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Involvement of cysteine residues in the electrophoretic mobility of histone H3 in acid-urea-Triton gels

Carbamylation of cysteines 96 and 110 in histone H3 increases the electrophoretic mobility of this histone in acetic acid-urea-Triton X-100 polyacrylamide gels but has no effect in gels lacking Triton. Residue 96 appears to be a major determinant in the affinity of histone H3 for the nonionic detergent Triton. Carbamylation and carboxymethylation of cysteine 96 caused a major loss of the gel retardation caused by Triton. Carbamylation of cysteine 110 did not affect Triton binding but prevented ionization of the thiol side-chain moiety in the acetic acid-urea-Triton X-100 gel.

1 Introduction

Core histones can be identified and distinguished from other proteins by their interaction with the nonionic detergent Triton X-100 in acetic acid-urea (AU) polyacrylamide gels [1–2]. The basis for this interaction is not understood, but it is observed for core histones of all species tested and has not been reported for other proteins. This study of the modification of cyste cysteine residues in histone H3 species with one or two cysteine groups has identified residue 96 as a location of major importance f for Triton binding. A much smaller role may be played by cysteine 110. An artifact was detected in two alfalfa histone H3 proteins which were prepared from isolated nuclei [3]. It was similar or identical to the early effects of carbamylation of cysteine 110 in histone H3. Analysis of this modification and its preventions has resulted in revised estimates for the steady state acetylation of alfalfa histone H3.1 and H3.2 variants.

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Abbreviations: **AU**, acetic acid-urea; **AUT**, acetic acid-urea Triton X-100; **HPLC**, high performance liquid chromatography; **TFA**, trifluoroacetic acid

2 Materials and methods

Calf thymus histone variant H3.1 with two cysteines and the lowest gel mobility in acetic acid-urea-Triton X-100 (AUT) gels [4–5] was purified to homogeneity from a commercial preparation of whole calf thymus histones (Worthington) by reverse phase chromatography on Zorbax Protein Plus (Dupont). Histones were solubilized in 7.2 M freshly deionized urea, 1 M dithiothreitol, 0.75 M ammonium hydroxide and 0.05 % phenolphtalein for 5 min at room temperature and acidified by the addition of 1/20 volume of glacial acetic acid. Up to 4 mg of histones were injected on a 4.6 mm × 25 cm column, equilibrated with 0.1 % trifluoroacetic acid (TFA) in

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water and eluted in 60 min at 1 mL/min between 42 % and 52 % acetonitrile. Histone H3.1 was localized by AUT gel analysis, pooled and lyophilized. Alfalfa (*Medicago sativa*) histones were prepared from whole callus cultures [1] and isolated nuclei [3], with 0.1 mM 2-mercaptoethanol in all solutions. Histone H3 was enriched by Bio-Gel P-60 chromatography [1], dialyzed into 2.5 % acetic acid – 0.1 mM 2-mercaptoethanol, lyophilized and prepared for high performance liquid chromatography (HPLC) as described above. Separated H3 variant preparations, judged homogeneous by AUT gel analysis, were used in this study. Protein sequence analysis of these proteins is reported elsewhere (unpublished results). Histones were solubilized as for HPLC sample preparation in alkaline urea sample buffer with addition of 0–500 mM potassium cyanate (KCNO). The carbamylation reaction was terminated by the addition of acetic acid and immediate application to discontinuous 18 % polyacrylamide AU and AUT gels (1 M acetic acid, 50 mM ammonium hydroxide and 8 M urea and with 9.5 mM Triton X-100 for AUT gels) [6–7]. Gels were stained with Coomassie Brilliant Blue, R-250, scanned and quantitated as described before [1]. Histone H3 was carboxymethylated with iodoacetate [8]. Gels combining AU and AUT conditions were prepared with a gradient from 0–10 mM Triton X-100 [1, 3].

3 Results

3.1 Carbamylation of bovine histone H3.1

Carbamylation of bovine histone H3.1 by cyanate progresses through three recognizable stages as analyzed by AU and AUT gel analysis (Fig. 1A/B). The unperturbed staining pattern of acetylated bands of histone H3.1 shows, in decreasing amounts, non-, mono- and diacetylated species. With each modification of lysine to acetyllysine the net positive charge of histone H3.1 at pH 3 decreases by one as seen by the discrete

