ROLE OF A HISTIDINE RESIDUE IN THE ACTIVE SITE OF CYCLIC AMP-DEPENDENT HISTONE KINASE

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1. Introduction

The cyclic AMP-dependent histone kinase [1] is an important enzyme that carries out phosphorylation of lysine-rich histones, in particular, histone H1. At the present time the molecular mechanism of phosphotransferase reaction is still unknown. In studying individual stages of the enzymic reaction it seems important to elucidate which functional groups of the active site participate in substrate binding and the catalytic act. This paper is concerned with the investigation of ATP binding in the active site of the histone kinase catalytic subunit and further transfer of the phosphate residue to a protein substrate.

2. Experimental

2.1. *Materials and methods*

The catalytic subunit of histone kinase from pig brain was isolated according to our previous paper [1]. 3-Phosphohistidine was synthesized as in [2]. γ-[^{32}P]ATP was obtained from Amersham. Phosphotransferase activity of histone kinase was assayed according to [1]. The absorption of protein solutions at 280 nm was measured with a Beckman A-25 spectrophotometer.

2.2. *Phosphorylation of the histone kinase catalytic subunit*

The catalytic subunit of histone kinase (1 mg) in 10 mM Tris–HCl, pH 7.4 (total volume 1 ml), was incubated with 0.125 μM γ-[^{32}P]ATP (0.1 μCi/nM) for 40 min at 30°C. The aliquots (100 μl) were applied to Millipore HA filters (0.45 μm). The filters were washed with 20 ml of 0.1 M Tris–HCl buffer, pH 8.0. The radioactivity of filters was measured with an SL-30 liquid-scintillation spectrometer (Intertechnique) in a toluene scintillator.

To obtain large quantities of the phosphoform of the catalytic subunit, the incubated mixture was passed through a Sephadex G-25 column (1 X 20 cm), equilibrated with deionized water, and then dialyzed against 10 litres of deionized water (pH of water was adjusted to 9.0 with concentrated ammonia) for 10 h at 4°C.

2.3. *Stability of the phosphate–protein bond at various pH values*

The samples of the phosphorylated catalytic subunit (100 μg) were incubated in solutions of different pH (final volume – 500 μl), namely 0.1 N HCl (pH 1.0); 0.2 M acetate buffer + 1 N HCl (pH 2.0); 0.2 M acetate buffer (pH 4.0); 0.2 M phosphate buffer (pH 6.0); 0.1 M Tris–HCl (pH 8.0); 0.1 M Tris–HCl (pH 10.0); 0.1 M NaOH (pH 14.0), for 40 min at 30°C. The samples were then applied to Millipore filters and treated as described before.

2.4. *Direct identification of* N-*phosphohistidine*

The catalytic subunit [^{32}P]phosphoform (3 mg), purified to remove excess ATP, was hydrolyzed in 3 N NaOH at 105°C for 12 h. The mixture was applied to 0.7 X 4 cm Biorex-70 column (H^+-form), equilibrated with deionized water. The elution was performed with 0.15 N NH_4OH. The eluate (15 ml) was lyophylized, then dissolved in 50 μl of water and subjected to electrophoresis on Whatman 3 mm paper

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(pyridine–acetate buffer, pH 6.5, U=4000 V, 30 min). The phoregram was dried, sprayed with acetate–formate buffer (pH 2.0) and dried at 105°C for 1 h, then treated with ninhydrin to detect amino acids and with Pauly reagent [3] to detect imidazole compounds. The position of the radioactive label was determined by autoradiography using RT-1 film (36 h).

2.5. *Polyacrylamide gel electrophoresis*

Electrophoresis was done as described in [4] with minor modifications. Runs were carried out in 7.5% polyacrylamide gel containing 0.1% SDS in 0.1 M Tris–HCl, pH 7.5.

The samples of the ^{32}P-phosphorylated catalytic subunit (20 μg) were incubated with a four-fold molar excess of histone H1 for 30 min at 20°C. The samples were then treated with SDS and mercaptoethanol and applied to gels (100 μl). Electrophoresis was carried out at 5 mA per tube for 3.5 h. The gels were then frozen to –40°C, cut into slices of 1 mm thick and dispersed in 0.5 ml of water. After 12 h elution the solutions were placed into 5 ml of dioxane scintillation liquid. The radioactivity of samples was measured with an SL-30 counter. To determine the catalytic subunit and histone H1 positions on the gel, pure samples of these proteins were subjected to electrophoresis under the same conditions. The gels were then stained with Coomassie Brilliant Blue for 1 h and washed with methanol/acetic acid/water mixture (50 : 5 : 150, v/v/v).

3. Results and discussion

Though many aspects of the biological role of protein kinases are now under investigation, the process of the phosphate residue transfer from ATP to histones still remains unclear.

We found that incubation of the pure catalytic subunit of histone kinase with γ-[^{32}P]ATP caused the incorporation of [^{32}P]phosphate into protein. That the radioactive label was not released from protein after gel filtration and dialysis, seemed indicative of the covalent character of phospho–protein bond.

