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THE KINETICS OF UNPHOSPHORYLATED, PHOSPHORYLATED AND PROTEOLYTICALLY MODIFIED FRUCTOSE BISPHOSPHATASE FROM RAT LIVER

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Phosphorylation of fructose-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) by the catalytic subunit of cyclic AMP-dependent protein kinase from pig muscle decreased the $K_{0.5}$ for fructose-bisphosphate from 21 to 11 μM. When the phosphorylated fructose-bisphosphatase was treated with trypsin the $K_{0.5}$ increased to 22 μM. The $K_{0.5}$ also increased when the phosphoenzyme was treated with a partially purified phosphatase from rat liver. There was no difference between the unphosphorylated and phosphorylated enzyme with respect to pH dependence, the pH optimum being about 7.0 for both. Limited treatment of fructose-bisphosphatase with subtilisin, which cleaves the enzyme at its unphosphorylatable N-terminal part, increased the pH optimum more than limited treatment with trypsin, which releases the phosphorylated peptide at the C-terminal part of fructose-bisphosphatase. The phosphorylated site on the phosphorylated fructose-bisphosphatase was more easily split off by trypsin treatment than the corresponding unphosphorylated site. The results suggest in addition to the glucagon-induced phosphorylation of fructose-bisphosphatase described by Claus et al. [1] that the phosphorylation-dephosphorylation of fructose-bisphosphatase could be of importance for the hormonal regulation of the enzyme in vivo.

Introduction

Rat liver fructose-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) has recently been shown to be phosphorylated by cyclic AMP-stimulated protein kinase both in vivo and in vitro [2]. Whether this phosphorylation effects the activity of the enzyme and thereby contributes to the regulation of glycolysis-glyconeogenesis is still uncertain. It therefore seemed of interest to investigate the effects of phosphorylation on the kinetics of the enzyme with regard to fructose-bisphosphate and pH.

Fructose-bisphosphatase can still be phosphorylated after treatment with subtilisin even though the N-terminal region has been split off as a peptide of molecular weight 6000 [3,4]. This treatment yields a fructose-bisphosphatase with an alkaline pH optimum [5]. The difficulties in preparing a fructose-bisphosphatase with a neutral pH optimum have been explained by the existence of a protease with activity similar to that of subtilisin during the first step in the purification procedure [5]. If precautions are taken to avoid this proteolysis a fructose-bisphosphatase with a native molecular weight of 140 000 and a neutral pH optimum can be obtained, but the difficulties in getting a phosphorylatable enzyme often remain [4]. Since Pilkis et al. [6] showed that the trypsin-treated fructose-bisphosphatase lost its phosphorylated site without any major change in maximal activity or molecular weight, it was of interest to include subtilisin- or trypsin-treated fructose-bisphosphatase in this investigation.

Materials and Methods

Materials

Bovine serum albumin, ATP, phenylmethylsulfonyl fluoride, Subtilisin Carlsberg (subtilopeptid-

ase A (14 U/mg)), dithiothreitol, NADP and fructose-bisphosphate were obtained from Sigma. Trypsin TPCK (228 U/mg) was bought from Worthington. Yeast glucose-6-phosphate dehydrogenase and phosphoglucose isomerase were purchased from Boehringer-Mannheim. [γ-^{32}P]ATP was obtained from New England Nuclear, Boston. Sephadex G-50 and G-200 were products of Pharmacia, Sweden. DE-52 cellulose was from Whatman and the collodion bags were from Sartorius GmbH, Göttingen. All other chemicals were of the highest commercial grade available.

Methods

Protein was estimated by the absorbance at 280 nm, using the coefficient $A^{0.1\%} = 1$.

Radioactivity in the samples was measured as Čerenkov radiation.

Fructose-bisphosphatase was purified from rat liver essentially as described by Riou et al. [2]. The exceptions were that the volume of the buffer added to the livers was increased from 2 to 4 vol. and that 1 mM EGTA and 0.5 mM phenylmethylsulfonyl fluoride were added to this buffer before use, to prevent proteolysis. The enzyme was 100% pure as judged from polyacrylamide gel electrophoresis in detergent as described by Dunker and Rueckert [7], and had a subunit molecular weight of 37 000. The purified enzyme had a specific activity of 10 units/mg when measured under optimal conditions.

