BINDING AND ARRANGEMENT OF NON-HISTONE PROTEINS IN CHROMATIN-LIKE STRUCTURES FROM MAMMALIAN CELLS

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1. Introduction

Intercalating agents bind strongly to DNA and inhibit nucleic acid function [1–3]. The analysis of the binding mechanisms has been the object of studies for many years [4–13] and it is believed that the mechanism can be described by the intercalation model [6].

We describe experiments which show that intercalating agents have an effect on protein–DNA complexes. Ethidium bromide (EB) and actinomycin D (AM) prevent non-histone proteins with high affinity for DNA but not histones from binding to a drug–DNA complex. Proflavine (PF) and acridine orange (AO) affect the binding of histones but to a lesser extent of non-histone proteins with high binding affinity. It is suggested that non-histone proteins which bind strongly to DNA recognise the DNA structure in the groove which contains the side groups of EB and AM. These proteins can sense base arrangements and may have a regulatory function.

The release of proteins from protein–DNA complexes by intercalating agents shows that in chromatin these proteins are associated with DNA which is complexed with histones.

2. Materials and methods

Reassociation in one step (Complex I) of cellular proteins from ascites tumour cells with double-stranded DNA from calf thymus by affinity chomatography was carried out as described previously [14]. This protein–DNA complex has a low histone content. Protein–DNA complexes with a histone content similar to that of chromatin were formed by passing DNA free cellular proteins in 1 M NaCl, 20 mM Tris, 2 mM EDTA, 1 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonylfluoride pH 7.5 (TEMP buffer) continuously through a double-stranded DNA–cellulose column and a dialysis chamber arranged in a closed circuit and the salt concentration reduced to 0.1 M NaCl. Double-stranded DNA was saturated with the various intercalating agents in 0.1 M NaCl TEMP buffer and drug concentrations of 250 μg/ml. Proteins were released stepwise from protein–DNA complexes by passing the drugs (250 μg/ml) in 0.1 M NaCl-TEMP buffer through columns followed by stepwise elutions with 0.4 M NaCl and 2.0 M NaCl-TEMP buffer.

CnBr activated sepharose 4-B (5 g) was reswollen, washed in 1 mM HCl and sintered on a glass filter. 100 mg of EB (4 mg/ml) in 0.5 M NaCl, 0.1 M $NaHCO_3$ pH 8.3 was mixed with the Sepharose and the coupling carried out overnight at 4°C, the coupling buffer removed and residual active groups on the gel inactivated by shaking in 50 ml 0.1 M glycine pH 8.0. EB (100 mg; 4 mg/ml in H_2O, pH 4.5) was coupled to CH-Sepharose 4-B in presence of 1 g carbodiimide by overnight rotation at room temperature. Excess EB was removed by washing in buffer containing 0.1 M NaCl, 20 mM Tris, 2 mM EDTA pH 7.5. Chromatography of proteins was in 1 cm × 20 cm columns after equilibration with 0.1 M NaCl-TEMP buffer. Release of bound proteins was with 2.0 M NaCl-TEMP buffer.

HeLa chromatin was prepared according to a method described previously [15]. Analysis of the

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proteins by electrophoresis in gels containing SDS was as described previously [14].

3. Results

Complexes consisting of murine proteins and DNA from calf thymus were formed on columns by a one step reassociation (Complex I) or a progressive reassociation (Complex II). The proteins bound were released stepwise by 0.4 M NaCl and 2.0 M NaCl (peak I and II respectively). In Complex I binding was about 6% and between 1 and 2% in peak I and II respectively, whereas in Complex II binding was about 7% and 6%. About 50 peptide bands can be assigned to non-histone proteins and 5 bands to histones in polyacrylamide gels containing SDS (fig.1 and 2A). Histones can only be reassociated by a progressive reduction of salt (Complex II) at a yield similar to that found in chromatin (table 1).

To study protein binding when the DNA is altered, EB was intercalated and proteins subsequently bound to the complex. Coelectrophoresis in gels containing SDS of polypeptides eluted by 0.4 M NaCl from double stranded DNA and from a complex of double-

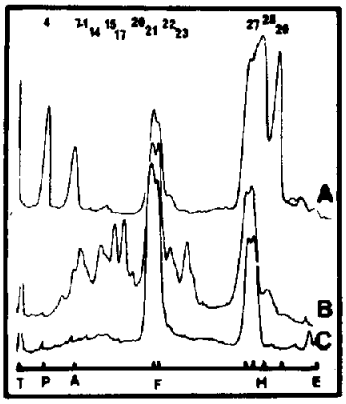


Fig.1. Densitometer tracings of stained proteins bound to ds-ct DNA and ds-ct DNA saturated with EB and eluted between 0.4 M NaCl and 2.0 M NaCl. Numbers of the bands as given previously [14]. No. 21, 22, 27, 28 and 29 are histones. (A) Control proteins phosphorylase A [P], bovine serum albumin [A] and histones from calf thymus [F, H]. (B) Proteins eluted from ds-ct DNA by 2.0 M NaCl elution buffer (peak II). Gel loading: 0.15 *A* 280 nm. (C) Proteins eluted from ds-ct DNA saturated with EB by 2.0 M NaCl elution buffer (peak II). Gel loading: 0.1 *A* 280 nm.

