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The interaction of core histones with DNA: equilibrium binding studies

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ABSTRACT

The binding of core histone proteins to DNA, measured as a function of [NaCl] is a reversible process. Dissociation and reassociation occurs in two stages. Between 0.7 and 1.2 M NaCl H2a and H2b bind non-cooperatively as an equimolar complex with ΔGo = 1.6 Kcals/mole at 4°C and 1.0 M NaCl. Between 1.2 and 2.0 M NaCl H3 and H4 bind cooperatively as an equimolar complex with ΔGo = 7.4 Kcals/mole at 4°C and 1.0 M NaCl. The proper binding of H2a and H2b requires the presence of bound H3 and H4. Nuclease digestion of H3-H4 DNA produces a tetramer of H3-H4 bound to fragments of DNA 145, 125 and 104 base pairs long. Thus an H3-H4 tetramer can protect fragments of DNA as long as those found in complete core particles and must therefore span the nucleosome core particle.

INTRODUCTION

Chromatin, the interphase form of the genetic material, is made up of a linear array of repeating substructures called nucleosomes. Each nucleosome is composed of eight core histones, two each of the histones H2a, H2b, H3 and H4 associated with about 200 base pairs of DNA and two molecules of histone H1 [1]. The nucleosomes interact with each other to form a linear fibre about 100 Å in diameter. Although a wealth of information has accumulated in the last few years relating to the structure and function of chromatin [2] there have been few quantitative studies on the binding of histones to DNA. Such studies are important because they provide a thermodynamic framework within which the properties of chromatin can be discussed. Thermodynamic information on the interaction of histones with DNA can be obtained from equilibrium binding curves and their variation with temperature, pH, ionic strength and so on. Histone-DNA interactions in chromatin are, however, highly complex in the sense that five different proteins interact with each other and with DNA to form a stoicheiometric complex in the nucleosome. Thus the construction of binding curves for the binding of individual histones or histone pairs to DNA may not be as revealing as the binding of all five histones. On the other

hand all the histones bind very tightly to DNA at physiological ionic strengths which makes the task of constructing equilibrium binding curves very difficult. It is known, however, that the histones may be dissociated differentially from chromatin by increasing concentrations of NaCl [3]. In the following the properties of the binding curves obtained in this way are described. In these experiments a sheared chromatin preparation has been used in which the higher-order structure has been destroyed since it does not give the characteristic pattern of DNA fragments on digestion with micrococcal nuclease [4,5]. These higher-order interactions may involve both H1 and the core histones. However, the same limit-digest pattern is obtained on extensive digestion with micrococcal nuclease whether the starting material is native chromatin or sheared chromatin [6,7]. In sheared chromatin, therefore, the core histone-DNA interactions are preserved. In what follows the binding of core histones to DNA in the presence of dissociated H1 are described.

EXPERIMENTAL METHODS

Chromatin was prepared from calf thymus tissue by the method of Zubay and Doty [8] and characterised as described previously [3,9,10]. Circular dichroic and viscosity measurements were made as previously described [9,10].

Digestion of chromatin with micrococcal nuclease (Worthington Biochem. Corp. Ltd.) was carried out at 37°C in Tris-HCl (1 mM, pH 7.0), $CaCl_2$ (10^{-4} M). The reaction was terminated by adding EDTA to 10 mM and cooling the reaction tubes in ice. The chromatin was then incubated with proteinase K (Boehringer Biochem. Corp.), 100 µg/ml, for 1 h at 37°C. The DNA was further deproteinised with chloroform-isoamyl alcohol (24:1) and then dialysed against Tris-HCl (40 mM) pH 8.0, sodium acetate (5 mM), EDTA (1 mM) for electrophoresis on agarose or polyacrylamide gels. After electrophoresis, the DNA was visualised by staining with ethidium bromide. The size of the DNA fragments was determined by comparison with fragments of PM2 or polyoma DNA of known size digested with restriction nuclease Endo R Hae III (gift from B. Ponder). Polyoma fragments were in turn calibrated against sequenced $\phi \times 174$ DNA fragments (B. Ponder, private communication); PM2 fragment sizes were according to Noll [11].

The dissociation of core histones from DNA was induced by increasing the concentration of NaCl over the range 0.7 to 2.0 Molar. In all cases the salt solutions contained sodium dihydrogen phosphate, 10 mM and disodium hydrogen phosphate, 10 mM, so that the final pH of the solutions was 6.2–6.4. The pH

meter was calibrated with sodium hydrogen phthalate, 0.1 M, pH 4.01 and sodium borate decahydrate, 0.1 M, pH 9.18, all measurements being made at 18–20°C. The construction of binding curves requires that the concentrations of the products and reactants in the dissociation reaction be measured without disturbing the equilibrium. This may be done by true equilibrium methods which measure the concentrations of the various species present in situ or by kinetic methods which separate the products and reactants and allow their concentrations to be measured separately. In the case of the system under study here the application of equilibrium methods is not possible since there is no easily measurable property of dissociated histone which distinguishes it from bound histone. A kinetic method has therefore been used which separates dissociated histone from depleted chromatin using gel filtration with Sepharose 4B. The method relies on the fact that the rate of dissociation and reassociation is slow compared to the time required to separate the products and reactants during their passage down the column. The equilibrium is thereby frozen. A typical elution profile is shown in Fig. 1 of ref. 12 which shows that dissociated histone can be completely separated from chromatin. In high salt there is a tendency for the histone to dissociate from the depleted chromatin peak which leads to a variation of histone to DNA ratio across the depleted chromatin peak. However, the histone-DNA ratio of the original depleted chromatin may be obtained by pooling all fractions across the peak and measuring the average histone-DNA content [12]. Chromatin (0.1–1.0 mg/ml) in 0.7 mM sodium phosphate, pH 7.0, was dialysed for 3 hours against salt solutions at either 4°C or 20°C. Aliquots (4 mls) were applied to a column of Sepharose 4B (50 × 1.0 cm) equilibrated with the appropriate salt solution; fractions (2 ml) were collected and analysed.