The catalytic subunit of cyclic AMP-stimulated protein kinase from pig muscle was prepared essentially as described by Bechtel et al. [8], but with the modifications as described [4]. 1 unit was defined as the amount of enzyme that could transfer 1 pmol phosphate from ATP to mixed histone per min at 30°C. The activity of fructose-bisphosphatase was estimated by coupling this reaction to phosphohexose isomerase and glucose-6-phosphate dehydrogenase. The increase in absorbance at 340 nm was measured by means of a Hitachi Perkin-Elmer double-beam spectrophotometer 124 and a Hitachi Perkin-Elmer recorder 165. The standard incubation mixture contained, unless otherwise stated, 10 mM potassium phosphate buffer, pH 7.5/25 mM 2-mercaptoethanol/ 0.1% bovine serum albumin/7.5 mM magnesium sulphate/ 0.4 mM NADP/0.7 and 0.35 unit of glucose-6-phosphate dehydrogenase and phosphohexose isomerase, respectively. Before use these enzymes were dialysed against 10 mM potassium phosphate buffer, pH 7.5/2 mM 2-mercaptoethanol. This mixture was preincubated with 25 μl fructose-bisphosphatase for 3 min at 30°C. The reaction was run at 30°C and was started by the addition of 50 μl 600 μM fructose-bisphosphate. The reaction volume was 600 μl. 1 unit was defined as the amount of enzyme that could dephosphorylate 1 μmol fructose-bisphosphate per min.

The phosphorylation of fructose-bisphosphatase (1 mg) was performed with 0.25 mM [^{32}P]ATP (spec. act. 50 000 cpm/nmol) and $1.6 \cdot 10^6$ units of the catalytic subunit of cyclic AMP-dependent protein kinase in the presence of 25 mM 2-(*N*-morpholino)-ethane sulfonic acid buffer, pH 6.9/4 mM magnesium acetate/2 μM cyclic AMP at 30°C for 120 min. After the addition of fructose-bisphosphate to a final concentration of 2 mM to stabilize the enzyme, the reaction mixture was chromatographed at 4°C on a Sephadex G-100 column, equilibrated and eluted with 0.1 M potassium phosphate buffer, pH 7.5/1 mM dithiothreitol/25 mM NaF/0.1 mM fructose-bisphosphate. The ^{32}P-labelled fructose-bisphosphatase was pooled and concentrated by dialysis against poly-(ethylene glycol).

The concentrated ^{32}P-labelled material and unphosphorylated fructose-bisphosphatase were equilibrated with 10 mM Tris-HCl buffer, pH 7.5/1 mM dithiothreitol, by chromatography on a Sephadex G-50 column. Bovine serum albumin was added to a concentration of 0.1% and the two enzyme forms were stored at −70°C before use.

The phosphoprotein phosphatase used was the low molecular weight phosphatase described by Titanji [9], purified through the second DEAE-cellulose chromatography step.

Partial proteolysis of phosphorylated fructose-bisphosphatase was performed at 30°C by the addition of trypsin in 50 mM Tris-HCl buffer, pH 7.0/0.2 mM dithiothreitol, the weight ratio of fructose-bisphosphatase to trypsin being 10 : 1. The incubation was interrupted by addition of either phenylmethylsulfonyl fluoride to a concentration of 1 mM or trichloroacetic acid to give a concentration of 10% (w/v). The former mixture was used for the kinetic studies or as samples in the polyacrylamide gel electrophoresis [7], and the latter for determination of the ^{32}P-labelling of fructose bisphosphatase as described [10]. The

digestion of fructose bisphosphatase with subtilisin was performed in the same way as that with trypsin, except for the temperature, which was 20°C, and for the buffer used, which was 50 mM sodium acetate buffer, pH 6.0/0.2 mM dithiothreitol.

All assays were repeated at least twice with similar or equal results.

