FRUCTOSE 1,6-DIPHOSPHATE, A REACTIVATOR OF Cu^{++}-INHIBITED PYRUVATE KINASE FROM LIVER[1]

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The key glycolytic enzymes, glucokinase (E.C. 2.7.1.2), phosphofructokinase (PFK) (E.C. 2.7.1.11) and pyruvate kinase (PK) (E.C. 2.7.1.40), which govern the conversion of glucose to lactate in liver, are regulated by sensitive mechanisms (Salas et al., 1963; Weber et al., 1965; Weber and Singhal, 1965). They are rate limiting and catalyze the strategic unidirectional reactions of glycolysis (Krebs, 1954, 1963). Recently Tanaka et al. (1965) reported the existence of at least two types of PK in rat liver, tentatively named type L and type M. They also showed that the former is under hormonal and dietary control, like the other key glycolytic enzymes.

In our search for regulatory mechanisms that could selectively and differentially control the activity of both types of PK, we found that the L type is strongly inhibited by Cu^{++}, and that this inhibition is reverted by fructose 1,6-diphosphate (FDP), whereas the M type enzyme is only slightly or not at all affected by this cation.

MATERIALS AND METHODS

Swiss Albino mice, maintained on a laboratory diet and water ad libitum

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were used throughout. The animals were killed by decapitation. The livers were rapidly excised, weighed and homogenized with two volumes of cold 0.1 M Tris-Cl-1 mM EDTA-1 mM mercaptoethanol, pH 7.5, in a glass homogenizer. The homogenate was centrifuged at 100,000 x g for 60 minutes. The supernatant fluid was purified by ammonium sulfate fractionation between 20-45% (type L) and 55-70% (type M) saturation. The precipitates were dissolved in a small volume of 0.1 M Tris-Cl-1 mM mercaptoethanol, pH 7.5.

PK was assayed by measuring the pyruvate formed from phosphoenol pyruvate (PEP) in the presence of ADP, according to Leloir and Goldemberg (1960). Incubations were carried out at 30° for 15 minutes.

RESULTS AND DISCUSSION

The effect of Cu^{++} concentration on the activity of both types of PK is shown in Fig. 1.

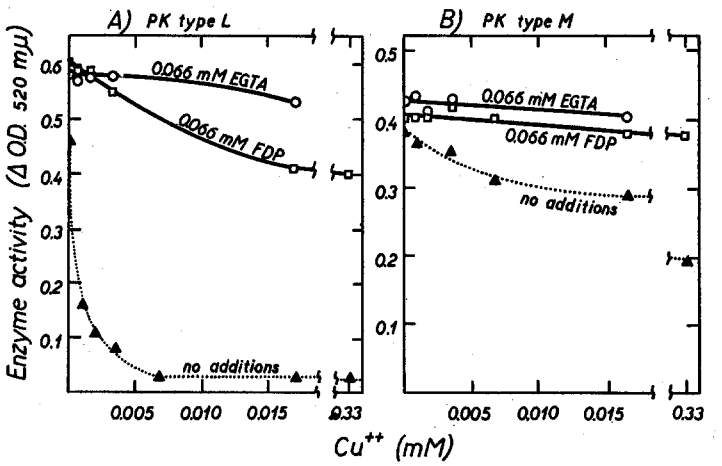

Fig. 1. Effect of copper concentration of PK activity (types L and M). The standard incubation mixture contained (in μmoles): 0.25 of PEP, 0.2 of ADP, 1.5 of $MgSO_4$, 10 of KCl, 10 of Tris-Cl, pH 7.5, varying $CuSO_4$ concentrations and freshly prepared enzyme in a total volume of 0.15 ml. Other additions as indicated on the figure. Pyruvate formation was measured as indicated under "Methods". A control without ADP was run simultaneously.

