CHROMATIN ACCESSIBILITY TO DNA MINOR GROOVE LIGANDS IN VITRO: ROLE OF LINKER HISTONES AND AMINO-TERMINAL DOMAINS OF OCTAMER HISTONES

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INTRODUCTION

The processes of eukaryotic gene expression must be considered in the light of the intrinsically dynamic properties of chromatin, and, in particular, of the basic level of it which is the nucleosome. It is now clear that in the process of chromatin condensation two structures have a prominent role: the amino-terminal domains of the histone octamer and the linker histones (H1, H5 and the other isoforms). Both act by favoring the coming-near of two adjacent nucleosomes, forming the high order structure of 30 nm fibers, the model of which (solenoid or zigzag) is not yet clearly known. The role of the amino-terminal domains in gene expression processes is firmly established; in fact these domains act as regulators of transcription with negative and positive effects either by restricting DNA accessibility to transcription factors (1) or through interactions with specific transcriptional regulatory proteins (2). Much less is known about the role of linker histones. Nucleosomes containing linker histones (H1-H5) and about 20 bp of linker DNA are called chromatosomes and can be considered as the fundamental units of the condensed chromatin fibers. The exact position of the linker histone in the chromatosome is not yet defined (3), but it is known to "lock" two complete turns of DNA on the histones octamer; so its presence, preventing DNA mobility, is expected to reduce also its accessibility toward outer molecules.

From the model of nucleosome positioning proposed by Travers (4) it is known that the AT sequences are preferentially positioned inward in the nucleosome structure, where the DNA is facing towards the histones. This situation encourages the use of minor groove, AT specific ligands to investigate the accessibility of the DNA in the nucleosome. In a previous work, based on binding-induced fluorescence, we presented evidence that DAPI is able to recognize its DNA sequence targets even when these are constrained in the nucleosome (5). We also investigated whether minor groove specific ligands can recognize DNA regions having different intrinsic curvature, with the aim to explain at the molecular level their different interactions with different chromosomal domains (6). We found that DAPI does not discriminate between intrinsically curved and non curved sequences as to the high affinity minor groove bindings, but Hoechst 33258 does, disfavoring non-curved sequences by a factor of about seven with respect to curved sequences.

On this ground, in the present report, based on ligand-induced circular dichroism spectroscopy, we measured more precisely the parameters of the interactions of DAPI and Hoechst 33258 in the nucleosome, while the accessibility of the DNA minor groove in the chromatosome and in a nucleosome devoided of the amino-terminal domains of the histone octamer (a model of hyper-acetylated chromatin) (7) was investigated with Hoechst 33258.

MATERIALS AND METHODS

Preparation of Core Particles, Chromatosomes and Trypsinized nucleosomes - Mononucleosome core particles and chromatosomes were prepared from chick erythrocytes according to the protocols of Kornberg (8) and Simpson (9), respectively, with minor modifications. The trypsinized nucleosomes were obtained by using free trypsin in a ratio of approximately 1:100 to nucleosomes (1.2 μg of trypsin for 100 μg of nucleosomes). Nucleosomes were digested at room temperature. After 30 min the reaction was stopped by adding the nucleosome solution to the immobilized trypsin-inhibitor (inhibitor bound to DITC glass, from Sigma)

in a ratio 1 μg of trypsin/1000 μg of glass beads. The immobilized inhibitor was preconditioned in 25 mM NaCl, 10 mM Tris-HCl pH 7.5 under mild rotary shaking at 4°C for 24 h, and washed thoroughly with TE buffer (0.01 M Tris, 0.001 M EDTA) pH 7.5 prior to use. As inhibitor binds trypsin in a covalent way, the enzyme-inhibitor complex is removed by centrifugation of the glass beads (12.000 rpm for 10 min). Finally, PMSF was added to a final concentration of 0.3 mM. The trypsinized nucleosomes were stored at 0-4°C.

