SUBTILISIN-CATALYZED REMOVAL OF PHOSPHORYLATED SITE OF PIG LIVER PYRUVATE KINASE WITHOUT INACTIVATION OF THE ENZYME

Gunnel BERGSTRÖM, Pia EKMAN, Ulla DAHLQVIST, Elisabeth HUMBLE and Lorentz ENGSTRÖM
Institute of Medical and Physiological Chemistry, Biomedical Center, University of Uppsala, Box 575, S-751 23 Uppsala, Sweden

Received 2 June 1975

1. Introduction

The activity of several enzymes is regulated by phosphorylation and dephosphorylation reactions catalyzed by protein kinases and phosphoprotein phosphatases, respectively [1]. In some cases it has been demonstrated that the phosphorylated sites of these enzymes are easily removed by proteolytic enzymes. Many years ago it was demonstrated that during a limited digestion of rabbit muscle phosphorylase *a* (EC.2.4.1.1.) with trypsin (EC.3.4.4.4.), phosphorylase *b'* is formed, which has properties similar to those of phosphorylase b [2,3]. Later it was shown that phosphopeptides from the phosphorylated site were cleaved off during this conversion [4,5]. The specifically phosphorylated serine residue of phosphorylase *a* subunits is located near the N-terminal end of the peptides [6].

Non-activated rabbit muscle phosphorylase kinase (EC.2.7.1.38) is irreversibly activated by treatment with trypsin, chymotrypsin (EC.3.4.4.5.) or a calcium-stimulated muscle protease (EC.3.4.99.–.) [7]. New NH_2-terminal amino acids arise on the phosphorylase kinase during proteolysis with the muscle protease. This protease also removes [^{32}P]phosphopeptides from ^{32}P-labelled phosphorylase kinase. A rapid release of such a peptide from the β-subunits of the enzyme occurs on treatment with trypsin [8].

Incubation of the glucose 6-P-dependent glycogen synthetase (EC.2.4.1.11.) from yeast with trypsin or subtilisin (EC.3.4.4.16.) converts the enzyme into an apparently glucose 6-P-independent form and concomitantly decreases its molecular weight by less than 10%. The proteases remove [^{32}P]phosphopeptides from the ^{32}P-labelled enzyme, which shows that the phosphorylatable sites are located near one end of the enzyme subunits [9]. When rabbit muscle glycogen synthetase phosphorylated with [^{32}P]ATP and cyclic 3',5'-AMP-stimulated protein kinase is treated with trypsin, [^{32}P]phosphopeptides are rapidly released [10].

Liver pyruvate kinase (ATP: pyruvate phosphotransferase, EC.2.7.1.40.) from the rat [11] and pig [12] was recently shown to be phosphorylated on incubation with [^{32}P]ATP and cyclic 3',5'-AMP-stimulated protein kinase, with a concomitant decrease of its activity, especially at low phosphoenolpyruvate concentrations. These results strongly indicate that the activity of pyruvate kinase from these sources is regulated by phosphorylation-dephosphorylation reactions. This view is further supported by the fact that the activity of the enzyme in rats in vivo decreases rapidly on injection of glucagon [13,14]. The aim of the experiments described in the present paper was to investigate whether the phosphorylated site of pig liver pyruvate kinase is especially sensitive to a proteolytic attack. ^{32}P-labelled enzyme was therefore incubated with a small amount of subtilisin. ^{32}P-labelled peptide material was released without inactivation of the enzyme.

2. Experimental

Pig liver pyruvate kinase was purified as previously described [12]. Assays were performed under optimal conditions by the method of Kimberg and Yielding [15] with some modifications [11]. Cyclic 3',5'-AMP-stimulated protein kinase from the same source was prepared as described elsewhere [16]. The protein

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kinase activity was measured as recently reported [17]. The radioactivity of ^{32}P-labelled components was estimated by measuring the Čerenkov radiation [18].

Pyruvate kinase (173 units/mg) was phosphorylated by incubation of the enzyme with 0.25 mM [^{32}P]ATP (spec. radioactivity 20 000–40 000 counts · min^{-1} · nmole^{-1}) and protein kinase for 60 min at 30°C in the presence of 5 mM potassium phosphate buffer (pH 7.0), 1 mM magnesium acetate, 10^{-5} M cyclic 3′,5′-AMP and 15% (v/v) glycerol. The final concentrations of the enzyme and the protein kinase in the incubation solution were 0.4 mg/ml and 0.2 units/ml, respectively. The incubation was interrupted by cooling in an ice-water bath and was followed by chromatography on a Sephadex G-50 column equilibrated and eluted with 1 mM potassium phosphate buffer (pH 7.0) containing 30% glycerol and 0.1 mM dithiothreitol. The extent of phosphorylation of the enzyme eluted was estimated as before [12] and was found to range between 0.8 and 1.0 mol of phosphate/mol of subunit.

