STUDY OF IN VITRO PHOSPHORYLATION OF HISTONES H3, H4 AND OF THE NON-ACETYLATED AND ACETYLATED TETRAMERS $(H3–H4)_2$

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Received 8 August 1981; revision received 15 September 1981

1. Introduction

The structures of the arginine-rich histones H3 and H4 which have been highly conserved during evolution, can be extensively modified by acetylation, methylation and phosphorylation [1].

Methylation is an irreversible post-synthetic modification which affects two lysine residues at positions 9 and 27 in histone H3 [2] and one lysine residue at position 20 in histone H4 [3].

Acetylation and phosphorylation are two post-synthetic reversible modifications of considerable interest which alter the charge of modified residues and thus modulate the histone–DNA interactions.

The acetylated lysines are at positions 14 and 23 in histone H3 [2] and at positions 5, 8, 12 and 16 in histone H4 [3].

If methylation and acetylation mainly affect histones H3 and H4, phosphorylation is generally related to histones H1, H2B and H2A which are the best substrates for kinases. Therefore, little is known about sites of phosphorylation of histones H3 and H4. The occurrence of phosphoserine in histone H3 from calf thymus has been reported in [4] and phosphorylation of histones H3 and H4 from trout testis has been pointed out in [5]. Histone H3 has been described as the best substrate with histone H2B, for the protein kinase from rabbit skeletal muscle [6]. Moreover, a cAMP-independent H3-specific histone kinase from bovine thymus chromatin has been reported to phosphorylate the threonine residue at position 3 [7].

Histones H3 and H4 are known to interact by structured apolar carboxyl regions (42–120 in H3 and 38–102 in H4) [8,9] to form a tetramer $(H3–H4)_2$ [10]. We have assumed that in the experimental conditions of phosphorylation, the equimolar complex (H3–H4) was a tetramer. This chromatin subunit structure can induce a phosphorylation specificity of the histones: thus, in the core particle, histone H3 is preferentially phosphorylated to a high degree (~65% of the total phosphate incorporated) by a protein kinase from rabbit skeletal muscle [6]. On the other hand, phosphorylation strongly reduces the association constants of the complex $(H3–H4)_2$ [11] and in this complex, basic N-terminal regions of histones where post-synthetic modifications are generally observed, are not involved.

This paper deals with in vitro phosphorylation of the calf thymus histones H3 and H4 and of the histone complex $(H3–H4)_2$ by a cAMP-dependent protein kinase from rat pancreas. This kinase was shown to phosphorylate only serine residues [12].

In comparison with lysine-rich histones, H4 does not incorporate any [^{32}P]phosphate and H3 is a poor substrate for this protein kinase [13]. In histone H3, one phosphoserine residue at position 10 has been identified. Surprisingly, the tetramer $(H3–H4)_2$ incorporates [^{32}P]phosphate more than the sum of each histone. Indeed, in the complex, H4 was a better substrate for the kinase than histone H3 and was phosphorylated on serine residue 47, whereas the phosphorylation of histone H3 takes place at the same serine residue as in the individual histone.

On the other hand, since extensive acetylation of histone H3 after Na-butyrate treatment stimulates its susceptibility to phosphorylation in presence of Ca^{2+} at physiological concentrations [14], it was of interest to investigate if the amount of incorporated [^{32}P]-phosphate was increased in the acetylated tetramer. Results show that the rate of phosphorylation of histones H3 and H4 was identical in the non-acetylated and acetylated $(H3–H4)_2$ complexes.

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

2. Materials and methods

Histone H4 was prepared as in [15,16]. Histone H3 and the non-acetylated and acetylated tetramers $(H3–H4)_2$ were isolated by ion-exchange chromatography (M.C., unpublished). We define as non-acetylated tetramer $(H3–H4)_2$ a complex in which histone H4 is non-acetylated and histone H3 partially acetylated (~20 nmol ε-acetyllysine/100 nmol H3); the acetylated tetramer is a complex in which histone H4 is fully acetylated on lysine residue at position 16 and histone H3 partially acetylated (~55 nmol ε-acetyllysine/100 nmol H3). It must be recalled that two sites of acetylation have been identified in calf thymus histone H3 at positions 14 and 23 [2].