RESULTS

The dissociation curve of histones from DNA as a function of NaCl concentration is shown in Fig. 1. Dissociation of histone takes place in three distinct stages. Between 0.7 mM sodium phosphate and 0.7 M NaCl H1 is selectively dissociated as shown previously [13]. Between 0.7 and 1.2 M NaCl H2a and H2b are selectively removed from the chromatin and between 1.2 and 2.0 M NaCl H3 and H4 are dissociated (Fig. 2 and refs. [3,12]). Dissociated H1 is always present in the system during the dissociation and re-association of the other four core histones.

(a) The Dissociation of H2a and H2b

The dissociation curve of H2a and H2b from chromatin is shown in detail

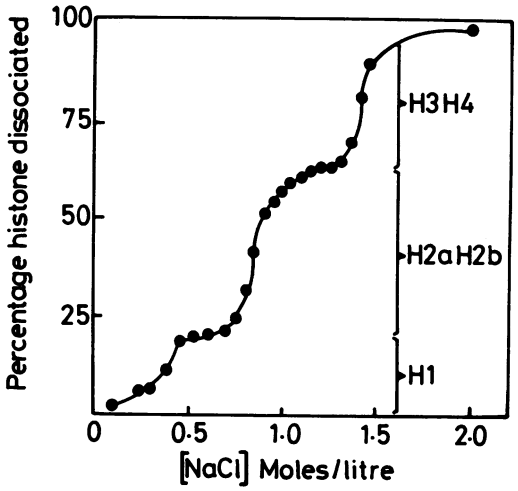

Fig. 1. The dissociation of histone types from chromatin as a function of NaCl concentration.

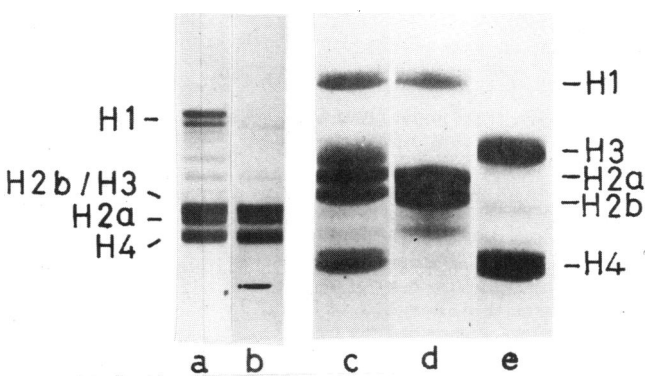

Fig. 2. Polyacrylamide gel electrophoresis patterns of histone types dissociated as a function of NaCl concentration: SDS-polyacrylamide gels of (a) histones in chromatin; (b) histones remaining bound to chromatin in 0.7 M NaCl; polyacrylamide-urea gels of (c) histones in chromatin; (d) histones dissociated in 1.2 M NaCl; (e) histones remaining bound to chromatin in 1.2 M NaCl.

in Fig. 3 together with the re-association curve obtained by dialysing samples of chromatin into 1.2 M NaCl, to dissociate completely H2a and H2b, followed by further dialysis into salt solutions of lower concentrations in the range 1.2 to 0.7 M. The two sigmoid curves are equivalent showing that the dissociating species are in reversible thermodynamic equilibrium.

The effect of chromatin concentration on the degree of dissociation was

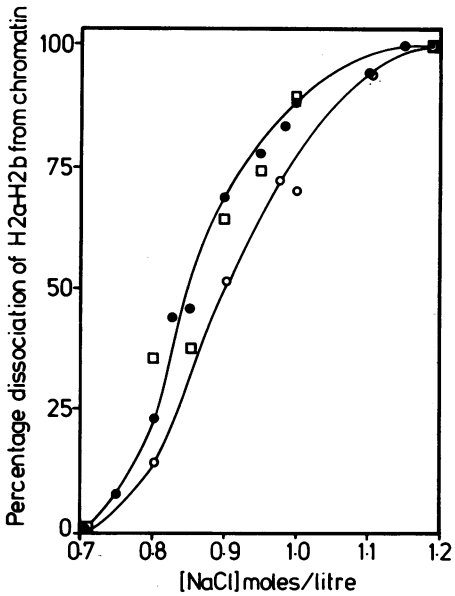

Fig. 3. The dissociation of H2a-H2b as a function of NaCl concentration: — ● — forward dissociation curve at 4°C; — □ — reverse dissociation curve at 4°C; — ○ — forward dissociation curve at 20°C.

investigated at a constant NaCl concentration of 0.85 M by decreasing the initial chromatin concentration. As would be expected from considerations of mass action effects on a disproportionation reaction, the degree of dissociation increases with increasing dilution as shown in Table 1. The right-hand column shows the dissociation constant, K_{app}, predicted by applying the simple mass action law. The degree of dissociation, α, is defined in the legend to Table 1. The relatively constant value of K_{app} over the four-fold change in initial concentration leads to the conclusion that H2a and H2b interact with chromatin non-cooperatively, that is, they bind to equivalent, independent, non-interacting sites. The effect of temperature on the dissociation is shown in Fig. 3. Increasing the temperature from 4°C to 20°C decreases the degree of dissociation over the whole dissociation range although the two dissociation curves are not parallel. The dissociation reaction is therefore exothermic but the enthalpy change may vary with the salt concentration possibly due to a contribution to the enthalpy change from salt-induced conformational changes in the components of the system.

The amounts of H2a and H2b which remained bound to the chromatin at various degrees of dissociation were monitored using polyacrylamide urea gel

TABLE 1

c_0	α	K_{app}
1010	0.26	9.2
725	0.28	8.2
520	0.29	6.1
250	0.38	5.9

Apparent dissociation constant, $K_{app} = \alpha^2 c_0^2/(1-\alpha)c_0$, as a function of degree of dilution of chromatin at NaCl = 0.85 M. c_0 = initial concentration; α = degree of dissociation of H2a-H2b. K_{app} in arbitrary units; temp. = 4°C.

electrophoresis. The results are shown in Table 2 and suggest that these two histones appear to dissociate concomitantly possibly as an equimolecular complex. This suggestion is supported by crosslinking studies which show that between 0.7 and 1.2 M NaCl dissociated H2a and H2b may be covalently cross-linked by formaldehyde to form dimers and higher polymers. No monomers were detected [3].