Figure 1 shows that after incubation of the catalytic subunit of histone kinase with fivefold excess of ATP the incorporation of [^{32}P]phosphate in protein was about 0.8–1.0 mol/mole of protein. The

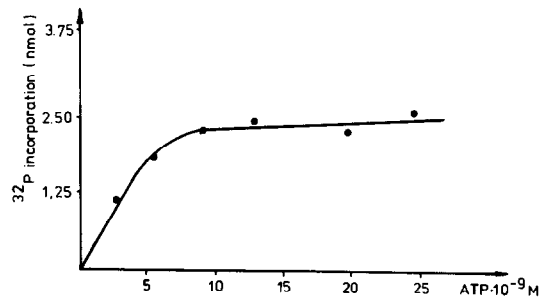

Fig.1. [^{32}P]Phosphate incorporation into the catalytic subunit in presence of ATP excess. Concentration of the histone kinase catalytic subunit: 1 mg/ml.

phosphoprotein bond was completely stable at neutral and alkaline pH (fig.2).

Lower pH values caused a considerable release of phosphate. The character of this pH dependence is similar to that observed with phosphohistidine residues in protein [5,6], which suggests that histidine is an acceptor of phosphate in the active site of histone kinase.

To identify the phosphohistidine residue directly, total alkaline hydrolysis of the [^{32}P]phosphorylated catalytic subunit was carried out. The hydrolysate was separated by high-voltage paper electrophoresis at pH 6.5. Calculations on the basis of the known pK values show that at pH 6.5 the histidine molecule carries an effective charge of +0.5, while that of N-phosphohistidine is approximately –0.7. These values allow one to distinguish N-phosphohistidine from other amino acids.

The electrophoretic pattern of alkaline hydrolysate of the phosphorylated catalytic subunit is shown in fig.3. It is seen that the electrophoretic zone with a

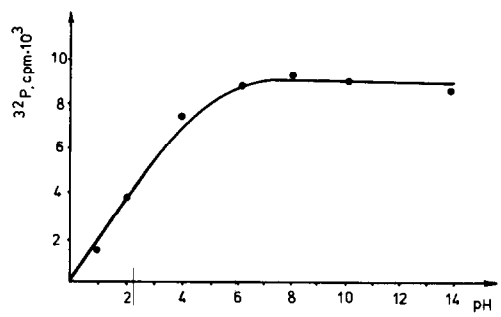

Fig.2. Stability of phospho–protein bond of ^{32}P-phosphorylated catalytic subunit at various pH values.

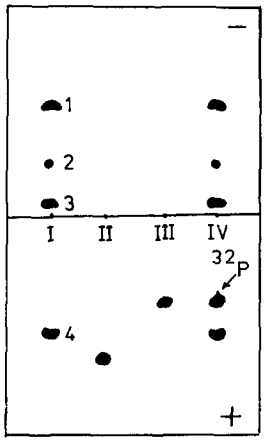

Fig.3. Paper electrophoresis of alkaline hydrolysate of ^{32}P-phosphorylated catalytic subunit. I, amino acid standard solution: 1, basic aminoacids; 2, histidine; 3, neutral amino acids; 4, acidic amino acids. II, *O*-phosphoserine. III, 3-*N*-phosphohistidine. IV, alkaline hydrolysate of ^{32}P-phosphorylated catalytic subunit.

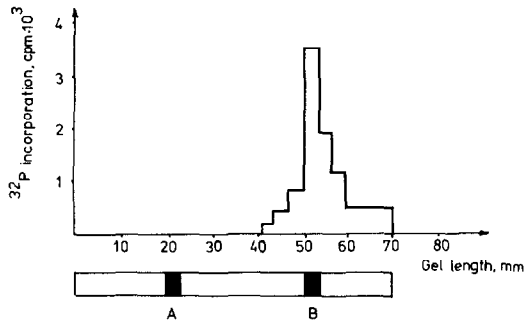

Fig.4. Polyacrylamide gel electrophoresis of ^{32}P-catalytic subunit histone H1 complex. A, histone kinase catalytic subunit, mol. wt 40 000. B, histone H1, mol. wt 22 000.

relative mobility of −0.7, coinciding with that of synthetic 3-phosphohistidine, contained radioactive phosphate and reacted positively with ninhydrin and Pauly reagent. On the basis of these data this substance was identified as *N*-phosphohistidine.

It seemed also important to find out whether the phosphoform of the histone kinase catalytic subunit was able to transfer its phosphate from histidine to a histone H1 serine residue. For this purpose approximately 20 μg of phosphoform, thoroughly purified from traces of ATP, were incubated with a 4-fold excess of histone H1. The incubation mixture was then separated by polyacrylamide gel electrophoresis (fig.4).

Figure 4 shows the distribution of [^{32}P]phosphate on the gel after separation. It can be seen that the radioactivity peak corresponded to the histone H1 zone, and, hence, transfer of the phosphate residue from histone kinase to histone had taken place.

The experimental data obtained allow one to draw the following conclusions on the mechanism of action of this type of enzyme:

1. The imidazole histidine residue in histone kinase serves as the active site catalytic group accepting the terminal phosphate of ATP.
2. The formation of an active intermediate – the phosphohistidine form of histone kinase – from the catalytic subunit and ATP is the main step in the phosphotransferase reaction.
3. The isolated phosphohistidine form of histone kinase is able to transfer its activated phosphate residue to the protein substrate, in our case to histone H1.

The detailed consideration of various stages of this phosphotransferase reaction and isolation of the active site phosphohistidine-containing peptide will be published elsewhere.

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