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EFFECT OF FRUCTOSE 1,6-BISPHOSPHATE ON THE ACTIVITY OF LIVER PYRUVATE KINASE AFTER LIMITED PROTEOLYSIS WITH CATHEPSIN B

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Treatment of rat liver-type pyruvate kinase with rabbit liver cathepsin B at pH 7.0 caused loss of activity in the standard assay with 0.6 mM of phosphoenolpyruvate. The modified enzyme exhibited about 10% of the original activity when assayed with 2.0 mM of the substrate. No detectable change in the subunit molecular weight of the enzyme occured during inactivation. On addition of 4 μM fructose 1,6-bisphosphate the activity of the treated enzyme was restored to that of the original enzyme. Limited proteolysis of the enzyme by cathepsin B appears to enhance the requirement for the positive effector, fructose 1,6-bisphosphate.

Limited proteolysis of enzyme molecules with tissue proteinases is considered to be an initial stage of protein degradation that may also contribute to the regulation of enzyme activity. Cathepsin B (EC 3.4.22.1) is defined as the hydrolase for α-N-benzoyl-L-arginineamide, α-N-benzoyl-DL-arginine-p-nitroanilide (BAPA), α-N-benzoyl-DL-arginine-β-naphtylamide and hemoglobin (1), and has been reported to inactivate some enzymes involved in glycolysis such as glucokinase, fructose 1,6-bisphosphate ($Fru\text{-}P_2$) aldolase and pyruvate kinase (1) and to modify fructose 1,6-bisphosphatase (2). With most protein substrates it appears to act as a peptidyldipeptidase (3-5).

We have shown that cathepsin B inactivated rabbit muscle aldolase for $Fru\text{-}P_2$ cleavage without loss of the activity for fructose 1-phosphate as a result of the limited proteolysis near the carboxyl-terminal region of the subunit molecule (6).

Pyruvate kinase (ATP: pyruvate 2-O-phosphotransferase, EC 2.7.1.40) catalyzes a key reaction in glycolytic pathway, and the activity of the liver-

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type enzyme, but not the muscle-type enzyme, has been shown to be affected by Fru-P_2 (7).

In the present paper we report that the requirement of the liver-type pyruvate kinase for the allosteric effector, fructose 1,6-bisphosphate is greatly enhanced by limited proteolysis with cathepsin B.

MATERIALS AND METHODS

Reagents: NADH, Fru-P_2 and BAPA were obtained from Boehringer-Mannheim. Phosphoenolpyruvate cyclohexylammonium salt and sodium ADP were purchased from Oriental Yeast Co. Japan. All other reagents were of the highest grade available.

Preparation of enzymes: Rat liver- and muscle-type pyruvate kinases were prepared from the tissues of the animals fed on a high carbohydrate diet (8) according to the method of Harada et al. (9). The enzymes preparations showed a single band on polyacrylamide slab gel electrophoresis in the presence of SDS. Specific activities of liver- and muscle-type of enzymes were 200 and 240 units/mg protein, respectively. These preparations were dissolved in 10 mM potassium phosphate buffer, pH 6.0, containing 5 mM each of 2-mercaptoethanol and $MgSO_4$, and then dialyzed aginst the same buffer in order to remove Fru-P_2 completely. Rabbit muscle-type pyruvate kinase was obtained from Boehringer-Mannheim and was dissolved and dialyzed as described above. Cathepsin B from rabbit liver was prepared as described (6), the specific activity was 2.5 units/mg protein.

Assay of enzymes: Except where otherwise indicated, pyruvate kinase activity was measured with a coupled assay system using lactate dehydrogenase (rabbit muscle-type: Boehringer-Mannheim) similar to that described by Harada et al. (6). The assay mixture contained final concentrations of 50 mM Tris-HCl buffer, pH 7.4, 100 mM KCl, 5 mM $MgSO_4$, 2 mM ADP, 0.2 mM NADH, 2 mM phosphoenolpyruvate and 4.6 units of lactate dehydrogenase in a total volume of 1.0 ml. The decrease in absorbance of NADH at 340 nm was measured at 30°C, using a Gilford 250 multiple spectrophotometer. One unit of pyruvate kinase was defined as the amount of enzyme that caused the oxidation of 1 μmole of NADH per min.

The activity of cathepsin B was measured with 0.1 M sodium phosphate buffer, pH 6.5, containing 0.8 mM BAPA, 1 mM each of 2-mercaptoethanol and EDTA at 37°C (1). Absorbance of p-nitroaniline released from BAPA by cathepsin B was measured at 410 nm using a Hitachi 100-10 spectrophotometer. One unit of cathepsin B was defined as the amount of enzyme that released 1 μmole of p-nitroaniline per hr.

