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INACTIVATION OF PHOSPHOFRUCTOKINASE 2 BY CYCLIC AMP - DEPENDENT PROTEIN KINASE

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SUMMARY : Treatment with the catalytic subunit of cyclic AMP-dependent protein kinase induced the following modifications in the kinetic properties of purified phosphofructokinase 2. The affinity for Fru-6-P, the V_{max} and the stimulatory effect of P_i were decreased; the inhibitory actions of *P-enol*-pyruvate and citrate were increased; the pH activity curve, measured in the presence of 5 mM Fru-6-P and 5 mM P_i was modified in the respect that the peak of activity normally measured at pH 6.6 was abolished whereas no effect of the treatment was observed at pH 8. Similar changes in the properties of phosphofructokinase 2 were also observed in a crude preparation obtained from hepatocytes incubated with glucagon.

Four groups of investigators have recently presented direct (1) or indirect (2-4) evidence that Fru-2,6-P_2 is formed in the liver by the transfer of the γ-phosphoryl group of ATP on to the hydroxyl group present on carbon 2 of Fru-6-P. The enzyme that catalyzes the reaction has been called PFK 2 in order to distinguish it from the classical PFK which forms Fru-1,6-P_2 and is now called PFK 1. The activity of PFK 2 is increased by AMP and by P_i and decreased by PEP and citrate in the physiological range of concentration (1). The activity is also decreased in hepatocytes incubated in the presence of glucagon (1,3,5). The fact that this change persists upon gel filtration (1, 3) suggests that it could be the result of a phosphorylation of PFK 2, or of another protein able to alter the activity of PFK 2, by cyclic AMP-dependent protein kinase. The present work was undertaken in order to investigate this problem.

ABBREVIATIONS : PFK, Phosphofructokinase; PEP, phospho*enol*pyruvate; bis-tris propane, 1,3-bis[tris(hydroxymethyl)-methylamino]-propane; DTT, dithiothreitol; cAMP, cyclic AMP; C subunit, catalytic subunit of cyclic AMP-dependent protein kinase

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MATERIALS AND METHODS

Hepatocytes were isolated from fed rats and treated as in (6).The pellets of hepatocytes were homogenized in 3 vol. of ice-cold 50 mM KCl, 50 mM KF, 25 mM Tris, pH 7.5 with a glass homogenizer. The homogenates were centrifuged for 30 min at 100,000 x g; before assay of PFK 2 activity the supernatants were passed through 20 vol. of Sephadex G-25 (Pharmacia) equilibrated with homogenization buffer. PFK 2 was prepared as in (1) and further purified on Blue Sepharose CL 6B (Pharmacia). Fractions containing PFK 2 obtained by DEAE cellulose chromatography (1) were diluted to give a medium containing 25 mM Tris-HCl, 100 mM KCl and 10 % glycerol, pH 7.5. The preparation was applied to a column of Blue Sepharose equilibrated with the same buffer. Protein was eluted with a linear gradient of KCl (0.1 M - 1.8 M) in the same buffer. PFK 2 came out at ca. 0.7 M KCl; it was concentrated by ultrafiltration through a Diaflo PM 30 Membrane (Amicon) and was stored frozen. The specific activity of the purified enzyme measured at 30°C was approximately 10 nmoles/min/mg protein corresponding to a 150-fold purification as compared to the high-speed supernatant. At each step of this purification procedure PFK 2 co-purified with a fructose 2,6-bisphosphatase activity, which, under certain assay conditions, had a specific activity comparable to that of PFK 2.

The inactivation of PFK 2 by protein kinase was carried out as follows; the purified PFK 2 (equivalent to 0.6 mg/ml protein) was incubated for 30 min at 30°C with 1 mM ATP-Mg, 1 mM DTT, 50 mM bis-tris propane (Sigma) pH 7.1 and 2 µg/ml of pure C subunit of cAMP-dependent protein kinase type II from beef heart (prepared as described in (7) and kindly provided by Dr. F. Hofmann, Heidelberg, Germany). It was checked that no further inactivation was obtained with a 5-fold higher concentration of the C subunit. In the experiment shown in Table I, similar incubations were carried out with crude beef heart protein kinase (Sigma). Control incubations were run without C subunit or crude protein kinase.

