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THE REACTIVE CYSTEINE RESIDUE OF PIG KIDNEY FRUCTOSE 1,6-BISPHOSPHATASE IS RELATED TO A FRUCTOSE 2,6-BISPHOSPHATE ALLOSTERIC SITE

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Modification of a highly reactive cysteine residue of pig kidney fructose 1,6-bisphosphatase with N-ethylmaleimide results in the loss of activation of the enzyme by monovalent cations. Low concentrations of fructose 2,6-bisphosphate or high (inhibitory) levels of fructose 1,6-bisphosphate protect the enzyme against the loss of monovalent cation activation, while non-inhibitory concentrations of the substrate gave partial protection. The allosteric inhibitor AMP markedly increases the reactivity of the cysteine residue. The results indicate that fructose 2,6-bisphosphate can protect the enzyme against the loss of potassium activation by binding to an allosteric site. High levels of fructose 1,6-bisphosphate probably inhibit the enzyme by binding to this allosteric site.

Monovalent cation activation and inhibition by high substrate concentrations are common regulatory properties of vertebrate fructose 1,6-bisphosphatases (EC 3.1.3.11, FBPase) (1,2). We have shown (3,4) that carbamoylation of a highly reactive cysteine residue of pig kidney FBPase causes the simultaneous loss of both potassium activation and substrate inhibition, indicating that both properties are closely related. Since potassium ions seem to inhibit the enzyme at low substrate concentrations (≤ 25 μM) (3,5,6) but to activate it at higher fructose 1,6-bisphosphate (Frul,6BP) concentrations (1,3,6,7), a model in which K^+ acts by decreasing the affinity of FBPase by Frul,6BP at both, the catalytic site and also an allosteric site for the substrate, was proposed (3). The posibility arising now that this second site correspond to an allosteric site for fructose 2,6-bisphosphate (Fru2,6BP) prompted us to study the effect of Fru2,6BP on the reactivity of thiol groups related with potassium activation.

The mechanism of the inhibition of FBPase by Fru2,6BP is controversial. Some authors (8-11) have proposed the existence of an allosteric site for Fru2,6BP, but others (12-17) favored an interaction of Fru2,6BP only at the active site of the enzyme. In this communication, data are presented which indicate that the highly reactive cysteine residue of pig kidney FBPase is in, or in close relation, with an allosteric site for Fru2,6BP. A preliminary report has been presented (18).

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EXPERIMENTAL PROCEDURE

Pig kidney FBPase purified to homogeneity was used (19,20), and its concentration was determined by absorbancy at 280 nm (21). A molecular weigth of 146.000 was considered (22). Modification of FBPase (12.5 μM) with N-ethyl-maleimide (NEM, 200 μM) was carried out at 4° in 50 mM triethanolamine-HCl buffer (pH 7.8), 0.1 mM EDTA with the additions indicated in the text. Samples were withdrawn at specific times and the reaction was stopped by dilution in cold 20 mM Tris-HCl buffer (pH 7.5), 0.1 mM EDTA, 0.5 mM dithiothreitol. Enzyme activity was measured at 30° either by the release of inorganic phosphate from the substrate or spectrophotometrically by following the rate of $NADPH^+$ production at 340 nm in the assay system coupled to the formation of fructose 6-phosphate (20). Unless otherwise stated, the assay mixture of 1 ml contained 50 mM Tris-HCl buffer (pH 7.5), 0.1 mM EDTA, 5 mM $MgSO_4$, 0.5 mM Fru1,6BP, and 0.1 ml of the enzyme solution (1-2 μg). The coupled assay medium was identical except for the addition of phosphoglucose isomerase (2.3 U/ml), glucose 6-phosphate dehydrogenase (0.6 U/ml) and $NADP^+$ (0.3 mM). A unit of enzyme activity is defined as the amount of enzyme which catalyzes the formation of 1 μmol of product per min.

