular temperature (Li, 1973).

Circular Dichroism of Nucleohistone IIb2. Typical CD spectra of nucleohistone IIb2 are given in Figure 5. As histone IIb2 is complexed with DNA, there are three obvious changes on the CD spectra. The positive band near 275 nm (λ_{max}) and the crossover near 255 nm (λ_c) shift to the red. There is a significant contribution of negative CD below 240 nm, presumably the CD of histone IIb2 in the complexes. The reduction in amplitude of the positive band near 275 nm is not so definite until a high ratio of histone to DNA is reached. As described in Materials and Methods, because of light scattering, the accuracy of the determination of DNA concentration in the complexes is estimated to be about 5–10% and is not as accurate as in pure DNA. This uncertainty affects the determination of the amplitude of the CD. Because of this we do not attempt to analyze the difference CD spectra in this report as we did on polylysine–DNA (Chang *et al.*, 1973) or protamine–DNA complexes (Yu and Li, 1974). In the last

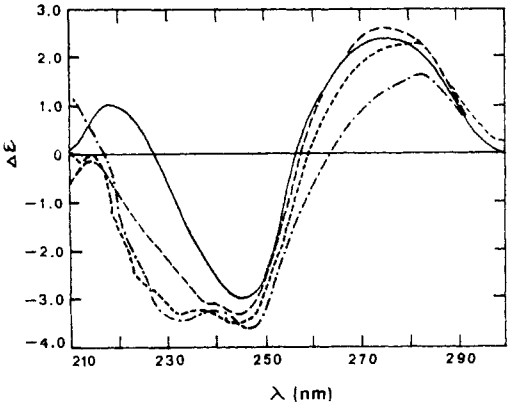

FIGURE 5: Circular dichroism spectra of nucleohistone IIb2. B = 0 (—), 1.0 (— —), 2.0 (- - - -), and 3.0 (—·—).

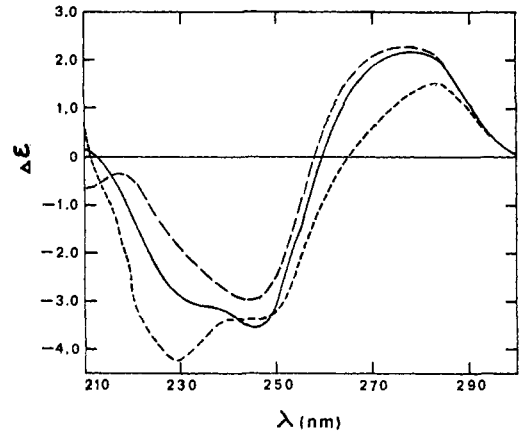

FIGURE 6: Circular dichroism spectra of nucleohistone IIb2 (B = 1.5): original complex (—), the pellet (- - - -), and the supernatant (— —).

two cases light scattering is negligible and accurate difference CD spectra can be calculated. As to be shown later, we try to interpret the CD results in terms of the parameters which are independent of DNA concentration determination, namely, λ_{max} of the positive band, λ_c of the crossover, and $\Delta\epsilon_{278}/-\Delta\epsilon_{246}$.

Figure 6 compares the CD of a regular complex, its pellet, and supernatant after centrifugation. Based upon the CD below 240 nm it is concluded that the supernatant has less and the pellet has more histone than the original complex. This is in agreement with the conclusion from thermal denaturation results (Figures 2–4). Referred to the CD spectrum of the original complex, the CD of the supernatant blue shifts its λ_{max} and λ_c, and has a slightly greater amplitude of the positive band near 275 nm. However, the pellet shows red shifts for λ_{max} and λ_c and a lower amplitude of the positive band. If it is explained that these CD changes are due to the binding of histone IIb2 on DNA, the results of Figure 6 indicate that more DNA base pairs are bound by histone IIb2 in the pellet than in the original complex than in the supernatant. This is in agreement with the conclusion based upon the results of thermal denaturation (Figure 2).

The CD spectra of the pellets of varied complexes are given in Figure 7 and those of the supernatants are given in Figure 8. As concluded earlier, the CD below 240 nm indicates more histone in the pellets than in the supernatants. The CD above 250 nm also indicates greater CD changes on DNA in the pellets than in the supernatants.