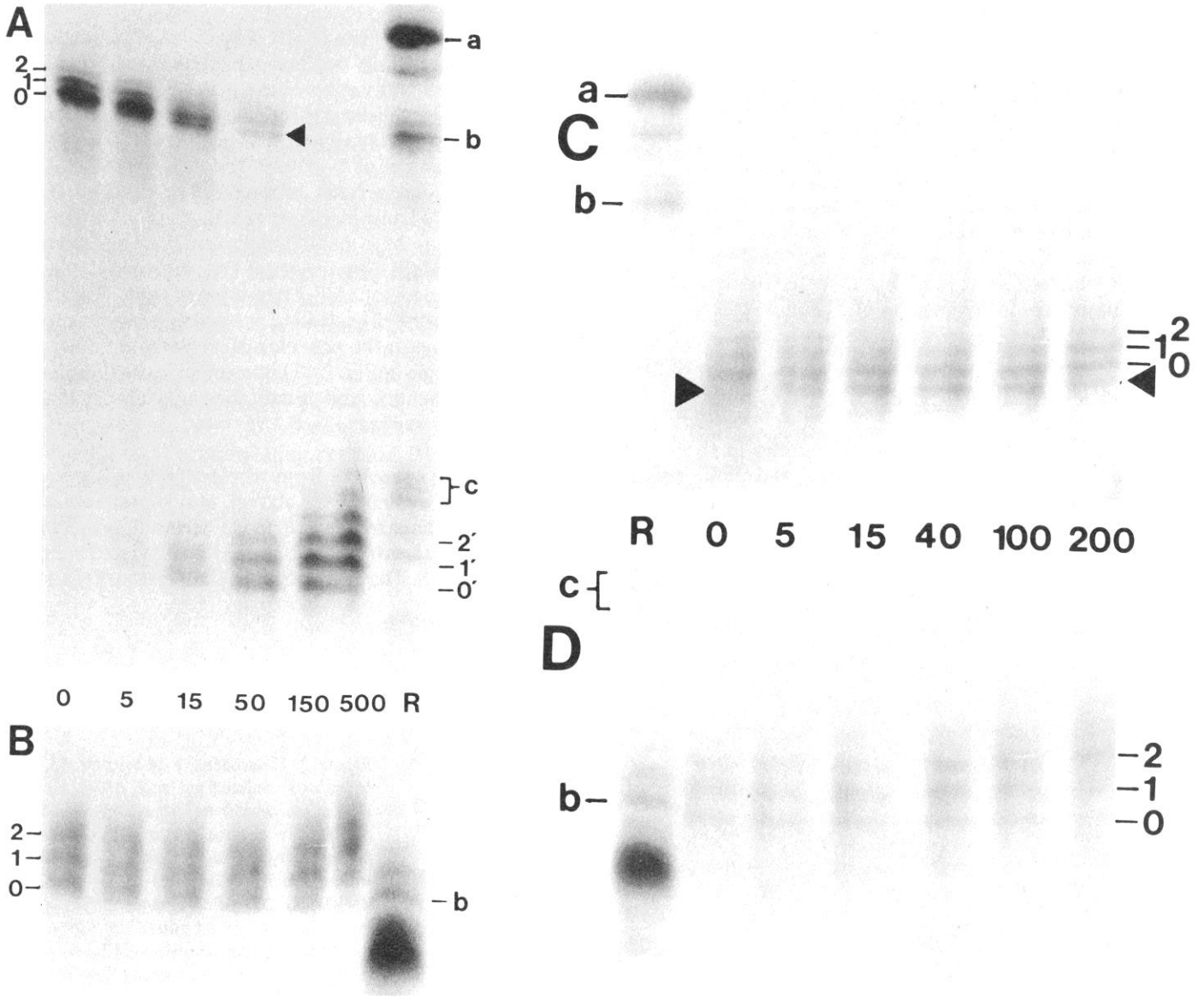

Figure 1. Gel analysis of carbamylation of histone H3. (A) AUT and (B) AU gel analysis of bovine H3.1. Untreated (0) or incubated as described in Section 2 with 5, 15, 50, 150 or 500 mM KCNO. (C) AUT and (D) AU gel analysis of alfalfa histone H3.2 untreated (0) or incubated with 5, 15, 40, 100 and 200 mM KCNO. The same results were obtained for alfalfa histone H3.1. The numbers 0 to 2 indicate non-through diacetylated histone H3 with unmodified cysteine. A solid triangle indicates nonacetylated H3 with one carbamylated cysteine. 0′ to 2′ indicate non-through diacetylated histone H3 with two carbamylated cysteines. Bovine histones indicated in the reference gel lane (R) are (a) H2A (b) nonacetylated histone H3 and (c) histone H1. Gel electrophoresis was from top (anode) to bottom (cathode).

reduction in mobility in AU (Fig. 1B) and AUT (Fig. 1A) gels. At low concentrations of cyanate the relative amounts in all bands were decreased while a new band appeared in the AUT gels with a gel mobility expected for unacetylated histone H3.1 modified to have one additional net positive charge (Fig. 1A). No effect was seen in AU gels (Fig. 1B). At intermediate concentrations of cyanate a major increase in AUT gel mobility was observed to a position equivalent to a loss of 80 % of Triton binding (Fig. 1A), again without detectable effect for AU gel electrophoresis (Fig. 1B). At high concentrations of cyanate, gradually slower moving bands were generated by discrete losses of net positive protein charge. This change was seen in AUT and in AU gels (Fig. 1A/B).

