CHEMICAL STUDIES ON HISTONE ACETYLATION USING A SYNTHETIC PEPTIDE FRAGMENT OF HISTONE H4

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1. Introduction

The enzymatic acetylation of ϵ-NH_2 groups of lysyl residues of histones may be important in the control of the structure and the function of chromatin [1]. All the histones that make up the chromatin core particle, i.e., histones H2A, H2B, H3 and H4, are subject to enzymatic acetylation [2].

Primary structure studies of histones show that only a few specific lysyl residues are involved and that the extent of acetylation depends on cellular activity [2–4]. The chemical modification of a specific lysyl residue by acetylation has been described as a molecular process which is able to regulate the structure of DNA in chromatin [5].

On the basis of sequence analogies, a classification into two types has been proposed [2] for the acetylation sites of histones:

(i) Type A with a single lysyl residue surrounded by two non-basic residues;
(ii) Type B comprising a basic doublet such as Lys–Lys,Lys–Arg and Arg–Lys.

This classification suggests that only a small fragment of the histone molecule is necessary for enzymatic recognition.

This work shows that an acetyltransferase acting on histone H4 is also able to acetylate the synthetic octapeptide AcGly–Ala–Lys–Arg–His–Arg–Lys–$ValNH_2$ which corresponds to the sequence fragment of histone H4 between residues 14 and 21 and includes two lysyl residues, one of which Lys^{16} is the major site of acetylation in calf thymus histone H4. Peptide [14–21] contains two basic doublets, i.e., Lys^{16}–Arg and Arg–Lys^{20}, which correspond to acetylation sites of type B. The ability of the acetyltransferase to recognize either both sites or one of them selectively has been investigated.

2. Materials and methods

Histone H4 was isolated from calf thymus by the method in [6] and its purity was checked by gel electrophoresis according to [7]. Sonicated DNA from calf thymus of mol. wt 2.7×10^5 was prepared as in [5]. Peptide (14–21), i.e., AcGly–Ala–Lys–Arg–His–Arg–Lys–$ValNH_2$, was prepared by stepwise solid-phase synthesis using:

(i) A benzylhydrylamine polystyrene resin (1% divinylbenzene) substituted with 0.16 mmol NH_2 groups/g resin (as in [8]);
(ii) The *t*-butyloxycarbonyl derivatives of L-valine, N^{ϵ}-carbobenzoxy–L-lysine, N^{ω}-tosyl–L-arginine and N^{im}-tosyl–L-histidine.

Treatment of the peptide–resin with liquid HF below 20°C gave the amide peptide which was purified by carboxymethylcellulose (CMC) chromatography with a linear NaCl gradient (0–0.4 M). The peptide, which is eluted at 0.32 M, was isolated as the hydrochloride and it was characterized by its amino acid content (Lys 1.85, His 1.02, Arg 2.0, Gly 1.15, Ala 1.0, Val 1.0), paper electrophoresis, field desorption mass spectrometry (M^+ =992) and high-field proton NMR spectroscopy [9].

All enzymatic reactions were carried out either in buffer A (30 mM Tris–HCl, 0.7 mM $MgCl_2$, 0.2 mM EDTA, 7 mM NH_4Cl, 50 mM KCl, 5 mM 2-mercaptoethanol, at pH 8.1) or in buffer B (buffer A diluted 10-fold).

2.1. *Histone acetyltransferase extraction and assay for ^{14}C-radioactivity incorporation*

Liver nuclei were prepared from Wistar male rats (150–200 g) as in [10]. Histone acetyltransferase activity was extracted from the nuclear preparations as in [11] and assayed by incubation at 37°C of 50 μg histone H4 in buffer A containing 0.05 μCi [1-^{14}C]-acetyl-CoA (spec. act. 52.4 Ci/mol; New England Corp; USA) and 40 μg nuclear protein in final vol. 0.5 ml. The enzymatic reaction was stopped with 2 ml cold 15% perchloric acid solution. The acid-insoluble radioactivity was counted in 10 ml Bray solution [12] using an Intertechnique (France) liquid scintillation spectrometer. After 20 min enzymatic reaction, 85% of the total radioactivity (measured at times much greater than 20 min) was incorporated.

2.2. *Enzymatic acetylation of peptide (14–21)*

Aliquots (0.25 ml) containing 0.175 μmol peptide (concentration by amino acid analysis) were incubated with 0.05 μCi [1-^{14}C]acetyl-CoA (spec. act. 2.5 Ci/mol) and 20 μg enzyme protein in buffer A for 20 min at 37°C. The reaction was stopped by cooling in ice and adding 0.1 M HCl to give pH 4. The reaction products were analyzed and purified by ion-exchange chromatography and gel filtration (see section 3).