The dissociation reaction is also reversible at the level of the secondary structure of the histones and DNA in the chromatin complex as judged by the CD spectra of partially dissociated and reconstructed chromatin. Data were obtained for four different salt concentrations between 0.7 and 1.2 M NaCl. The spectra of the dissociated and reassociated chromatins were identical in all cases; that for 0.85 M NaCl is shown in Fig. 4.

(b) The Dissociation of H3 and H4

The dissociation and reassociation curves of H3 and H4 from DNA is shown in Fig. 5. As in the case of H2a and H2b the curve is sigmoidal and thermodynamically reversible. It is important to realise that the dissociation of H3 and H4 from DNA has here been studied in the presence of unbound H1, H2a and H2b, which have completely dissociated before H3 and H4 begin to dissociate above 1.2 M NaCl (Fig. 1).

The effect of protein concentration on the degree of dissociation was investigated at 1.35 M NaCl, as before, by decreasing the initial concentration. The equilibrium constants, calculated by assuming a simple mass action effect on the reaction, are shown in Table 3. The degree of dissociation α is defined as before. Over a five-fold range of initial chromatin concentration, the value of K_{app} changes by two orders of magnitude, showing that K_{app} is itself a function of concentration. This indicates that the binding of H3 and

TABLE 2

[NaCl],M	α	[H2a]/[H2b]
0.8	.25	1.04
0.9	.70	1.02

The ratio of H2a to H2b remaining bound to chromatin at two different degrees of dissociation, α. Temp. = 4°C.

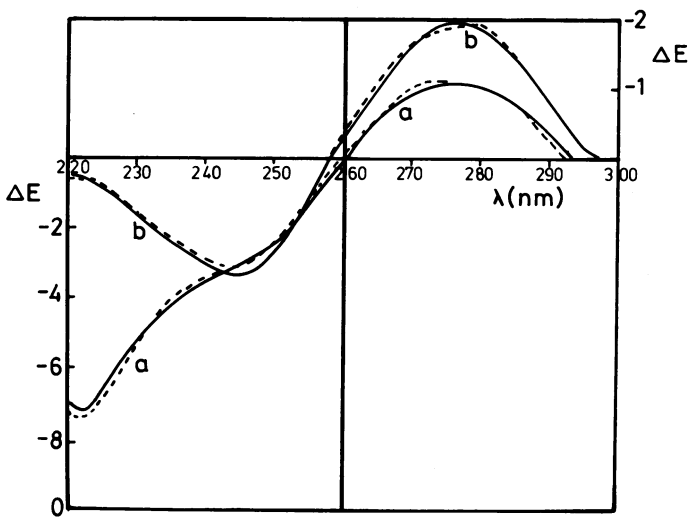

Fig. 4. Circular dichroic spectra of partially dissociated and reassociated chromatin in (a) 0.85 M NaCl and (b) 1.35 M NaCl: —— dissociated species, ---- reassociated chromatin; ΔE is the difference in extinction per mole of nucleotide.

H4 to DNA is accompanied by cooperative interactions between the reacting species.

Increasing the temperature from 4°C to 20°C provokes a decrease in the degree of dissociation showing, as with H2a and H2b, that the reaction is exothermic. The two dissociation curves are not parallel which suggests that there may be a contribution to the overall enthalpy change from salt-induced conformational changes.

An analysis of the histone types which are dissociated over the range 1.2 to 2.0 M NaCl by gel electrophoresis has shown that H3 and H4 are dissociated and reassociated in equimolar amounts, probably as an equimolar com-

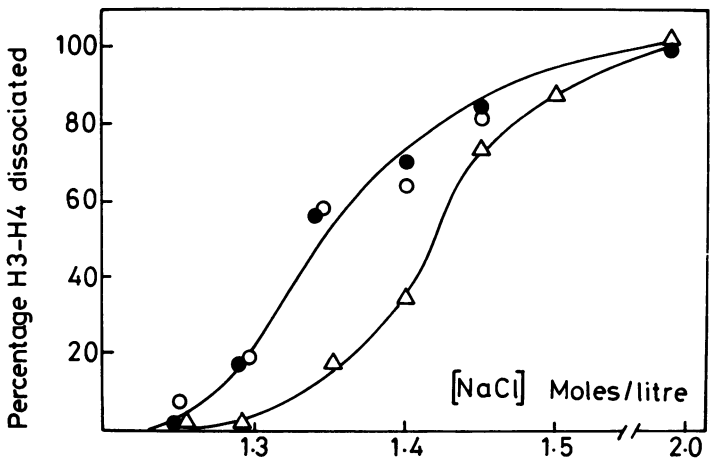

Fig. 5. The dissociation of H3-H4 as a function of NaCl concentration: — ● — forward dissociation curve at 4°C; — ○ — reverse dissociation curve at 4°C; — △ — forward dissociation curve at 20°C.

plex, which must therefore be at least a dimer [12]. This result is supported by crosslinking studies of H3-H4 DNA in 2 M NaCl which show that the histones exist as heterotypic dimers, tetramers and higher polymers [3]. Circular dichroic spectra of partially dissociated complexes and re-associated complexes at the same NaCl concentration show that as regards the secondary structure of the nucleoprotein the interaction is completely reversible (Fig. 4).

The intrinsic viscosity of the native, sheared chromatin preparation was 20 dl/gm, in 0.7 mM sodium phosphate, pH 7.0. The intrinsic viscosity of the DNA in the same solvent was 105 dl/gm. A sample of chromatin was made 2 M in NaCl by the addition of solid and the solution was then dialysed into 0.7 mM sodium phosphate. The intrinsic viscosity of the reconstructed sample was 20 dl/gm. This shows that dissociation and re-association of all five histones takes place reversibly with respect to viscosity which is a measure of the supercoiling and compaction of the molecule [14].

