SITES OF 'IN VITRO' ENZYMATIC ACETYLATION OF HISTONE H3

B. H. THWAITS, W. F. BRANDT and C. VON HOLT

Department of Biochemistry, C.S.I.R. Chromatin Research Unit, University of Cape Town, Rondebosch 7700, Cape Town, South Africa

Received 4 October 1976

1. Introduction

Acetylation, one of the post-synthetic modifications of histones, weakens histone–DNA interactions and may play a regulatory role in gene control of eukaryotes by either (a) allowing RNA synthesis on the previously repressed DNA [1]; or (b) permitting newly synthesised histones to find their specific binding site on DNA [2]. Various histone transacetylating enzymes have been recently prepared from nuclei [3–6], chromatin [7] or cytoplasm [8–10] and characterised with respect to their affinity to the various histone subfractions. However nothing is known about the actual sites of acetylation in the various histone molecules under in vitro conditions.

The present communication is concerned with the sites of acetylation of histone H3 by histone transacetylase(s).

2. Materials and methods

[^{3}H]Acetyl coenzyme A was prepared by the method of Stadtman [11] with two specific activities (1.03 or 2.7 Ci/mmol) for routine histone transacetylase assays and the preparation of [^{3}H]acetyl histone for sequencing purposes respectively.

Nuclei were isolated [6] from rat liver (male Wistar rats, 150–200 g).

Total histones from calf thymus were prepared either by 0.25 M HCl extraction of nucleoprotein, or via protamine displacement [12]. Electrophoretically pure histone subfractions were prepared as described previously [13].

Histone transacetylase was assayed at the following millimolarities in nuclei (a) and in solution (b): (a) sucrose 172, Tris–HCl (pH 7.9) 58, KCl 60, $MgCl_2$ 1.6, (b): NH_4Cl 47, Tris–HCl (pH 7.9) 29, EDTA 0.15, mercaptoethanol 0.78, KCl 4.3, $MgCl_2$ 0.6; acetyl coenzyme A in (a) and (b): 3.3×10^{-5} (0.02 μCi). The concentration of added substrate (total acid extracted histone) was 1.7 mg/ml and the total volume 0.58 ml. Start of the reaction, addition of acetyl-coenzyme A; incubation time, 30 minutes at +37°C. One unit enzyme activity, 1 pmol acetate incorporated under these conditions. Termination of the reaction, addition of 1 N HCl to a final concentration of 0.25 N. Protein was precipitated with 10 vol. of acetone, the precipitate heated at +90°C for 15 min in 2 ml of 18% (w/v) trichloroacetic acid (TCA), washed twice with 1 ml of 18% (w/v) TCA and once with methanol/ethanol/ether (2:2:1). The precipitate was then redissolved in 0.5 ml 0.25 N HCl, mixed with a suitable solubilizer–scintillator solution and the radioactivity determined in a liquid scintillation counter. Non-enzymatic histone acetylation was determined and found to amount to 13% of the total incorporation.

Total rat liver protamine displaced histone [12] was incubated with partially purified histone transacetylase and [^{3}H]acetyl-CoA, separated into subfractions on Biogel P-60 [13], the crude histone H3 was rechromatographed after dimerization with iodozobenzoate [14] to remove other contaminating histones and non-histone proteins.

Polyacrylamide electrophoresis of total histone after enzymatic acetylation was done according to Panyim and Chalkley [15]. The stained gels were scanned with a densitometer at 615 nm. At suitable intervals dextran blue markers were injected into the gels to facilitate the alignment of slices cut sub-

sequently with a gel cutter. The slices (approximately 1 mm thickness) were oxidized in a sample oxidizer, the tritium containing water trapped and its radioactivity determined.