Preparation of nucleosomal and chromatosomal DNA - DNA was obtained by adding TE buffer to 100 μg of nucleosomes (or chromatosomes) to a final volume of 200 μl. Then 1800 μl of 1% CTAB, NaCl 0.3 M were slowly added, shaking continuously by vortex. After 30 min at room temperature, the sample was centrifuged for 15 min at 15.000 rpm and the pellet was resuspended in 500 μl of a TE, 1 M NaCl. After 45 min at room temperature, 1 ml of absolute EtOH was added and the solution was put on ice for 15 min. After centrifugation at 15.000 rpm for 15 min, the pellet was resuspended in 70% EtOH and washed twice or more with the same. Finally, the DNA was dried, resuspended in TE and stored at 4°C.

Electrophoretic Mobility Assay - Nucleosomal and chromatosomal DNA were run on a native 10% polyacrylamide gel (20 x 20 x 0.5 cm) in 1 x TBE buffer (90 mM Tris-Borate, 2.5 mM EDTA) at a constant voltage of 200 V. The gel was then colored for 30 min with an EtBr solution and washed with distilled water. The electrophoretic mobility of the samples was compared with that of a molecular weight standard. Chromatosomes, mononucleosomes and trypsinized nucleosomes were run on a 1% agarose gel in 1% TBE buffer at a constant voltage of 70 V for about 2 h. After running, the gel was colored with EtBr solution for 10 min and then washed with distilled water. To be sure that ligands (DAPI and Hoechst 33258) do not induce the dissociation of the DNA-histones complex, electrophoretic mobility assays were repeated comparing samples with and without drugs. The drug/DNA ratio used was 1/5.

Circular Dichroism measurements - CD spectra were recorded at room temperature on a Jasco 715 spectropolarimeter. Starting samples were

prepared with DNA-drug concentration ratio ([bp]/[D]) of about 50. Subsequently [DNA]/[Drug] rations were made to decrease by sequential substitution of volumes with drug at the same concentration. Spectra were recorded for each [bp]/[D] ratio thus obtained.

RESULTS

It is known that the binding of the minor groove ligands to DNA can cause a little distortion in the double helix and so it could have an effect on nucleosome stability. To be sure that Hoechst 33258 and DAPI bind to nucleosomes, chromatosomes and trypsinized nucleosomes without causing the dissociation of these DNA-histones complexes, we compared the electrophoretic migration of the complexes with and without drugs, obtaining no differences (data not shown). This result demonstrates that these ligands do not cause the disruption of the nucleosomes and of the other DNA-histones complexes used in our experiments, even if they seem to inhibit the nucleosome assembly (10).

The binding of the ligands to DNA (naked and organized with the histones) was studied by circular dichroism spectroscopy, using the spectra induced in the visible range by the specific interactions of DNA with the drugs (fig. 1). A further control of the integrity of the complexes through the experiment was indeed provided by the CD spectra in the UV. The CD spectrum of naked DNA is strongly different from that of the DNA-histones complexes. This situation is substantially conserved also in the presence of the ligands; furthermore, the DNA molar ellipticity spectra are almost independent on [D]/[bp] ratios in the range from 0 to 0.1. These features confirm that the binding of the drugs does not cause dissociation.

The complexes with DAPI show an isodichroic point at about 351 nm (11) in the same range of [D]/[bp] ratios both in the case of free DNA and of core nucleosome. This indicates that only two types of strong bindings are present, and they are conserved in the nucleosome. No isodichroic point is found with Hoechst 33258 in all complexes, suggesting that the types of strong bindings are either one or more than two. Again, it can

be reasonably affirmed that these strong bindings are conserved in all forms of nucleosome examined.

In order to construct the binding isotherms, we considered the variations of molar ellipticity as a function of DNA/ligand molar ratios at a fixed wavelength (fig. 2, top). A suitable wavelength is the λ_{max} of the visible absorption spectrum of the ligands. Direct isotherms are shown in the bottom of fig. 2, where r (the bound ligand molecules per DNA base pair) is plotted against C/[D] (ligand free concentration relative to total concentration). The plots of free DNA and of purified mono-nucleosome devoid of linker DNA and of H1-H5 histones (core nucleosome) are almost parallel along the abscissa. This strongly suggests that the number of sites for both ligands are conserved for each binding type, while the association constants are the most affected parameters. The plots of Hoechst 33258 complexes with trypsinized nucleosomes and chromatosomes lay within the limits defined by those of free DNA and core nucleosome. It appears that following partial trypsinization a core nucleosome maintains the strong modes of Hoechst binding, while the weaker bindings are barely distinguishable from those of free DNA. On the contrary, the chromatosome displays features of the strong bindings that are intermediate between those of free DNA and those of the core nucleosome, while joining the latter as regards the weaker bindings.