The intrinsic viscosity of H3-H4-DNA in sodium phosphate (0.7 mM, pH 7.0) was 42 dl/gm, indicating that the arginine-rich histone DNA complex has a conformation intermediate between the fully supercoiled native structure and free DNA. H3-H4-DNA which had been dissociated in 2M NaCl and then re-associated by dialysis into 1.1 M NaCl had an intrinsic viscosity of 51 dl/gm (measured in 0.7 mM sodium phosphate). Thus the intermediate, partly supercoiled conformation is recovered after dissociation and re-association of the arginine-rich histone complex.

TABLE 3

c_0	α	K_{app}
106	0.10	1.17
97.5	0.16	3.02
76.1	0.42	23.0
66.7	0.52	37.5
46.9	0.70	76.4

Apparent dissociation constant, $K_{app} = \alpha^2 c_0^2/(1-\alpha)c_0$, as a function of degree of dilution of chromatin at NaCl = 1.35 M. c_0 = initial concentration; α = degree of dissociation of H3-H4. K_{app} in arbitrary units; temp. = 4°C.

(c) Analysis of Binding Curves

The equation describing the equilibrium between dissociated histone and chromatin as a function of NaCl may be written as:

$$DNH + nNaCl = DNH' + mH$$

where DNH is the molar concentration of undissociated chromatin, H is the molar concentration of dissociated histone, DNH' is the concentration of depleted chromatin, n is the number of moles of NaCl bound to depleted chromatin when m moles of histone are dissociated. The equilibrium constant, K_{eq}, may then be written:

$$K_{eq} = \frac{(DNH')(H)^m}{(DNH)(NaCl)^n} \quad . \qquad (1)$$

It follows that

$$\log K_{eq} = \log \left[\frac{(DNH')(H)^m}{(DNH)}\right] - n \log (NaCl).$$

For equivalent, independent, non-interacting sites for histone binding, m = 1; cooperativity is indicated if $m > 1$. It is assumed that the number of binding sites for histone molecules in native chromatin is equal, on a molar basis, to the number of moles of histone bound. That is, in the native state, for core histones, the binding sites are fully saturated and binding is stoicheiometric. If c_0 is the total concentration of a given histone type bound in native chromatin the concentration of dissociated histone is αc_0 where α is the degree of dissociation determined from the binding curves; αc_0 is the

concentration of free binding sites on the chromatin and $(1-\alpha)c_0$ is the concentration of bound sites on the chromatin. It follows from (1) that

$$K_{eq} = \frac{(\alpha c_0)^{m+1}}{(1-\alpha)c_0\,(NaCl)^n} \cdot \qquad (2)$$

Since it has already been shown that the binding of H2a-H2b to DNA is non-cooperative (Table 1), m = 1 and a graph of log (DNH')(H)/(DNH) against log (NaCl) will have a slope of n. Such a graph is shown in Fig. 6, for the data at 4°C and 20°C. The relationship is linear in both cases with n = 30 ± 1 per dimer of H2a-H2b. The intercept at 4°C gives K_{eq} = .072 M and ΔG_0 = 1.6 Kcals/mole at 1.0 M NaCl. The temperature-dependence of K_{eq} yields values of ΔH_0 and ΔS_0 equal to -10 Kcals/mole and -31 cals/mole/° respectively at 4°C and 1.0 M NaCl.

Thus the dissociation of one mole of H2a-H2b dimer results in the net binding of 30 moles of NaCl. The standard free energy of dissociation is positive which is to be expected since molar concentrations of NaCl are required to dissociate histones from chromatin in micromolar concentrations. Since the enthalpy change is negative, the positive free energy term is dominated by the large negative entropy change on dissociation. This presumably

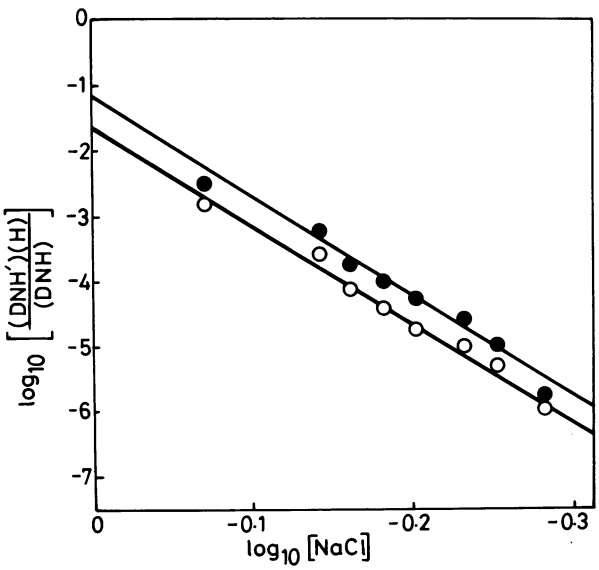

Fig. 6. The variation of $\log_{10}[(DNH')(H)/(DNH)]$ with $\log_{10}$ (NaCl): — ● —, 4°C; — ○ — 20°C.

arises primarily as a result of the ordering of solvent water molecules on breaking electrostatic bonds and hydrophobic interactions in the dissociation reaction. The number of sodium chloride molecules bound as one molecule of histone dimer dissociates is very close to the number expected (27) if all the lysine and arginine side chains in the amino terminal domains of the histones defined as in ref. [15] form salt links with phosphate groups on the DNA.

Since the dissociation of H2a-H2b is non-cooperative with respect to histone the sigmoidal nature of the dissociation curve (Fig. 3) shows that the dissociation with respect to NaCl is cooperative. It is important to realise that n in Equation (1) is not a Hill coefficient and is therefore not a measure of the degree of cooperativity. It is not possible to construct Hill plots directly from the data presented here because there is no direct measure of the amount of sodium chloride bound as a function of the sodium chloride concentration. However, by making the assumption that the amount of sodium chloride bound (to either DNA or histone or both) is directly measured by the degree of dissociation of the histone it is possible to construct a Hill plot with a slope of 11.5, which would indicate a high degree of co-operativity for sodium chloride binding.