Cyanate will react readily with cysteine thiol side chains and with primary amino groups to generate acid-stable derivatives [8]. This made it likely that the two initial stages of carbamylation involved reactions with cysteine residues 96 and 110 in bovine histone H3.1, and that subsequent changes involved carbamylation of lysine side chains.Carbamylation of lysines will remove the potential for ionization at low pH and will cause the reduction in gel mobility seen in AU and AUT gels (Fig. 1A/B). To distinguish between the effects of carbamylation of cysteine 96 and cysteine 110, histone H3 variants of alfalfa were used which have single cysteine residues at position 110 (unpublished results). At low concentrations of cyanate, alfalfa histone variant H3.2 generated an additional, more positively charged gel band in AUT gels (Fig. 1C) but not in AU gels (Fig. 1D). At high concentrations of cyanate, lysine carbamylation became apparent by AU and AUT gel electrophoresis (Fig. 1C/D). Major loss of Triton binding to alfalfa histone H3 was not observed. This indicated that cysteine 96 plays a major role in the binding of Triton X-100 to bovine H3.1.

3.2 Effect of carboxymethylation

To substantiate the involvement of cysteine in these carbamylation reactions, bovine histone H3.1 and alfalfa histone H3.1 and H3.2 were exhaustively carboxymethylated with iodoacetate to remove the possibility of cysteine reactions with cyanate. This carboxymethylation resulted in a major loss of binding of Triton X-100 to bovine H 3.1 in AUT gels but less than that observed after the carbamylation shown in Fig. 1A. The resulting H3.1 bands showed approximate coelectrophoresis with histone H1 (marked by c in Fig. 1A). The absence of any effect in AU gels (results not shown) indicates that the carboxyl group introduced at the cysteine residues does not become ionized in these gel systems. A qualitatively similar although smaller increase in AUT gel mobility, equivalent to approximately 2 unit charges, was observed for alfalfa histones H3.1 and H3.2, again without effect on AU gel mobility. A similar small change has been observed previously when tobacco histone H3 was carboxymethylated [2]. These observations suggest that cysteine 110 could play a minor role in the binding of Triton X-100 to histone H3. Subsequent attempts to carbamylate these carboxymethylated histone H3 species by cyanate failed to cause any of the early changes induced by cyanate in unmodified histone H3. This proved that cysteine residues were the first targets of cyanate modification of histone H3.

3.3 Preparation of histones

Preparation of nuclei from alfalfa cells [3] prior to extraction of histones has generally resulted in histone H3 preparations, which, to a small and variable degree, in AUT and AU gels, behaved similarly to histone H3 exposed to 5 mM cyanate (Fig. 1C). This is most readily shown in an acetic acid-urea gel with a transverse gradient of 0–10 mM Triton X-100 (Fig. 2). A preparation of unseparated histone H3 variants at 0 mM Triton is separated only by differences in acetylation (0 to 3 in Fig. 2). Each of these bands splits into four distinct and overlapping bands in the presence of Triton: an initial split into histone H3.1 and histone H3.2 bands (open triangle in Fig. 2) followed by splitting of each band at a higher concentration of Triton X-100 due to the presence of partially modified cysteine (solid triangle in Fig. 2). This complexity has resulted in incorrect identification and quantitation of acetylation of alfalfa histone H3 variants [3].