In the experiments with crude extracts the PFK 2 assays were performed as described in (1), except that 20 mM KF was present in the incubation mixture, and Fru-2,6-P_2 was assayed by its property to stimulate PFK 1. In the experiments with the purified enzyme, Fru-2,6-P_2 was assayed as acid-labile Fru-6-P after destruction of the hexose 6-monophosphates in hot alkali. Purified PFK 2 was incubated for 20 min at 30°C in a vol. of 0.25 ml containing 5 mM ATP, 100 mM KCl, 7 mM $MgCl_2$, 50 mM bis-tris propane at the specified pH and concentrations of Fru-6-P and P_i. The reaction was stopped by the addition of 0.5 ml of 0.2 M NaOH. A 0.5 ml portion of this alkaline mixture was incubated at 100°C for 15 min; it was then cooled to 25°C and acidified with 0.1 ml of M HCl. After 10 min the samples were neutralized with 0.2 ml of M Tris pH 8 and Fru-6-P was assayed with an Aminco Chance spectrophotometer (8). In this procedure, less than 0.01 % of the initial hexose 6-monophosphates and more than 95 % of Fru-2,6-P_2 were assayed as Fru-6-P. Usually amounts ranging between 0.2 and 10 nmoles of Fru-2,6-P_2 were measured. In some experiments PFK 2 was assayed by the production of ADP. The incubation mixture contained 50 mM bis-tris propane, 100 mM KCl, 0.25 mM PEP, 1 mM ATP, 3 mM $MgCl_2$, 0.15 mM NADH, 20 µg/ml of pyruvate kinase and 25 µg/ml of lactate dehydrogenase (desalted on Sephadex G-25) and Fru-6-P as indicated.

Fru-2,6-P_2 was synthesized as described in (9). Other biochemicals were from Boehringer (Mannheim, Germany).

RESULTS

Effect of treatment of hepatocytes with glucagon on the activity of PFK 2 in a high-speed supernatant. The incubation of hepatocytes in the presence of glucagon induced several changes in the kinetic properties of PFK 2 which

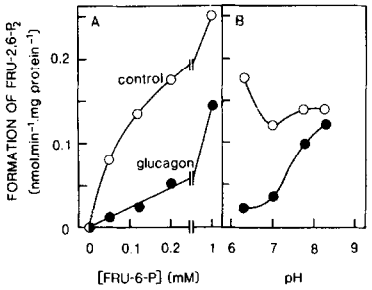

Fig. 1. Kinetic properties of PFK 2 in the high-speed supernatant obtained from hepatocytes incubated with or without 10^{-6}M glucagon. (A) Effect of the concentration of Fru-6-P on the activity of PFK 2 measured at pH 7.1 and at 5 mM P_i. (B) Effect of pH on the activity measured at 0.5 mM Fru-6-P and at 20 mM P_i. All assays were performed at 37°C.

could be detected in the high-speed supernatant. As exemplified in Fig. 1A, the apparent K_m for Fru-6-P, measured at pH 7.1 and in the presence of 5 mM P_i, was increased several fold and the V_{max} was decreased 1.5-3-fold by glucagon. The pH activity curve, measured in the presence of 20 mM P_i, indicated that the loss of activity caused by glucagon was much greater in the acid than in the alkaline range of pH (Fig. 1B).

Inactivation of purified PFK 2 by cyclic AMP-dependent protein kinase. The activity of the purified PFK 2, measured at pH 6.6 and at a saturating concentration of Fru-6-P, was decreased several fold by the action of either protein kinase and cyclic AMP, or of the C subunit of protein kinase in the absence of cyclic AMP. The activity was not significantly affected by either protein kinase or cyclic AMP alone. None of these treatments decreased the activity of PFK 2 measured at pH 8 (Table 1).