Type L is strongly inhibited by this cation; nearly full effect can be achieved at a Cu^{++} concentration of 0.006 mM. This inhibition is completely reverted by 0.066 mM FDP or ethyleneglycol bis(aminoethylether) tetraacetic

acid (EGTA). In the absence of inhibitor, FDP and EGTA are also capable of enhancing the activity of the enzyme (see Fig. 1A and also Fig. 3).[1]

On the other hand, these effects were nearly absent with PK type M (Fig. 1B). Several other metabolites were tested as possible antagonists of Cu^{++} inhibition on type L, but only FDP was active. The following compounds were found to be ineffective: P_i (2.0-20 mM), glycerophosphate (0.02-0.2 mM), 3-P-glyceric acid (0.033-0.2 mM), cyclic 3',5'-AMP (0.066-0.33 mM), citrate (0.33-3.3 mM), lactate (0.166-0.66 mM) and AMP (0.166-0.99 mM). Some reactivation was obtained with fructose-6-P (0.066-0.260 mM) but its nature is under study, since it might be mediated through newly formed FDP.

As indicated in Fig. 2, FDP produces more than half its full effect at a concentration of 0.005 mM. This level of metabolite also counteracts the ATP inhibition of PFK of brain and liver (Passoneau and Lowry, 1964).

The inhibition produced by 0.0066 mM Cu^{++} can be reverted by EGTA (Fig. 2) as well as by other chelating agents like EDTA and 8-hydroxyquinoline. However FDP is by far the most effective, since more than 60% reactivation can be obtained at a FDP concentration 5 times lower than that of Cu^{++}. Furthermore, while 0.013 mM FDP reverts the inhibition produced by 0.133 mM Cu^{++}, EGTA does not overcome it at all (Fig. 2). These results seem to indicate that FDP does not act as a simple chelating agent, but rather has a direct effect on the enzyme.

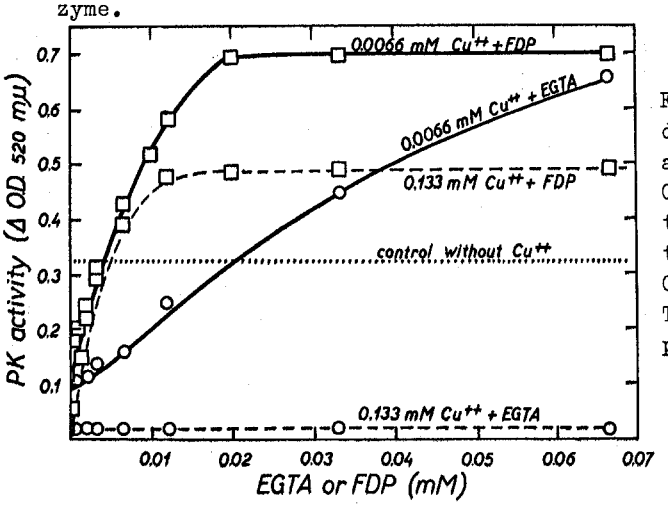

Fig. 2. Effect of FDP or EGTA at different concentrations on the PK activity (type L) inhibited by Cu^{++}. Assay conditions were identical to those in Fig. 1, except that the cation levels were: 0.0066 mM (—) and 0.133 mM (- -). The activity was measured in the presence of EGTA (o) or FDP (□).

[1] It is not known if this effect is a direct action of these compounds on the enzyme or if it is due to traces of Cu^{++} which might contaminate some of the reagents. At this respect it may be of interest to mention that we found Cu^{++} as an impurity in several samples of commercial ADP.

A series of other metal ions were tested on the activity of PK type L. At a concentration of 0.066 mM Zn^{++} gave an inhibition of more than 70%, while Ca^{++}, Ba^{++}, Mn^{++}, Fe^{++}, Cd^{++}, Pb^{++} and Co^{++} showed no appreciable effect. At a concentration 10 times higher the last four cations also inhibited the enzyme. None of these inhibitions were reverted by FDP.

