The intrinsic substrate specificity of a cyclic nucleotide-independent protein (histone) kinase

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1. INTRODUCTION

Phosphorylation and dephosphorylation of proteins play a pivotal role in the regulation of intracellular processes (reviewed in [1–3]). Beside several well-characterized protein kinases a number of protein phosphorylating enzymes have been found; however, their natural substrates and regulatory properties are not known. A possible way to identify these enzymes is the determination of their intrinsic substrate specificity; i.e., the most important residues of the amino acid sequence that they recognize in their substrate.

A cyclic nucleotide-independent histone kinase (designated as HK II) was described in [4,5]. We have also reported a type of cyclic nucleotide-independent histone kinase found in the cytoplasm and nucleus of human tonsillar lymphocytes [6,7] and in the extract of bovine thymus [8]. This enzyme phosphorylates the Ser-32 residue of calf thymus H2b histone and a synthetic peptide containing the amino acid sequence of H2b from Gly-26–Lys-34, but it does not act on Ser-36 which is phosphorylated preferentially by the catalytic subunit of the cyclic AMP-dependent protein kinase [9–11].

Here, we demonstrate that the histone kinase investigated by us is identical with that designated as HK II in [4,5]. Comparing the amino acid sequences around the phosphorylated serines of histones H1 and H2b we concluded that this enzyme recognized substrates containing lysine located 2 positions toward the COOH terminus from the serine. Studies on the phosphorylation of a series of synthetic peptides proved this conclusion.

2. EXPERIMENTAL

Separation of the cyclic nucleotide-independent histone kinase from the catalytic subunit of the cyclic AMP-dependent protein kinase was carried out by DEAE-cellulose chromatography in the presence of cyclic AMP, as in [8]. The histone kinase fraction obtained by this procedure from bovine thymus was dialyzed against 0.005 M potassium phosphate (pH 7.5) and it was applied onto a hydroxylapatite (Bio-Gel HTP) column. The column was washed by 5 vol. of each of 0.01 M, 0.07 M, 0.085 M, 0.1 M and 0.4 M potassium phosphate (pH 7.5). The histone kinase was eluted by the last volumes of 0.07 M and by the first volume of 0.085 M potassium phosphate. The specific activity of the enzyme preparation obtained was 10–12 nmol phosphate transferred mg protein^{-1} . min^{-1}, as measured in the presence of 1 mg histone H1/ml and 2×10^{-5} M ATP. This preparation yielded a single (M_r 55 000) histone kinase peak on Sephadex G-100 gel-chromatography.

Phosphorylation of H1 histone and tryptic digestion of the phosphorylated sample was done as in [8]. The ^{32}P-labelled peptide material was applied onto a DEAE-Sephadex A-25 column equilibrated with 0.005 M ammonium acetate (pH 7.5). Stepwise elution was carried out with 0.025 M ammonium acetate (pH 6.0), 0.125 M ammonium acetate (pH 4.5) and 0.5 M acetic acid: ~80% of the radioactivity was found in a peptide fraction eluted at pH 4.5; 20% of the radioactivity was found in 3 minor fractions. The main radioactive fraction yielded a single peak on Sephadex G-15 gel-chromatography

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(in 0.2 M acetic acid containing 1% *n*-butanol) corresponding to an ~1000-M_r peptide. This radioactive peptide was further purified (using a Waters HPLC system) on a μBondapak C 18 column. It was eluted by 0.01 M ammonium acetate (pH 4.0) containing 10% methanol, as in [8]. The analysis of the amino acid composition of the purified peptide was performed using a Durrum D 500 amino acid analyzer.

Synthetic peptides were produced by solid phase method, they were purified and their amino acid composition was controlled as in [8]. Phosphorylation of the synthetic peptides was performed in triplicate samples, in a reaction mixture containing 0.0125 M $MgCl_2$, 0.01 M Tris–HCl (pH 7.5), 10^{-5} M [γ-^{32}P]ATP (7.4 MBq/μmol) and ~10 μmol protein of the histone kinase preparation in 0.8 ml final vol. Synthetic peptide levels varied from 2×10^{-4}–1.5×10^{-3} M. The reaction was stopped by the addition of 0.3 ml glacial acetic acid. One sample was lyophylized and analyzed by paper-electrophoresis at pH 1.9 (formic acid:acetic acid:water = 35:100:865, by vol.) at 33 V/cm. The position of the ^{32}P-labelled peptide was determined by radioautography. Two samples were treated with Norit A and measured for radioactivity as in [8].

3. RESULTS AND DISCUSSION

Table 1 shows the amino acid composition of the tryptic peptide of histone H1 phosphorylated preferentially by the histone kinase. A tryptic peptide of histone H1 phosphorylated by HK II: Gly–Thr–Gly–Ala–Ser–Gly–Ser(PO_4)–Phe–Lys and HK II was characterized by this phosphorylation site in [4,5]. The amino acid composition of the tryptic peptide of H1 phosphorylated by our histone kinase agrees completely with that of the peptide obtained with HK II and it agrees with no other tryptic fragment of the known sequences of histone H1. Therefore the cyclic nucleotide-independent histone kinase investigated by us is identical with HK II in [4,5].

