Circular dichroism as a probe of DNA structure inside reconstituted nucleohistones

A. Garel, A. M. Kovacs, M. Champagne and M. Daune

Institut de Biologie Moléculaire et Cellulaire du CNRS, Groupe de Biophysique,
15, rue Descartes, 67000 Strasbourg, France

Received 5 July 1976

ABSTRACT

Reconstituted nucleohistones were obtained by mixing in given conditions acid extracted histones and eukaryotic DNA. The histone/DNA ratio (w/w) was in the range 0.35 - 0.95. With the four histones ($H_{2A} + H_{2B} + H_3 + H_4$) we have been able to obtain subunits (nucleosomes or v-bodies). The variation of circular dichroism signal with temperature at 280 nm was measured to follow structural changes of the DNA inside the complex. The true change of ellipticity $\Delta[\theta]_{280}^{*T}$ of histone - bound DNA regions, is similar for reconstituted nucleohistone and H_1 -depleted chromatin, and is therefore a physical probe of the presence of nucleosomes.

INTRODUCTION

The organization of the DNA inside chromatin into globular subunits of definite shape and dimensions, can be considered now as definitely established from many experimental evidences (1 - 9). Such a structure was also found with circular DNA, in so-called minichromosomes isolated in-vivo (10) or reconstituted in-vitro (11). Similar organization appears to exist in plants (12). The subunit structure is observed as well after H_1 removal (8).

Electron microscopy (2, 8, 13) and controlled enzymatic digestion (1, 4, 5, 14-16) were generally the main techniques used to check the chromatin structure. We have shown however, when following the change with temperature of the CD signal at 280 nm, that a cooperative change of structure occurred in the premelting

Footnote

This work was presented in part at the 1975 FEBS Meeting (PARIS) and at the 1975 Symposium on chromatin (GLASGOW).

region of the histone-bound DNA (7). This increase of molar ellipticity was interpreted as a release of constraint upon DNA which fully recovers its B-structure.

In the present work, we have obtained a reconstituted nucleoprotein, by mixing eukaryotic DNA and different histone mixtures. We have then used the change of CD signal to follow the appearance of subunit structure in this reconstituted nucleoprotein and compared our results with those obtained in the case of chromatin.

MATERIAL AND METHODS

The histones were prepared by three different manners :

a) Total histones were extracted from chicken erythrocytes chromatin with HCl 0.25 N, dialysed against distilled water and lyophilised. H_1 and H_5 were extracted selectively and quantitatively from this preparation, with perchloric acid 0.5 M (18). The resulting sediment which contains a stoichiometric mixture of $H_{2A} + H_{2B} + H_3 + H_4$ as bound inside nucleosomes (or v-bodies) will be called H_N in this paper. It was redissolved in HCl 0.2 N, dialysed against distilled water and freeze-dried.

b) The three histones ($H_{2A} + H_3 + H_4$) were extracted from the chromatin by the method N°2 of Johns but simplified (19, 20).

c) The complete separation of the four histones (a) was achieved by gel chromatography on Biogel P 60, followed by Sephadex G-100 (21). They were electrophoretically pure (22) and the aminoacid analyses agreed with that expected. The concentrations of the free histones were determined by dry weight or from their absorption spectrum. In the DNA - histones complexes, the concentrations of the histones were controlled by the method of Lowry (23). The protein/DNA ratio (w/w) was always called r. Purified DNA from chicken erythrocytes was obtained using the procedure of Kay et al. (24). The concentration of DNA in the complex was determined spectrophotometrically, the molar absorption coefficient per nucleotide residue being taken as equal to $6600 \text{ M}^{-1} \text{ cm}^{-1}$ at 260 nm.

Reconstitution of nucleohistone is obtained as followed : the four histones are dissolved together in HCl 10^{-3} M with Dithio-treitol 10^{-3} M in the cold room, where the solutions are main-

tained during about two hours. The buffer (NaCl 4 M, citrate 2.10^{-3} M, EDTA 4.10^{-4} M, pH 5.6) was added to the histone solution (vol/vol).

Histones and DNA in the same solvent (NaCl 2 M, citrate 10^{-3} M, EDTA 2.10^{-4} M) were slowly mixed with gentle stirring in the cold room.

Then followed a step gradient dialysis in the cold room against NaCl solution of decreasing ionic strength, citrate 10^{-4} M, EDTA 2.10^{-4} M, in the following order : NaCl 1.5 M ; 1.1 M ; 0.75 M ; 0.5 M ; 0.4 M ; 10^{-3} M (two times). Each dialysis takes place during 12 hours but the last one can be shortened. The passage through 0.1 M is made as short as possible. The histones-DNA ratio (w/w) was in the range 0.35 - 0.95 with a DNA concentration of 50 $\mu\text{g/ml}$.