[^{3}H]acetyl histone H3 (1 mg containing 3.2×10^6 dpm) together with unlabelled carrier rat histone H3 (2 mg) was subjected to sequential Edman degradation [16]. Details of the conditions of degradation, the gas chromatographic identification of residues and hydrolysis of PTH-derivatives with subsequent amino acid analysis have been described previously [17]. In addition aliquots of all residues were analysed by thin layer chromatography on silical gel (Merck F_{254}) in chloroform/methanol/ethanol (90:7:1), a system which clearly separated the PTH-derivative of ϵ-*N*-acetyllysine. Fractions were located via fluorescence quenching. The radioactivity of aliquots of the PTH-derivatives of the amino acids for each position was determined in a liquid scintillation counter. In addition 1 cm sections of the silical gel coating of the thin layer plates were scraped off, transferred into vials, suspended in scintillator solution and the radioactivity determined. The repetitive yield of 91% during the sequential degradation caused a gradually increasing appearance of [ϵ-*N*-^{3}H]acetyllysine PTH at each cycle. This background radioactivity amounted at residue 3 and 5 to 0.112×10^5 and 0.31×10^5 dpm to increase at residue 22 and 24 to 2.64×10^5 and 2.63×10^5 dpm respectively. Acetylation was considered significant if the radioactivity was either at least twice that of the preceding degradation cycle or returned after a lysine residue to the value preceding the lysine.

3. Results and discussion

Histone transacetylase was prepared from rat liver nuclei. After sonication of nuclei in unbuffered 0.25 M sucrose for one minute the viscous solution was adjusted to 0.14 M NaCl–0.001 M $MgCl_2$–0.05 M Tris–HCl (pH 8.0) and insoluble nucleoprotein removed by centrifugation at 7200 × *g* for 30 min. The supernatant was then dialysed extensively against 0.3 M NH_4Cl–0.03 M Tris–HCl (pH 7.9)–1 mM EDTA–5 mM 2-mercaptoethanol, centrifuged at 198 000 × *g* for 15 min to remove any particulate matter and applied to a linear 5–20% sucrose gradient in the above buffer. After centrifugation for 30 h at +4°C at 284 000 × *g* fractions with histone transacetylating activity were pooled (fig.1 and table 1). Though further purification on a DEAE column with a linear NH_4Cl gradient is possible (0.03 M Tris–HCl, pH 7.9–5 mM β-mercaptoethanol, 0.01 M–0.3 M NH_4Cl), this results in a low yield (15% of the activity recovered from the gradient centrifugation). On gel electrophoresis the fraction isolated from the sucrose gradient is free of histones.

This fraction has been used to produce acetylated histone H3 for the determination of the site undergoing acetylation under 'in vitro' conditions. 170 mg unfractionated histone complex isolated as described previously [12] was incubated with the transacetylase preparation at an enzyme:substrate ratio of 1:20 to result in 125 mg total histone with a specific activity

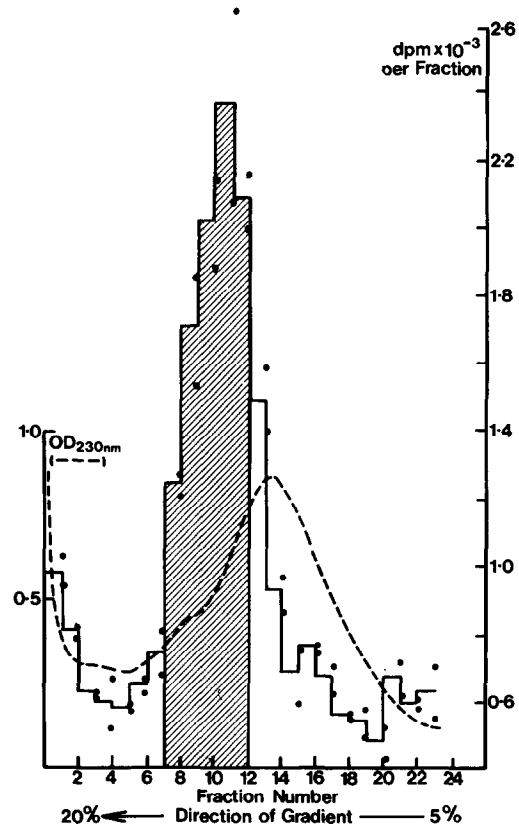

Fig.1. Sucrose gradient centrifugation of crude histone transacetylase: (•——•) Histone transacetylase fractions, (▨) Pooled histone transacetylase fractions, (- - - -) Protein concentration (A_{230} nm).

