INVESTIGATION OF THE SITES PHOSPHORYLATED IN LYSINE-RICH HISTONES BY PROTEIN KINASE FROM PIG BRAIN

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1. Introduction

The specificity of a large class of enzymes, which transfer γ-phosphate of ATP to protein substrates, the so-called protein kinases (ATP: protein phosphotransferases, EC 2.7.1.37), has not been investigated yet. A large number of protein kinases from different tissues has been described; these enzymes phosphorylate a wide variety of proteins like albumin, casein, ribosomal and nuclear acidic proteins, histones, etc. [1–3]. An outstanding enzyme in this respect is the pig brain cyclic-AMP-dependent protein kinase (histone kinase) which specifically phosphorylates lysine-rich histones only [4].

The present communication is concerned with the sites of phosphorylation of F1, F2a2, and F2b histones by this enzyme.

2. Materials and methods

Cyclic AMP was obtained from Sigma, [γ-^{32}P]-ATP (0.1 Ci/mmol) from Amersham, and DNS-Cl* from Merck; the latter was twice recrystallized from benzene.

Pig brain histone kinase was isolated according to the method described elsewhere [4]. Histone fractions were obtained from calf thymus as described by Jones [5].

The phosphorylation of histones by homogeneous histone kinase (0.003 mg) was run for 20 hr at 30°C in 3 ml of reaction mixture containing 5×10^{-2} M Tris-HCl, pH 7.4, 5×10^{-6} M cyclic AMP, 1×10^{-2} M $MgCl_2$, 1×10^{-3} M dithiothreitol, 3×10^{-4} M EGTA, 2×10^{-3} M theophylline and 3×10^{-4} M [γ-^{32}P]-ATP. The amount of radioactive phosphate incorporated into histone was determined under the conditions described elsewhere [4].

The tryptic digest of phosphohistones (37°C, 20 hr, histone: trypsin 80 : 1) was fractionated by fingerprinting on Whatman No.3 MM paper; in the first direction electrophoresis (80 V/cm) at pH 6.5 for 45 min and in the second chromatography in pyridine–butanol–acetic acid–water (10:15:3:12). The positions of radioactive peptides in the fingerprints were determined by radioautography using RT-2 X-ray film (2 hr exposure).

The amino acid composition of eluated [^{32}P] phospho-peptides was studied using a quantitative method of amino acid analysis [6] based upon their dansylation, followed by thin-layer chromatography on polyamide plates. DNS-amino acid were scanned using a PMQ-II (Opton) spectrophotometer with 6 × 0.1 mm slit.

3. Results and discussion

Studies of the substrate specificity of pig brain histone kinase showed that the highly purified preparation phosphorylates only lysine-rich histones F1, F2a2 and F2b. Arginine-containing histones F2a1 and F3, as well as standard protein substrates of various protein kinases of different origin, are not phosphorylated by this enzyme [4].

Abbreviations: DNS-Cl, *N,N*-dimethylaminonaphthalene-5′-sulphochloride. DNS-OH, *N,N*-dimethylaminonaphthalene-5′-sulphonic acid. EGTA, ethyleneglycoltetraacetic acid.

