ENZYMATIC DEACETYLATION OF A SYNTHETIC PEPTIDE FRAGMENT OF HISTONE H4

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1. Introduction

Acetylation and deacetylation of the ϵ-amino groups of specific histone lysyl residues occurs in the course of the cell cycle. The overall level of acetylation at any given time involves an equilibrium between acetate uptake catalysed by nuclear acetyltransferases [1,2] and acetate removal catalysed by nuclear deacetylases [3–5]. All the histones that make up the chromatin core particle, i.e., histones H2A, H2B, H3 and H4 are subject, in vivo, to acetylation and deacetylation but no acetylation sites have been observed on the very lysine-rich histone H1 [2].

On the basis of sequence analogies, a classification of the acetylation sites into two types (A,B) was proposed [2]. In type A there is a single lysyl residue surrounded by two non-basic residues and in type B 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. However, chemical studies on the activity of a calf thymus deacetylase using histone H4 fragments, including the monoacetylated peptide (15–21) H–Ala–Lys^{16} (Ac)–Arg–His–Arg–Lys–Val–OH, led to the conclusion that a rather long sequence of the histone molecule is required for enzyme recognition [6].

Here we report the enzymatic deacetylation of the diacetylated histone H4 fragment (14–21) Ac–Gly–Ala–Lys–(Ac)–Arg–His–Arg–Lys(Ac)–Val–NH_2, with the N- and C-terminal groups protected by an acetyl and an amide group, respectively, in conditions similar to those used for the deacetylation of the whole histone H4 molecule.

2. Materials and methods

2.1. *Preparation of substrates*

2.1.1. (i) [1-^{14}C]Acetate-labelled histone H4

Histone H4, isolated by the method in [7], was incubated in the presence of [1-^{14}C]acetyl-CoA and nuclear acetyltransferase from rat liver as in [8]. The labelled histone H4 was purified by carboxymethyl cellulose (CMC) chromatography and lyophilized.

2.1.2. Diacetylated peptide [14–21]: Ac–Gly–Ala–Lys([2-$^3H_3$]acetyl)–Arg–His–Arg–Lys-([2-$^3H_3$]acetyl)–Val–NH_2

The peptide (14–21) was synthesized as in [8] and the ϵ-NH_2 groups acetylated using tritiated acetic anhydride (spec. act. 50 mCi/mol) by the method in [9]. Purification of the diacetylated peptide was carried out by gel filtration on Sephadex G-10 and analysis by CMC chromatography showed the peptide to be fully acetylated.

2.2. *Histone deacetylase extraction*

Calf thymus nuclei were prepared by the method in [10]. The histone deacetylase was extracted as in [11] and partially purified by DEAE-cellulose chromatography using a 0.01–0.4 M NH_4Cl gradient in buffer A (15 mM Tris–HCl (pH 7), 10 mM NH_4Cl, 0.2 mM EDTA, 5 mM 2-mercaptoethanol, 20% glycerol, v/v). Fractions showing deacetylase activity with labelled histone H4 as a substrate were pooled, concentrated by ultrafiltration (Amicon membrane UM10), dialysed against 25 mM Tris–HCl (pH 7), 50% glycerol, v/v and stored at –20°C. The protein

content was measured by the Lowry method [12]. SDS–polyacrylamide gel electrophoresis showed the fraction to be histone free.

2.3. *Assay of deacetylase activity with labelled histone H4 as substrate*

The method in [3] was used to follow the enzymatic deacetylation of histone H4. Labelled histone H4 (0.8 mg, spec. act. 3870 dpm/mg) was incubated at 37°C for 60 min in 0.5 ml 25 mM Tris–HCl (pH 7) in the presence of varying amounts of enzyme (50–1200 μg total protein). The reaction was stopped by adding 0.1 ml 1 M HCl containing 0.16 M non-radioactive acetic acid as a carrier. Acetic acid was extracted from the aqueous phase in 3 ml ethylacetate. The radioactivity in 1 ml organic layer, corresponding to the [1-^{14}C]acetate, was measured after adding 10 ml Bray solution [13] using an Intertechnique (France) liquid scintillation spectrometer.

