Unmasking of Histone Amino Groups in Chromatin at High pH

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The reactivity of the amino groups of histones in chromatin towards acetic anhydride was determined as a function of pH. In the pH range 7–10 the vast majority of amino groups in all five histones are buried. However, at higher pH values some of the histone amino groups become exposed, and the higher the lysine:arginine ratio for the histone the greater was the degree of unmasking observed. At pH 11.8 histone I appears to be completely dissociated, histones IIB1 and IIB2 have approx. 55% of the amino groups unmasked, and histones III and IV have approx. 25% of the amino groups unmasked.

In a previous study (Malchy & Kaplan, 1974) we have shown by competitive labelling that at pH 8.4 the ϵ -amino groups of histones in chromatin react with acetic anhydride at a much lower rate than that expected for fully exposed amino groups. It was concluded that the protonated amino groups of histones are neutralized in salt linkages and are buried, i.e. not accessible to solvent, in the major conformational states of chromatin. An earlier titration study by Walker (1965) on the total chromatin proteins claims that at pH 11.8 20% of the total basic residues (lysine plus arginine) could be titrated. This result has been interpreted (Elgin & Weintraub, 1975) as being at variance with the results of our competitive labelling study. In fact, however, no discrepancy may exist since the titration observed at high pH could reflect a disruption of the chromatin structure, which exposes previously buried groups in histones or other groups from non-histone chromosomal proteins. Since histones can be selectively removed from chromatin by salt treatment (Ohlenbush *et al.*, 1967), it is likely that the addition of base will also differentially affect the ionic interactions that are responsible for the binding of histones in chromatin. We decided to determine which histones, if any, have altered properties at high pH values and to what extent the effect of pH parallels the effect of salt.

Materials

Chromatin was prepared from calf thymus as described previously (Malchy & Kaplan, 1974). [^3H]Acetic anhydride (specific radioactivity 2.6 Ci/mmol) and [^{14}C]acetic anhydride (22 mCi/mmol) were from Amersham/Searle, Don Mills, Ont., Canada. All other reagents were high-purity preparations obtained from commercial sources.

Methods

Labelling procedure

A sample of chromatin (15.0 ml of solution in 5 mM- KH_2PO_4 /5 mM-sodium veronal, pH 7.5, with an E_{260} of 24) containing 500 nmol of phenylalanine was adjusted to the desired pH value with 1 M-NaOH. Then 50 μl of acetonitrile containing 0.48 μmol of [^3H]acetic anhydride was added to the vigorously stirred chromatin solution. After 30 min the samples were centrifuged at 143 000 *g* for 4.5 h. The supernatant containing the internal standard (phenylalanine) was separated from the chromatin. The histones were then isolated from the chromatin by sulphuric acid extraction (Malchy & Kaplan, 1974) and added back to the supernatant containing the labelled internal standard, which had been concentrated into 4.0 ml of 8 M-urea. This mixture was fully acetylated with 50 μl of [^{14}C]acetic anhydride (500 μCi diluted to 0.5 ml with acetic anhydride).

Purification of acetylphenylalanine

After reaction with [^{14}C]acetic anhydride, the double-labelled acetylphenylalanine was extracted with ethyl acetate and purified by high-voltage electrophoresis as previously described (Kaplan *et al.*, 1971).

Purification of acetylated histones

The aqueous phase was dialysed and freeze-dried. The acetylated histones were separated in 7.5% (w/v) acrylamide/0.05% methylenebisacrylamide in 0.9 M-acetic acid containing 8 M-urea, following the procedure of Panyim & Chalkley (1969). After staining with 0.2% Amido Black, the histone-

containing fractions were cut out and the acetylated histones were extracted with 0.05% sodium dodecyl sulphate (Bray & Brownlee, 1973).

Determination of $^3\text{H}/^{14}\text{C}$ ratios

The purified acetylphenylalanines and extracted acetylated histones were placed in scintillation vials containing 18 ml of Aquasol (New England Nuclear, Boston, MA, U.S.A.), and the ^3H and ^{14}C radioactivities determined on a Univex scintillation counter.

Calculation of reactivity indexes

The reactivity index (R.I.) has been defined previously (Visentin *et al.*, 1973). With the present double-labelling procedure, the equation for the calculation of the reactivity index at any pH value is:

$$\text{R.I.} = \frac{\text{Protein specific radioactivity} \times \frac{f_{\text{Phe}}}{f_{\text{Lys}}} \times \frac{1}{2.5}}{\text{Acetylphenylalanine specific radioactivity}} \quad (1)$$

where f_{Phe} and f_{Lys} are the fractions of the amino groups of phenylalanine ($\text{p}K_a$ 9.5) and lysine ($\text{p}K_a$ 10.3) in the deprotonated form at the particular pH value of the labelling. The specific radioactivities are expressed as ^3H radioactivity (d.p.m.)/ ^{14}C radioactivity (d.p.m.). A reactivity index of approx. 1 indicates that the ϵ -amino groups are fully exposed to solvent and a reactivity index of less than 1 indicates that some of these groups are buried. A value substantially greater than 1 indicates that groups other than free ϵ -amino groups are reacting with the label.