RESULTS AND DISCUSSION

Effect of K^+ and Fru2,6BP on the substrate saturation function of pig kidney FBPase. Potassium ions seem to have a dual role on Fru1,6BP saturation curves. It appear to activate the enzyme at high substrate concentrations (>20 μM), by interacting with an allosteric site (3,6), and to inhibit at lower Fru1,6BP concentrations (Fig. 1), by affecting the catalytic site (3,6). On the other hand, pig kidney FBPase is inhibited by 2 μM Fru2,6BP in the presence or absence of K^+ at non-inhibitory substrate concentrations (Fig. 1). Moreover, Fru2,6BP produces a shift of the saturation curves to higher Fru1,6BP

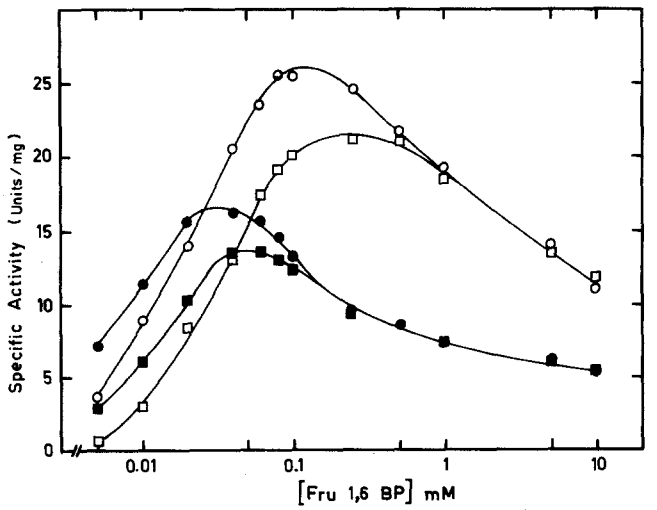

Figure 1. **Effects of K^+ and Fru2,6BP on the saturation function of pig kidney FBPase with Fru1,6BP.** Enzyme activity was assayed spectrophotometrically as described under EXPERIMENTAL PROCEDURE, and the reactions were initiated by addition of enzyme (0.1-0.2 μg). Assays in the absence (●) or presence of 150 mM K^+ (○), 2 μM Fru2,6BP (■) or 150 mM K^+ plus 2 μM Fru2,6BP (□).

concentrations. However, the inhibition by K^+ at low substrate concentrations is maintained (Fig. 1) even at higher (up to 40 μM) Fru2,6BP levels, suggesting that K^+ and Fru2,6BP inhibitions occur by the interaction of both effectors at different sites of the enzyme. Furthermore, Fru1,6BP may compete with Fru2,6BP only at inhibitory substrate concentrations (Fig. 1). These two facts could be interpreted as Fru2,6BP interacting with the postulated (2,3, 23) allosteric site responsible for the inhibition by excess of substrate, rather than with the allosteric site.

Modification of native FBPase with NEM. Carbamoylation of the enzyme diminished the ratio of activities measured in the presence and absence of 150 mM K^+ (R value) from its initial value of 2.4 to 1.1, with no decrease in activity in the absence of the monovalent cation activator (3,4). This alteration was related to the modification of one reactive cysteine residue on the enzyme subunit (4). We selected NEM to further characterize this reactive cysteine, because its high specificity for thiol groups. The results of the treatment of native enzyme with NEM are shown in Fig. 2 and Table I. The addition of NEM to FBPase in a 4:1 molar ratio of reagent to enzyme subunit causes a total loss of potassium activation in about 30 min (Fig. 2). In the NEM-treated enzyme potassium ions became inhibitor since the ratio R decreases to values lower than 1, and always minimal values of about 0.4 were obtained (Fig. 2 and Table I). Under this experimental condition, 1.2 mol [^{14}C] NEM/mol monomer was incorporated (to be published). Higher concentrations of NEM (up to 0.6 mM) affected the rate but not the final extent of the modification. These results strongly support the postulated inhibitory effect of monovalent cations at the catalytic site of FBPases (3,6).