3. Results

Diacetylated peptide (14–21) 5 mg, with spec. act. 50 mCi/mol, was divided into fractions of 0.2 mg. Each fraction was incubated in the same conditions used for the deacetylation of H4 (see section 2). Analysis of the bulked reaction products was carried out by CMC chromatography using a linear NaCl gradient (fig.1). The A_{210} peaks, named P_2, P_1 and P_0, which were eluted at 0.14 M, 0.22 M and 0.29 M NaCl correspond to the di-, mono- and non-acetylated peptides (14–21), respectively. ^{3}H-radioactivity is found in fractions eluted below 0.1 M NaCl as well as in the mono- and diacetylated species. This corresponds to free acetate which is not retained in the column and to protein–peptide complexes which are eluted at 0.07–0.1 M NaCl.

It can be seen from fig.1 that the diacetylated peptide (14–21) acts as a substrate for a nuclear deacetylase and that both acetyl groups on the side-chains of Lys^{16} and Lys^{20} are accessible to the enzyme. Previous results on the enzymatic acetylation of peptide (14–21) also showed that both lysyl residues are accessible to a nuclear acetylase [8].

As radioactive peptide–protein complexes are formed in the conditions described in fig.1, a quantitative analysis using this experimental approach is

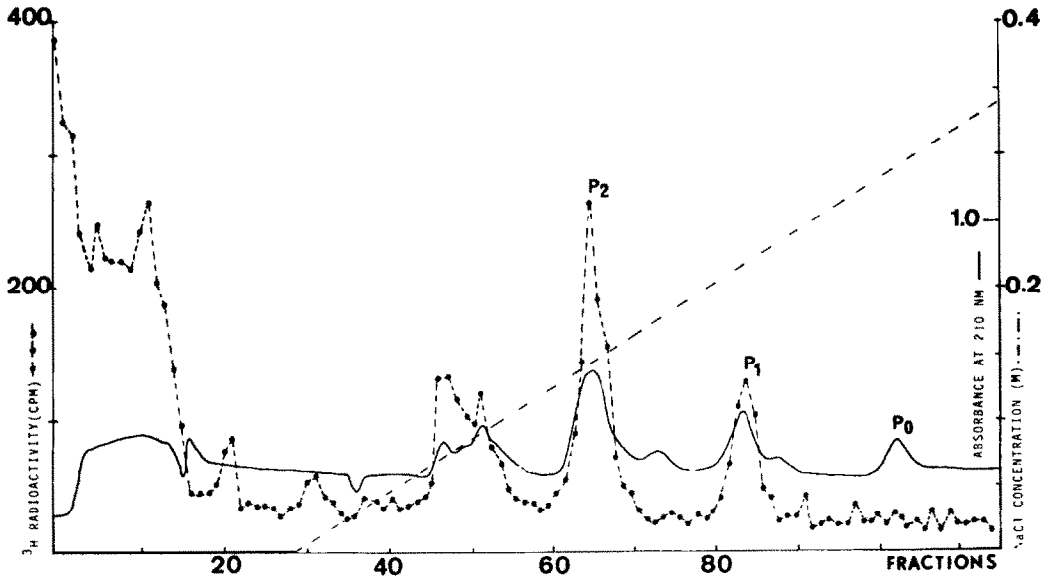

Fig.1. Carboxymethyl cellulose (MC) chromatography. After enzymatic deacetylation, the labelled diacetylated peptide (4.39 μmol) was applied to a CMC column (1.8 × 9 cm) and eluted with a linear NaCl gradient (0–0.4 M) with a flow rate of 43.6 ml/h. Fractions (7 ml) were collected and the radioactivity of 0.4 ml aliquots was measured in 10 ml Bray solution.