Preparation of histone H3 from whole alfalfa cells prevents modification of cysteine (see above), and separation of the histone H3 variants by reverse phase chromatography prevents the overlap between H3.1 and H3.2 acetylated bands in AU and AUT gels. The acetylation levels of histones H3.1 and

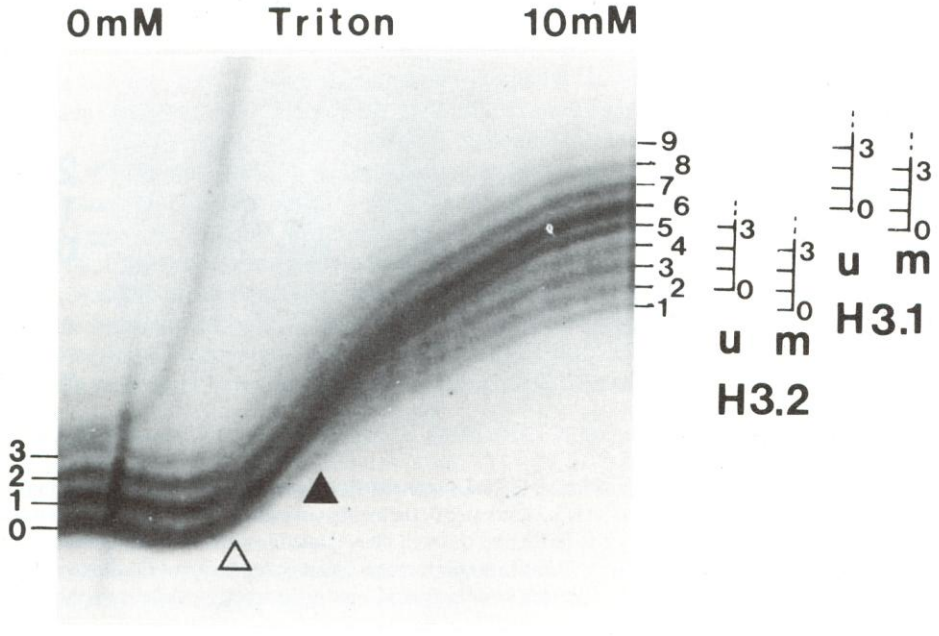

Figure 2. Heterogeneity of histone H3, prepared from isolated nuclei of alfalfa. Histone H3 was extracted from isolated nuclei of alfalfa, purified by reverse phase HPLC to near homogeneity and electrophoresed in an AU gel with a transverse gradient of Triton X-100 (0–10 mM). Distinctive changes in gel mobility due to histone variant differences become apparent at lower concentrations of Triton X-100 (open triangle) than changes in gel mobility due to modification of cysteine (solid triangle). 0–3 indicate non-through triacetylated total histone H3 in the absence of Triton. 1–9 indicate distinct electrophoretic forms, interpreted to the right as histone variants H3.1 and H3.2 with unmodified (u) or modified (m) cysteine with at least 0 to 3 acetylated lysines.

H3.2 were quantitated separately in AU and AUT gels and showed 0.44-0.56 and 0.97–1.01 acetylated lysines per protein molecule, respectively (results not shown). In all analyses, the ratio of acetylation of H3.2 relative to H3.1 was approximately 2. These new determinations of the steady state level of histone H3 variant acetylation represent a correction on the previously published values [3].

4 Discussion

The major loss of Triton binding to bovine histone H3.1 when cysteine 96 is carbamylated or carboxymethylated does not prove that cysteine is important for Triton binding. Most histone H3 species, including both variants of histone H3 in alfalfa, do not have cysteine residues at position 96 in their sequence. Still, they all show a significant binding of Triton in AUT gels with an affinity characteristic of histone H3. This affinity is less than that of typical H2A histones but more than that observed for core histones H2B and H4 [1]. All these core histones lack cysteine; thus, it is most likely that the sequence localization of residue 96 in histone H3 is important for the binding of Triton X-100 in AUT gels.

Carbamylation at low concentrations of potassium cyanate (KCNO) generated a histone H3 protein species increased by one net positive charge in AUT gel electrophoresis without any effect on AU gel mobility (Fig. 1). The reaction proceeded with similar reaction rates at alkaline pH, as described above, and in 1 M acetic acid and 7.2 M urea (results not shown). Such a change can only occur if cyanate reacts with a group which is negatively charged in AUT gels but neutral under AU gel conditions, or if cyanate reacts with a neutral group which creates a positive charge only in AUT gels. No neutral molecular structure is known which might react with cyanate to generate a product which might become positively charged. Carboxymethylation of histone H3 indicated that cysteine 110 was the target of this carbamylation reaction. Thiol groups can become ionized but the pK value of 8.3 [9] is so hight that ionization is insignificant under the aqueous, acidic conditions of AU gel electrophoresis. However, within the hydrophobic environment created around each histone molecule by Triton X-100 in an AUT gel, such ionization cannot be excluded. If correct, it is most likely that the negatively charged cysteine side chain is stabilized in a salt bridge by another, positively charged group of histone H3. Carbamylation of cysteine 110 modifies the thiol and creates a non-ionizable group. This increases the net positive charge of carbamylated histone H3 by the positive group which in unmodified histone H3 is part of the salt bridge with cysteine 110.

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