ETHIDIUM BROMIDE AS A PROBE OF CHROMATIN STRUCTURE

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Received 15 December 1973
Revised version received 24 January 1974

1. Introduction

Chromatin is made of an association of proteins and DNA. Among proteins, histones are the most important in mass, and qualitatively the best known. There are five main kinds of histones and some of them have an important role in maintaining the tertiary configuration of the DNA [1–4].

The biological function of the structure of DNA in chromatin has been emphasized by several authors but the insufficiency of stuctural data does not allow a choice among the proposed models [5, 6].

Experiments which consider the physico-chemical state of the DNA in chromatin may be a source of information about the possible heterogeneity of the DNA associated with proteins. We have used the property of the DNA to bind a fluorescent label, ethidium bromide (E.B.) [7, 8], to study the physico-chemical state of the DNA in chromatin and the role of some proteins.

The present paper describes the binding isotherms of EB to calf-thymus chromatin and to partially-histone-depleted chromatin by mean of salt extraction. The heterogeneity of DNA is shown by the presence of two types of binding sites in chromatin; the role of histone f_1 is also examined.

2. Experimental

Most of the experimental conditions may be found in an earlier publication [9]. A particular attention is brought to the blending during the extraction process of chromatin so that 98 to 100% of the chromatin is found in the gel form. The integrity of the chromatin fiber is controlled by means of electron microscopy according to the Bram and Ris' method [4].

Histone extraction is performed by the method described by Ohlenbush et al. [10], and histones remaining on the pellet are analysed, after HCl-extraction, by means of polyacrylamide gel electrophoresis [11]. The results are identical to those already described [9].

The dissociation–reassociation experiments are made in the following way: a solution of chromatin ($OD_{260} \simeq 6$) is allowed to equilibrate (12 hr) by dialylis against the appropriate sodium chloride. When the equilibrim is reached the reassociation is achieved by dialysing again the mixture against three changes of distilled water.

Reconstituted complexes of f_1 and DNA are made by the dialysis method of Touvet-Poliakov et al. [12]. The histone f_1 was generously provided by Miss M. Champagne from the University of Louis Pasteur de Strasbourg.

EB binding is measured as described by Le Pecq and Paoletti [7], with a spectrofluorimeter Zeiss with two monochromators (λ excitation = 490 nm, λ emission = 590 nm). All measurements are made at room temperature on solution containing 10^{-3} M NaCl. The fluorescent data give the amount of bound dye, r, and that of free dye, C, which are plotted in terms of Scatchard representaion ($r/C = f(r)$). In the case of a two sites binding process, the graphical resolution method of Ohnishi et al. [13] is used to determine the number of each binding sites and their respective association constant.

3. Results

The binding isotherms of EB to chromatin is shown in fig. 1a. Contrary to the binding of EB to DNA (fig. 2a) this isotherm may be analysed with a two sites model: sites I: $K_1 = 6\ 10^7, N_1 = 0.038$, sites II: $K_2 = 2.5\ 10^5\ N_2 = 0.24$. If the error on the evaluation of the number of binding sites is estimated to 10%, all the binding sites of chromatin DNA are available to the dye, since one reaches the maximum number of binding sites of the DNA according to the excluded site model [14, 15].

The association constants are very different from that of DNA ($K_1/K_{DNA} = 14, K_2/K_{DNA} = 0.06$).

Sites I disappear just as one extracts histone f_1 (fig. 2b) and the number of binding sites now allowed to the dye is lowered by 40%. This phenomenon could simply be due to a partial restructuration of the system after the extraction process. If this is the case it would be at least reversible: fig. 1b showas, indeed, the binding isotherm obtained after a dissociation–reassociation process of histone f_1 and one can calculate (table 1) the number of binding sites of each type and their respective binding constants which are not significantly different from that of native chromatin.

A different result is found if the dissociation process takes place in 1 M NaCl, where in addition to histone f_1, histone f_{2a2} and f_{2b} are removed. After reassociation the two sites binding process is still present but the number of sites II is lowered by 20% while the number of sites I increases slightly. The existence of two types of binding sites appears to be correlated with the presence of histone f_1. One has tried therefore to see if the same phenomenon occurs when this histone is bound alone to DNA.

Fig. 2c, d, e display the binding isotherms of EB to reconstituted DNA–f_1 complexes with protein/DNA ratios of 0.1, 0.45 and 0.7 respectively.

In each case a linear isotherm is found, charateristic of a single type of binding sites. The corresponding association constants are markedly lowered, even in the case of a f_1/DNA ratio, but the total number of binding sites is roughly the same (~0.2).