Histones H3 and H4 and the non-acetylated and acetylated $(H3–H4)_2$ complexes were dissolved at 1 mg/ml, in the phosphorylation buffer containing 30 mM Tris–HCl (pH 7.8), 12 mM $MgCl_2$ and 1 mM dithiothreitol.

Individual histones and the $(H3–H4)_2$ complexes were phosphorylated by a cAMP-dependent protein kinase isolated from rat pancreas as in [12] but at 0.2–0.3 μg enzyme/ml in the presence of 0.4 mM [γ-^{32}P]ATP (10 Ci/mmol) prepared as in [17].

The presence of an equimolar complex (H3–H4) in the experimental conditions of phosphorylation was assessed by circular dichroism spectroscopy. Increase of α-helix content typical of histone–histone interaction is seen at 222 nm by comparison with individual histone spectra (not shown).

Electrophoresis of native and phosphorylated histones H3, H4 and of non-acetylated and acetylated complexes $(H3–H4)_2$ was performed on polyacrylamide (17%) slab gel (14 × 14 × 0.15 cm) at pH 2.7, in 2.5 M urea [18] for 6 h at 10 mA/slab.

Tryptic hydrolysis of labelled histones and complexes and identification of the phosphorylated sites were performed as in [12].

3. Results and discussion

The kinetic study of the phosphate incorporation into individual histones and histones complexes in the presence of the catalytic subunit of a cAMP-dependent histone kinase from rat pancreas is shown in fig.1.

Histone H3 was slightly phosphorylated whereas histone H4 was ineffective as acceptor and very poorly phosphorylated. In the $(H3–H4)_2$ complex, the [^{32}P]-phosphate incorporation is higher than the sum of ^{32}P incorporated in individual histones. Both non-

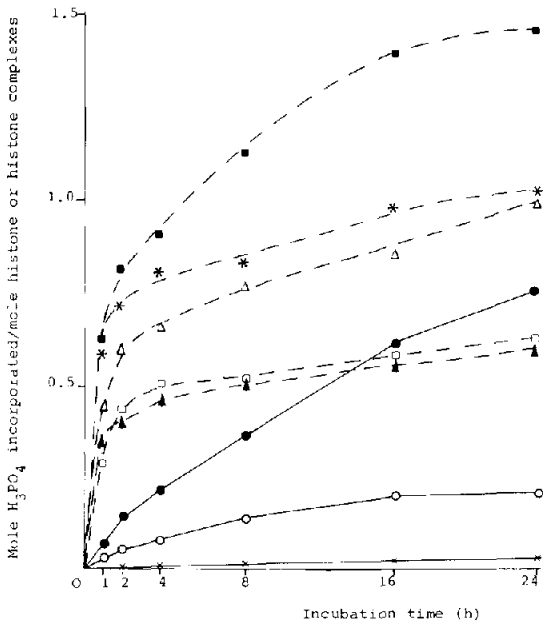

Fig.1. Kinetics of [^{32}P]phosphate incorporation into individual histones and histone complexes. The phosphorylation reaction by the catalytic subunit of the rat pancreas protein kinase was done as in [12] with different incubation times. [^{32}P]-Phosphate incorporation for: H4 (×); H3 (○); non-acetylated and acetylated tetramers $(H3–H4)_2$ (●); H1 (▲); chicken erythrocyte H5 (△); H2A (□); H2B (*); dimer H2A H2B (■).

acetylated and acetylated complexes gave the same kinetic curves of [^{32}P]phosphate incorporation.

Histones H1 and H2A were found equally phosphorylated to a higher extent than histones H3 and H4 but histones H2B and H5 were indeed the best substrates for the cAMP-dependent protein kinase.

In contrast with the tetrameric complex $(H3–H4)_2$, the dimer H2A–H2B incorporates an amount of ^{32}P approximately equal to the sum of ^{32}P incorporated by individual histones H2A and H2B.

Fig.2 shows the analytical gel electrophoreses of histones H3 and H4 and of their complexes, before and after ^{32}P-labelling. By comparison of the stained gel (fig.2A) and of the autoradiogram (fig.2B), it is obvious that individual histone H3 was phosphorylated whereas individual histone H4 was not. In contrast to this, both histones were found phosphorylated in the complexes. These results corroborate the kinetic studies of the [^{32}P]phosphate incorporation.