The binding isotherms were reduced to putative components by simultaneous fittings to one hyperbolic and one or more logistic functions, that are representative, respectively, of the first, strongest binding to a class of independent sites, and of a number of weaker bindings to cooperative sites (fig. 2, bottom). The parameters calculated from these deconvolutions are reported in tables I and II. The analysis confirms the number of bindings suggested by the spectra. In particular, only two specific bindings are found for DAPI, and three for Hoechst. A less specific, weaker binding of both ligands is found only for free DNA. This means that the minor groove of free DNA can be saturated by DAPI with a frequency of about 5 and a relative affinity of 2.4, by Hoechst 33258 about every 6 bp with a relative affinity of 1.2.

The main result of this analysis is that all the specific sites are "seen" by both ligands in the core nucleosome. Hoechst 33258 recognizes them also in the chromatosome and in the partially trypsinized nucleosome; actually, in the latter structure the third binding of free DNA is substituted by a couple of bindings with higher frequency. It is noteworthy that the first two specific bindings take place with the same periodicity, that is every 13 bp for DAPI and every 33 bp for Hoechst 33258 (bindings I and II in tables 1 and 2). The second important result is that only the association constant for the first, strongest binding is drastically reduced, by an order of magnitude, with both ligands. This effect is moderated partially by the removal of amino-terminal domains of octamer histones, but, surprisingly, even more so by the presence of the linker histone.

Tab. 1 - Equilibrium binding properties of DAPI to nucleosomes and free DNA.

	n (bp)			$K_a[D]$		
	I	II	III	I	II	III
146 bp nucleosomal DNA	13	13	5	50.9	5.4	2.4
core nucleosome	13	13	-	6.6	4.3	-

Tab. 2 - Equilibrium binding properties of Hoechst 33258 to nucleosomes and free DNA.

	n (bp)				$K_a[D]$			
	I	II	III	IV	I	II	III	IV
146 bp nucleosomal DNA	33	33	25	6	39.4	2.5	1.6	1.2
chromatosome	33	33	25	-	9.0	1.8	1.3	-
trypsinized core nucleosome	33	33	17	17	4.3	1.9	1.5	1.2
core nucleosome	33	33	25	-	3.6	1.7	1.2	-

DISCUSSION

From our results we can assert that small molecules like DAPI and Hoechst 33258 are able to bind their specific sites even when these are organized in a nucleosome. In fact, in the presence of histones we found a decrease in binding affinity, but the number of binding sites remain unmodified with respect to free DNA. To explain this unexpected result we can draw two hypotheses. First, minor groove ligands may have the capacity to follow the groove and so to arrive at their sites without suffering the steric inhibition of the histones. This situation implies a consideration of a "relaxed" nucleosome structure, in which the contacts between histones and DNA leave the possibility for specific interactions with other molecules. The second hypothesis is supported by the model of a dynamic nucleosome proposed by Widom (12). According to this model, the DNA around the histones could change his rotational and translational phases, exposing the internal sites to the outside of the structure, thus making them available for the contacts with the ligands. This DNA mobility is confirmed by Fox (13), who found a DNA rotation of 180° on the histones surface, after its interactions with Hoechst 33258. He interprets this rotation as an effect inducted by Hoechst, but it could also be explained by the intrinsic DNA mobility found by Widom; both explanations can account for the high accessibility of the nucleosome found in our experiments. In the case of trypsinized nucleosome, the removal of the amino-terminal domains increases the affinity of the specific binding and allows for the presence of a couple of bindings with lower specificity. This is a reasonable result if we consider that the amino-terminal domains may stabilize the DNA around the octamer. What is more surprising is the behavior of the chromatosomes. In fact the linker histone should lock the DNA on the octamer surface, thereby condensing the nucleosome structure. In this way, each DNA movement should be inhibited and so the binding of the ligands remains possible only if they can reach the inside of the nucleosome. With these premises, it should be interesting to verify if we can obtain the DNA rotation found by Fox in the nucleosome

also in presence of the linker histones, thus gaining a possibility to discriminate between our two initial hypotheses.

