The interaction of fructose 2,6-bisphosphate with an allosteric site of rat liver fructose 1,6-bisphosphatase

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Rat liver fructose 1,6-bisphosphatase can be protected against partial inactivation by *N*-ethylmaleimide by low concentrations of fructose 2,6-bisphosphate or high concentrations of fructose 1,6-bisphosphate. The partially inactivated enzyme has a much reduced sensitivity to high substrate inhibition and has lost the sigmoid component of the inhibition by fructose 2,6-bisphosphate; this compound is a simple linear competitive inhibitor of the modified enzyme. The results suggest that fructose 2,6-bisphosphate can bind to the enzyme at two distinct sites, the catalytic site and an allosteric site. High levels of fructose 1,6-bisphosphate probably inhibit by binding to the allosteric site.

Fructose 1,6-bisphosphatase *Fructose 2,6-bisphosphate* N-*Ethylmaleimide* *High substrate inhibition* *Allosteric site*

1. INTRODUCTION

Fructose 2,6-bisphosphate ($F2,6P_2$) is thought to play a key role in the regulation of carbohydrate metabolism in liver [1]. Its level in liver varies in response to hormones and to changes in dietary status. It is an activator of phosphofructokinase and an inhibitor of fructose 1,6-bisphosphatase ($F1,6P_2$ase) and its level could therefore control glycolysis and gluconeogenesis at the level of the fructose 6-phosphate-fructose 1,6-bisphosphate ($F1,6P_2$) cycle [1].

There has been considerable controversy regarding the mechanism of the inhibition of liver $F1,6P_2$ases by $F2,6P_2$. In [2] it was reported that $F2,6P_2$ was a simple competitive inhibitor of rat liver $F1,6P_2$ase with respect to $F1,6P_2$ and they concluded that $F2,6P_2$ interacted with the enzyme at the catalytic site. Similar conclusions have been drawn from kinetic and chemical modification data for the $F1,6P_2$ases from rabbit and pig liver [3,4]. However, authors in [5] argued that the inhibition of rat liver $F1,6P_2$ase by $F2,6P_2$ was more complex than this. They showed that in kinetic experiments $F2,6P_2$ reduced the affinity of the enzyme for $F1,6P_2$ and also induced co-operativity in the response to $F1,6P_2$. There is also general agreement that the inhibitions of liver $F1,6P_2$ases by AMP and $F2,6P_2$ are synergistic [2–6]. Moreover, $F2,6P_2$ but not $F1,6P_2$ can protect the AMP-binding properties of rat liver $F1,6P_2$ase against modification by acetylimidazole [6]. These 3 findings are consistent with the idea that $F2,6P_2$ interacts with an allosteric site on $F1,6P_2$ase distinct from both the catalytic site and the AMP site.

We report here observations that suggest that $F2,6P_2$ can interact with two distinct sites on rat liver $F1,6P_2$ase and we propose that high substrate inhibition of liver $F1,6P_2$ases results from binding of $F1,6P_2$ to the allosteric site for $F2,6P_2$.

2. MATERIALS AND METHODS

Glucose 6-phosphate dehydrogenase, phosphoglucose isomerase, $NADP^+$, $F1,6P_2$ and

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triethanolamine hydrochloride were obtained from Boehringer (London). *N*-Ethylmaleimide (NEM) was obtained from Aldrich (Gillingham). 5,5[1]-Dithio-bis-(2-nitrobenzoate) (Nbs_2) was obtained from Sigma (London). F2,6P_2 was prepared using a minor modification of the method in [7]; during the hydrolysis of F1,6P_2 by F1,6P_2ase, 10 mM $MgCl_2$ was used instead of 0.5 mM $MnCl_2$. All other chemicals were of the highest available purity.

F1,6P_2ase was purified to homogeneity from rat liver by a method involving fractionation with $(NH_4)_2SO_4$ and chromatography on DEAE-cellulose, Procion red-Sepharose and Sephadex G200 [8]. The purified enzyme was free from proteolytic degradation as judged by its subunit molecular mass, its sensitivity to AMP and its pH-activity profile [8]. For treatment with *N*-ethylmaleimide the enzyme (0.125 mg/ml) was incubated in 50 mM triethanolamine hydrochloride–KOH (pH 7.5) containing 100 mM KCl and the additions indicated in the text at 0°C. At zero time NEM was added to a final concentration of 25 μM. Samples were removed at various times and assayed for F1,6P_2ase using the standard assay.