The data of Table 3 show that the binding of H3-H4 to DNA is cooperative and that $m > 1$ in Equation (1). It follows from (2) that

$$K_{eq} = \frac{\alpha}{1-\alpha} \cdot (\alpha c_0)^m \cdot \frac{1}{(NaCl)^n} ,$$

and $\log K_{eq} = \log \alpha/1-\alpha + m \log (\alpha c_0) - n \log (NaCl)$. At constant (NaCl)

$$\log K_{eq} = \log \frac{\alpha}{1-\alpha} + m \log (\alpha c_0) + \text{Constant.}$$

Thus m may be obtained from the slope of a graph of $\log \alpha/1-\alpha$ versus $\log \alpha c_0$. This is shown on Fig. 7 and gives slope of $m = 2$. From equation (2),

$$\log K_{eq} = \log \left[\frac{(\alpha c_0)^{m+1}}{(1-\alpha)c_0} \right] - n \log (NaCl).$$

Thus a graph of $\log [(\alpha c_0)^{m+1}/(1-\alpha)c_0]$ with $m = 2$, versus log (NaCl) gives a slope of n. This is shown in Fig. 7 and gives $n = 14.0$ per mole of H3-H4 dimer. As before this number represents the net number of sodium chloride molecules bound when one mole of H3-H4 dissociates. It may be compared to the number of basic amino acid side chains (29) in the N-terminal domains of H3-H4 (see ref. [15]) and indicates that, in contrast to H2a-H2b, displacement of the histone-DNA salt linkages by bound counter ion is not stoicheiometric.

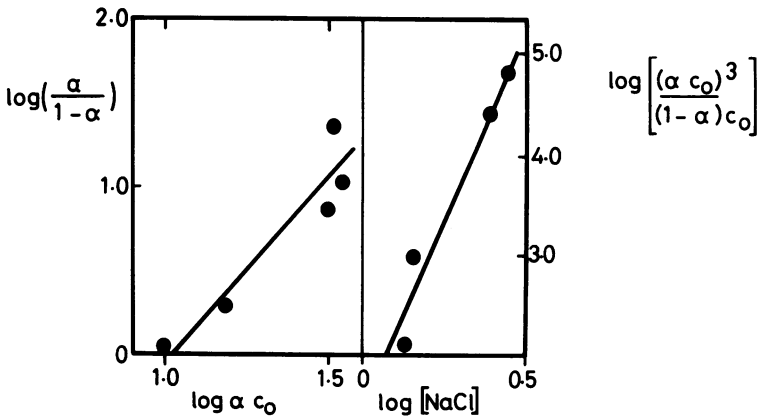

Fig. 7. Left: the variation of log $(\alpha/1-\alpha)$ with log αc_0; right: the variation of log $[(\alpha c_0)^3/(1-\alpha)c_0]$ with log (NaCl).

The apparent equilibrium constant at 1 M NaCl is 4.3×10^{-6} M, corresponding to ΔG_0 = 7.4 Kcals/mole; ΔH_0 = -15 Kcals/mole at 1.4 M NaCl.

(d) Circular Dichroism Studies

It is well known that the circular dichroic spectrum of nucleohistone in the region 300–260 nm is very much reduced in intensity compared to that of DNA [10,16]. This difference has been attributed to short range effects of the bound histones on the DNA conformation [10]. The separate contributions of the lysine rich and the arginine rich pair to the decrease in dichroism at 280 nm is shown as a function of the amount of each histone type bound in Fig. 8. The removal of H2a-H2b caused a proportional increase in the dichroism which is consistent with the non-cooperative mode of binding revealed by the binding curves. By contrast the dissociation of H3-H4 caused a disproportionate change in the dichroism of DNA showing that the interaction of H3 and H4 with DNA produces long range cooperative changes in the conformation of the DNA. These conformational changes may be related to the cooperative binding curves described earlier.

(e) Nuclease Digestion Studies

Nuclei, sheared chromatin, H1-depleted and H1-H2a- and H2b-depleted chromatin were digested with micrococcal nuclease for various times, the DNA extracted and the fragments separated and sized on acrylamide gels (Fig. 9). The nucleosome repeat length for native chromatin in nuclei was 200 base pairs, in good agreement with previous estimates [1]. As others have found,

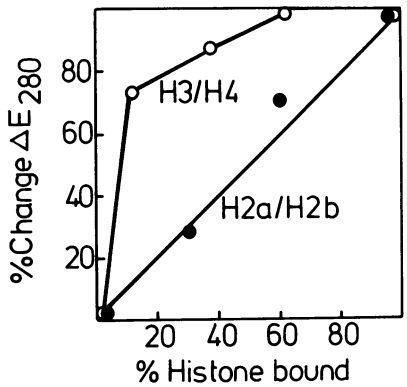

Fig. 8. The percentage change in ΔE at 280 nm for the dissociation of H2a-H2b over the range 0.7 to 1.2 M NaCl and for the dissociation of H3-H4 over the range 1.2 to 2.0 M NaCl. The latter spectra were all measured at 1.30 M NaCl. ΔE is the difference in extinction for left and right circularly polarised light per mole nucleotide.

sheared chromatin and depleted chromatin did not give the regular ladder of base pair repeats observed for the native material [5,6]. H1-depleted chromatin showed discrete bands at 570, 420, 290 and 160 base pairs. The larger fragments decreased in amount with time to produce a limit digest with bands at 145, 130, 108, 99, 77, 65, 55 and 45 base pairs. At early stages in the digestion the oligonucleosome patterns indicate an internucleosome spacing of 140–150 base pairs which is considerably less than the repeat length in the native chromatin (200 base pairs). Thus, removing H1 appears to have caused the nucleosome core particles to slide closer together, as observed by others [28]. An alternative explanation might be that the process of digestion and degradation of the DNA itself promotes a rearrangement of the core particles so that they move closer together. The oligomer bands are very broad with a sharper leading edge than trailing edge. For example the width of the trimer band corresponds to 50 base pairs and the material in the band is clearly very heterogeneous arising from fragments as large as 450 base pairs and as small as 390 base pairs. It is possible that at very early times of digestion the repeat length could approach that of the native chromatin. After longer digestion times core particles are produced with maximum DNA lengths of 145 base pairs, as observed by others [6,7]. Extensive cuttings of intranucleosomal DNA has occurred to give the well-characterised subnucleosomal lengths.