Finally, one has to look for a possible role of non-histone proteins. Indeed, when f_1 is removed after treatment of chromatin in 0.6 M NaCl, the majority of non-histone proteins is extracted from chromatin

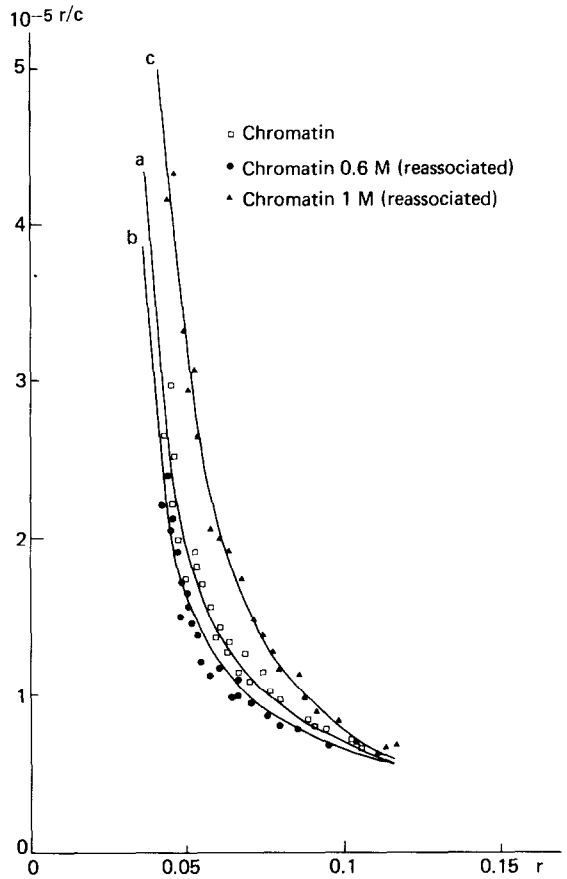

Fig. 1. Binding isotherms to chromatin – (▫); to dissociated–reassociated chromatin in 0.6 M NaCl – (•); to dissociated–reassociated chromatin in 1 M NaCl (▲).

at the same time. In the case of f_1–DNA artificial complexes, non-histone proteins are no more present.

In order to rule out the role played by non-histone proteins, we have stusied the binding of EB to chromatin washed three times in 0.35 M NaCl, a well-known procedure [16] to remove non-histone proteins. A curved isotherm and two types of binding sites are found again (fig. 3), with K and n values very close to those observed with native chromatin (see table 1).

4. Discussion

Experimental results present two distinctive fea-

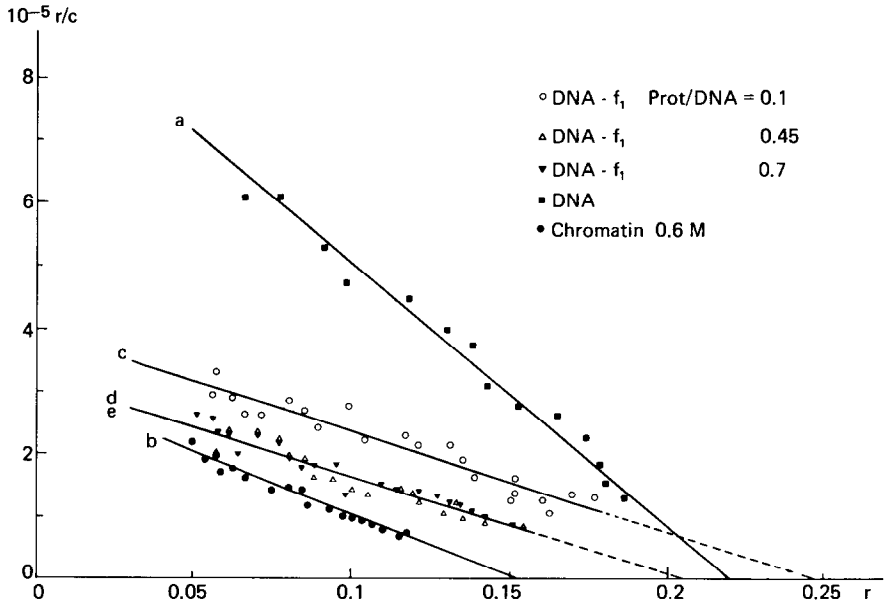

Fig. 2. Binding isotherms of EB to DNA (▪); chromatin 0.6 M (•) and to reconstituted complexes DNA–f_1 with protein/DNA ratio of 0.1 (○), 0.45 (△), 0.7 (▼).