Absorbance melting curves were registered at 260 nm and circular dichroism melting curves were obtained at 280 nm for DNA and at 227 nm for proteins as described previously (25).

The results are presented in terms of molar ellipticities $[\theta]$ (degrees. cm^2 . dmol^{-1}) based on either the molar nucleotide concentration at 280 nm, or the molar aminoacid concentration at 227 nm. The true change of ellipticity $\Delta[\theta]_{280}^{*T_2}^{\circ\text{C}}$ of histone bound DNA regions was calculated as previously indicated (17).

RESULTS

A. Melting behaviour of reconstituted nucleohistone

In figure I are gathered the different melting profiles obtained with a complex H_N - DNA ($r = 0.68$). Changes of absorbance at 260 nm and of circular dichroism at 280 nm and 227 nm respectively, were followed in function of temperature. The general features of figure I are similar to those already found with H_1 -depleted chromatin (17), except for slight changes of T_m due to small differences in ionic strength.

a) The absorbance melting profile is biphasic and indeed this appearance is generally found in any case of basic protein - DNA interactions. According to a preceding paper (25) we define h/H as the ratio of the change of absorbance in the second transition to the total change of absorbance during the melting pro-

Figure 1 - Circular dichroism at 280 nm and 227 nm (—) and absorbance at 260 nm (----) versus temperature in NaCl 10^{-3} M, citrate 10^{-4} M, EDTA $2 \cdot 10^{-4}$ M, of a complex H_N - DNA ($r = 0.68$).

cess. A linear variation of h/H with r is expected.

In our case, $h/H \sim 0.5 r$, a relationship similar to that found with H_1 -depleted chromatin (17) or artificial complexes between H_{2A} and DNA (25).

b) In the dichroic melting curve at 280 nm, one finds again the typical behaviour already described in the case of H_1 - depleted chromatin (17). The premelting of naked DNA (between 20°C and 45°C) is followed by its melting and then a cooperative increase of θ_{280} takes place. A maximum occurs around 70°C, in the range of temperature separating the two transitions as observed in the absorbance melting profile.

c) The melting curve followed at 227 nm (a wavelength for which the major part of the CD signal is coming from pro-

tein moiety) is highly cooperative. This feature is only found in the second part of the corresponding curve obtained with H_1 -depleted chromatin (see fig. 9 or ref. 17). The slight increase of θ_{227} , which was observed at the beginning of the process is never found with H_N - DNA complexes.

This difference beeing taken apart, it is clear that the T_m of proteins (60°C) is much lower than the temperature of the maximum of θ_{280} (70°C).

B. Variation of $\Delta[\theta]_{280}^{T_2}$ with the protein/DNA ratio (r)

In figure 2 are given the CD melting profiles at 280 nm of H_N - DNA complexes corresponding to increasing values of r ($0 < r < 0.95$). The two temperatures (denoted T_1 and T_2) are practically constant whatever r, but the corrected value $\Delta[\theta]_{280}^{*T_2}$ ($T_2 = 70^\circ\text{C}$) is an increasing function of r (see table I).

Figure 2 - Circular dichroism melting curves at 280 nm of different H_N - DNA complexes and of DNA. 1 : DNA ; 2 : r = 0.35 ; 3 : r = 0.60 ; 4 : r = 0.80 ; 5 : r = 0.95.

Table I

Dichroic melting data of reconstituted nucleohistones

Histones in the complex	r	T ₁ °C	T ₂ °C	θ ₂₈₀ 20°C	Δ θ *T ₂ °C 280	
$ H_{2A}+H_{2B}+H_3+H_4 $ $= H_N^{(a)}$	0	41	-	8,400	0	
	0.35	40	70	7,700	200	
	0.60	42	70	6,500	1100	
	0.68	45.5	70.5	6,000	1600	
	0.80	42	70	5,300	1750	
	0.95	-	70	4,800	2350	
x $ H_{2A}+H_3+H_4 ^{(b)}$ $+H_{2B}^{(c)}$	0	0.68	43.5	70.5	6,000	650
	1/8	0.67	45	70.5	6,600	800
	2/8	0.70	43.5	70	6,300	1150
	3/8	0.70	45.5	70.5	6,300	1400
$H_{2A}+H_{2B}+H_3+H_4^{(c)}$	0.76	-	-	6,000	1600	

(a) (b) (c) : see Material and Methods

C. Influence of histone/histone ratio

Keeping constant the protein/DNA ratio, the relative amount of H_{2B} is modified by increasing the ratio $x = |H_{2B}|/|H_{2A} + H_3 + H_4|$ from 0 to 3/8 (table I).

