ACETYLATION OF HISTONE H4 IN CHICKEN ERYTHROCYTE AND CUTTLE-FISH TESTIS CHROMATIN

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1. Introduction

Acetylation is a post-synthetic modification which mainly occurs in the NH_2-terminal and basic region of the four nucleosomal core histones. This reversible modification affects their binding to DNA, as evidenced by circular dichroism studies [1], and is thought to control the changes in the structure of chromatin during the transcriptional process [2,3]. Indeed, it has been shown that the degree of histone acetylation is related to the transcriptional activity of the cell. In *Tetrahymena pyriformis*, highly acetylated forms of histone H4 occur in the transcriptionally active macronucleus, while the micronucleus contains only the non-acetylated form of this protein [4]. Moreover, the degree of acetylation seems to be variable according to the species, the tissue and the stage of development of a given tissue [5]. For example, a program of histone acetylation has been observed during spermatogenesis in trout [6], rat [7] and locust [8].

Four sites of acetylation have been identified in histone H4. The major site is the lysine residue at position 16 which is ~50% acetylated in calf thymus histone H4 [9]. Three other sites have been identified in trout testis histone H4 at lysine residues 5, 8 and 12 [10]. Each of these 4 lysine residues can be acetylated independently from the others and in reactions catalysed probably by different enzymes [11]. Acetyltransferase can recognize the immediate neighborhood of the specific lysine residues to be acetylated. The lysine residue at position 16 is adjacent to a basic residue, whereas the other sites of acetylation in histone H4 are surrounded by glycine residues. These two classes of acetylation sites are acetylated by two distinct histone acetyltransferases [12].

Here, we report the determination of the sites and the rates of acetylation of histone H4 from chicken erythrocyte and cuttle-fish testis. The mature chicken erythrocyte is a highly differentiated cell which is characterized by an almost complete cessation of RNA synthesis and a comparable decrease in the rate of histone acetylation. Cuttle-fish is a cephalopod which exhibits an intermediary type of evolution. The testis is well differentiated and contains, essentially, when the animal is mature, primary and secondary spermatocytes and spermatids. Differentiation of the spermatozoa is achieved in the genital tract. The cuttle-fish testis chromatin, in which no protamines have been identified so far, is characterized by an extensive acetylation of histone H4.

Since acetylation occurs in the NH_2-terminal region of histone H4, which contains the primary DNA binding sites, the NH_2-terminal peptide (residues 1–53) of histone H4 was used as starting material for our studies. This peptide was easily obtained by cleavage of the protein at the glutamyl residue in position 53, with the V8 staphylococcal protease. The peptide 1–53 was further hydrolyzed by trypsin, and tryptic peptides containing acetyl lysine were identified.

Three residues of lysine (in positions 5, 12 and 16) were found to be acetylated to an extent of 25–35% in cuttle-fish testis histone H4, whereas the same residues were acetylated to a lesser extent (6–12%) in chicken erythrocyte histone H4.

2. Materials and methods

Mature cuttle-fish testes were frozen in solid CO_2 after excision and kept at −20°C until use.

Abbreviations: DFP, diisopropylfluorophosphate; TPCK, *N*-tosyl phenylalanine chloromethyl ketone; PTH, phenylthiohydantoin

Chromatin was isolated from testes as in [13], in the presence of 0.1 mM DFP. Histone fraction F2a was extracted from chromatin according to [14].

Chromatin was isolated from chicken erythrocyte nuclei as in [15]. After extraction of the very lysine-rich histones H1 and H5 with 5% perchloric acid, the F2a histone fraction was isolated from the chromatin and subsequently fractionated into F2a1 and F2a2 subfractions according to [14].

In both cases, histone H4 was obtained in pure form from the fraction F2a1 by gel filtration chromatography on a Biogel P10 column (150 × 5 cm) equilibrated and eluted with 0.01 N HCl, saturated with chloroform.

The purity of the proteins was assessed by electrophoresis on polyacrylamide gels in 2.5 M urea, at pH 2.7, according to [16], using a 17% acrylamide concentration. The amino acid composition was established on 24 h and 72 h hydrolysates.