Results

Dependence of enzyme activity on fructose bisphosphate. The maximal phosphorylation of fructose bisphosphatase to 1 mol/mol enzyme subunit decreased its $K_{0.5}$ for fructose bisphosphate from 21 μM for the unphosphorylated enzyme to 11 μM (Fig. 1). Treatment of the phosphoenzyme with a partially purified phosphoprotein phosphatase [9] reversed this activation, giving a $K_{0.5}$ of 20 μM. The dephosphorylation decreased the [^{32}P]phosphate content of fructose bisphosphatase to 0.02 mol/mol enzyme subunit. A similar increase in $K_{0.5}$ (to 22 μM) was seen when phosphorylated fructose bisphosphatase was incubated with small amounts of trypsin. The V value for all four enzyme forms was about 65 μmol fructose bisphosphate transformed/mg and min.

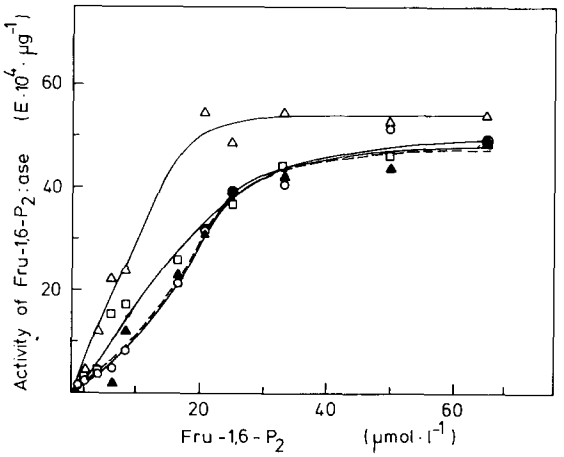

Fig. 1. The activity of unphosphorylated (○———○), phosphorylated (△———△), dephosphorylated (▲- - - - -▲) and trypsin-treated (□———□) fructose bisphosphatase (fru-1,6-P_2:ase) at different concentrations of fructose bisphosphate. The dephosphorylation was achieved by incubating 20 μl phosphorylated fructose bisphosphatase with 20 μl of the purified phosphoprotein phosphatase in the presence of 1.25 mM Mn^{2+} for 5 min. The total volume was 80 μl. Half of the material was used for activity measurements and the other half was extracted for determination of released phosphate by the method of Martin and Doty [11]. The weight ratio of phosphorylated fructose bisphosphatase to trypsin was 10 : 1 and the incubation time 5 min. About $6.5 \cdot 10^{-3}$ units of the different enzyme forms were used in each test.

In supplemental studies when the buffer used in the assay was changed to 10 mM Tris-HCl, pH 7.5, the $K_{0.5}$ values decreased. The $K_{0.5}$ for the phosphorylated fructose bisphosphatase was then 3 μM and that of the unphosphorylated enzyme 8 μM.

Dependence of enzyme activity on pH. The effect of pH was studied in phosphate buffer titrated with HCl to the respective pH values. It was ascertained that the pH did not change during the reaction time. As seen in Fig. 2, the pH optimum was the same, about 7.0, for unphosphorylated and phosphorylated fructose bisphosphatase, and was significantly shifted to the alkaline side only for the subtilisin-treated enzyme, for which it was 8.2. The pH optimum for the trypsin-treated enzyme was 7.5. This pH profile did not change when the four enzyme forms were assayed at 4.2 and 8.3 μM fructose bisphosphate, respectively (data not given).

Limited proteolysis of unphosphorylated and

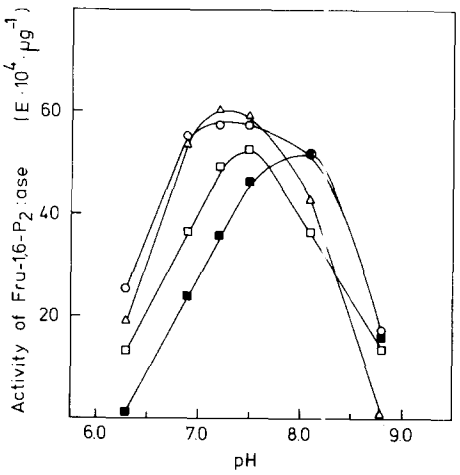

Fig. 2. The activity of unphosphorylated (○———○), phosphorylated (△———△), trypsin-treated (□———□) and subtilisin-treated (■———■) fructose bisphosphatase as a function of pH. The assay was performed as described in Materials and Methods. The partial subtilisin and trypsin digestions were achieved by incubation for 20 min at 20°C and 2 min at 30°C, respectively. The amount of the different forms of fructose bisphosphatase used in each test was $6.5 \cdot 10^{-3}$ units.