2.3. *Chemical acetylation of peptide (14–21)*

The lysyl residues were acetylated as in [13] at about 0°C with an excess of Ac_2O (~120-fold) while maintaining the pH constant at 7.5 by automatic addition of 0.4 M NaOH in a pH-stat apparatus (Radiometer, Denmark).

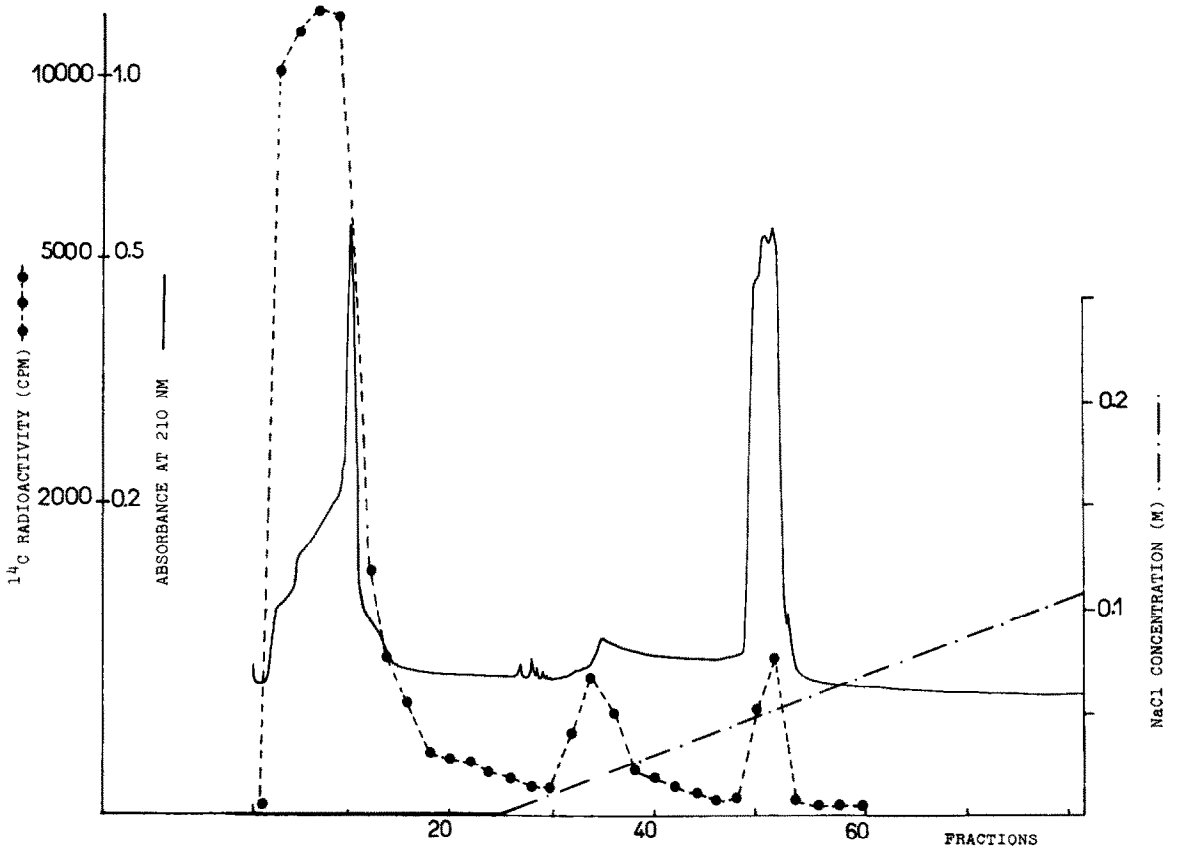


Fig.1. Carboxymethylcellulose chromatography. After enzymatic acetylation the peptide (14–21) (3.5 μmol) was applied to a CMC column (1.8 × 10 cm) and eluted with a linear HCl gradient (0–0.1 M) with a flow rate of 30 ml/h. Fractions of 6 ml were collected and the radioactivity of 0.5 ml aliquots was measured in 10 ml of Bray solution.