The initial ellipticities $\theta_{280}^{20^\circ}$ are roughly identical ($6000 < \theta_{280}^{20^\circ} < 6600$) and independent of x values, but $\Delta|\theta|_{280}^{*T_2}$ is increasing with x. In other words, to the same initial decrease (~ 2100) of $\theta_{280}^{20^\circ}$ relative to the DNA value (8400), the cooperative increase of ellipticity as measured at 70°C (T₂) is the highest when the stoichiometric amount of histones (as found in H_N (20)) is associated to DNA. The two temperatures T₁ and T₂ are the same as in figure 2.

All of the data relative to $\Delta|\theta|_{280}^{*T_2}$ are plotted versus r and compared in figure 3 with previous results obtained with chromatin and partially histone - depleted chromatin (table 4 of ref. 17). The two sets of points are both well fitted by straight lines with an identical slope.

Figure 3 - Variation of $\Delta|\theta|_{280}^{*T_2}$ of reconstituted nucleohistones (—) and partially depleted nucleoprotein (----) as a function of r .

Nucleohistones were reconstituted with :

■ H_N (a)

○ $|H_{2A} + H_3 + H_4| + H_{2B}$ (b)

△ $H_{2A} + H_{2B} + H_3 + H_4$ (c)

(see Material and Methods, a, b, c).

DISCUSSION

A. Presence of nucleosomes

In view of the results we have obtained the first question to be raised is relative to the effectiveness of the reconstitution process. What type of nucleoprotein are we dealing with, after properly mixing together histones and DNA ? At the present time two evidences of a subunit structure of the chromatin are generally given : the visualization of a subunit (nucleosomes or nu-bodies) under the electron microscope, and a definite electrophoretic pattern on acrylamide gel after partial digestion with staphylococcal nuclease. Unfortunately this latter technique which appears to be suitable and reproducible in the case of native

chromatin, gives, with artificial complexes, a not yet well understood band pattern.

On the other hand, and for all of our H_N - DNA reconstituted complexes, electron microscopy reveals the presence of nucleosomes (P. OUDET, personal communication). The general appearance is similar to that described with H_1 - depleted chromatin, and the amount of subunit is roughly proportional to r values. If the same experiment is made as a control on nucleoprotein complexes made with only two or three histones, no presence of subunit could be revealed.

B. $\Delta|\theta|_{280}^{*T_2}$ as a physical probe of the nucleoprotein structure

One of the aim of our present investigation is to point out the interest of measuring the variation of CD signal with temperature at 280 nm, in order to correlate this specific structural changes of the DNA inside chromatin with the presence of a subunit structure.

As previously suggested in our study of chromatin and H_1 - depleted chromatin (17) the increase of θ_{280} observed in the premelting region of histone - bound DNA corresponds to the total recovery of its B-structure, since the corrected value $\Delta|\theta|^*$ was equal to the difference of θ_{280} between DNA and chromatin as measured at 20°C.

This conformation change was triggered by the partial denaturation of histone molecules inducing an irreversible partial disruption of the subunit structure.

As seen in figure 1, reconstituted nucleoprotein presents a similar behaviour. Moreover, it was possible to show (table I and figure 2) (i) the increase of $\Delta|\theta|_{280}^{*T_2}$ with r , (ii) the necessary presence of a stoichiometric amount of the four histones, since the closer x is to the ratio 0.55 inside H_N , the higher is the $\Delta|\theta|^*$ value, (iii) the equivalence between H_N and a mixture of the four histones previously isolated and purified. In each case there is a good correlation between the amplitude of $\Delta|\theta|_{280}^{*T_2}$ and the amount of nucleosomes as qualitatively estimated from electron microscopy.

The difference between the results obtained with H_1 -depleted chromatin and those given above, lies in the value of $\Delta|\theta|_{280}^{*T_2}$. With reconstituted material, the difference of molar ellipticities at

280 nm and 20°C between DNA and nucleoprotein complex is always greater than the amplitude of $\Delta|\theta|_{280}^{*T_2}$. In other words, the DNA does not fully recover its B-structure as it was the case in chromatin and H_1 -depleted chromatin.

This observation raises immediately two questions about the yield of reconstitution and the exact meaning of $\Delta|\theta|_{280}^{*T_2}$.