Using the circular dichroism spectra, induced in the visible range by the binding of minor groove ligands to DNA, we found that two drugs, DAPI and Hoechst 33258, are able to occupy their specific sites even when these are located inside the nucleosome structure. This high accessibility of the binding sites in the nucleosome is not modified by the removal of the amino-terminal domains of the octamer histones and, surprisingly, it is not reduced by the presence of linker histone. Interesting and reasonable differences were found in the association constants, that reveal the "reluctance" of the ligands to bind the DNA-minor groove when the histones are present.

1) LEE D.Y., HAYES J.J., PRUSS D., WOLFFE A.P., Cell, 1993, 72, 73-84.
2) BORTVIN A., WISTON F., Science, 1996, 272, 1473-1476.
3) TRAVERS A.A., Trends in Biological Science, 1999, 24, 4-7.
4) DREW H.R., TRAVERS A.A., J. Mol. Biol., 1985, 186, 773-790.
5) CANEVA R., ROSSETTI L., SAVINO M., Biochim. Biophys. Acta, 1996, 1306, 117-121.
6) CANEVA R., DE SIMONI A., MAYOL L., ROSSETTI L., SAVINO M., Biochim. Biophys. Acta, 1997, 1353, 93-97.
7) AUSIO' J., DONG F., VAN HOLDE K.E., J. Mol. Biol., 1989, 206, 451-463.
8) KORNBERG D.R., Methods in Enzymology, 1989, 170, 3-14.
9) SIMPSON R.T., Biochemistry, 1978, 17, 5524-5531.
10) FITZGERALD D.J., ANDERSON J.N., J. Biol. Chem., 1999, 274, 27128-27138.
11) KUBISTA M., AKERMAN B., NORDEN B., Biochemistry, 1987, 26, 4545-4553.
12) POLACH K.J., WIDOM J., J. Mol. Biol., 1995, 254, 130-149.
13) BROWN P.M., FOX K.R., J. Mol. Biol., 1996, 262, 671-685.

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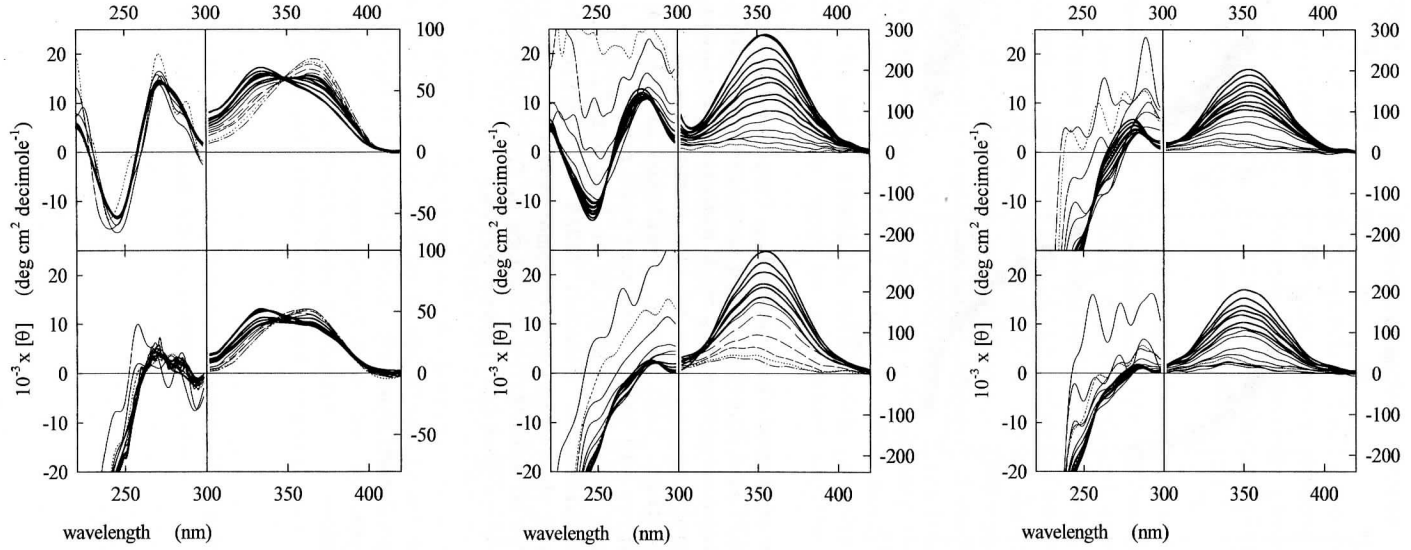