[^{32}P]phosphorylated or unphosphorylated pyruvate kinase was incubated at 20°C with subtilisin (subtilopeptidase A = subtilisin Carlsberg; Sigma Protease Type P 5380) in the presence of 10 mM potassium phosphate buffer (pH 7.0), 10% glycerol, 0.1 mM dithiothreitol and the additives indicated in table 1 and fig. 2. The total incubation vols were 1.5 ml. The reactions were started by the addition of subtilisin dissolved in 10 mM potassium phosphate buffer (pH 7.0). At varying intervals 50 μl samples were taken for enzyme activity measurements and in addition, when ^{32}P-labelled pyruvate kinase was used, 0.2 ml samples for determination of residual protein-bound radioactivity. The 50 μl samples were diluted with 0.95 ml of 20 mM potassium phosphate buffer (pH 7.0) containing 30% glycerol, 0.1 mM fructose 1,6-diphosphate and 0.1 mM dithiothreitol. The pyruvate kinase activity of the diluted samples was determined within 2 hr. Control experiments showed that the enzyme activity did not change during this time. The 0.2 ml samples were added to 2 ml of 10% trichloroacetic acid and 0.1 ml of 1% bovine serum albumin (Sigma) dissolved in water was then added. The protein precipitates were collected by centrifugation and their radioactivity measured. Control experiments without addition of subtilisin were performed in parallel.

Polyacrylamide gel electrophoresis of the pyruvate kinase and of the subtilisin-treated enzyme was performed according to Shapiro et al. [19] with the modifications of Duncker and Rueckert [20]. Subtilisin was inactivated by the method of Adkins and Foster [21].

3. Results

3.1. *Proteolytic removal of [^{32}P]phosphopeptides from ^{32}P-labelled enzyme without inactivation of the enzyme*

Five units of pyruvate kinase per ml were treated with 0.05 μg of subtilisin/ml as described above. The activity and the ^{32}P-content of the enzyme were determined after different periods of digestion. A rapid decrease of the radioactivity of the enzyme and a slight increase of the enzyme activity took place during digestion (Fig. 1). [^{32}P]P_i was not removed from the pyruvate kinase during the incubation, as demonstrated by the method of Martin and Doty [22]. Chromatography of a sample (0.8 ml) incubated with

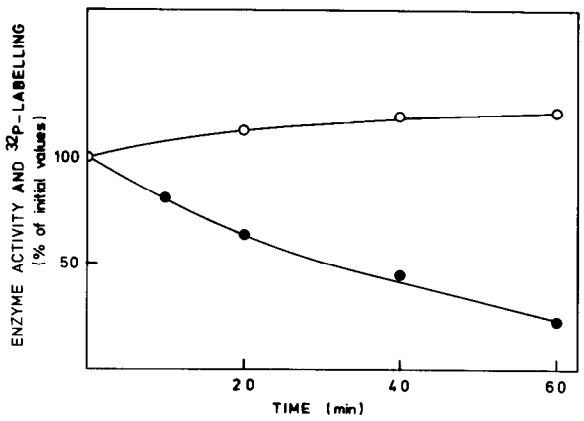

Fig. 1. Treatment of ^{32}P-labelled pyruvate kinase with a low concentration of subtilisin (0.05 μg/ml). The graph shows the time course of release of [^{32}P]phosphopeptides from the enzyme (●—●) and the change of enzyme activity (○—○) during incubation with subtilisin as described in the text.

subtilisin for 60 min on a 1.2 X 45 cm Sephadex G-25 column in 5 mM Tris-acetic acid buffer (pH 7.5) revealed that [^{32}P]phosphopeptide material had been released, since the radioactivity appeared as a peak after 0.5 column volume.

On polyacrylamide gel electrophoresis in detergent the ^{32}P-labelled enzyme which had been treated with subtilisin for 60 min appeared as one distinct band in the same position as that of untreated enzyme, demonstrating that within the experimental error the modified enzyme subunits had the same size as unmodified subunits.

The influence of different ions, as well as of substrates and some modulators, was examined. As seen in table 1, the enzyme was stabilized against the proteolytic attack to a considerable extent by 100 mM KCl and 10 mM magnesium acetate. The effect of the other substances used was hardly significant. It was shown that the additives had no effect on the subtilisin activity when this was tested according to Herriott [23], using 2% casein in 50 mM potassium phosphate (pH 7.0) as substrate.