phosphorylated fructose bisphosphatase. Unphosphorylated and phosphorylated fructose bisphosphatase were treated with trypsin for different times as described in Materials and Methods. The remaining radioactivity of the trypsin-treated phosphorylated enzyme and the remaining phosphorylatable sites of the unphosphorylated fructose bisphosphatase were determined. The phosphorylation in the latter case was performed as described in Materials and Methods, and the amounts and volumes were converted to the phosphorylation of 10 μg fructose bisphosphatase. This phosphorylation was interrupted by the addition of 10% trichloroacetic acid and the pellet was washed as described [12], after which the radioactivity was measured.

No [^{32}P]phosphate was released, as estimated by the method of Martin and Doty [11].

The phosphorylated site of the phosphorylated enzyme was more easily split off than the corresponding phosphorylatable sites of the unphosphorylated enzyme (Fig. 3). To give the same rate of peptide cleavage, the amount of trypsin had to be increased 4-fold for the unphosphorylated fructose bisphosphatase as compared with the phosphorylated form. The maximal activity of the two enzyme forms was not changed after any of the incubation times shown in Fig. 3 (data not given). Phosphorylated fructose bisphosphatase was treated with trypsin or subtilisin as described in Materials and Methods and in the legend of Fig. 4. The reactions were interrupted with 1 vol. 8 M urea, pH 7/2% sodium dodecyl sulfate (w/v)/2% 2-mercaptoethanol (v/v) at the times indicated in Fig. 4. These mixtures were then heated for 60 min at 60°C before analysis by polyacrylamide gel electrophoresis.

As seen in Fig. 4, trypsin removed a [^{32}P]phosphopeptide that was so small that a decrease in molecular weight of fructose bisphosphatase could not be de-

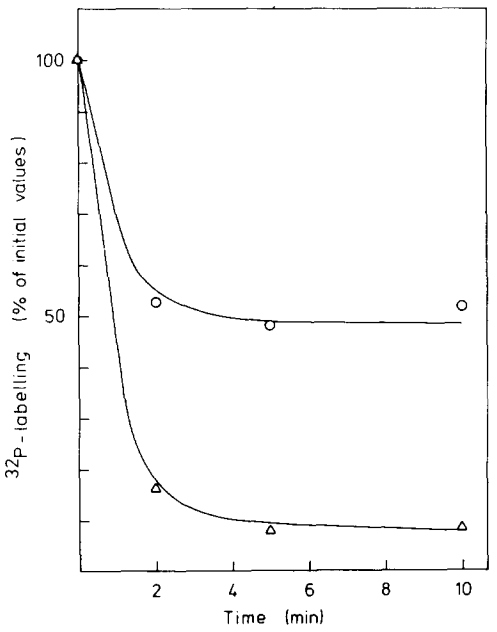

Fig. 3. The remaining phosphorylatable sites (o———o) and phosphorylated sites (Δ———Δ) after different times of digestion with trypsin. The weight ratio of fructose bisphosphatase to trypsin was 10 : 1 for both enzyme forms. The amount of enzyme used in each test was about 65 pmol.