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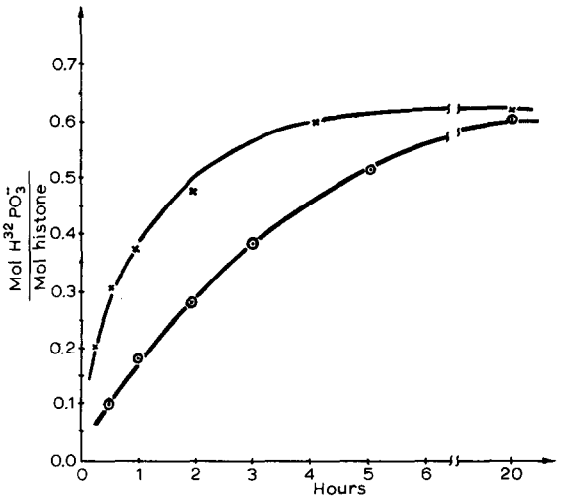
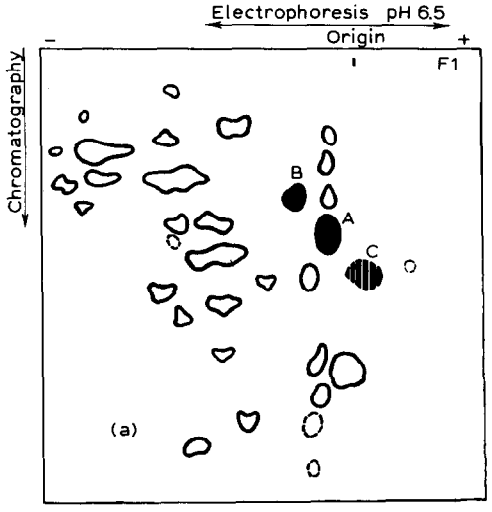
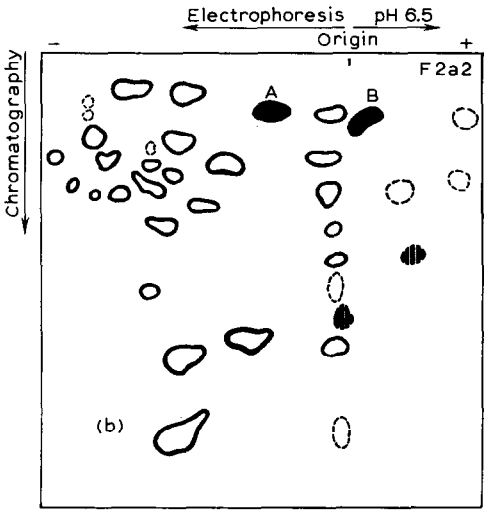


Fig.1. Kinetics of the incorporation of radioactive phosphorus into F2a2 histone.
–x– concentration of histone kinase 10 μg/ml
–o– concentration of histone kinase 1 μg/ml

The kinetics of the phosphorylation of F2a2 histone at different enzyme: substrate ratios are shown in fig.1. The maximum extent of phosphorylation of lysine-rich histones F1 and F2a2 does not exceed one mol of phosphate per mol of protein. This fact suggests high selectivity of the enzyme to the structure of the phosphate-accepting centers of histones. Studies of the peptides of the tryptic digests of ^{32}P-labelled substrates revealed the phosphorylation sites in the primary structures of lysine-rich histones.

The tryptic fingerprints of histones F1, F2a2 and F2b are shown in fig.2 (a,b,c). Table 1 shows the amino acid compositions of radioactive peptides, the





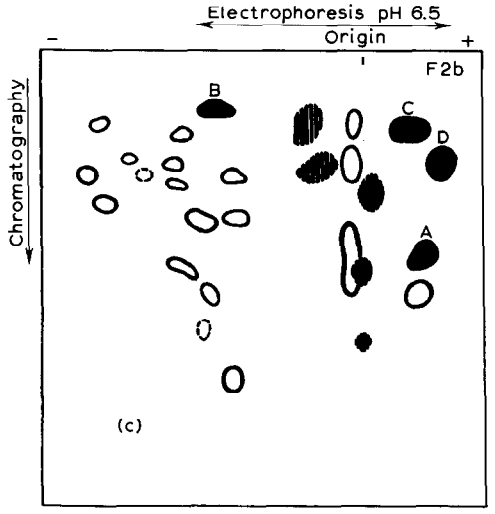


Fig.2. Peptide maps of the tryptic hydrolysates of ^{32}P-phosphohistones F1 (a), F2a2 (b), F2b (c).
-peptides, strongly stained by ninhydrin;
-weakly stained by ninhydrin;
-highly P^{32} labelled products;
-weakly P^{32} labelled products. Fingerprints were stained by ninhydrin after cutting off the central part of radioactive spots.