C. Yield of the reconstitution process

We have to analyse the different factors affecting the yield of the reconstitution which could be conceptually defined as the ratio of the observed number of nucleosomes to the expected one.

a) the structural integrity of all of the histone molecules could be questioned since they are obtained after acidic treatment. It was claimed (3) that such drastic methods of preparation must lead to proteins which are no longer in their "native" state.

b) According to others (26, 27) the method of isolation is not so critical but rather the step order of the mixing process and the pH at which it takes place.

Our reconstitution procedure takes place indeed at a pH (5,6) at which the homotypic dimer is present but lower than the pH required to get an heterotypic tetramer (27). Our mixing pH is also definitely lower than the optimum pH (> 8) which seems necessary to obtain a fair amount of octamer (28). However after mixing with DNA both complexes are similar as far as the digestion pattern is concerned.

Actually, inside the mixture of histones and DNA and during the process, series of kinetics take place simultaneously giving rise to heterologous oligomer or homologous aggregates, but so far their relative rate constants are unknown. However, the quality of the reconstituted material, when referred to amount of nucleosomes, is not increased when the concentration of the reactants is five times higher.

D. Histone - DNA association inside nucleosome and the meaning of $\Delta|\theta|_{280}^{*T_2}$

To interpret all of our experimental results one is leading to postulate the existence of at least two types of associations between histones and DNA.

A specific association with a tetramer ($H_{2A} + H_{2B} + H_3 + H_4$) or an octamer, leading to nucleosome formation and an unspecific association, similar to that observed with a single histone, or groups of two or three histones bound to DNA.

These two binding processes give actually the same change of $\Delta|\theta|_{280}$, whatever the type or the number of histone molecules bound to DNA, as measured at 20°C , as it is clear from the inspection of figure 4 in which $|\theta|_{280}^{20^\circ}$ was plotted against r .

Figure 4 - Molar ellipticity $|\theta|_{280}$ of histones - DNA complexes as a function of r .

- H_N (a)
 - $|H_{2A} + H_3 + H_4| + H_{2B}$ (b)
 - ▲ $H_{2A} + H_{2B} + H_3 + H_4$ (c)
 - × $H_{2A} + H_3 + H_4$ (b)
 - +
- (see Material and Methods, a, b, c).

It simply means a similar modification of the secondary structure of the DNA (i.e. a slight winding of the helix) when basic proteins (as well as cations) are bound to phosphate groups. However, in the case of the specific association and of the building of a subunit, the local changes of the secondary structure of DNA induce the formation of a tertiary turn in the DNA. Such a process was clearly demonstrated in the case of covalently bound circular DNA to which H_N (or any stoichiometric mixture of the four histones) was added (11). It was possible to create along the circular DNA, as many nucleosomes as negative tertiary turns were initially present.

In order to create a nucleosome, we can therefore assume necessary either to induce a negative superhelical turn by winding a certain number of base pairs, either to use available ones as in the case of twisted circular DNA. In each case the tertiary structure and the whole complex can only be maintained by a proper association of histone molecules. Since the initial structural change is always a winding process, the change of θ_{280} is the same and therefore the ellipticity of the chromatin cannot be used as a probe of its internal organization. However, upon heating, the cooperative increase of ellipticity as measured by $\Delta|\theta|_{280}^*$ and the total recovery of the B-structure would only occurred, when histones and DNA interact inside a nucleosome since then one observes the irreversible disappearance of a tertiary turn.

In such a model, it is implicitly assumed that the regions of histone molecules interacting with DNA are different whether the association is specific or not. The superhelical turn can only be organized and then stabilized by a proper tertiary structure of the histone inside a definite heterotypic oligomer.

The parallelism between the two variations of $\Delta|\theta|_{280}^*$ with r (figure 3) in the case of histone - depleted chromatin and reconstituted nucleoprotein respectively deserves two comments :

a) at any given value of r , $\Delta|\theta|_{280}^*$ value of histone - depleted chromatin is always higher than the corresponding one of the reconstituted material. According to our model, it only means a partial reconstitution, i.e. a mixture of well-organized subunits around a tertiary turn of DNA and of non-specific histo-

ne - DNA complexes. Under electron microscope, these latter appear no more as nucleosomes but as a thickened thread of DNA similar to that observed with complexes of DNA with two or three histones.

After heat disruption of histone - histone interactions, the complete recovering of the B-structure of DNA occurs only by removal of the tertiary turn inside the nucleosome. Anywhere else, DNA is maintained in a "B-type" structure characterized by a lower value of θ_{280} .

b) In the case of histone - depleted chromatin, it is somewhat puzzling to still observe a good linear relationship at low values of r , i.e. when histones H_{2A} , H_{2B} and H_3 have been partially or even totally removed.