The proteins (100 mg) were cleaved at glutamyl residues by V8 staphylococcal protease (Miles) in 0.05 M ammonium acetate (pH 4.0), 10 ml, at 37°C for 16 h, using an enzyme:substrate ratio of 1/50 (w/w). The digestion was stopped by adding 0.5 ml glacial acetic acid and freeze-drying.

The digest was fractionated on a Sephadex G-100 column (200 × 2.5 cm) equilibrated and eluted with 0.01 N HCl, saturated with chloroform. The N-terminal fragment (residues 1–53) was further hydrolyzed by TPCK-treated trypsin for 2 h, at 37°C, in 0.1 M *N*-methyl-morpholine acetate (pH 8.0) using an enzyme:substrate ratio of 1:50 (w/w). The tryptic peptides were fractionated on a Chromobeads P column (Technicon) with pyridine–formate and pyridine–acetate buffers [17]. The purity of the peptides was checked by paper chromatography and electrophoresis. When necessary, peptides were purified as in [18].

Amino acid compositions of the peptides were determined on 24 h total acid hydrolysates. ϵ-*N*-Acetyl lysine was determined on the analyser after complete digestion of the peptides with aminopeptidase M [19]. Methylated derivatives of the lysine were separated at 26°C on a M-71 resin column (Beckman, France) (0.9 × 22 cm) equilibrated and eluted with 0.35 M sodium citrate buffer (pH 5.28) at 70 ml/h.

Sequence of peptides was performed by manual Edman degradation with identification of PTH-amino acids by high-pressure liquid chromatography [20].

3. Results and discussion

When submitted to polyacrylamide gel electrophoresis, cuttle-fish testis histone H4 migrated as 4 bands which correspond to non-acetylated and differentially acetylated forms of the protein. Chicken erythrocyte histone H4 showed two bands, one major with identical mobility to the non-acetylated form of calf thymus histone H4 and one minor corresponding to the mono-acetylated form of calf histone H4 (fig.1).

The amino acid compositions of histone H4 from cuttle-fish and chicken are similar to that of calf histone H4. From these results and [9,21–24], we can reasonably assume that their amino acid sequences are identical to that of calf histone H4.

The peptides obtained by hydrolysis of the cuttle-fish and chicken histones H4 with the V8 staphylococcal protease were separated by chromatography on a Sephadex G-100 column (200 × 2.5 cm) equilibrated and eluted with 0.01 N HCl (fig.2). The amino-terminal fragment of histone H4 (residues 1–53) is eluted

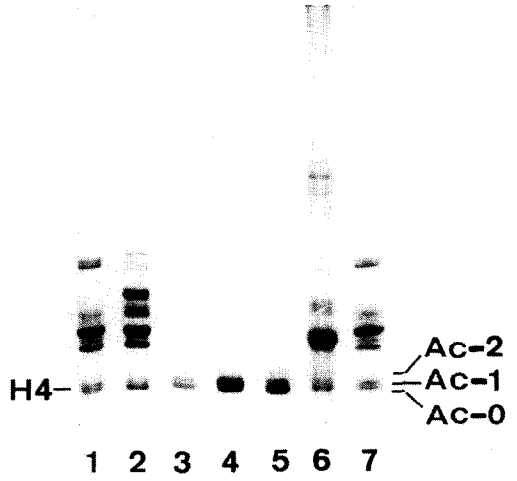

Fig.1. Polyacrylamide gel electrophoresis of chicken erythrocyte and cuttle-fish testis histone H4: (1) whole histone from calf thymus; (2) whole histone from chicken erythrocyte; (3) chicken erythrocyte histone H4; (4) calf thymus histone H4; (5) cuttle-fish testis histone H4; (6) whole histone from cuttle-fish testis; (7) whole histone from calf thymus. Electrophoresis was performed at 22 mA, for 210 min, at pH 2.7, in 2.5 M urea [16]. Gels were stained for 60 min with 0.5% Coomassie blue R 250 in acetic acid/ethanol/water (1:2:7, v/v/v) and destained for 24 h by diffusion in the same mixture. The differently acetylated forms of histone H4 are indicated Ac-0 (non acetylated, Ac-1 (monoacetylated) and Ac-2 (diacetylated).