Treatment of pyruvate kinase with cathepsin B: The reactin of pyruvate kinase with cathepsin B was carried out at 30°C in 0.2 M triethanolamine-acetate buffer at indicated pHs in the presence of 5 mM each of 2-mercaptoethanol and EDTA.

Protein determination: Protein concentrations were determined by the method of Lowry et al. (10) using bovine serum albumin as a standard.

Electrophoresis: SDS polyacrylamide slab gel electrophoresis was carried out by the method of Laemmli (11). The gel was stained with 0.2% Coomassie brilliant blue and scanned at 580 nm with a Gilford 250 multiple spectrophotometric gel scanner.

RESULTS

Figure 1 shows the effect of pH on the activity of liver pyruvate kinase after incubation with cathepsin B in triethanolamine-acetate buffer. Liver pyruvate kinase was stable at neutral pH region after incubation for 30 min at 30°C. Modification of the enzyme by cathepsin B was rapid at neutral pH. The activities of rat and rabbit muscle pyruvate kinases were not affected by cathepsin B under the condition described above (data not shown).

The activity of liver pyruvate kinase as a function of the concentration of phosphoenolpyruvate is shown in Fig. 2A. The enzyme treated with cathepsin B showed little activity at concentrations of phophoenolpyruvate below 0.6 mM, but approximately 10% of the original enzyme activity was observed in the presence of 2.0 mM substrate. The addition of 4 μM Fru-P_2 to the assay system caused remarkable activation; pyruvate kinase modified by cathepsin B exhibited a hyperbolic type of curve with a Hill coefficient of 1.2 similar to that of the original enzyme (Fig. 2C), and in the presence of 1.0 mM phophoenolpyruvate the activity was identical to that of the untreated enzyme. From Lineweaver-Burk plots (Fig. 2B), the Km of the native enzyme for phosphoenolpyruvate in the presence of 4 μM Fru-P_2 was calculated to be 0.17 mM and that of the treated enzyme with cathepsin B was 0.27 mM.

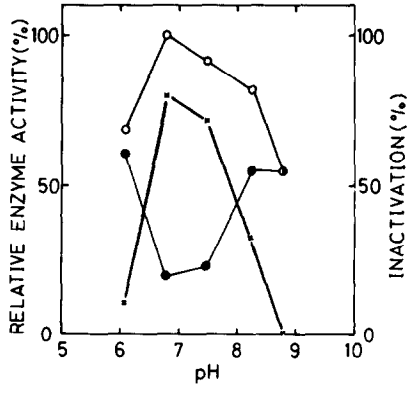

Fig. 1. Loss of activity of liver-type pyruvate kinase, measured under standard assay condition, on digestion with cathepsin B at several pHs. Rat liver pyruvate kinase (0.225 unit/ml) was incubated with (closed circles) and without (open circles) cathepsin B (0.05 unit/ml) as described in MATERIALS AND METHODS. Relative inactivation is shown by the line (—×—).

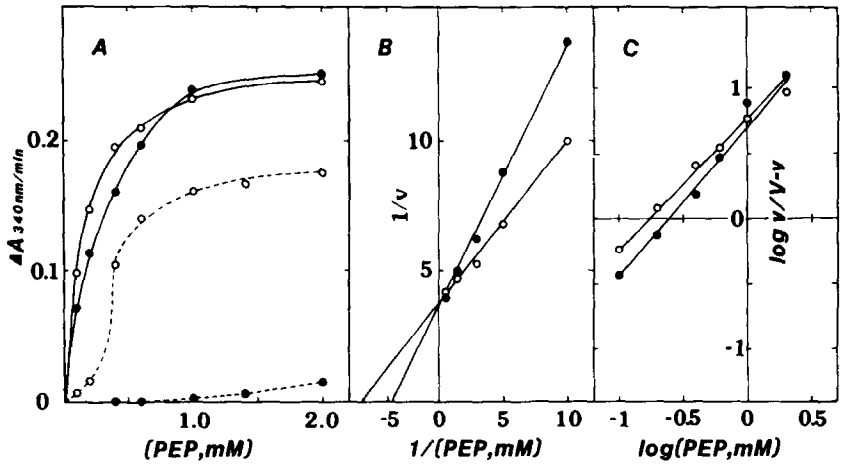

Fig. 2. Effect of Fru-P_2 on the inactivated liver-type pyruvate kinase with cathepsin B (0.05 unit/ml) at pH 7.0 for 2 hr at 30°. (A); The modified enzyme (closed circles) and the original enzyme (open circles) were assayed at various concentrations of phosphoenolpyruvate in the presence (solid line) and the absence (dotted line) of 4 μM Fru-P_2. Other assay conditions were as described in MATERIALS AND METHODS. (B); Lineweaver-Burk plots obtained from data of (A). (C); Replots of data from (A) for calculation of the Hill coefficients.