It is clear, in this case, that the integrity of the subunit structure is no longer maintained. We are thus leaded to assume that the tertiary turn is still maintained by histone H_4 which is known to be the last to be removed. In other words, a well-defined tetramer (or octamer) structure is necessary to "shape" the nucleosome and to impose a tertiary structure to the DNA, but such a constrain could be maintained thereafter, by only one histone molecule (H_4) acting as a clamp.

This peculiar role of H_4 could be relevant to the previous observations of a greater change of θ_{280} (per gr of protein) when H_4 is removed from DNA (17). One has also to point out the importance of H_4 acetylation in spermiogenes (29) which could be understood if acetylation would act as a triggering process to release the tertiary structure of the DNA.

In conclusion, we have been able to obtain subunits (nucleosomes or ν -bodies) in a reconstituted nucleoprotein by mixing in given conditions histones and DNA. We have developped a simple physical method (change with temperature of the CD signal at 280 nm) to detect and measure the amount of structurally modified DNA inside these subunits. Improvement of the reconstitution procedure and development of new techniques of measurement, and especially kinetic ones, are now in progress in our laboratory.

Acknowledgement

This work was supported by a grant (n° 75.7.0187) of the
Délégation Générale à la Recherche Scientifique et Technique.

REFERENCES

1. Sahasrabuddhe, C.G., Van Holde, K.E. (1974) J. Biol. Chem. 249, 152-156.
2. Olins, A.L., Olins, D.E. (1974) Science 183, 330-332.
3. Kornberg, R.D., Thomas, J.O. (1974) Science 184, 865-868.
4. Noll, M. (1974) Nature 251, 249-251.
5. Oosterhof, D.K., Hozier, J.C., Rill, R.L. (1975) Proc. Nat. Acad. Sci. US 72, 633-637.
6. Baldwin, J.P., Boseley, P.G., Bradbury, E.M., Ibel, K. (1975) Nature 253, 245-249.
7. Senior, M.B., Olins, A.L., Olins, D.E. (1975) Science 187, 173-175.
8. Oudet, P., Gross-Bellard, M., Chambon, P. (1975) Cell 4, 281-300.
9. Bakayev, V.V., Melnickov, A.A., Osicka, V.D., Varshavsky, A.J. (1975) Nucleic Acids Res. 2, 1401-1419.
10. Griffith, J.D. (1975) Science 187, 1202-1203.
11. Germond, J.E., Hirt, B., Oudet, P., Gross-Bellard, M., Chambon, P. (1975) Proc. Nat. Acad. Sci. US 72, 1843-1847.
12. McGhee, J.D., Engel, J.D. (1975) Nature 254, 449-450.
13. Van Holde, K.E., Sahasrabuddhe, C.G., Schaw, B.R., Van Bruggen, E.F.J., Arnberg, A.C. (1974) Biochem. Biophys. Res. Comm. 60, 1365-1370.
14. Clark, R.J., Felsenfeld, G. (1971) Nature New Biol. 229, 101-106.
15. Hewish, D.R., Burgoyne, L.A. (1973) Biochem. Biophys. Res. Comm. 52, 504-510.
16. Weintraub, H. (1975) Proc. Nat. Acad. Sci. US 72, 1212-1216.
17. Wilhelm, F.X., De Murcia, G., Champagne, M.H., Daune, M.P. (1974) Eur. J. Biochem. 45, 431-443.
18. Oliver, D., Sommer, K.R., Panyim, S., Spiker, S., Chalkley, R. (1972) Biochem. J. 129, 349-353.
19. Johns, E.W. (1964) Biochem. J. 92, 55-59.
20. Mazon, A., Champagne, M. (1972) Biochimie 54, 1273-1279.
21. Van der Westhuyzen, D.R., Böhm, E.L., Von Holt, C. (1974) Biochim. Biophys. Acta 359, 341-345.
22. Panyim, S., Chalkley, R. (1969) Arch. Biochem. Biophys. 130, 337-346.
23. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
24. Kay, E.R.M., Simmons, N.S., Dounce, A.L. (1952) J. Amer. Chem. Soc. 74, 1724-1726.
25. Garel, A., Kovacs, A.M., Champagne, M., Daune, M. (1975) Biochim. Biophys. Acta 395, 16-27.
26. D'Anna, J.A., Isenberg, I. (1974) Biochem. Biophys. Res. Comm. 61, 343-347.
27. Weintraub, H., Palter, K., Van Lente, F. (1975) Cell 6, 85-110.
28. Thomas, J.O., Kornberg, R.D. (1975) Proc. Nat. Acad. Sci. US 72, 2626-2630.
29. Louie, A.J., Dixon, G.H. (1972) Proc. Nat. Acad. Sci. US 69, 1975-1979.