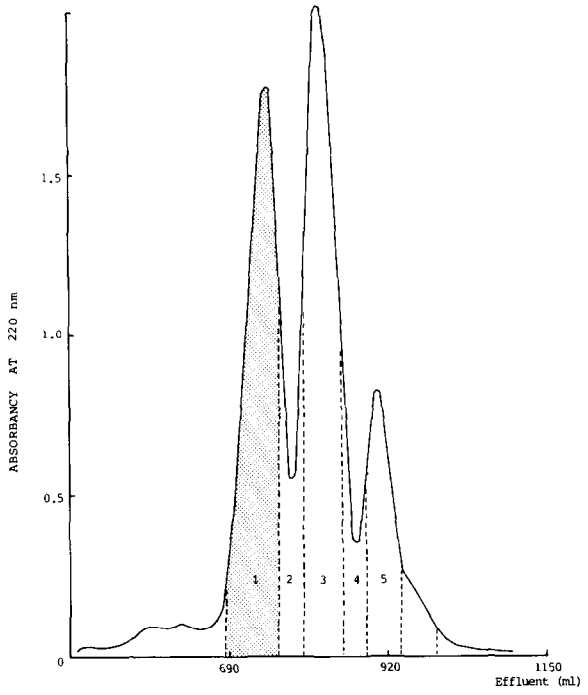

Fig.2. Separation by gel filtration chromatography of the peptides obtained by cleavage of histone H4 with V8 staphylococcal protease. The hydrolysate was freeze-dried and dissolved in 3 ml 0.01 N HCl–4 M urea. The solution was then centrifuged and applied to a Sephadex G-100 column (200 × 2.5 cm) equilibrated and eluted with 0.01 N HCl saturated with chloroform. Fractions of 4.5 ml were collected at 11 ml/h.

in the first fraction and has the following amino acid composition: Asp_2, Thr_1, Ser_2, Glu_3, Pro_1, Gly_{12}, Ala_3, Val_2, Ile_5, Leu_4, Tyr_1, His_1, Lys_7, Arg_9. This composition is identical to that of the fragment 1–53 from calf histone H4.

The elution diagram of the tryptic peptides obtained by hydrolysis of the NH_2-terminal peptide with TPCK-treated trypsin and fractionated on a Chromobeads P column, is shown in fig.3. After purification of the 19 fractions, 24 peptides were obtained which overlay the 53 residues of the sequence. Among these peptides, 3 from chicken H4 and 4 from cuttle-fish H4 were found acetylated at a lysine residue (table 1). In chicken histone H4, lysine residues 5 and 12 were found acetylated to ~6% whereas lysine residue 16 was found acetylated to 12%. In cuttle-fish histone H4, lysine residues 5, 12 and 16 were found acetylated to about the same extent (25%, 35% and 29%, respectively). In both histones, the lysine residue 8 was found non-acetylated. Moreover, the lysine residue 20 was found methylated with the dimethyl:monomethyl derivative ratio being ~7:1.

The low rate of acetylation of chicken erythrocyte histone H4 must be related to the condensed state of chromatin and to the decrease of its transcriptional activity [5]. On the other hand, the high level of acetylation of cuttle-fish testis histone H4 could be involved either with an extensive histone synthesis in the early stages of spermatogenesis or with the removal of histones from DNA in the late spermatid cells prior to their replacement by protamines, as quoted in [6]. Acetylation of histone H4 has also been observed in the testicular tissue, synthetically active, of the Echinoderm *Arbacia lixula*, whereas in the mature sperm cells, only the non-acetylated form of this protein is found [25].

The non-acetylated form, as well as the mono-, di- and triacetylated forms, of cuttle-fish histone H4 have been isolated in our laboratory (M. Couppez, P. S., unpublished). These subfractions will be used in reconstitution experiments of the nucleosome in order to determine the influence of acetylation on the stability of the nucleosome [26].