Autoradiography of the peptide map of the tryptic hydrolysate of the tetramers $(H3–H4)_2$ labelled with [^{32}P]phosphate showed 3 weakly radioactive spots,

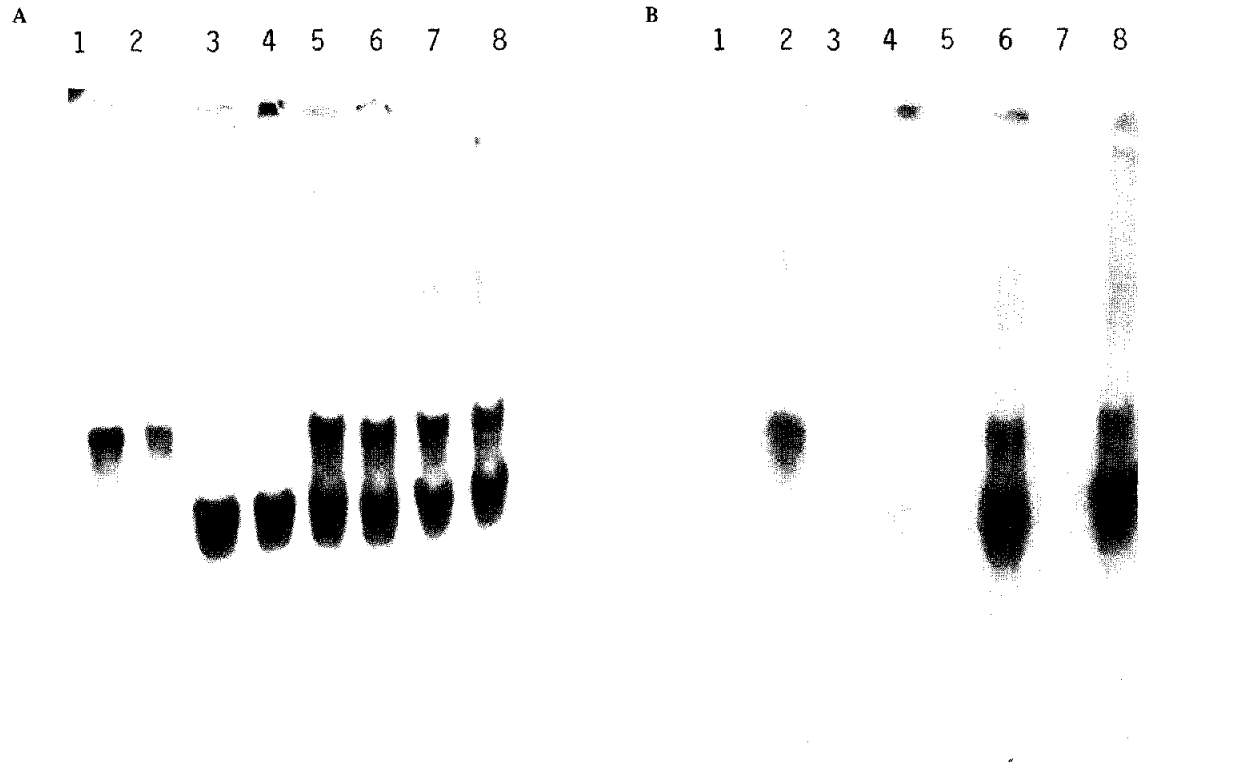

Fig.2. Polyacrylamide slab gel electrophoresis of histones H3 and H4 and of complexes $(H3-H4)_2$ at pH 2.7, in 2.5 M urea: (1) native histone H3; (2) phosphorylated histone H3; (3) native histone H4; (4) phosphorylated histone H4; (5) native tetramer $(H3-H4)_2$; (6) phosphorylated tetramer $(H3-H4)_2$; (7) native acetylated tetramer $(H3-H4)_2$; (8) phosphorylated acetylated tetramer $(H3-H4)_2$; (A) staining with Coomassic brilliant blue; (B) autoradiography.

identical to those observed in the tryptic hydrolysate of phosphorylated histone H3. However, the tryptic hydrolysate of the tetramers is characterized by the presence of a highly labelled phosphopeptide which migrated rapidly on paper chromatography and was later on shown to originate from H4. No difference was observed between the autoradiograms of the tryptic peptides generated from non-acetylated and acetylated $(H3-H4)_2$ complexes.