Results and Discussion

In order to determine the reactivities of each histone in chromatin in a completely rigorous manner, it was necessary to convert the histones

which had been trace-labelled with [^3H]acetic anhydride into a chemically homogeneous form by reaction with excess of acetic anhydride. This step necessitated the development of a separation procedure for the acetylated histones. Plate 1 shows the separation obtained after electrophoresis. The fully acetylated histones separated as expected, according to their charge and size.

Although the use of polyacrylamide gels provided a rapid and convenient separation procedure, two technical difficulties arose. The first was that it became necessary to quantify the amount of amino groups in each protein band. The second was that acetylated histone I could not be detected in the gels without overloading to the point where the other four histones were not completely resolved. The first difficulty was solved by using [^{14}C]acetic anhydride to fully acetylate the histones (Visentin & Kaplan, 1975). Although the absolute amount of each histone could be determined from the amount of ^{14}C label, this was unnecessary as all that is required to determine the reactivity is the $^3\text{H}/^{14}\text{C}$ ratio (see eqn. 1). The second problem was solved by adding a sufficient amount of unlabelled acetylated histone I to the double-labelled mixture, to enable detection of this component.

The reactivity index of each histone was calculated by determining the $^3\text{H}/^{14}\text{C}$ ratio of each histone after extraction from the gel. This ratio was substituted into eqn. (1) along with the $^3\text{H}/^{14}\text{C}$ ratio for acetylphenylalanine; these are summarized in Table 1, along with the calculated reactivity indexes.

At pH values between 7 and 9.8, the reactivity indexes obtained for each of the histones, with the exception of histone IIb2, are very low, indicating that the vast majority of their amino groups are buried in chromatin. We have previously shown (Malchy & Kaplan, 1974) that the *N*-terminal proline residue of histone IIb2 is responsible for most of the observed reactivity and, as in the case of the other four histones, the ϵ -amino groups are also buried. Histone III exhibits a reactivity as low as any of the

Table 1. Summary of reactivity data for the reaction of histones in calf thymus chromatin with acetic anhydride
For details see the text.

pH Histone	Acetylphenylalanine ³ H/ ¹⁴ C ratio						Reactivity index					
	...	I	I ib 2	I ib 1	III	IV	Acetylphenyl- alanine	I	I ib 2	I ib 1	III	IV
7.00		3.01	9.60	2.58	4.54	2.32	49.1	0.15	0.49	0.13	0.23	0.12
7.50		3.89	15.8	4.02	6.52	2.72	75.5	0.13	0.52	0.14	0.22	0.090
8.01		4.40	22.7	4.62	7.38	3.49	86.5	0.13	0.65	0.13	0.21	0.094
8.50		13.3	83.4	13.1	15.1	7.74	245.5	0.13	0.79	0.13	0.14	0.074
9.40		24.1	128	18.3	16.3	11.6	282	0.16	0.87	0.13	0.11	0.079
9.80		38.2	140	26.8	19.0	14.7	257	0.17	0.60	0.12	0.081	0.063
10.70		96.8	90.0	74.2	37.6	34.2	107	0.48	0.45	0.37	0.19	0.17
11.80		144	62.9	67.9	30.3	27.0	46.3	1.3	0.56	0.60	0.27	0.24

EXPLANATION OF PLATE I

Electrophoretic separation of fully acetylated histones by polyacrylamide-gel electrophoresis using the system of Panyim & Chalkley (1969)

The identity of each of the bands was determined by electrophoresis of the individual fully acetylated histones. (a) Fully acetylated histone I (50 μ g); (b) fully acetylated total histone (100 μ g).

Fig. 1. pH-profile for the reaction of histones with acetic anhydride

○, Histone I; □, histone IIb1; △, histone IIb2; ■, histone III; ●, histone IV. For details see the text.

other histones at pH9; however, it appears to have a higher reactivity at lower pH values. This somewhat higher reactivity may be caused by the contribution of its unblocked alanine amino terminus to the overall reactivity. The main conclusion to be drawn from the present results is that between pH7 and 9.8 the reactivity indexes remain relatively constant and therefore no large conformational changes occur in chromatin over this pH range. At pH values above 9.8, however, the reactivity data show that large changes are occurring in the environment of the histone molecules in chromatin (Fig. 1).

The increase in reactivity of the histones in chromatin at high pH was found to vary among the histone classes. The greatest increase was observed for histone I, whose reactivity index is approx. 1 at pH 11.8, indicating complete exposure of its amino groups. The moderately lysine-rich class of histones IIb1 and IIb2 have approx. 50% of the reactivity of histone I, whereas the arginine-rich class histones III and IV have 25% of the reactivity of histone I. Thus, as in the case of the salt effect on chromatin (Ohlenbush *et al.*, 1967), there is a parallel between the lysine/arginine ratio and the extent of unmasking of the histones from chromatin.

These observations help to explain the results obtained by Walker (1965) in a detailed titration study of calf thymus chromatin. He observed, on titration up to pH 11.8, that 20% of the basic residues could be titrated and interpreted this as indicating a significant degree of exposure of these residues in native chromatin. The present results and those of an

earlier study (Malchy & Kaplan, 1974) indicate that at neutral pH only a very small number of ϵ -amino groups are exposed. At high pH, however, the results of this study show that a significant number of the basic groups become exposed, as initially demonstrated by Walker (1965). It is now clear that the fact that basic groups can be titrated at high pH is due in large part to the unmasking of the basic groups of histone I, which results from a disruption of the native chromatin structure in which these groups are buried.

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