We have identified in the tryptic digest of the fragment 1–53 obtained by cleavage of chicken and cuttle-fish histones H4 with V8 staphylococcal protease, the peptide Ser–Gly–Arg which obviously derives from the amino-terminal peptide *N*-acetyl-Ser–Gly–Arg of histone H4. The non-acetylated peptide and the acetylated peptide were found in the ratio 1:4. Indeed, acetylation of the NH_2-terminal serine of histones is a cytoplasmic modification of newly synthesized histone molecules and is essentially irreversible in contrast to the acetylation of internal lysine residues [5]. This finding prompted the following important question. Were 20% of the histone H4 molecules non-acetylated at their amino-terminal end, or could this partial deacetylation be due to the presence of a deacetylase activity in the V8 staphylococcal protease preparation? In the latter case, the concomitant deacetylation of the internal lysine residues would have to be considered in evaluating our acetylation results. We therefore submitted cuttle-fish histone H4 to automated Edman degradation on a Beckman 890 C Sequencer. No PTH-amino acid was obtained, indicating that the amino-terminal end of the protein was completely blocked. On the other hand, when intact cuttle-fish histone H4 was hydrolyzed with trypsin, and the tryptic digest fractionated

Table 1
Sites of in vivo acetylation of chicken and cuttle-fish histone H4

A. Tryptic peptides obtained from the NH_2-terminal fragment (1-53) of chicken erythrocyte histone H4	Amounts of acetylation[a]
T-1 Ac-Ser-Gly-Arg (1)	
T-2 Gly-Lys (5)	
T-3 Gly-Gly-Lys (8)	
T-2a Gly-Lys-Gly-Gly-Lys	
T-2b Gly-Lys(Ac)-Gly-Gly-Lys (5)	5.4
T-4 Gly-Leu-Gly-Lys (12)	
T-5 Gly-Gly-Ala-Lys (16)	
T-5a Gly-Gly-Ala-Lys-Arg	
T-5b Gly-Gly-Ala-Lys(Ac)-Arg (16)	11.8
T-4a Gly-Leu-Gly-Lys(Ac)-Gly-Gly-Ala-Lys (12)	5.8

B. Tryptic peptides obtained from the NH_2-terminal fragment (1-53) of cuttle-fish testis histone H4	Amounts of acetylation[a]
T-1 Ac-Ser-Gly-Arg (1)	
T-2 Gly-Lys (5)	
T-3 Gly-Gly-Lys (8)	
T-2a Gly-Lys-Gly-Gly-Lys	
T-2b Gly-Lys(Ac)-Gly-Gly-Lys (5)	25.6
T-4 Gly-Leu-Gly-Lys (12)	
T-5 Gly-Gly-Ala-Lys (16)	
T-5a Gly-Gly-Ala-Lys(Ac)-Arg (16)	28.8[b]
T-4a Gly-Leu-Gly-Lys(Ac)-Gly-Gly-Ala-Lys (12)	35.6[c]
T-4b Gly-Leu-Gly-Lys(Ac)-Gly-Gly-Ala-Lys(Ac)-Arg (12, 16)	

[a] Results are expressed as mol acetylated lysine/100 mol lysine at a given position (5, 12 and 16)
[b] The amount of acetylation for lysine 16 takes into account peptides T-5a and T-4b
[c] The amount of acetylation for lysine 12 takes into account peptides T-4a and T-4b