Table 1
Structure of tryptic peptides of ^{32}P-phosphohistones

Histone	Peptide	Yield	N-terminal A.A.	Amino acid composition and sequence of tryptic peptides	Site of phosphorylation
F1	A	100	Lys	Lys–Ala–$\overset{38}{\text{Ser}}$–Gly–Pro–Pro–Val–Ser–Glu–Leu–Ile–Thr–Lys 1.5 1.0 2.3 1.5 1.8 1.1 0.7 1.2 0.8 1.0 *	Ser^{38}
	B	20	Arg	Arg–Lys–Ala–$\overset{38}{\text{Ser}}$–Gly–Pro–Pro–Val–Ser–Glu–Leu–Ile–Thr–Lys 1.3 1.8 1.0 2.4 1.5 2.1 0.7 1.1 0.9 0.8 1.0 *	Ser^{38}
	C	7	Ala	Ala–$\overset{38}{\text{Ser}}$–Gly–Pro–Pro–Val–Ser–Glu–Leu–Ile–Thr–Lys **	Ser^{38}
F2a2	A	100	Thr	Thr–Arg–Ser–$\overset{19}{\text{Ser}}$–Arg 1.0 2.2 2.4	Ser^{19} (Ser^{18})
	B	25	Ser	Ser–$\overset{19}{\text{Ser}}$–Arg 1.9 1.0 *	Ser^{19}
F2b	A	100	Lys	Lys–Glu–$\overset{36}{\text{Ser}}$–Tyr–Ser–Val–Tyr–Val–Tyr–Lys	Ser^{36}
	B	50	Lys	Lys–Gly–$\overset{14}{\text{Ser}}$–Lys 2.1 1.2 1.0	Ser^{14}

The amount of the amino acid underlined in the text was assumed to be one mole per mole of peptide and the contents of other amino acids were determined relative to it.

* The chromatographic separation of DNS-amino acids was run under the conditions which do not resolve completely DNS-Ala and DNS-OH. The number of alanine residues evaluated was for this reason rather approximate.

** Only a limited amount of the peptide was available; the data are qualitative.

N-terminal amino acid residues and the relative yields of peptides, determined by scanning the corresponding spots on the developed autoradiographic X-ray film.

Comparison of the data obtained with the known primary structure of the histones [7] demonstrates that the phosphorylated peptides of the tryptic hydrolysates are:

Lys–Ala–$\overset{38}{\text{Ser}}(HPO_3^-)$–Gly–Pro–Pro–Val–Ser–Glu–Leu–Ile–Thr–Lys in the case of F1;
Thr–Arg–Ser–$\overset{19}{\text{Ser}}(HPO_3^-)$–Arg in the case of F2a2
and Lys–Gly–$\overset{14}{\text{Ser}}(HPO_3^-)$–Lys
and Lys–Glu–$\overset{36}{\text{Ser}}(HPO_3^-)$–Tyr–Ser–Val–Tyr–Lys in the case of F2b.

The other labelled phosphopeptides detected on the same fingerprints are fragments of the same peptides and arise from the incomplete selectivity of the tryptic hydrolysis. The analysis of the electrophoretic mobility of the peptides obtained and their structures leads to the conclusion that Ser^{38} is phosphorylated in F1 histone, Ser^{19} in F2a2 histone and Ser^{14} and Ser^{36} in F2b histone.

Thus, the pig brain protein kinase shows high selectivity and phosphorylates definite sites of the polypeptide chains of protein substrates. The structure of the phosphorylated centers in the histones is characterized by a common feature. In all the lysine-rich histones phosphorylated, it is the serine residue in the sequence X-Y-Ser, where X is always a lysine or arginine residue, and Y can be an acidic or neutral amino acid residue. Neither serine nor threonine residues which are not in this regular sequence are phosphorylated in lysine-rich histones. But it does not necessarily follow that the enzyme-substrate recognition is limited by this strict rule. The real specificity of histone kinase is determined probably by the local microenvironments of these serine residues in the tertiary structure of histones.

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