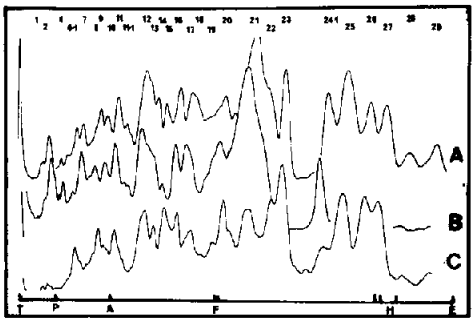


Fig.2. Densitometer tracings of stained proteins eluted stepwise from protein–DNA Complex I with 0.1 M NaCl elution buffer containing EB or 0.4 M NaCl. Protein DNA–Complex I was formed as described previously [14]. Proteins which did not bind to ds-ct DNA were removed by 0.1 M NaCl elution buffer. Bound proteins were then eluted stepwise by EB (250 μg/ml) in 0.1 M NaCl elution buffer and by 0.4 M NaCl. (A) Proteins eluted by 0.4 M NaCl elution buffer from ds-ct DNA as control. Gel loading: Approx. 10% of total bound protein (5 *A* 280 nm) from 100 *A* 280 nm of cellular protein loaded on to the column. (B) Proteins eluted by EB in 0.1 M NaCl elution buffer from ds-ct DNA. Gel loading: 0.13 *A* 280 nm. Approx. 5 to 6% of total protein eluted from ds-ct DNA by EB. (C) Proteins eluted from ds-ct by 0.4 M NaCl elution buffer after the column had been eluted by EB. Gel loading: Approx. 10% of total protein eluted by 0.4 M NaCl.

stranded DNA and EB showed that EB intercalation did not alter the affinity of the proteins in this fraction. However, the polypeptides with high affinity for DNA which are eluted by 2.0 M NaCl could not be bound effectively to the EB–DNA complex (fig.1). A large number of proteins can also be removed from Complex I by EB intercalation (fig.2) but only a minor fraction is removed from Complex II and chromatin when EB is bound to it (fig.3).

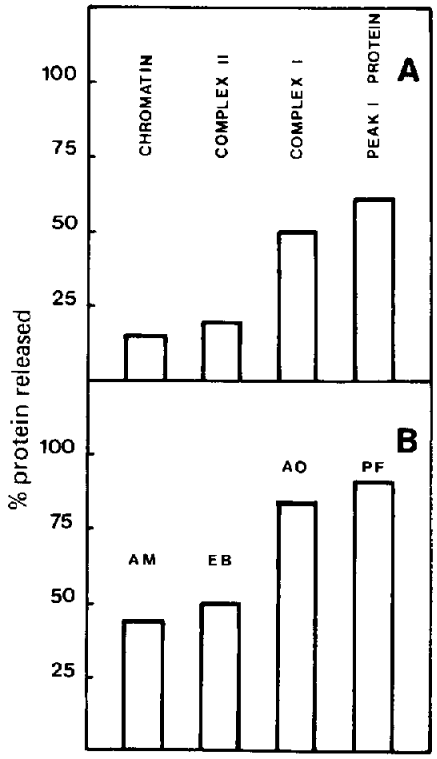
When proteins are bound to DNA saturated with actinomycin D about 4% and 0.8% can be released by increased salt respectively. The protein profiles of gels containing SDS are essentially the same as shown for EB. Similarly about 40% of the non-histone protein fraction was removed from Complex I when AM was bound to the complex (fig.3B).

In order to determine whether steric hindrance or base extension is the reason for the reduced binding of proteins to EB–DNA and AM–DNA similar experiments were carried out with the intercalating agents

Table 1
Relative distribution of non-histone proteins and histones in reconstituted protein-DNA complexes and chromatin

Fraction	Percentage of total protein bound		
	One-step reassociation	Progressive reassociation	Native chromatin
Non-histone proteins eluted by 0.4 M NaCl	76 ± 10	43 ± 5	42 ± 5
Non-histone proteins eluted by 2.0 M NaCl	10 ± 4	10 ± 3	
Histone F1	6 ± 3	4 ± 1	10 ± 2
Residual histones	7 ± 2	43 ± 7	48 ± 5
Histone/Non-histone	1.0/6	1.0/1.1	1.0/0.7
Residual histone/F1	1.2/1.0	10/1.0	5/1.0

Each figure is the mean percentage from 3 experiments and was derived from densitometer tracings from stained polyacrylamide gels containing SDS. The areas below the peaks were compared on a weight per weight basis. For the DNA-binding proteins, figures were corrected to give the exact proportions of total proteins bound in peak I and II.