not possible. In order to establish the relative percentage of deacetylation of each residue (Lys^{16} or Lys^{20}), a parallel experiment was carried out using a non-radioactive peptide sample. The reaction mixture after enzymatic deacetylation was analyzed by CMC chromatography using a linear HCl gradient (0–0.1 M). It has been established that in these conditions all peptides, whatever their degree of acetylation, are eluted in a single peak [8]. Because of the highly basic character of the peptides P_1 and P_0, no protein–peptide complexes are likely to occur in acidic conditions. The peptide mixture was then treated with [^{3}H]acetic anhydride [8] and the pure diacetylated peptide (14–21) was obtained in quantitative yield. This corresponds to a selective radioactive labelling of the enzymatic products P_0 and P_1. The radioactive peptide thus obtained allowed the specific activity of the enzyme to be estimated at ~70 pmol acetate released $.h^{-1}$ $.mg\ protein^{-1}$. The diacetylated peptide (14–21) was then digested with trypsin and the fragments resulting from the selective cleavage of the polypeptide chain at arginine residues were separated by CMC chromatography as in [8]. The amount of radioactivity present in the different tryptic peptides indicates that the degree of deacetylation of Lys^{16}(Ac) and Lys^{20}(Ac) is nearly the same (54% and 46%, respectively).

4. Discussion

The present results show that the acetylated Lys^{16} in the octapeptide Ac–Gly–Ala–Lys^{16}(Ac)–Arg–His–Arg–Lys^{20}(Ac)–Val–NH_2 can be deacetylated by a calf thymus histone deacetylase in conditions similar to those used to deacetylate the histone H4. The octapeptide represents the histone H4 fragment between residues 14 and 21 with the N- and C-extremities protected by an acetyl group and a primary amide group, respectively. The monoacetylated peptide (15–21) H–Ala–Lys^{16}(Ac)–Arg–His–Arg–Lys–Val–OH with unprotected N- and C-extremities was found [6] not to be deacetylated by the calf thymus histone deacetylase and they concluded that recognition by the enzyme requires an histone fragment much larger than an heptapeptide. However, the results obtained with the protected peptide (14–21) suggest that the failure to deacetylate Lys^{16} of the unprotected peptide (15–21) is due to the presence of charged groups at N- and C-extremities which are very close to the deacetylation site. This is not encountered when the enzyme interacts with the intact histone molecule. The protected octapeptide (14–21) offers a better analogy with the situation that occurs in vivo since both the N- and C- extremities are uncharged. As the peptide (14–21) and the peptide (15–21) differ in length and substitution of their sidechains, the possibility that the presence of an N-terminal glycine residue and/or an N^{ϵ}-acetyl group on Lys^{20} might affect the deacetylation of Lys^{16} cannot be excluded. However, the fact that deacetylation of Lys^{16} readily occurs with the protected peptide (14–21) suggests that the histone deacetylase does not require a long fragment of the histone H4 in order to recognize the deacetylation site in the whole histone molecule.

The observation that Lys^{20} is also deacetylated in the diacetylated peptide (14–21) suggests that the sequence specificity of the histone deacetylase is not very strict. Both the lysyl residues belong to type B acetylation sites and only differ by their sequences: Lys^{16}–Arg and Arg–Lys^{20}, respectively. In chromatin the Lys^{20} of histone H4 is never acetylated and is substituted by methyl groups which are likely to prevent any acetylation at this site [14]. However, Arg–Lys occurs as an acetylation site in the histone H3 [2]. We reported [8] that the Lys^{16} and Lys^{20} in the unacetylated peptide (14–21) can both be acetylated enzymatically to a similar extent. This suggested that the sequence Arg–Lys^{20}, if unmethylated, would be a potential acetylation site in the histone H4. Preliminary results also show that Lys^{91} of the histone H4, which is adjacent to an arginyl residue (Lys–Arg), can be acetylated when the C-terminal fragment of the histone H4 (residues 85–102) is used as a substrate of the histone acetyltransferase (unpublished). However, no acetylation has been detected on Lys^{91} in vivo [15].

It appears that both the acetylating and deacetylating enzymes which act on B sites have similar recognition requirements when interacting with histone substrates and that only a restricted sequence of the histone molecule is needed for the enzyme–substrate complex to be formed. The fact that a restricted number of lysyl residues are acetylated in chromatin histones is probably related to structural

features of the nucleoprotein complex which affect the accessibility of the enzymes. The different levels of chromatin structure (tertiary structure of the chromatin core particle [16] and the superstructure of the nucleofilament [17]) are likely to be important factors in maintaining the specificity of the enzymatic acetylation–deacetylation processes in vivo. Histone deacetylases and acetylases should therefore be useful probes to detect different conformational states of chromatin.

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