In order to determine whether cathepsin B caused a change in molecular weight of the enzyme subunits, the modified enzyme was subjected to SDS slab gel electrophoresis (Fig. 3). No significant changes in the subunit molecular weight were observed during the modification.

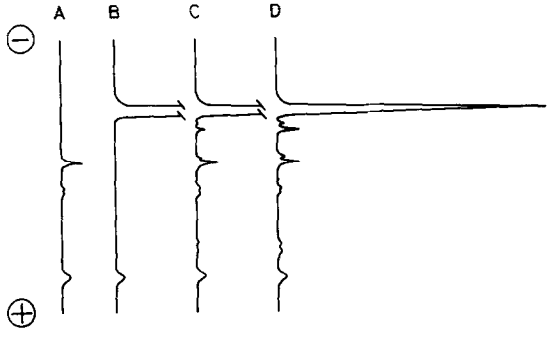

Fig. 3. SDS polyacrylamide (15%) slab gel electrophoretic patterns of the native and modified pyruvate kinases. Liver pyruvate kinase (0.61 mg/ml) was treated with cathepsin B (0.05 mg/ml) at pH 7.0. Cathepsin B, native pyruvate kinase and 50% and 80% "inactivated" enzymes were analyzed on SDS slab gels. (A); Cathepsin B, (B); native enzyme, (C) and (D); 50% and 80% "inactivated" enzymes, respectively.

DISCUSSION

The pH optimum of cathepsin B has been reported to be acidic region; 6.5 for hydrolysis of synthetic substrates such as BAPA and 4.5 for proteins such as hemoglobin (1). The optimal pH for the inactivation of aldolase was tentatively determined to be 5.3 in a citrate buffer (6), and that for the modification of fructose 1,6-bisphosphatase was 5.0 (2). In contrast, modification of liver pyruvate kinase by cathepsin B was most effective at neutral pH, which is also the pH of greatest stability for the native enzyme. Thus, the difference in pH optima for proteolytic modification of these enzymes may depend on the quarternary structure of the substrate proteins, which may also explain the lack of effect of cathepsin B on muscle pyruvate kinase activity.

Under the standard assay condition with high concentration (2.0 mM) of phosphoenolpyruvate, liver pyruvate kinase treated with cathepsin B showed about 10% of the activity observed with the native enzyme. Unexpectedly, the modified enzyme was fully reactivated by the addition of Fru-P_2 in the presence of 1.0 mM substrate. Limited proteolysis, probably at the COOH-terminus of pyruvate kinase, appears to cause a change in conformation that makes the enzyme completely dependent on the allosteric modifier, Fru-P_2. The nature of the proteolytic modification of pyruvate kinase by cathepsin B is now under investigation.

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REFFERENCES

1. Otto, K. (1971) in Tissue Proteinases (Barrett, A.J. and Dingle, J.J., eds), pp. 1-28, North Holland Publishing Co., Amsterdam.
2. Nakashima, K. and Ogino, K. (1974) J. Biochem. 75, 355-365.
3. Aronson, N.N.Jr. and Barrett, A.J. (1978) Biochem. J. 171, 759-765.
4. Bond, J.S. and Barrett, A.J. (1980) Biochem. J. 189, 17-25.
5. Hannappel, E., MacGregor, J.S., Stephen, D. and Horecker, B.L. (1982) Arch. Biochem. Biophys. 214, 293-298.

6. Nakai, N., Wada, K., Kobashi, K. and Hase, J. (1978) Biochem. Biophys. Res. Communs. 83, 881-885.
7. Bailry, E., Stirpe, F. and Taylor. C.B. (1968) Biochem. J. 108, 427-436.
8. Tanaka, T., Harano, Y., Sue, F. and Morimura, H. (1967) J. Biochem. 62, 71-91.
9. Harada, K., Saheki, S. Wada, K. and Tanaka, T. (1978) Biochim. Biophys. Acta 534, 327-339.
10. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
11. Laemmli, U.K. (1970) Nature (London) 227, 680-685.