The gels of H3-H4 DNA showed a smear at early times of digestion but

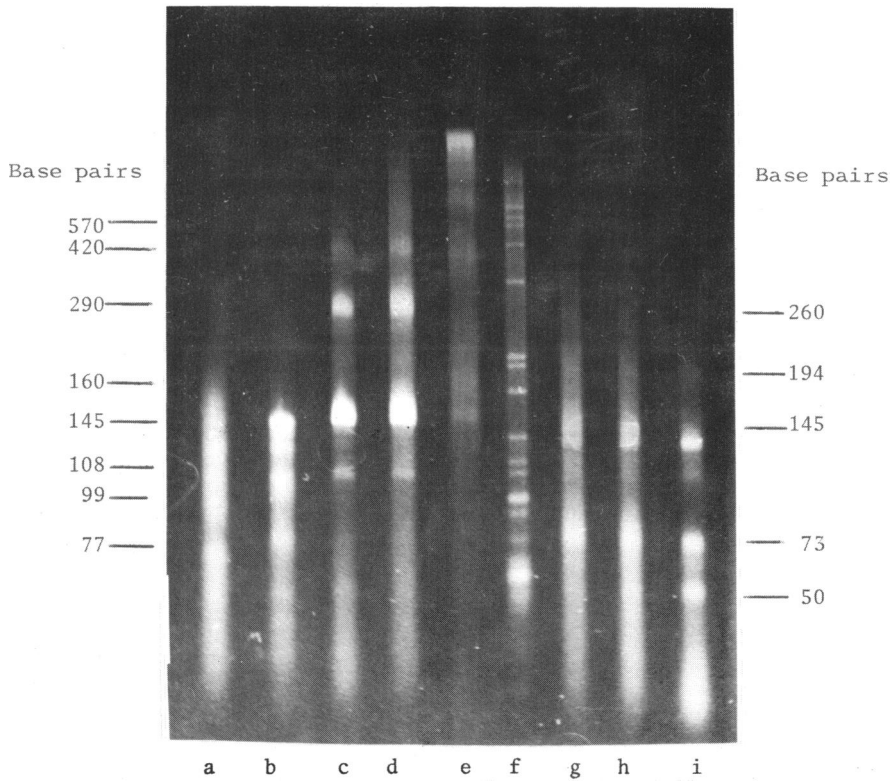

Fig. 9. Digestion of H1 depleted chromatin and H3 H4 DNA with micrococcal nuclease followed by electrophoresis on 5% polyacrylamide gels. a, Calf thymus limit fragments; b-d, time course of digestion of H1-depleted chromatin; b, 60 mins (36% digestion); c, 15 mins (24% digestion); d, 10 mins (14% digestion); e, partial digestion native chromatin; f, Polyoma DNA digested with Hae III; g-i, time course of digestion of H3-H4 DNA; g, seven mins (36% digestion); h, 10 mins (38% digestion); i, 40 mins (56% digestion). The extents of digestion were determined in all cases from the absorbance of the sample at 260 nm, after digestion and dialysis, relative to a zero-time control.

later on discrete bands were observed at 260, 194, 145, 135, 129, 105, 73 and 50 base pairs. A metastable limit digest was reached at about 60% digestion with major bands at 145, 135, 73 and 50 base pairs. It was not possible to estimate a repeat length for the H3-H4 core particle from this data but it is clear that the arginine-rich complex is capable of transiently protecting DNA fragments which are longer than the DNA lengths associated with complete nucleosome core particles (140 base pairs) and longer even than the DNA repeat in native chromatin. From the size of the limit-digest bands it is

clear that H3-H4 alone is capable of protecting DNA lengths the size of the complete nucleosome core particle.

DNA, reconstituted by salt dialysis with H2a-H2b or H1, H2a-H2b, and then digested with micrococcal nuclease gave rise to smears on gels; no discrete bands were seen in keeping with other reports [17]. The explanation for this may lie in the observation that the secondary structure of the bound histone, as measured by circular dichroism at 222 nm, decreased by over 50% when the ionic strength was decreased from 2 M NaCl to 1 mM Tris. Thus the DNA is unable to stabilise the structure of H1, or H1, H2a and H2b at low ionic strength. On the other hand in the presence of H3 and H4, the secondary structure of all four core histones when bound to DNA remains intact when the ionic strength is decreased.

(f) Separation of Limit-digest Products

H3-H4 DNA in 0.7 mM sodium phosphate, pH 7.0, was digested for 30 mins at 37°C with micrococcal nuclease, the reaction terminated by the addition of 10 mM EDTA, pH 7.0, and the products applied to a column of Sephadex G-200 equilibrated with 10 mM EDTA, pH 7.0 at 4°C. The elution profile showed that the column had resolved three fractions of material absorbing at 260 nm. The protein and DNA content of the three fractions were determined and the size of the DNA fragments in each fraction were measured by electrophoresis on polyacrylamide gels (Fig. 10). These experiments showed that the first fraction which eluted from the column had a DNA protein ratio of 1.9. The DNA fragments in this fraction were resolved into three components of length 145, 125 and 104 base pairs present in approximately equal amounts. The amount of protein associated with these fragments suggests that a tetramer of H3-H4 is associated with 104-145 base pairs of DNA. The sedimentation coefficient of fraction I was 7.2 in 10 mM Tris, pH 7.0.