AO and PF. Both drugs do not have side groups but form strong secondary complexes with DNA [4,5]. When proteins are bound to an AO–DNA complex and the bound fraction released by 0.4 M NaCl and analyzed electrophoretically fewer proteins are observed as compared to the profile obtained from an EB–DNA complex. The reduction in protein binding seems to be determined by the secondary binding of AO. The non-histone proteins with high affinity for DNA, however, could be bound effectively to AO–DNA complexes, (fig.4) except for the bands no. 17 and 18. Histones even in small quantities present in Complex I could not be bound effectively.

The effects described are not caused by a strong protein–drug interaction. This is shown by analysing

Fig.3. Release of proteins from protein–DNA complexes and chromatin by drugs. (A) Amount of proteins released from various DNA–protein complexes by EB. Chromatin was incubated with 250 μg EB in 0.1 M NaCl TEMP buffer for 15 min and the high speed supernatant analysed. Proteins of peak I where complexed with DNA in the absence of histones and treated as Complex I and II. (B) Amount of protein released from Complex I by various intercalating agents.

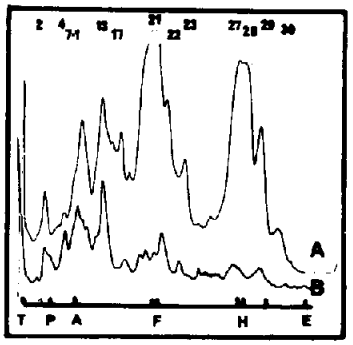


Fig.4. Densitometer tracings of stained proteins bound to a ds-ct DNA–AO complex and eluted by salt in a stepwise fashion. (A) Proteins eluted from ds-ct DNA by 2.0 M NaCl elution buffer. Gel loading: 0.15 *A* 280 nm. (B) Proteins eluted from ds-ct DNA complexed with AO by 2.0 M NaCl elution buffer. Gel loading: Approx. 0.075 *A* 280; 60% of total protein in peak II.

the proteins which are selectively retained on columns containing EB. Soluble cellular proteins as prepared for affinity chromatography [14] were bound to EB-Sepharose in 0.1 M NaCl-TEMP buffer. The column was washed with 4 to 5 column vols of 0.1 M NaCl-TEMP buffer and bound proteins eluted with the above buffer containing 2.0 M NaCl. About 25 proteins equivalent to 6% of the input were bound to EB-Sepharose as determined electrophoretically (fig.5A). Rechromatography of proteins which were released from DNA on EB-Sepharose however showed that this protein fraction was not retained on EB (fig.5B). When the complete 2.0 M NaCl eluate from the EB-Sepharose was subjected to electrophoresis only a small amount of histones was detected. When cellular

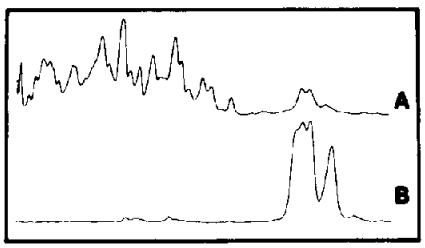


Fig.5. Binding of cellular (A) and DNA-binding (B) proteins to EB-Sepharose 4-B.

proteins were chromatographed on EB-CH-Sepharose 4-B it was found that the spacer which was introduced did not alter the affinity of proteins for EB. The protein fraction of peak II which is not bound to EB–DNA was also chromatographed on EB-CH-Sepharose 4-B. It was found that none of these proteins were specifically retained by EB.

4. Discussion

Various models describe aspects of protein–DNA interactions [16–20]. Common to all the models is the supposition that binding involves several different modes of interaction and that for highly specific recognition to occur, a protein has to envelope the DNA in one of its cavities or has to be enveloped by the DNA in one of the grooves. Since non-histone chromosomal proteins are largely regarded as being involved in the regulation of gene expression [21–25] a close fit for their binding to DNA is required. Intercalating agents are therefore likely to interfere with their binding. The primary binding to DNA of EB [13] and AM [11] is believed to occur via the minor groove whereas AO and PF completely intercalate between the bases. Studies with reporter molecules suggest that chromosome proteins are principally bound to the major groove of the DNA [26]. Our results show that at least one class of non-histone proteins seems to recognise the DNA where the side groups of EB and AM are inserted. They are therefore likely to be involved in the regulation or enzyme functions requiring sequence recognition.

The number of primary binding sites for intercalating agents is lower in chromatin than in DNA [27] but primary binding sites increase as salt soluble proteins or histones are removed [28,29]. Our results show that the release of protein from protein–DNA complexes depends upon the amount of histone in the complex which suggests that the release is correlated with the number of primary binding sites. In chromatin non-histone chromosome proteins should therefore be associated with DNA which is also complexed with histones.

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