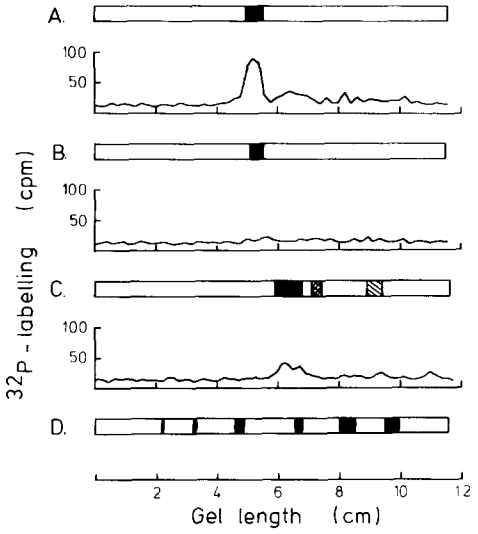

Fig. 4. Polyacrylamide gel electrophoresis in detergent under reducing conditions of [^{32}P]phosphorylated fructose bisphosphatase treated with trypsin or subtilisin. The gel was loaded with 7 μg fructose bisphosphatase in a total volume of 110 μl in each case. A. Phosphorylated fructose bisphosphatase. B. Phosphorylated fructose bisphosphatase treated with trypsin for 10 min at 30°C, the weight ratio of the enzyme to trypsin being 20 : 1. C. Phosphorylated fructose bisphosphatase treated with subtilisin for 5 min at 20°C; the weight ratio was 1 : 5. D. Reference proteins: phosphorylase *b* (M_r 94 000) bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soya bean trypsin inhibitor (20 000) and α-lactalbumin (14 400). The dark bands indicate the proteins in the gel when stained with Coomassie blue. ■, ■ and ■ correspond to the visually decreasing intensity of the blue colour.

tected with the method used. This method can discriminate between two protein molecules with a difference of about 2000 in molecular weight.

Subtilisin cleaved the molecule at the other end, as the radioactivity remained in the larger part with a molecular weight of 29 000. During these digestions there was no change in the activity of the phosphorylated fructose bisphosphatase as measured at pH 7.5 at a fructose bisphosphate concentration of 50 μM.

No [^{32}P]orthophosphate was released, as determined by the method of Martin and Doty [11].

Discussion

Extensive investigation has revealed that glucagon increases the flux through the gluconeogenetic pathways [13]. Rognstad and Katz [14] and Taunton et al. [15] have shown that activation of the fructose bisphosphatase step may be partially responsible for this activation. Claus et al. [1] demonstrated that glucagon increased the incorporation of phosphate into fructose bisphosphatase. Concomitant with this phosphorylation they noticed a slight increase in the activity of fructose bisphosphatase measured under optimal conditions [2]. Taunton et al. [15] observed an increase in the activity of the enzyme measured at a fructose bisphosphate concentration of 1.2 mM at pH 8.8, in rat livers perfused with glucagon, and a decrease when insulin was injected into the portal vein. We obtained an increase in V of the phosphorylated enzyme of about the same order of magnitude as those described [2,15], but consider the change in $K_{0.5}$ more significant. This difference persisted when Tris-HCl buffer was used, although the absolute values decreased. The choice of buffer has been shown to influence the activity of enzyme before, e.g., pyruvate kinase from pig liver [16]. Therefore, we chose a buffer as close to the in vivo conditions as possible.

The concentration of free fructose bisphosphate in the hepatocytes is not fully known but is probably in the low μM range during gluconeogenesis [17].

The fact that we were able to phosphorylate the purified fructose bisphosphatase to 1 mol/mol enzyme subunit and get a pH optimum of around 7.0 indicates that the inclusion of the proteolytic inhibitors EGTA [10] and phenylmethylsulfonyl fluoride early in the purification was successful.

It was also shown that the phosphorylation and trypsin treatment did not change the pH optimum for the enzyme very much, while the subtilisin treatment shifted it to the alkaline side. The trypsin digestion also resulted in a very small change in molecular weight, which might explain the difficulties sometimes encountered in attempts to phosphorylate the enzyme even when it has a native molecular weight of 140 000 and a neutral pH optimum. However, in rat liver cell sap there is a Ca^{2+}-activated protease which among its substrates has phosphorylated pyruvate kinase [10] and fructose bisphosphatase (data not given). The activity of this proteolytic enzyme might, in addition to the lysosomal (subtilisin-like) protease, be responsible for the proteolysis that occurs during purification [5,18].

That the phosphorylated fructose bisphosphatase was the most sensitive enzyme form to tryptic attack supports the hypothesis [12] that phosphorylation not only effects the activity of enzymes but might also be of importance in the regulation of their degradation.

In addition to the fact that fructose bisphosphatase is phosphorylated both in vivo and in vitro [2] and that its activity and phosphorylation are increased upon glucagon injection [1,14] the present finding that the phosphorylation in vitro is followed by an increase in the activity of the enzyme supports the view that fructose bisphosphatase is subjected to hormonal regulation through a phosphorylation-dephosphorylation mechanism.

Acknowledgement

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