Table 1
Summary of purification of histone transacetylase (S)

Fraction	Specific activity (U/mg)[a]	mg Protein in fraction	Overall purification
Nuclei	0.3	305	1
Crude extract	3.5	71	12
Gradient fractions	33.3	16	111
DEAE cellulose fractions	100.1	0.8	334

[a]Definition of U see Materials and methods

of 4×10^6 dpm/mg. Polyacrylamide electrophoresis (fig.2A) as well as the fractionation on Biogel P-60 [13] show that histone H3 and H4 are the main acetyl acceptors. The crude histone H3 fraction isolated after Biogel P-60-exclusion chromatography was further purified via its dimer after oxidation with iodozobenzoate followed by exclusion chromatography [14] to result in approximately 6 mg histone H3 dimer with a specific activity of 3.2×10^6 dpm/mg. The minor electrophoretic heterogeneity of the dimer (fig.2) may be due to the presence of histone H3 species with 1 or 2 cystein residues which, as has been suggested, occur in rodents [18] or to the formation of a hybrid dimer in which only on of the partners is histone H3 or one of its degradation products [19]. On reduction with mercaptoethanol the radioactivity which co-electrophoreses and co-chromatographs with the dimer appears in the monomer (fig.2B and 2C). The specific activity of histone H3 corresponds to approximately 0.01 nmol acetate/nmol.

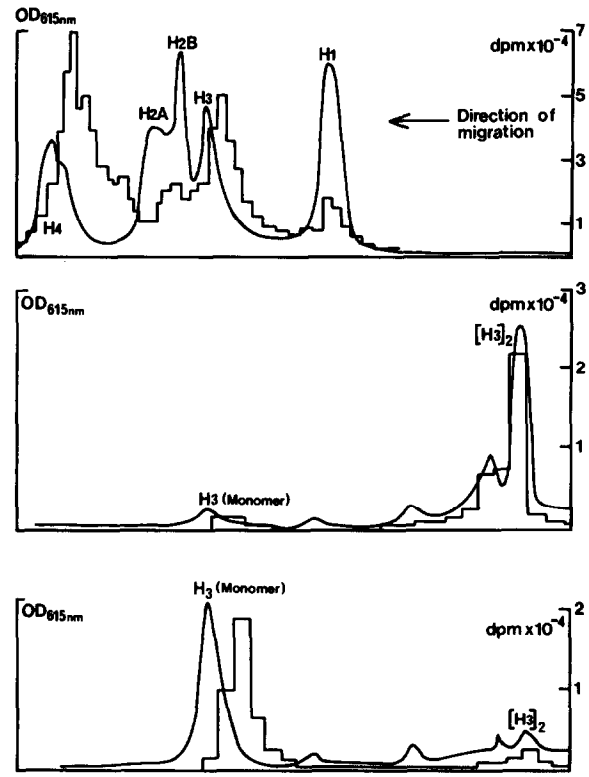

Fig.2. Polyacrylamide electrophoresis of total histone after enzymatic acetylation (A), histone H3 dimer (B) and histone H3 monomer (C): (———) Densitometer scan at 615 nm, (⎍⎍) Radioactivity, ($[H3]_2$) Histone H3 dimer.

To determine the sites of acetylation this histone H3 was subjected to sequential Edman degradation. All of the radioactivity occurred in the N-terminal region (fig.3). In all lysine positions in which significant radioactivity was present the radioactive compound co-migrated with the PTH-derivative of ϵ-*N*-acetyl-

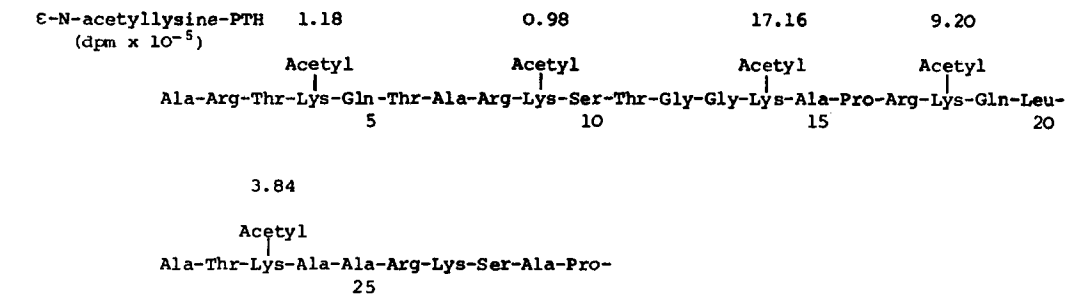

Fig.3. Sites of enzymatic 'in vitro' acetylation of lysine residues in histone H3.