Three CD parameters, $\Delta\epsilon_{278}/-\Delta\epsilon_{246}$, λ_{max}, and λ_c of the original complexes, their pellets, and supernatants are given in Table II. Also included in Table II are these three parameters of chromatin (Shih and Fasman, 1970), $\Delta\epsilon_b$ of polylysine-bound (Chang *et al.*, 1973), and protamine-bound base pairs (Yu and Li, 1974) in direct mixed complexes. For the original complexes, as B is raised, $\Delta\epsilon_{278}/-\Delta\epsilon_{246}$ decreases while λ_{max} and λ_c increase. For the supernatants, except the one from the complex with B = 3.0, these three parameters are close to those of pure DNA (Table II).

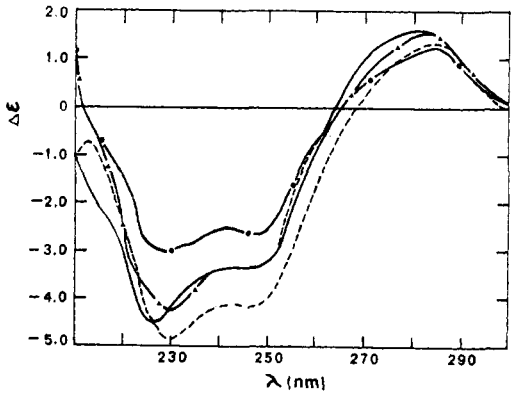

FIGURE 7: Circular dichroism spectra of the pellets of nucleohistone IIb2. B values of the original complexes are respectively 1.0 (—), 1.5 (—▲—), 2.0 (—●—), and 3.0 (- - - -).

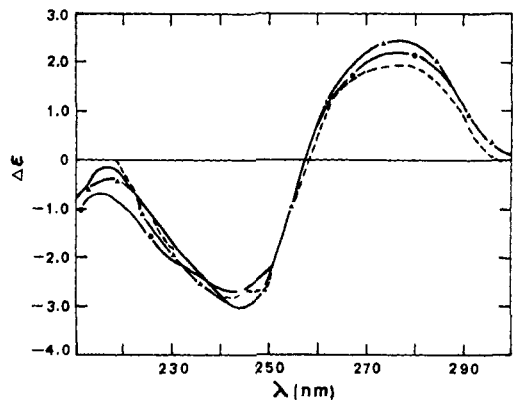

FIGURE 8: Circular dichroism spectra of the supernatants of nucleohistone IIb2. B values of the original complexes are respectively 1.0 (—), 1.5 (—▲—), 2.0 (—●—), and 3.0 (- - - -).

As seen from Table II, these three CD parameters of the pellets are nearly constants irrespective of B values of their original complexes. Based upon $\Delta\epsilon_{278}/-\Delta\epsilon_{246}$, the CD of DNA in these pellets is close to that of direct mixed polylysine–DNA complex with 70% base pairs bound or of pure DNA in 3.3 M NaCl (Figure 7 in Chang *et al.*, 1973). In addition to the fact

TABLE II: CD Characteristics of Nucleohistone IIb2.[e]

Complexes	B (Amino Acid)/ Nucleotide)	$\Delta\epsilon_{278}/-\Delta\epsilon_{246}$	λ_{max}	λ_c
Originals	0	0.83	275	256.5
	0.5	0.83	275.5	257
	1.0	0.79	275.5	257.5
	1.5	0.72	277	258.5
	2.0	0.63	280	260
	3.0	0.49	282.5	262.5
Pellets[a]	0.5	–	–	–
	1.0	0.42	280	265
	1.5	0.46	282	264
	2.0	0.43	282	264.5
	3.0	0.36	283	266.6
Supernatants[a]	0.5	0.82	275	256.5
	1.0	0.79	275.5	257
	1.5	0.77	275.5	257
	2.0	0.80	276	256.5
	3.0	0.70	278.5	259
Chromatin[b]		0.33	277	259.5
Polylysine–DNA[c]		0.16	283	273
Protamine–DNA[d]		0.55	278	261

[a] See footnote *a* of Table I. [b] From Shih and Fasman (1970). [c] CD characteristics of polylysine-bound DNA base pairs (Chang *et al.*, 1973). [d] CD characteristics of protamine-bound DNA base pairs (Yu and Li, 1974). [e] The average values are obtained from three preparations.

that these three CD parameters remain constant in the pellets, their melting curves are also nearly the same (Figure 3). It is assumed that melting band II has its symmetric peak at 65° and each melting curve of the pellets is resolved into three melting bands. The fraction of base pairs bound by histone IIb2 in the pellets can therefore be calculated as equal to the fraction of area under melting bands II and III, namely, $(A_{T_{m,II}} + A_{T_{m,III}})/A_T$, where $A_{T_{m,II}} + A_{T_{m,III}}$ is the sum of area under bands II and III and A_T the total area. It is then obtained that about 60–80% of base pairs are bound by histone IIb2 in these pellets. In other words, compared with the CD of direct mixed polylysine–DNA complexes, the CD of base pairs bound by histone IIb2 in reconstituted nucleohistone IIb2 is approximately the same as that of polylysine-bound base pairs (Chang *et al.*, 1973).