Table I shows that the modification also causes a fall (36%) in enzyme activity. However, since the potassium activation is expressed as a ratio between the activities measured in the presence and absence of K^+, any loss of enzyme activity is corrected. As pointed out by Chatterjee *et al.* (24) this fall was probably due to the increase of the K_a value for Mg^{2+}. In our conditions, the K_a changed from 0.25 mM for the native enzyme to 2.85 mM in the NEM-modified enzyme.

Fru2,6BP has been described as a potent inhibitor of FBPases (8,12,13) and when included in a 1:1 molar ratio of hexose to enzyme subunit showed a remarkable protective effect against the loss of K^+ activation (Fig. 2). In contrast, the inclusion of Fru1,6BP in the same amount gave only a partial protection of K^+ activation. This results suggest that the reactive cysteine residue is not locate in the catalytic site and due to the high affinity of the enzyme by its substrate (K_m<5 μM) the partial protection probably reflect some conformational change induced by the substrate at the catalytic site. In addition, Fru1,6BP at a concentration which is about 100-fold the amount of

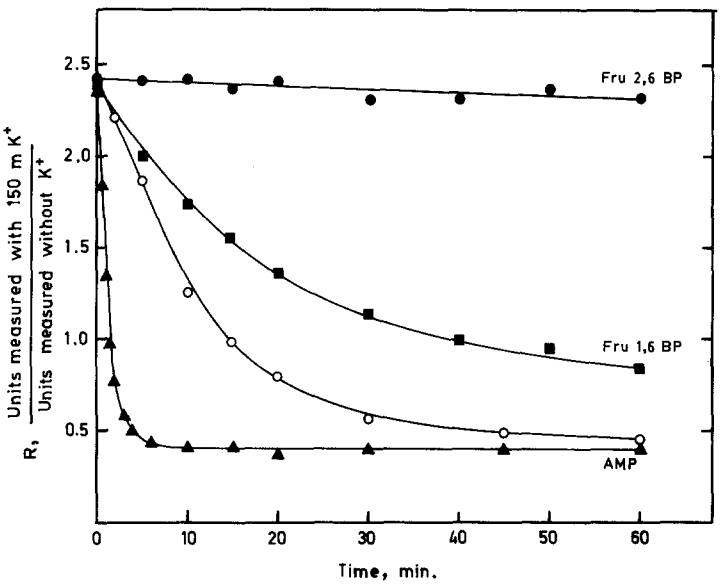

Figure 2. **Modification of potassium activation of FBPase by incubation with NEM.** Aliquots of enzyme (50 μM subunit) were incubated with NEM as described in EXPERIMENTAL PROCEDURE, in the absence (O) or presence of different additions: (●) 50 μM Fru2,6BP, (■) 50 μM Fru1,6BP, and (▲) 200 μM AMP. Enzyme activity was measured at 30° by the rate of formation of inorganic phosphate. R represent the ratio of activities measured in the presence and absence of 150 mM K^+ (75 mM K_2SO_4).

TABLE I

Effects of Fru2,6BP, Fru1,6BP and AMP on the loss of enzyme activity and potassium activation caused by modification of pig kidney FBPase with NEM[a]

	Enzyme activity[b]	Potassium activation[c]
	%	R
no modification	96.8	2.45
no addition	65.3	0.42
100 μM Fru2,6BP	95.6	2.35
100 μM Fru1,6BP	66.0	0.94
200 μM Fru1,6BP	62.0	1.61
5000 μM Fru1,6BP	63.4	2.08
200 μM AMP	63.5	0.39
200 μM AMP + 200 μM Fru1,6BP	65.0	1.63
200 μM AMP + 100 μM Fru2,6BP	92.0	2.35