Table 1
Influence of different ions, substrates and modulators on the removal of the phosphorylated site of ^{32}P-labelled pyruvate kinase

Additions	Residual ^{32}P-labelling of the enzyme (% of initial value)
None	23
100 mM KCl	62
10 mM magnesium acetate	63
1 mM phosphoenolpyruvate	36
1 mM pyruvate	29
1 mM ADP	37
1 mM ATP	38
0.1 mM fructose 1,6-diphosphate	30
2 mM alanine	24
2 mM phenylalanine	23

Pyruvate kinase (5 units/ml) incubated at 20°C for 60 min with subtilisin (0.05 μg/ml) in a total volume of 0.2 ml, and protein labelling determined as described under Experimental. There was no decrease of ^{32}P-labelling of the enzyme in a control incubated in parallel without subtilisin.

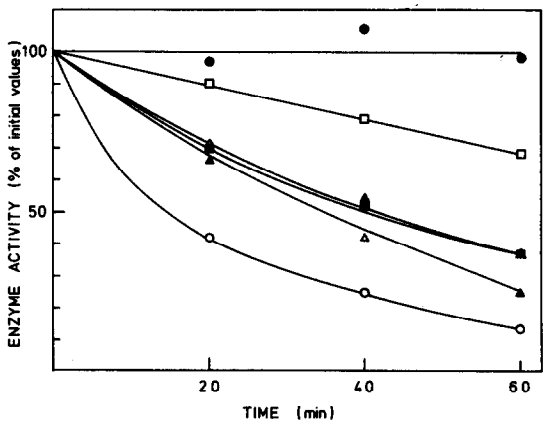

Fig. 2. Treatment of pyruvate kinase with a high concentration of subtilisin (20 μg/ml). The graph shows the enzyme activity after different periods of incubation with subtilisin under various conditions as described in the text. (○—○) No additions; (●—●) 0.1 mM Phosphoenolpyruvate; (□—□) 10 mM Magnesium acetate; (■—■) 1 mM Pyruvate; (▲—▲) 1 mM ADP and (△—△) 100 mM KCl present during proteolytic digestion.

3.2. *Inactivation of pyruvate kinase by proteolytic digestion*

When pyruvate kinase (6 units/ml) was incubated with a high concentration of subtilisin (20 μg/ml) under the same conditions as those described in table 1, there was a rapid inactivation of the enzyme (fig.2). In this respect there was no difference between phosphorylated and unphosphorylated enzyme (data not given here). As seen in fig.2, 0.1 mM phosphoenolpyruvate completely protected the enzyme from inactivation. Partial protection was obtained, in order of decreasing efficiency, by 10 mM magnesium acetate, 1 mM ADP, 1 mM pyruvate, 100 mM KCl and 1 mM ATP. In another set of experiments, it was shown that neither 2 mM alanine, 2 mM phenylalanine, nor 0.1 mM fructose 1,6-diphosphate altered the rate of inactivation significantly.

4. Discussion

The results reported demonstrate that the phosphorylated site was easily removed from ^{32}P-labelled pyruvate kinase, without inactivation of the enzyme, on incubation with subtilisin. Four hundred times

more subtilisin was necessary to inactivate the pyruvate kinase at approximately the same rate. The substrate phosphoenolpyruvate protected the enzyme completely from inactivation, most probably by binding to the active site. The fact that phosphoenolpyruvate did not prevent the removal of the phosphorylated site indicates that this site is separated from the active site. The gel electrophoresis experiments clearly demonstrated that the phosphorylated site is located near one end of the peptide chains, since the size of the subunits did not decrease measurably by removal of the phosphopeptides. Such a location of the phosphorylated site has also been shown in rabbit muscle phosphorylase [6], yeast glycogen synthetase [9] and the β-subunits of native phosphorylase kinase [8]. The amino acid sequence of the phosphorylated site of pig liver pyruvate kinase is similar to that of the corresponding sites of phosphorylase and glycogen synthetase, with hydrophobic amino acid residues surrounding the specific serine residue, which is phosphorylated, and basic residues in the vicinity [24]. The same type of sequence has recently been found in the α-subunits of rabbit muscle phosphorylase kinase [8].

With the work cited in the introduction and the present results as a background it seems possible that a prerequisite for a protein to be a substrate for cyclic 3′,5′-AMP-stimulated protein kinase is that at one end of the peptide chains it contains a structure which is sensitive to proteolytic enzymes and exhibits a specific type of amino acid sequence.

Acknowledgements

This research was supported by the Swedish Medical Research Council (Project No. 13X–50). The protein kinase preparation was kindly supplied by Drs Olle Ljungström and Lars Berglund at this institute.

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