lysine on thin layer chromatography. Also the increasing background radioactivity can be ascribed by that criterion to ϵ-*N*-acetyllysine. The tritium content of the remainder of histone H3 after 30 sequential degradation cycles amounted to 6% of initial radioactivity. The significantly acetylated lysine sites under 'in vitro' conditions are the same as those reported to undergo acetylation 'in vivo' [20]. This indicates a fair degree of retention of enzyme specificity and also suggests a substrate conformation near the acetylation sites closely resembling that existing 'in vivo'. The observation that residue 9 shows the lowest degree of acetylation (fig.3) may be due to the fact that this particular lysine residue, in a wide variety of organisms, is also consistently methylated to a fairly high degree [21].

Acknowledgements

One of the authors (B.H.T.) acknowledges a C.S.I.R. postgraduate scholarship. We thank Mrs M. Morgan for expert technical assistance and the C.S.I.R. and the University of Cape Town Research Committee for their support.

References

[1] Pogo, B. G. T., Allfrey, V. G. and Mirsky, A. E. (1966) Proc. Natl. Acad. Sci. USA 55, 805–812.
[2] Louie, A. J., Candido, E. P. M. and Dixon, G. H. (1973) Cold Spring Harbor Symp. Quant. Biol. 38, 803–819.
[3] Gallwitz, D. and Sures, I. (1971) Biochim. Biophys. Acta 236, 315–328.
[4] Lue, P. F., Gornall, A. G. and Liew, C. C. (1973) Can. J. Biochem. 51, 1177–1194.
[5] Candido, E. P. M. (1975) Can. J. Biochem. 53, 796–803.
[6] Gallwitz, D. (1968) Biochem. Biophys. Res. Commun. 32, 117–121.
[7] Racey, L. A. and Byvoet, P. (1972) Exp. Cell Res. 73, 329–334.
[8] Pestana, A., Sudilovsky, O. and Pitot, H. C. (1971) FEBS Lett. 19, 83–86.
[9] Horiuchi, K. and Fujimoto, D. (1972) J. Biochem. 72, 433–438.
[10] Harvey, S. R. and Libby, P. R. (1976) Biochim. Biophys. Acta 429, 742–749.
[11] Stadtman, E. R. (1957) in: Methods in Enzymol. (Colowick, S. P. and Kaplan, N. O. eds) Vol. III, pp. 931–932, Academic Press, New York.
[12] van der Westhuyzen, D. R. and von Holt, C. (1971) FEBS Lett. 14, 333–337.
[13] Böhm, E. L., Strickland, W. N., Strickland, M., Thwaits, B. H., van der Westhuyzen, D. R. and von Holt, C. (1973) FEBS Lett. 34, 217–221.
[14] Brandt, W. F. and von Holt, C. (1971) FEBS Lett. 14, 338–342.
[15] Panyim, S. and Chalkley, R. (1969) Arch. Biochem. Biophys. 130, 337–346.
[16] Edman, P. and Begg, G. (1967) Eur. J. Biochem. 1, 80–91.
[17] Brandt, W. F. and von Holt, C. (1974) Eur. J. Biochem. 46, 419–429.
[18] Garrard, W. T. (1976) FEBS Lett. 64, 323–325.
[19] Brandt, W. F., Böhm, E. L. and von Holt, C. (1975) FEBS Lett. 51, 88–93.
[20] Dixon, G. H., Candido, E. P. M., Honda, B. M., Louie, A. J., Macleod, A. R. and Sung, M. T. (1975) in: The Structure and Function of Chromatin (Ciba Symposium) pp. 229–258, Assoc. Scientific Publishers, Amsterdam.
[21] Brandt, W. F., Strickland, W. N., Morgan, M. and von Holt, C. (1974) FEBS Lett. 40, 167–172.