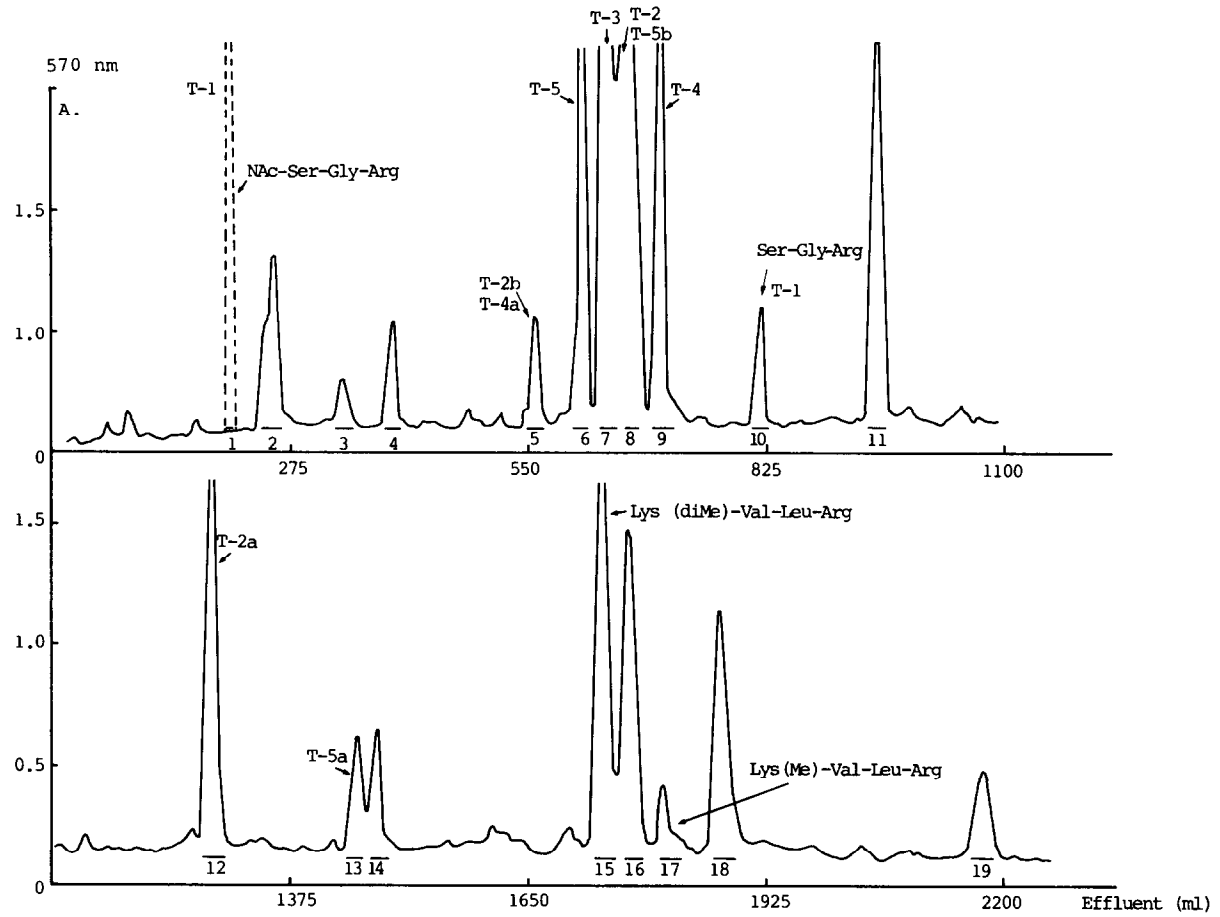

Fig.3. Elution pattern of tryptic peptides from the amino-terminal fragment (residues 1–53) of histone H4, fractionated on a column of Chromobeads P (60 × 0.635 cm) with pyridine–formate and pyridine–acetate buffers [17]. Fractions of 5.5 ml were collected at 33 ml/h. The pooled fractions indicated by solid bars are numbered by order of elution from 1–19. The peak in dotted line (peak 1) corresponds to a ninhydrin-negative reaction and to a Sakaguchi-positive reaction. Only peptides containing acetylation sites are mentioned on the elution pattern and numbered according to their position in the sequence of the protein (e.g. T-1, T-2, . . .).

as described previously [17], no unblocked peptide Ser–Gly–Arg was detected. Furthermore, the ε-acetyl lysine containing peptides were identified, and the extents of acetylation of lysine residues 5, 12 and 16 were found to be identical to those calculated earlier.