Possible Modification of Histone IIb2 in CM-Cellulose Chromatography. Since 17.6% residues of histone IIb2 molecule are hydroxyamino acids (serine and threonine), the possibility of formyl ester formation in the process of CM-cellulose chromatography performed in 2.6 M formic acid has been seriously considered. As described in Materials and Methods, ^{14}C-labeled formic acid was used to react with histone IIb under the same condition as that of CM-cellulose chromatography; 0.8 μmol in amino acid residue of treated histone IIb (about 130 μg) was applied to the gel for electrophoresis. From the specific radioactivity of [^{14}C]HCOOH in the incubation mixture (4.8×10^4 cpm/μmol of HCOOH), 0.8 μmol of amino acid residues of histone IIb with a single formyl modification per histone molecule is expected to have a radioactivity of about 310 cpm. It was found that none of the gel slices had detectable radioactivity. A parallel gel was stained in which protein bands were clearly resolved. The conclusion from this experiment is that there is no appreciable modification of histone IIb2 by formylation in the process of CM-cellulose chromatography (Senshu and Iwai, 1970).

Discussion

One of the main concerns of chromatin structure is its DNA conformation, the conformation of histone-bound regions in particular. Conformational change of DNA in chromatin was first discovered by optical rotatory dispersion (Tuan and Bonner, 1969) and more clearly demonstrated by circular dichroism (Shih and Fasman, 1970; Simpson and Sober, 1970; Permogorov *et al.*, 1970). The CD changes on DNA can be either due to the existence of C-form structure or supercoil in the chromatin (Shih and Fasman, 1970).

In order to find the role of each histone in inducing CD changes on DNA in chromatin, Simpson and Sober (1970) further studied the CD of chromatin in salt which dissociates histones from DNA. They found that removal of histone I by 0.6 M NaCl had little effect on CD at 280 nm while the removal of histone II by NaCl between 0.6 and 1.2 M brought the CD to its maximum. They found no significant CD change when NaCl was greater than 1.2 M and concluded that other histones (III and IV) were not responsible for conformational change on DNA in chromatin. When their experiments were examined more carefully we found that the last conclusion was not well justified. In the presence of high salt, the CD of DNA at 280 nm is grately reduced (Tunis-Schneider and Maestre, 1970; Li *et al.*, 1971). The constant CD of chromatin at NaCl >1.2 M can be a combination of two effects, an increase of CD due to the removal of histones from DNA and a simultaneous decrease of CD due to salt effect on free DNA. Our recent studies (manuscript in preparation) showed that after the removal of histone I by 0.6 M NaCl, there is a linear relation between $\Delta\epsilon_{278}$ and the fraction of base pairs bound by histones in EDTA buffer for NaCl-treated nucleohistones with NaCl >0.6 M. It implies that histone II as well as III and IV are responsible for induced CD changes on DNA in chromatin.

Another approach to investigating conformational effect on DNA by histone binding is to study the CD of reconstituted nucleohistones. For reconstituted nucleohistone I (f1–DNA complexes) at 0.14 M NaF CD changes are very big at high B values. At low B values the CD are similar to that of chromatin (Fasman *et al.*, 1970). Nevertheless, at very low salt CD effect on DNA by histone I is negligible (Olins, 1969; Fasman *et al.*, 1970). For nucleohistone IV, no CD changes on DNA were observed at low salt (Wagner, 1970; Shih and Fasman, 1971; Li *et al.*, 1971) while some significant effects were found at 0.14 M NaF (Shih and Fasman, 1971). In this report, it is found that the CD changes on DNA in nucleohistone IIb2 are similar to those found in chromatin, direct mixed polylysine–DNA (Chang *et al.*, 1973), and protamine–DNA complexes (Yu and Li, 1974). If these CD changes are referred to base tilting, such as in C-structure of DNA, induced by protein binding (histones, polylysine or protamine), the results reported here suggest that the binding of histone IIb2 on DNA also leads to base tilting similar to that in C-structure.