Another interesting feature is shown in Fig. 3, which presents the enzyme activity as a function of time of preincubation at 37°. The fresh enzymic preparation shows a variable activation (ranging from 20 to 100%) by FDP or EGTA and is strongly inhibited by Cu^{++}. During the preincubation the enzyme declines in sensitivity towards Cu^{++} while the activation by FDP in the absence of inhibitor is markedly increased, and the initial enhancing effect of EGTA is lost after 8 hours of treatment.

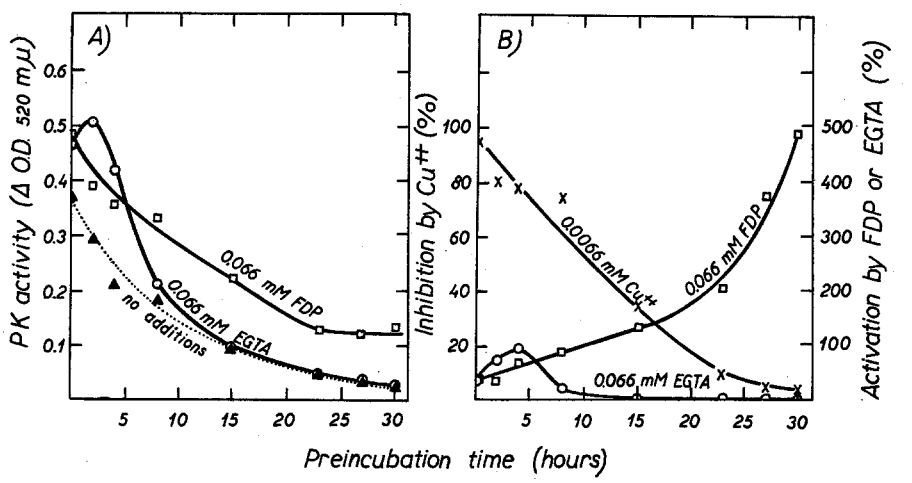

Fig. 3. Time course of changes in PK activity (type L) during preincubation at 37°. Aliquots of the extract were conveniently diluted and tested as indicated under "Methods" in the presence of 0.0066 mM Cu^{++} (x), 0.066 mM FDP (□) or 0.066 mM EGTA (o). Dotted line: control with no additions.

It must be mentioned that although the qualitative pattern was reproducible from one preparation to another, there was a certain variability in the stability of the enzyme. For example, preincubation times varying from 30 to 100 hours were necessary to obtain the same degree of inactivation on different batches.

As can also be seen in Fig. 3A, the curves for the decrease in activity measured in the absence and in the presence of FDP are approximately parallel. Therefore, it cannot be discarded that two forms of the enzyme, one FDP dependent and one independent, are present in the fresh preparation and that only the latter form is destroyed during incubation at 37°. A more detailed study of this problem is in progress.

The physiological significance of the findings reported in this communication is obscure, partly due to the fact that the concentration of free Cu^{++} in the hepatic cell is unknown. However the levels of total copper in the liver of several adult mammals are between 0.06 and 0.12 μmole/g wet weight of tissue (Lorenzen and Smith, 1947; Parr and Taylor, 1964). These figures are well above the range that produce the maximum inhibition of the L form of the PK "in vitro". Therefore, one may tentatively speculate with the possibility that this form of the enzyme is normally inhibited in the liver, so that its activity "in vivo" could be controlled by small variations in the FDP levels.

Another possibility that cannot be ruled out is that Cu^{++} in our "in vitro" system takes the place of another metabolite which could be the true inhibitor of the enzyme.

In any case, FDP seems to have a selective effect on the L form of the hepatic PK. In this respect, it is important to point out that Hess et al. (1966) found a striking activation of the yeast enzyme by this metabolite.

It is now assumed that the reciprocal control of fructose 1,6-diphosphatase (E.C. 3.1.3.11) and PFK by FDP could dictate the balance between glycolysis and gluconeogenesis in liver. Furthermore, if this key metabolite increases the activity of PK "in vivo", it would reinforce the complex control system deciding the direction of carbohydrate metabolism in this organ.

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