Kinetics of purified PFK 2, treated or untreated with the C subunit of protein kinase. The incubation of PFK 2 with the C subunit of cyclic AMP-dependent protein kinase in the presence of ATP changed several of the kinetic properties of the enzyme. When measured at pH 7.1, there was a 4-fold increase in the K_m for Fru-6-P and a decrease in the V_{max} (Fig. 2A). These two changes were much less apparent when the activity was assayed at pH 8 (Fig. 2B). Little or no change in the K_m for ATP was observed (results not

Table 1. Inactivation of PFK 2 by cyclic AMP-dependent protein kinase

Additions	Formation of Fru-2,6-P_2 (nmol.min^{-1}.mg $protein^{-1}$)	
	pH 6.6	pH 8.0
None	13.1	7.7
+ Protein kinase (0.1 mg/ml)	14.0	8.1
+ cAMP (0.25 mM)	12.0	8.0
+ Protein kinase + cAMP	4.5	8.5
+ Catalytic subunit (2 μg/ml)	2.8	8.1

PFK 2 was assayed in the presence of 5 mM Fru-6-P and 5 mM P_i

shown). The effect of pH and of P_i on the activity of the two forms of PFK 2 measured at 5 mM Fru-6-P is illustrated in Fig. 3. In the case of the untreated enzyme, there was a peak of activity at pH 6.6, which was dependent on the presence of P_i. This peak was abolished by treatment of the enzyme with the C subunit although the treated preparation remained, to some extent, sensitive to the stimulatory effect of P_i. The treatment had little or no effect on the activity of PFK 2 measured at pH 8 or above.

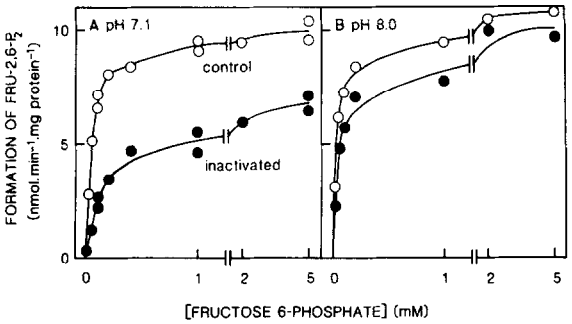

Fig. 2. Effect of inactivation of purified PFK 2 by the C subunit of protein kinase on its affinity for Fru-6-P. PFK 2 activity was measured in the presence of 5 mM P_i at pH 7.1 (A) and at pH 8 (B). The calculated K_m values for the control and the inactivated enzyme were, respectively 65 and 270 μM at pH 7.1 and 65 and 90 μM at pH 8.

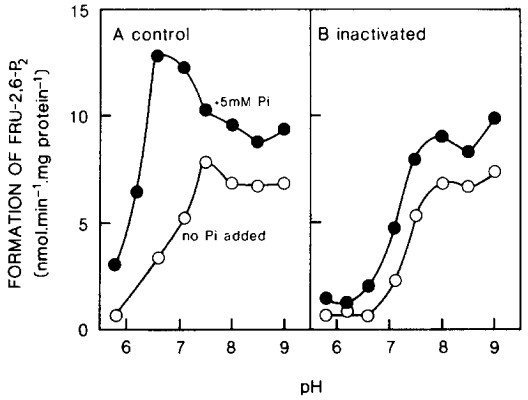

Fig. 3. Effect of pH and of P_i on the activity of control and the inactivated PFK 2. Purified PFK 2 was inactivated by treatment with the C subunit of protein kinase. Its activity was measured at 5 mm Fru-6-P.

Another change induced by the C subunit treatment was an increased sensitivity of the enzyme to inhibition by PEP (Fig. 4) and by citrate (results not shown). It can be seen in Fig. 4 that in the presence of 5 mM P_i and at 0.2 mM Fru-6-P, 0.5 mM PEP caused a 40 % inhibition of the control PFK 2 and a 95 % inhibition of the treated enzyme.