From these data, it was therefore concluded that a deacetylase activity, with a specificity strictly limited to the α-acetyl amino group, was present in the V8 staphylococcal protease. This observation can be of major interest for sequence work on proteins with an acetylated amino-terminal residue. In this respect V8 protease has been successfully used, in our laboratory, to unblock cuttle-fish histone H2A which has an identical amino-terminal sequence (residues 1–8) to that of cuttle-fish histone H4. Work is in progress to assay the deacetylase activity of this protease on different proteins and peptides of known structure which have their amino-terminal end blocked by an acetyl group.

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References

[1] Adler, A. J., Fasman, G. D., Wangh, L. J. and Allfrey, V. G. (1974) J. Biol. Chem. 249, 2911–2914.

[2] Allfrey, V. G. (1971) in: Histones and Nucleohistones (Phillips, D. M. P. ed) pp. 241–280, Plenum, London.

[3] Hnilica, L. S. (1972) in: The Structure and Biological Function of Histones, pp. 79–92, CRC Press, Cleveland, OH.

[4] Gorovski, M. A., Pleger, G. L., Keevert, J. B. and Johmann, C. S. (1973) J. Cell Biol. 57, 773–781.

[5] Allfrey, V. G. (1977) in: Chromatin and Chromosome Structure (Jei, H. and Eckhardt, R. eds) pp. 167–191, Academic Press, New York.

[6] Candido, E. P. M. and Dixon, G. H. (1972) J. Biol. Chem. 247, 5506–5510.

[7] Grimes, S. R., Chae, C. B. and Irvin, J. L. (1975) Arch. Biochem. Biophys. 168, 425–435.

[8] Bouvier, D. and Chevaillier, P. (1976) Cytobiol. 12, 287–304.

[9] De Lange, R. J., Fambrough, D. M., Smith, E. L. and Bonner, J. (1969) J. Biol. Chem. 244, 319–334.

[10] Candido, E. P. M. and Dixon, G. H. (1971) J. Biol. Chem. 246, 3182–3188.

[11] Sures, I. and Gallwitz, D. (1980) Biochemistry 19, 943–951.

[12] 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 (Fitzsimons, D. W. and Wolstenholme, G. E. W. eds) Ciba Found. Symp. vol. 28 (new ser.) pp. 229–258, Elsevier/Excerpta Medica, Amsterdam, New York.

[13] Goodwin, G. H., Nicolas, R. H. and Johns, E. W. (1975) Biochim. Biophys. Acta 405, 280–291.

[14] Johns, E. W. (1967) Biochem. J. 105, 611–614.

[15] Champagne, M., Mazen, A. and Wilhelm, X. (1968) Bull. Soc. Chim. Biol. 50, 1261–1272.

[16] Panyim, S. and Chalkley, R. (1969) Arch. Biochem. Biophys. 130, 337–346.

[17] Sautière, P., Tyrou, D., Laine, B., Mizon, J., Ruffin, P. and Biserte, G. (1974) Eur. J. Biochem. 41, 563–576.

[18] Sautière, P., Moschetto, Y., Dautrevaux, M. and Biserte, G. (1970) Eur. J. Biochem. 12, 222–226.

[19] Wouters-Tyrou, D., Sautière, P. and Biserte, G. (1976) FEBS Lett. 65, 225–228.

[20] Hermann, J., Titani, K., Ericsson, L. H., Wade, R. D., Neurath, H. and Walsh, K. A. (1978) Biochemistry 17, 5672–5679.

[21] De Lange, R. J., Fambrough, D. M., Smith, E. L. and Bonner, J. (1969) J. Biol. Chem. 244, 5669–5679.

[22] Wilson, R. K., Starbuck, W. C., Taylor, C. W., Jordan, J. and Busch, H. (1970) Cancer Res. 30, 2942–2951.

[23] Sautière, P., Tyrou, D., Moschetto, Y. and Biserte, G. (1971) Biochimie 53, 479–483.

[24] Sautière, P., Lambelin-Breynaert, M. D., Moschetto, Y. and Biserte, G. (1971) Biochimie 53, 711–715.

[25] Ruiz-Carillo, A., Wangh, L. J. and Allfrey, V. G. (1975) Science 190, 117–128.

[26] Bode, J., Henco, K. and Wingender, E. (1980) Eur. J. Biochem. 110, 143–152.