DISCUSSION

The results presented here show that the interaction of core histones with DNA is a thermodynamically reversible process with respect to the amount and type of histone bound and with respect to the secondary and tertiary structure of the nucleo-protein complex. Other studies have shown that it is also reversible with respect to the intimate association of the histones with DNA as revealed by the protection to digestion of the DNA with micrococcal nuclease [17]. Dissociation and re-association of core histones takes place in two discrete stages. When the NaCl concentration is increased above 0.7 M, the histones H2a and H2b dissociate as an equimolar complex,

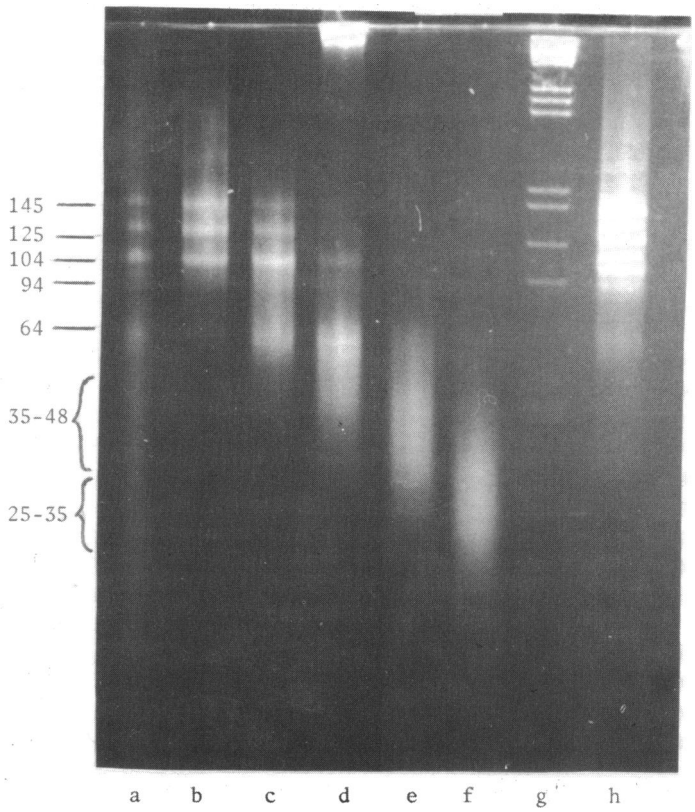

Fig. 10. Gel electrophoresis patterns of H3-H4 DNA digested with micrococcal nuclease and then fractionated by gel filtration with Sephadex G-200: a, unfractionated digestion mixture; b, c, d, fractions across Fraction I (1st peak from column); e, f, fractions across Fraction II (2nd peak from column); g, PM2 DNA digested with Hae III; h, same as b.

probably a dimer, in a non-cooperative manner. When the dissociation of this histone pair is complete, H3 and H4 dissociate cooperatively at higher NaCl concentrations as an equimolar complex. The cooperativity is manifested not only in the free energy of interaction between the histones and DNA but also in the conformational change produced in the DNA of the nucleoprotein complex, which shows that the H3-H4 complex is capable of inducing long range changes in the DNA to produce a conformation which is intermediate between free DNA and the conformation it adopts in tightly supercoiled chromatin. It is possible that DNA in 2 M NaCl exists in a variety of conformational states some of which may approximate to the supercoiled conformation found in chromatin. If H3-H4 binds preferentially to supercoiled DNA, the arginine-rich complex

could stabilise such a conformation thus accounting for the changes in circular dichroism. The binding would be further stabilised by histone-histone interactions between bound tetramers. Such a mechanism would also explain the decreases in viscosity of the H3-H4 DNA which, on this view, result from the folding of the DNA into a supercoiled conformation. Evidence in support of direct histone-histone interactions has been obtained from crosslinking studies on H3-H4 DNA at various ionic strengths [3]. It is interesting to note in this context that Bina-Stein and Simpson [26] have recently reported that H3-H4 histones can compact SV40 DNA by 2.6 fold into nucleosome-like particles.

On extensive digestion with micrococcal nuclease a tetramer of H3-H4 complexed to 104, 125 and 145 base pair lengths of DNA may be isolated. The larger of these protected fragments is identical in size to the DNA protected in nucleosome core particles (145 base pairs). This suggests that the H3-H4 tetramer spans the nucleosome core particles, thus anchoring the ends of the DNA. The time course of digestion with nuclease suggests that lengths of DNA greater than the H3-H4 core particle, 145 base pairs, or even the complete nucleosome, 200 base pairs, can be transiently protected from digestion. This implies that there are long range interactions between H3-H4 tetramers and higher polymers which may generate ordering or phasing of the H3-H4 core along the DNA. The phasing interval cannot be less than 140 base pairs and, on average, cannot be greater than 200 base pairs, the repeat length in native calf thymus chromatin.

The proper binding of the H2a-H2b complex to DNA appears to require the presence of H3-H4. The binding of H2a-H2b and/or H1 to DNA by itself does not lead to the discrete protection of DNA fragments from nuclease digestion, unlike H3-H4, as observed by others [17]. This difference appears to be due to the differential stability of the secondary structure in the two histone pairs. Whereas, at low ionic strength, the secondary structure of the histones in the H3-H4-DNA complex is retained, the H2a-H2b-DNA complex loses structure at low ionic strength. Thus in the former case DNA maintains the stability of the bound histones, whereas in the latter it does not. On the other hand, when H2a-H2b binds to the H3-H4-DNA, the secondary structure is stabilised at low ionic strength, presumably due to histone-histone interactions between the arginine-rich and the lysine-rich pair. This suggests that the binding site for the H2a-H2b dimer is created by and formed from the H3-H4-DNA complex, such that binding occurs not only to the histone but also to the DNA. These conclusions confirm and support the observations of Sollner-Webb et al.

[17], Camerini-Otero et al. [18] and Oudet et al. [19] on the central importance of the H3-H4 arginine-rich complex in the structure of the nucleosome core particle.

The thermodynamic analysis shows that the binding of H2a-H2b is non-cooperative with respect to histone-histone interactions but highly cooperative with respect to NaCl. A further indication of cooperativity comes from considerations of the rates of dissociation and association. The rate of dissociation of core histones from DNA is a relatively slow process with a half time of at least one hour. This property has been utilised in separating the products of the reaction by gel exclusion chromatography in order to construct the binding curves. Thus the dissociation rate constant is of the order of 10^4 sec^{-1}. From this and the equilibrium constant (in 1.0 M NaCl equal to 0.072 M), we estimate that the association rate constant must be in the range 10^5-10^6 M sec^{-1}. Both rate constants are small compared to the values expected for simple ionic reactions involving unlike charges. These have very high frequency factors, low activation energies and large negative activation free energies. By contrast, the histone-DNA binding, which is dominated by ionic interactions, appears to involve large free energies of activation. This may be interpreted to mean that a large number of weak bonds have to be made or broken simultaneously in the activated complex for interaction to occur. In other words, the reaction is highly cooperative.