[a] The enzyme was treated with NEM for 60 min as described in EXPERIMENTAL PROCEDURE, in the presence of the additions indicated. Aliquots were assayed for activity in the presence and absence of 150 mM K^+ by the formation of inorganic phosphate.
[b] Enzyme activity assayed in the absence of K^+. A relative value of 100% was given to the activity at zero time.
[c] R is defined as in the legend of figure 2.

enzyme subunit (5 mM, inhibitory concentration) gave a significant protection against the loss of K^+ activation (Table I). On the other hand, Fru1,6BP is a better protector than Fru2,6BP when the active site Lys-274 is modified by pyridoxal-phosphate (13). Thus, the cysteine residue essential for monovalent cation activation is in, or in close relation, with a high affinity binding site for Fru2,6BP, which is not the catalytic site. We believe that this site really corresponds to an allosteric site for Fru2,6BP. Furthermore, the lack of inhibition by Fru2,6BP at high levels of Fru1,6BP, along with the fact that high concentrations of the substrate seem to mimick the protective effect of low concentrations of Fru2,6BP on the loss of K^+ activation, indicate that the allosteric site for Fru2,6BP can also bind Fru1,6BP with lower affinity. This binding site could then be reponsible for the classical high substrate inhibition of vertebrate FBPases.

It has been reported (10) that inactivation of rat liver FBPase with NEM can be protected by Fru2,6BP. However, this partial inactivation (about 50%) should be due mainly to a loss of monovalent cation activation rather than to a loss in enzyme activity. In contrast to the results of Meek and Nimmo (25), the allosteric inhibitor AMP appears to interact with the enzyme in the absence of ligands (e.g. Fru1,6BP or Fru2,6BP), since AMP alone markedly increases the reactivity of the cysteine residue related with K^+ activation (Fig. 2). With 200 μM AMP nearly complete reaction with NEM was observed in less than 5 min. The K_i of the enzyme for AMP was not altered by the modification, and the presence of AMP does not affect the protective effect exerted by either Fru1,6BP or Fru2,6BP (Table I), indicating that the enzyme binding sites for AMP or both sugar-bisphosphates are different. A similar conclusion has been derived by others (8,10,13). We believe that the increase in thiol reactivity elicited by AMP, when it is bound to its allosteric site, is due to an AMP-induced conformational change at the Fru2,6BP allosteric site. This agrees with the known synergistic effect of AMP on the binding of Fru2,6BP on FBPases (16,17).

Recently, Chatterjee *et al.* (24) also characterized a highly reactive thiol group on pig kidney FBPase, whose reactivity towards thiol-directed reagents increased in the presence of AMP. They did not relate this reactive group (Cys-128) with the monovalent cation activation property. Although the identification of the reactive Cys-128 was based upon modification with iodoacetamide, it would appear that the same residue reacts specifically with NEM, since the FBPase modified with iodoacetamide (24) or NEM exhibited higher V_{max} than the native enzyme when Mn^{2+} was used as the activating divalent cation (data not shown). Likewise, in the presence of Mg^{2+}, the activity of the NEM-treated enzyme decreased as a result of an increased K_a for Mg^{2+}, which also occurs in the iodoacetamide-modified pig kidney FBPase (24).

The question if the reactive cysteine is adjacent or is part of the allosteric site for Fru2,6BP cannot be solved at the present time, but it is interesting that the region of the enzyme molecule which contains the reactive Cys-128 is highly conserved in different FBPases (24). Thus, it is conceivably that this region corresponds to the binding site for Fru2,6BP.

Therefore, this report should be added to the growing number of observations appeared recently (8-11) that support the existence of the Fru2,6BP allosteric site on FBPases. Yet, there are disagreements in some points, since François et al. (8) concluded that Fru2,6BP interacts only with the allosteric site on rat liver FBPase, while Meek and Nimmo (10,25) suggest that Fru2,6BP can interact at both, the catalytic and an allosteric site for Fru2,6BP. Although our results favor the interaction of Fru2,6BP with a specific allosteric site, further studies will be required to solve the controversy.

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