The inactivation of PFK 2 was also demonstrable when the enzyme was assayed, either at pH 6.6 or at pH 7.1, by the production of ADP (results not shown).

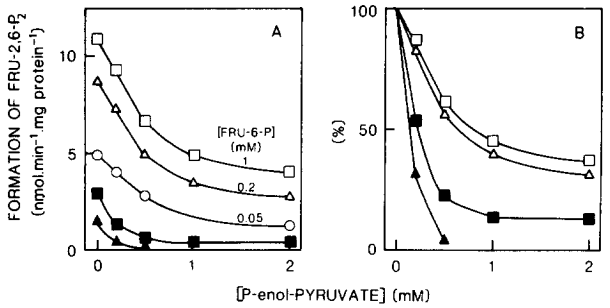

Fig. 4. Effect of PEP on the activity of PFK 2 treated (closed symbols) or untreated (open symbols) with the C subunit of protein kinase. The assays were performed at pH 7.1 and in the presence of 5 mM Pi and 1 (□,■), 0.2 (△,▲) and 0.05 (○) mM Fru-6-P. In (B), the activity is expressed as % of that measured in the absence of PEP.

DISCUSSION

The effect of protein kinase on PFK 2. Since the purified PFK 2 was presumably far from homogeneity, no attempt has been made to demonstrate the phosphorylation of the enzyme by the C subunit. Because the effects of cyclic AMP-dependent protein kinases are related to their ability to phosphorylate proteins, we can safely assume that the changes that we have observed in the kinetic properties of PFK 2 are caused by the transfer of the γ-phosphoryl group of ATP on to one or several amino acid residues of a protein. Considering the degree of purification, this protein is most likely to be PFK 2.

The inactivation of PFK 2 by protein kinase is the result of a series of kinetic modifications which can be summarized by saying that the phosphoenzyme is less sensitive to its positive effectors, P_i and Fru-6-P and more sensitive to its inhibitors, PEP and citrate. The decrease of the P_i effect, which was greatest in the acid range of pH, results in the disappearance of the pH 6.6 activity peak.

Since the purified preparation of PFK 2 displayed fructose-2,6-bisphosphatase activity, an apparent loss of the kinase activity, as measured by the amount of Fru-2,6-P_2 which has accumulated, could result solely from an activation of the phosphatase. This possibility has been discarded by the demonstration of a similar loss of the kinase activity, measured by the formation of ADP.

Physiological implications. Incubation of hepatocytes with glucagon induced changes in the properties of PFK 2, measured in a crude preparation, similar to those observed with the purified system. This similarity allows us to conclude that the effects discussed above have a physiological significance.

In isolated hepatocytes, the addition of glucagon causes, after a period of latency of about 1 min, the almost complete disappearance of Fru-2,6-P_2 within about 5 min (6). The problem under discussion is to know if this effect can be explained by the changes in the kinetic properties of PFK 2 which are induced by treatment of the enzyme with the C subunit of protein kinase.

At pH 7.1 and at 50 μM Fru-6-P, the increase in K_m for Fru-6-P would cause a loss of approximately 70 % of the enzymic activity. However, in cells obtained from fed rats, the enhancement of glycogenolysis by glucagon causes a 3 to 4-fold increase in the concentration of Fru-6-P which would compensate for the effect of the K_m modification. Another effect of protein kinase on PFK 2, also observed at neutral pH, is to increase its sensitivity to the inhibitory action of PEP and citrate. Furthermore, glucagon increases by 2 to 3-fold the concentration of PEP in the liver cell (10) so that in this case, the two modifications would act synergistically to decrease the activity of PFK 2. This mechanism would also account for the short period of latency observed with the glucagon effect.

In order to explain the disappearance of Fru-2,6-P_2 by an inhibition of PFK 2, one has also to assume that the basal activity of the degradative system, recently recognized to be a fructose 2,6-bisphosphatase (11), would be sufficient to cause the complete disappearance of Fru-2,6-P_2 within a few minutes, when its biosynthesis has been arrested.

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