2.4. *Peptide digestion by trypsin*

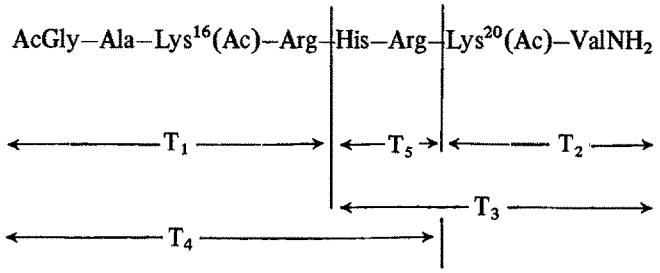
The reaction was carried out at 37°C under nitrogen with a trypsin/peptide molar ratio around 30, at pH 8.0. After addition of dilute HCl to pH 5, the final mixture was analyzed by CMC chromatography.

2.5. *Peptide (14–21)-DNA complexes*

Formation of the complex was carried out in buffer B by gradually adding the peptide solution to a 5.2 mM DNA–phosphate solution. It was monitored by ultraviolet spectroscopy at 350 nm to detect turbidity effects and by thermal denaturation at 260 nm.

appear at 0.24 M and peptide (14–21) at 0.32 M NaCl). After freeze-drying the excess of NaCl was removed by Sephadex G-10 filtration.

3. After tryptic digestion of the diacetylated peptide, the fragments were separated by CMC chromatography using an adapted NaCl gradient (fig.2). Because of the absence of free amino groups on the lysyl side-chains, fragmentation of peptide bonds by trypsin is expected only at the arginyl residues [13]. This leads to a theoretical fragmentation pattern of 5 tryptic peptides as shown in the diagram:



3. Results

3.1. *Enzymatic acetylation of peptide (14–21)*

Because of the low molecular weight of peptide (14–21) it is not possible to follow the incorporation of radioactive acetyl groups by simple precipitation as for the histone H4. The following strategy was therefore adopted:

1. The products from the enzymatic reaction were separated by CMC chromatography (fig.1). The unreacted acetyl-CoA is eluted first with water. Fractions 49–54 correspond to a mixture of unreacted peptide and radioactive acetylated derivatives.

2. The diacetylated peptide AcGly–Ala–Lys(Ac)–Arg–His–Arg–Lys(Ac)–$ValNH_2$ was obtained by chemical acetylation of the peptide mixture with Ac_2O and purified by CMC chromatography using a linear NaCl gradient (0–0.4 M). A major peak containing almost all the radioactivity was apparent at 0.15 M NaCl suggesting that the acetylation was complete (monoacetylated species would

Five peaks, labelled S_1–S_5 in order of increasing positive charge densities, appeared on the CMC chromatogram (fig.2). The small peak S at 0.15 M NaCl is the unreacted diacetylated peptide. The structure of the tryptic fragments was readily established by amino acid analysis. S_1,S_2,S_4 and S_5 correspond to peptides T_1,T_2,T_4 and T_5, respectively. The occurrence of the peak S_3, which contains only arginine, is not well understood. The expected peptide T_3 was not observed, undoubtedly because of extensive enzymatic hydrolysis. The radioactivity present in peptides T_1 and T_4 together represents ~48% of the total radioactivity incorporated in peptide (14–21). This corresponds to the percentage of acetylation of Lys^{16}. The remaining radioactivity appears on peptide T_2 and therefore the percentage of acetylation of Lys^{20} is 52%.

3.2. *Enzymatic acetylation of a peptide (14–21)–DNA complex*

The enzymatic acetylation of peptide (14–21) bound to double-stranded DNA was studied in conditions similar to those used for the free peptide except that the ionic strength was decreased by a