Since melting band II is not well separated from band I, especially for complexes with higher B values and all pellets, it is difficult to analyze the thermal denaturation results as quantitatively as done before (Li and Bonner, 1971; Li, 1973; Li *et al.*, 1973). However, melting band III is well separated and its area can be evaluated. It is seen that the melting area of band III of the complex is not proportional to the input ratio of histone to DNA (Figure 1). For instance $A_{T_{m,III}}/A_T$ are respectively 10, 14, 13, and 16% for complexes of B equal to 1.0, 1.5, 2.0, and 3.0, where $A_{T_{m,III}}$ is the melting area of band III and A_T is the total melting area. It indicates a saturation of binding sites on DNA which result in a stabilization near 88° by histones. On the other hand, approximately the increase of the amplitude of melting band II at about 65° is proportional to the B value (Figure 1).

It is interesting to compare the CD changes in the original complexes (Figure 5 and Table II) and the increases of melting areas in bands II and III (Figure 1). The major changes in CD occur when B is greater than 1.0 while the major increase in melting band III occurs when B is lower than 1.0. It indicates that it is the lower melting band of the histone-bound regions which correspond to the main CD changes on base pairs in nucleohistone IIb2. This is in agreement with the results of our recent CD studies on chromatin treated by trypsin or bound by polylysine (manuscript in preparation). It is opposite to the proposal that the base pairs melted at higher melting band are in C-form while those at lower melting band are in B-form (Johnson *et al.*, 1972).

Though there is some similarity between reconstituted nucleohistone IIb2 and chromatin in thermal denaturation and circular dichroism, there are still some differences. For instance, soluble chromatin has very little light scattering. On the other hand, light scattering is significant in nucleohistone IIb2 especially at high B values. In chromatin, as an average, there are 3.5 amino acid residues per nucleotide in histone-bound regions (Li, 1973; Li *et al.*, 1973). In other words, at $B = 3.0$ it is expected that 85% of base pairs will be bound by histones in chromatin. Nevertheless, only about

60% of base pairs in nucleohistone IIb2 with $B = 3.0$ are bound by histone IIb2, estimated from the melting curve in Figure 1. This difference can possibly be due to the saturation of binding sites corresponding to melting band III (Figure 1). In addition, melting results imply that binding of histone IIb2 to calf thymus DNA by using the method of salt gradient dialysis with urea is not random. Some DNA molecules are more favorably bound by histone IIb2 while the others are not. This is shown by the separation of an original complex into a pellet with more binding and a supernatant with less binding. It may indicate some cooperativity of binding of histone IIb2 to DNA which is similar to that in reconstituted polylysine–DNA complexes (Olins *et al.*, 1967). It can also imply heterogeneous distribution of binding sites on different calf thymus DNA molecules for histone IIb2.

Artifacts on CD of macromolecules due to light scattering have been reported recently. For examples, it has been demonstrated that differential light scattering of large optically active particles can have significant CD effects (Gordon and Holzwarth, 1971; Gordon, 1972; Dorman and Maestre, 1973). In particular it causes a flattening effect and a red-shift of the CD spectrum. The particulate suspension also results in diminished concentration of chromophores due to the shadowing effect. The possibility of these artifacts in the measured CD spectra of nucleohistone I and IV has previously been discussed (Shih and Fasman, 1972).

Though the scattering artifacts just mentioned on the measured CD of nucleohistone IIb2 cannot completely be ruled out in this report, we have the following reasons to argue against these artifacts. First, the decrease of CD below 240 nm correlates well to the presence of histone in the complexes. Second, there is also a good correlation between the CD changes above 255 nm and the melting bands II and III. The latter is a measurement of the fraction of DNA base pairs bound by histones in the complexes. Third, recently Maestre and Dorman (1973) showed that a correction of light scattering on the CD spectra of reconstituted nucleohistone I prepared by the method used in Fasman's laboratory reduces the amplitude by about 30%, while the spectra are qualitatively not changed. Based upon the previous discussion we believe that though light scattering may have some contribution to the amplitude of the CD, the use of those three parameters, λ_{max}, λ_c, and $\Delta\epsilon_{278}/-\Delta\epsilon_{246}$, to interpret the CD results is quite reasonable.

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