Structural studies of the phosphorylated tryptic peptides isolated by chromatography on Chromobeads P (Technicon Corp.) allowed us to identify the sites of phosphorylation in histone H3 and in the tetramer $(H3-H4)_2$ (table 1). No phosphopeptide was found in the tryptic digest of individual histone H4.

In histone H3 alone or interacting with histone H4, one serine residue at position 10 was phosphorylated by the cAMP-dependent protein kinase. This site was located next to a residue of methylated lysine in the highly basic amino-terminal sequence of the molecule.

On the other hand, it is worth noticing that the

Table 1
Sites of in vitro phosphorylation of histones

Histones	Sequences of ^{32}P-labelled tryptic peptides
H3, individual or in the tetrameric complex	^{a}(Arg–Lys(Me))–Ser(P)$_{10}$–Thr–Gly–Gly–Lys
H4, in the tetrameric complex only	(Arg)–Ile–Ser(P)$_{47}$–Gly–Leu–Ile–Tyr–Glu–Glu–Thr–Arg

[a] Residues in parentheses are not included in the tryptic peptides. They are written to show that the phosphorylation sites are placed in a sequence B–X–Ser(P) where B is a basic amino acid and X any amino acid

serine residue at position 28 of histone H3 which is located in the same sequence as Ser_{10}: Ala–Arg–(Me)Lys–Ser was not found phosphorylated. Since Ser_{28} is located outside the region of the molecule involved in protein–protein interactions, the sequence just after the serine residue must be essential for the specific recognition by the protein kinase.

In the tetramer $(H3–H4)_2$ acetylated or not, histone H4 was a better substrate than histone H3 and was phosphorylated at Ser_{47}.

Phosphorylated sites by a cAMP-dependent protein kinase occur generally on a serine residue located in a sequence B–X–Ser where B is lysine or arginine and X any amino acid (table 1): Ser_{10} in histone H3 and Ser_{47} in histone H4 interacting with histone H3 are placed in such a sequence.

Phosphorylated Ser_{47} in histone H4 is located at the edge of the structured apolar region which interacts with histone H3 to form the complex [8]. This fact has already been observed in the dimer H2A–H2B where phosphorylation sites (Ser_{19} in histone H2A and Ser_{32}, Ser_{36} in histone H2B) are located in a similar manner at the edge of the interacting regions [12].

Individual histone H4 was not phosphorylated. This was not surprising because histone H4 self-associates in solution to form aggregates and circular dichroism and nuclear magnetic resonance spectroscopic work [19,20] has clearly shown that the C-terminal part of the molecule (residues 38–102) is involved in the formation of secondary and tertiary structures. Therefore, in the aggregated state of H4, the Ser_{47} was not accessible to the protein kinase.

On the other hand, we have observed that, although histones H4 and H2A have an identical amino-terminal sequence (residues 1–5) the amino-terminal residue (N-acetylserine) was not phosphorylated in histone H4 or in the tetramer $(H3–H4)_2$ as it was in individual histone H2A and in the dimer H2A–H2B [12].

The only possible explanation is that the structural conformation of the tetramer $(H3–H4)_2$ or of H4 in the aggregated form is such that the amino-terminal sequence of histone H4 is not accessible to the enzyme. However, this is in contradiction with the fact that basic N-terminal regions of histones are free and mobile in solution and thus available for interaction with DNA [9].

Acknowledgements

We are very much indebted to Dr J. Pichon (Institut de Chimie Biologique, Marseille) for the preparation of γ-[^{32}P]ATP. We acknowledge with pleasure the skilful technical assistance of Mrs D. Belaïche, M. J. Dupire, T. Ernout and A. Hemez. We thank Dr M. H. Loucheux for giving us access to the CD spectrophotometer. This work was supported by grants ATP 4206 and LA 268 from the Centre National de la Recherche Scientifique.

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