Table 1

Amino acid composition of the ^{32}P-labelled tryptic peptide of H1 histone phosphorylated by the histone kinase

Amino acid	Amount (nmol)	Molar ratio to lysine
Lys	1.37	1.0
Thr	1.11	1.0
Ser	2.35	2.0
Gly	4.40	3.0
Ala	1.50	1.0
Phe	1.22	1.0

The sequence of the synthetic fragment of H2b histone phosphorylated by the histone kinase is: Gly–Lys–Lys–Arg–Lys–Arg–Ser–Arg–Lys–(Ala) [8]. Comparing the sequences of H1 and H2b around the phosphorylated serines the only similar feature was that in both sequences lysine residues were located two positions toward the COOH terminus from the serine. We presumed that this lysine was an important determinant of the substrate specificity of the histone kinase and therefore a peptide was synthesized with the sequence $(Gly)_4$–Ser–Arg–Lys–Gly. This peptide was phosphorylated by the histone kinase and it was not phosphorylated by the catalytic subunit of the cyclic AMP-dependent protein kinase. It was also a competitive inhibitor of the histone kinase with respect to histone H1 as

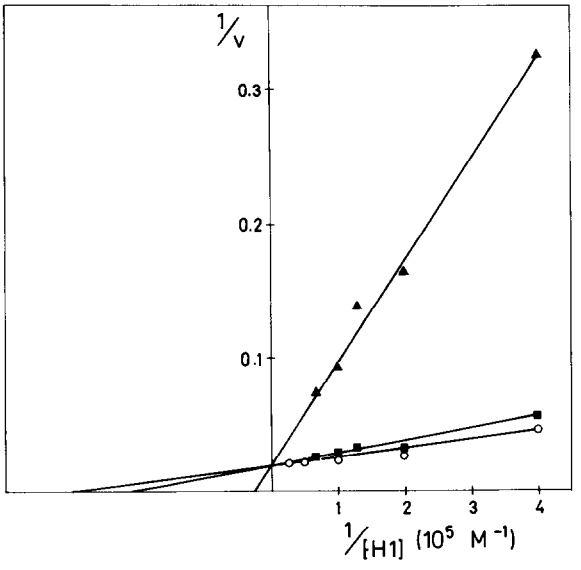

Fig.1. Inhibition of H1 histone phosphorylation by the synthetic peptide $(Gly)_4$–Ser–Arg–Lys–Gly. Reaction velocity (v) is expressed as pmol phosphate transferred/min. Phosphorylation of H1 in the absence (○) and presence of 7×10^{-4} M (■) or 4×10^{-3} M (▲) peptide.

Table 2

Phosphorylation of synthetic peptides

Peptide	Relative migration at pH 1.9 (Arg = 1.0)	Phosphorylation by	
		Histone kinase	Catalytic subunit
Gly–Lys–Lys–Arg–Lys–Arg–Ser–Arg–Lys–Ala	1.15	+	+
Gly–Lys–Lys–Arg–Lys–Arg–Ser–Arg–Lys–Ala (P)	1.03		
Gly–Gly–Gly–Gly–Ser–Arg–Lys–Gly	0.83	+	–
Gly–Gly–Gly–Gly–Ser–Arg–Lys–Gly (P)	0.59		
Glp–His–Trp–Ser–Tyr–Lys–Leu–Arg–Pro–etilamide	0.65	+	–
Glp–His–Trp–Ser–Tyr–Lys–Leu–Arg–Pro–etilamide (P)	0.43		
Glp–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–amide	0.50	–	–
Glp–His–Trp–Ser–Tyr–D-Lys–Leu–Arg–Pro–Gly–amide	0.65	–	–

a substrate (fig.1) although the detailed analysis of the kinetics of this inhibition (not shown) suggested that >1 molecule of peptide was involved in the inhibition (parabolic inhibition [12]). The complex nature of the competition agrees well with the finding that $(Gly)_4$–Ser–Arg–Lys–Gly was not only an alternative substrate for the histone kinase but caused also the inhibition of its own phosphorylation (the maximal rate of phosphorylation was achieved when peptide was at $\sim 7 \times 10^{-4}$ M).

Further we studied the phosphorylation of the luteinizing hormone releasing hormone (LHRH) and its synthetic analogues. While the serine residue of the synthetic LHRH was not acted on by the histone kinase the analogue containing Lys in position 6 instead of Gly (Lys-6 LHRH) was phosphorylated by the enzyme. However, D-Lys-6 LHRH (containing the D enantiomer of Lys) was not phosphorylated (table 2).

Our results suggest that this histone kinase (HK II) is a protein kinase recognizing the sequence –Ser–X–Lys–. Naturally the affinity of the enzyme and the rate of phosphorylation are influenced by other determinants of the local sequence of a substrate, too.

REFERENCES

[1] Krebs, E.G. and Beavo, J.A. (1979) Annu. Rev. Biochem. 48, 923–959.
[2] Cohen, P. (1982) Nature 296, 613–620.
[3] Weller, M. (1979) Protein Phosphorylation, Pion Limited, London.
[4] Langan, T.A. (1971) Fed. Proc. FASEB 30, 1089.
[5] Langan, T.A. (1978) Methods Cell Biol. 19, 127–142.
[6] Faragó, A., Antoni, F., Takáts, A. and Fábián, F. (1973) Biochim. Biophys. Acta 297, 517–526.
[7] Faragó, A., Antoni, F. and Fábián, F. (1974) Biochim. Biophys. Acta 370, 459–467.
[8] Romhányi, T., Seprödi, J., Antoni, F., Nikolics, K., Mészáros, G. and Faragó, A. (1982) Biochim. Biophys. Acta 701, 57–62.
[9] Faragó, A., Romhányi, T., Antoni, F., Takáts, A. and Fábián, F. (1975) Nature 254, 88.
[10] Yeaman, S.J., Cohen, P., Watson, D.C. and Dixon, G.H. (1977) Biochem. J. 162, 411–421.
[11] Glass, D,B. and Krebs, E.G. (1979) J. Biol. Chem. 254, 9728–9738.
[12] Cleland, W.W. (1963) Biochim. Biophys. Acta 67, 173–187.