It is generally assumed that the interaction between histone and DNA involves electrostatic bonds between the positively charged lysine and arginine side chains and the phosphate groups in the DNA. The positive charges on the core histones are clustered mainly in the N-terminal and C-terminal parts of the molecules [20]. It has been suggested on the basis of nmr studies that these regions are structureless and exist, in the uncombined histones, as random coils which are thought to wrap around the grooves of the DNA when the histones interact [21,22]. If the extent of the tails is as defined by Hyde and Walker [18] then the number of basic side chains varies between 12 in H2a and 15 in H2b and H4. If these basic side chains neutralise adjacent phosphates on both chains of DNA, they will interact with at least one half to three quarters of a turn of DNA helix. Thus, on the assumption that the histone tails wrap into the DNA grooves, the tails must be flexible enough to wrap around at least half a turn of DNA and the binding mechanism must involve a 'zipper' interaction in which successive segments of the molecule bind consecutively. Such binding would involve fast 'on' and 'off' rate constants and show a low degree of cooperativity [23]. Alternatively, binding may occur

in a single step with the ligand (the histone basic tail) rigidly and correctly positioned with respect to the phosphate binding site. This mode of binding could occur if binding takes place to one face of the DNA helix only so that the tails do not wrap into and round the DNA grooves. Thus, interaction takes place on that surface of the DNA which faces into the histone core leaving the other, outer-facing half freely accessible to enzymes and other small molecules. Such a binding process would be slower than the 'zipper' mechanism and highly cooperative since all the corresponding subsites on the macromolecules interact simultaneously. Both 'on' and 'off' rate constants would in this case be small. The experimental data, such as it is, is more consistent with the latter kinetic model.

It is evident from the data presented here that the formation of core particles from free histone and DNA by dialysis from high salt is an example of a self-assembling process. The key step in this assembly appears to be the association of an equimolar H3-H4 complex with DNA; this is followed by the binding of H2a-H2b. Evidence has been presented to show that the core histones in 2 M NaCl exist in the form of a heterotypic tetramer or octamer [24,25]. If such species do exist in high salt, it is clear that they are not on the main thermodynamic pathway of assembly of core particles and are not a necessary requirement for the in vitro assembly. The situation in vivo will presumably depend very strongly on local concentrations of histones, DNA and counter ion at or close to the DNA replication points.

Several models for chromatin structure have been described which have as their basis the symmetry elements which arise as a result of specific interactions between the eight histones in the nucleosome core particle [15,27,28]. The model described by Hyde and Walker [15] is based on a central core of histones composed of two helical polymers of H3-H4 and H2a-H2b. The DNA is supercoiled around the central core with a pitch determined by the pitch of the helical polymers. The models described by Weintraub et al. [27] and Worcel et al. [28] for the 100 Å fibre are generally rather similar to that described in [15] and will not be discussed further. It is, however, instructive to see how the assembly of core particles can be explained in terms of, for example, the structure described in [15]. In high ionic strength solutions DNA may be visualised as an equilibrium mixture of different conformations one of which may approximate to the supercoiled conformation found in chromatin. The core histones also exist as an equilibrium mixture of heterotypic dimers, tetramers and higher polymers in equilibrium with homotypic species. As the salt concentration is decreased these equilibria are disturbed by the cooperative forma-

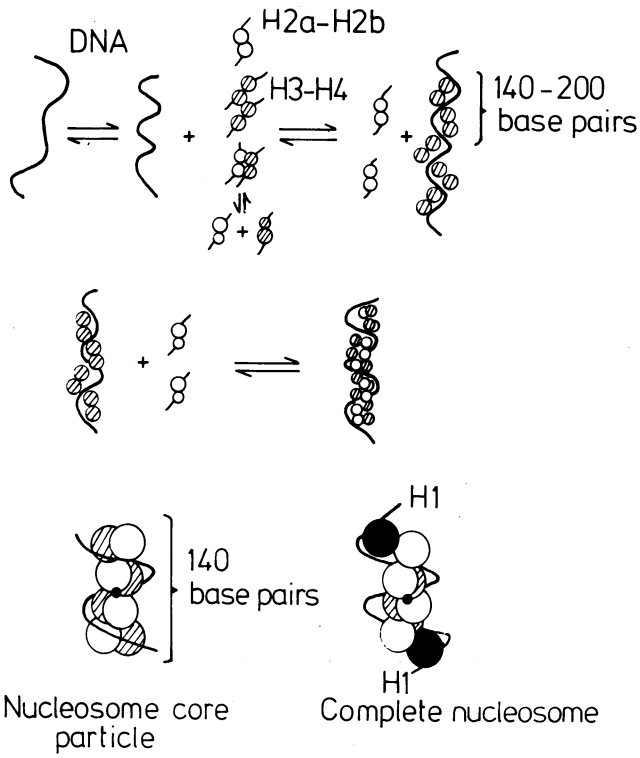

Fig. 11. The association of the arginine-rich and lysine-rich histone pairs to form the 100 Å fibre in terms of the chromatin model described in ref. [15]. Bottom left: details of a nucleosome core particle; bottom right: a complete nucleosome with molecules of bound H1.

tion of the H3-H4-DNA complex. In 1.2 M NaCl the H3-H4 dimers bound to DNA are in contact and may be crosslinked with HCHO to form high polymers [3]. The core particles are completed, on further lowering the salt concentration, by the association of H2a-H2b oligomers (probably dimers) to specific non-interacting sites formed by the H3-H4 DNA. This results in a tightening of the structure, shown in a decrease in viscosity, a further tightening of the supercoil which leads to changes in the circular dichroic spectrum and the formation of an interacting, linear array of nucleosome cores to form the 100 Å fibre (Fig. 11).

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