Fig. 1 - CD spectra induced by the bindings of DAPI and Hoechst 33258 to DNA at [bp]/[D] ratios decreasing from 50 to 1. Left panel: complexes of DAPI with free nucleosomal DNA (top) and core nucleosome (bottom). Central panel: the same as in left panel, with Hoechst 33258. Right panel: complexes of Hoechst 33258 with trypsinized core nucleosome (top) and chromatosome (bottom). The molar ellipticity of DNA is reported in the UV range, that of the drug in the visible range.

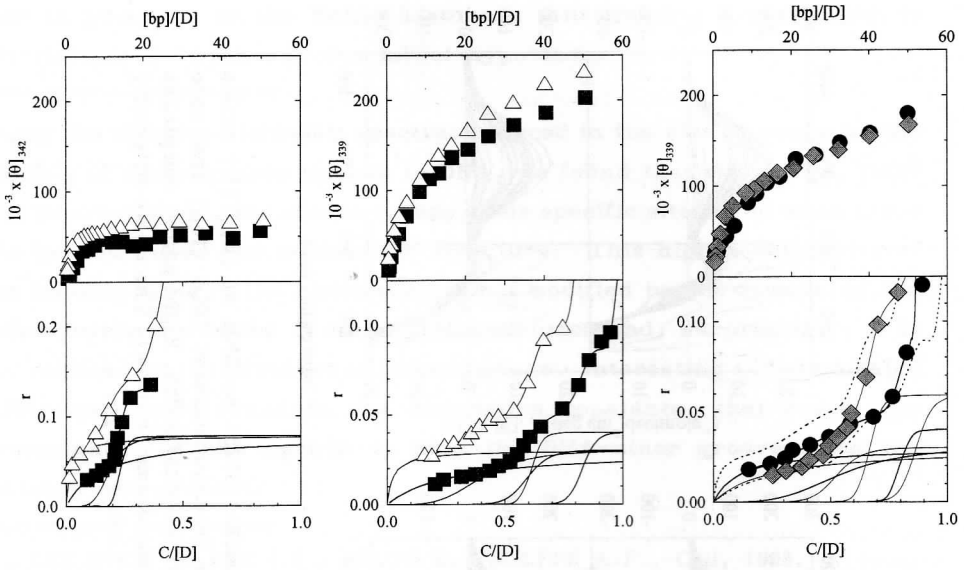

Fig. 2 - Molar ellipticity at ligand absorption λ_{max} (top) and direct binding isotherm (bottom) of DAPI and Hoechst 33258 complexes with DNA. Points are means of at least three experiments, with standard deviations in the order of 10%. Deconvolutions and fits of the cumulative bindings are reported as continuous lines. Left panel: complexes of DAPI with free nucleosomal DNA (triangles and thin lines) and core nucleosome (squares and thick lines). Central panel: the same as in left panel, with Hoechst 33258. Right panel: complexes of Hoechst 33258 with trypsinized core nucleosome (diamonds and thin lines) and chromatosome (circles and thick lines); dotted lines refer to cumulative bindings of free nucleosomal DNA (upper curve) and core nucleosome (lower curve).