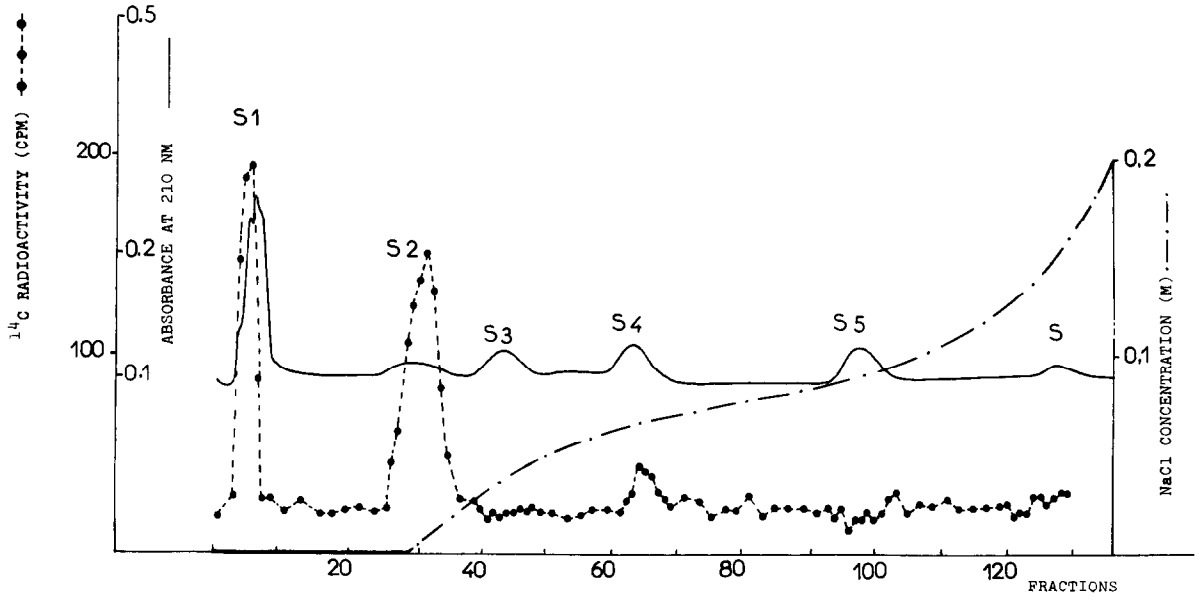


Fig.2. Carboxymethylcellulose chromatography. The diacetylated peptide was treated with trypsin and the digested fragments separated on a CMC column (1.8 × 10 cm) using an adapted NaCl gradient (11300 Ultrograd, Gradient Mixer, Pharmacia).

factor of 10 (buffer B) in order to ensure a high stability of the complex. The peptide/DNA-phosphate molar ratio was kept at about 0.1 to avoid formation of aggregates. Under these conditions 56% and 44% of the ϵ-NH_2 groups of Lys^{16} and Lys^{20}, respectively, were acetylated. This result does not differ significantly from that observed with the free peptide.

3.3. *Non-enzymatic acetylation of peptide (14–21) by acetyl-CoA*

Parallel experiments carried out in the absence of any enzyme show acetylation corresponding to ~18% of that observed in the presence of the enzyme. For histone H4 the non-enzymatic acetylation represents 4% of the total incorporated radioactivity. However, chemical acetylation gives rise to the diacetylated peptide exclusively while in the presence of the enzyme a mixture of monoacetylated species is obtained. As the peptide concentration is much larger than that of acetyl-CoA, a plausible explanation is that a peptide–acetyl-CoA complex containing more than one coenzyme molecule is formed owing to electrostatic interaction. Acetyl transfer would occur within the complex.

4. Discussion

It is of particular interest that a short segment of histone H4, the peptide (14–21), which contains the main acetylation site Lys^{16}, can be acetylated by a nuclear acetylase under the same conditions under which acetylation occurs with the whole histone molecule. The steric requirements for enzymatic recognition by nuclear acetyltransferases can therefore be studied using synthetic peptides which mimic sequence fragments of the histones. Peptide (14–21) contains two basic doublets, Lys^{16}–Arg and Arg–Lys^{20}, which correspond to acetylation sites of type B [2]. The two lysyl residues are acetylated to nearly the same extent both with the peptide free in solution and when bound to a double-stranded DNA. Lys^{16} in histone H4 is found acetylated in the chromatin of several tissues [2]. However, Lys^{20} is not acetylated in vivo as the ϵ-NH_2 group is substituted by methyl groups [14]. It is likely that Lys^{20}, which is the single site for methylation in histone H4, is also a potential site of acetylation. Because of the competition with methylation, acetylation does not occur. It has been shown that the methyl turnover of

histones in chromatin is very slow in comparison to that of an acetyl group substituting the ϵ-NH_2 of a lysyl residue [15]. It has been reported that the reaction products due to enzymatic acetylation of isolated histones H3 and H4 differ from those obtained under in vivo conditions [16]. These results were interpreted by assuming that the native conformation of histone in chromatin is necessary to ensure the specificity of the enzymatic reaction [17]. Although recognition requires only a short segment of a histone molecule, as suggested by the present work, specificity may nevertheless be dependent on the native conformation. Acetylation sites available in the free histone may be inaccessible to the enzyme when the histone molecule is part of the chromatin nucleoprotein complex.

The N-terminal part of histone H4 (residues 1–23) is subject in the cell to several post-synthetic modifications involving the five lysyl residues at positions 5,8,12,16 (acetylation) and 20 (methylation). It is very likely that owing to its basic character this part of the histone is intimately associated with DNA in chromatin [5]. This does not preclude the recognition of the histone molecule by the nuclear enzyme that is suggested by the results reported here for the peptide (14–21) bound to a double-stranded DNA.

Acknowledgements

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