Table 1

Samples	Prot/DNA	Number of types of sites	K_1	N_1	K_2	N_2	K	N
Chromatin	1.6	2	$6\ 10^7$	0.038	$2.5\ 10^5$	0.24	–	–
0.6 M reassociated chromatin	1.6	2	$6\ 10^7$	0.035	$2.5\ 10^5$	0.24	–	–
1 M reassociated chromatin	1.6	2	$6\ 10^7$	0.044	$4\ 10^5$	0.18	–	–
DNA–f_1	0.1	1	–	–	–	–	$1.65\ 10^6$	0.246
DNA–f_1	0.45	1	–	–	–	–	$1.55\ 10^6$	0.204
DNA–f_1	0.70	1	–	–	–	–	$1.50\ 10^6$	0.210
DNA	0	1	–	–	–	–	$4.2\ 10^6$	0.220
Chromatin 0.35 M	1.48	2	$6\ 10^7$	0.047	$2.5\ 10^5$	0.25	–	–

tures:

1) The binding of histones to DNA involves at least electrostatic interactions between negative phosphate and positive animo groups, thus preventing the local opening and detorsion of the helix and decreating the number of EB binding sites. At the same time, in view of the surrounding of DNA by the positivly charged histone molecules, the absolute value of its negative potential is decreased. The binding constant of the positively charged dye must therefore be lower if intercalation occurs in the vicinity of histone molecules. However, in the case of f_1 depleted nucleohistone, for which 50% of the DNA appears stripped according to melting profiles, a linear isotherm and only one binding constant is observed. Actually EB binding to partially depleted nucleohistone is never similar to that observed with free DNA, which is likely to be due to the existence of a tertiary structure even for low protein/DNA ratio.

2) The two sites binding process (a) is obtained with chromatin, only if f_1 is present and does not depend of the amount of non-histone proteins (0.35 M extractable) (b) is not observed when f_1 alone is bound to DNA.

It can be thus concluded that the interaction of f_1 with other histones, induces the information of

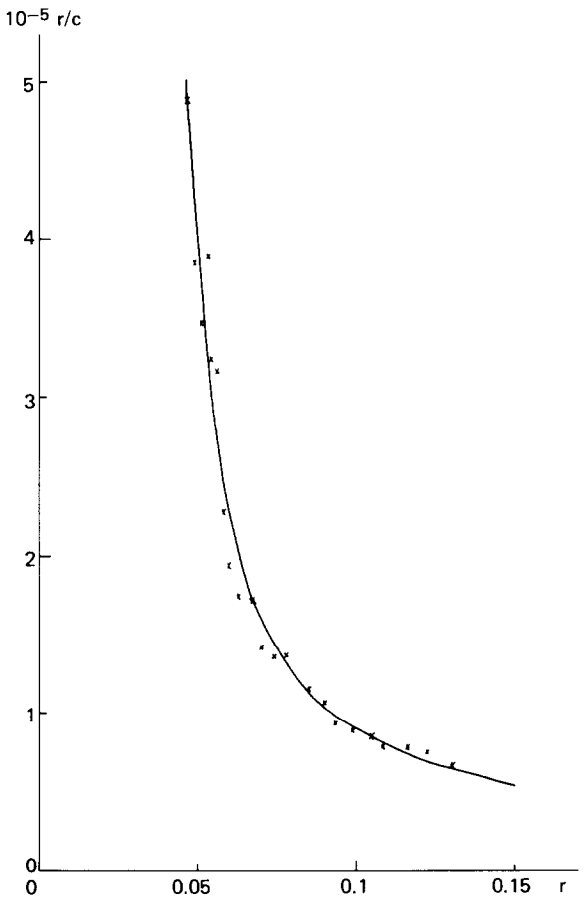

Fig. 3. Binding of EB to chromatin washed three times with 0.35 M NaCl.

regions along the DNA where EB is strongly bound.

Since EB binding is not base-pair specific, one can assume that some parts of the double-stranded DNA are maintained in a physical constraint favorable to intercalation and somewhat similar to that observed in the beginning of the binding process to covalently twisted circular DNA.

The bound dye/nucleotide ratio could then be higher than the normal value of 0.25 (but of course lower than 0.5) and one can say that 8 to 15% of the DNA in the chromatin presents this high reactivity with respect to EB intercalation.

These easily opened regions appear to be possible sites of transcription of the chromatin.

Acknowledgements

We wish to thank Professor C. Mouriquand for the constant interest he brings to this study.

We are grateful to Professor Daune for numerous and so fruitful discussions.

Loeey Roby has been a critical judge for our English translation. We thank her for this generous task.

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