F1,6P_2ase was assayed by coupling the production of fructose 6-phosphate to the reduction of $NADP^+$. The standard assay conditions were those given in [9]. For kinetic experiments (fig.2,3) a slightly modified method was used; each cuvette contained, in 2 ml, 50 mM triethanolamine hydrochloride–KOH (pH 7.2), 100 mM KCl, 10 μM EDTA, 0.15 mM $NADP^+$, 5 units of phosphoglucose isomerase and 3 units of glucose 6-phosphate dehydrogenase. The concentrations of free Mg^{2+}, F1,6P_2 and F2,6P_2 were as stated in the text. The reaction was initiated by the addition of F1,6P_2ase and the reduction of $NADP^+$ was monitored at 25°C using a Hitachi Perkin-Elmer MPF 2A spectrofluorimeter. The excitation and emission wavelengths were 340 nm and 460 nm, respectively, and the instrument was adjusted to give a full scale deflection corresponding to 0.5 μM NADPH. The association constant for the Mg^{2+}–F1,6P_2 complex was taken to be 250 M^{-1} [10] and that for the Mg^{2+}–F2,6P_2 complex was found to be 350 M^{-1} using the method in [11]. Kinetic plots were analysed using a weighted least mean squares linear regression method [12].

3. RESULTS AND DISCUSSION

Liver F1,6P_2ases are inhibited by high levels of F1,6P_2 (e.g., [10,13]). Treatment of an ox liver F1,6P_2ase with *p*-chloromercuribenzoate abolished the sensitivity of the enzyme to this high substrate inhibition but did not greatly affect its catalytic properties or its allosteric response to AMP [10]. Moreover, inhibitory levels of F1,6P_2, but not non-inhibitory levels, protected the enzyme against thiol group reagents [10]. These observations suggest that the inhibition of liver F1,6P_2ases by high levels of F1,6P_2 may be mediated by low affinity binding of F1,6P_2 to a site distinct from the catalytic site; this site could be an allosteric F2,6P_2 site.

Titration of the thiol groups of rat liver F1,6P_2ase with a large excess of Nbs_2 revealed the presence in the native enzyme of 0.8 rapidly reacting thiol groups per subunit (not shown). Titrations carried out in the presence of 8 M urea showed the presence of 7.0 thiol groups per subunit; this is consistent with the cysteine content determined by amino acid analysis [8]. The rapid reaction of native F1,6P_2ase with Nbs_2 correlated with a 50% decrease in the activity of the enzyme. However, the activity of the enzyme continued to decline slowly even after the reaction with Nbs_2 had been terminated by the addition of excess dithiothreitol and so the kinetic properties of Nbs_2-modified F1,6P_2ase were not examined. These results show that rat liver F1,6P_2ase resembles other F1,6P_2ases in containing one particularly reactive thiol group per subunit.

The results in fig.1 show that the addition of NEM to rat liver F1,6P_2ase causes 50–55% inactivation of the enzyme in a reaction that is complete after about 20 min; no further decrease in activity took place over 1 h. F2,6P_2 (100 μM) afforded complete protection against inactivation, and F1,6P_2 (5 mM) gave significant, though not complete, protection (fig.1). The results from a series of protection experiments are summarised in table 1. While 4 μM F2,6P_2 gave considerable protection and 10 μM F2,6P_2 gave complete protection, 200 μM F1,6P_2 gave no protection; this concentration is about 100-times the K_m of the enzyme for F1,6P_2 [14]. These results show that the target for modification by NEM is a high affinity binding site for F2,6P_2 and a low affinity binding site for

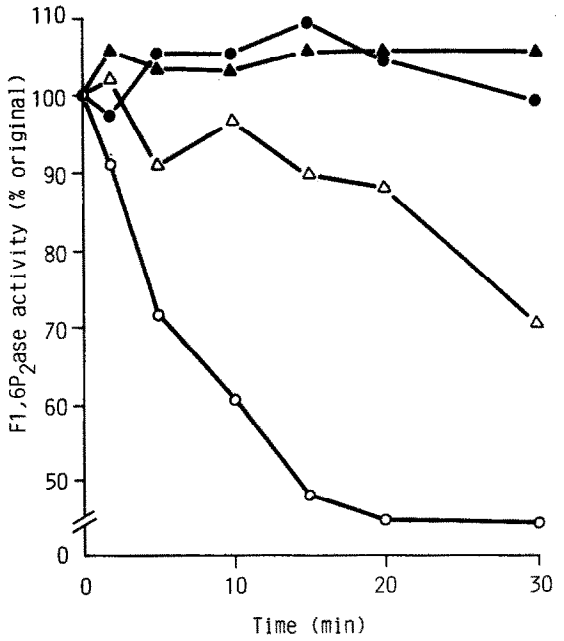


Fig.1. The effect of NEM on the activity of rat liver F1,6P₂ase. F1,6P₂ase was incubated as described in section 2. The standard assay for F1,6P₂ase was used. Symbols represent: no NEM (●), NEM with no other additions (○), NEM plus 5 mM F1,6P₂ (△), NEM plus 100 μM F2,6P₂ (▲).

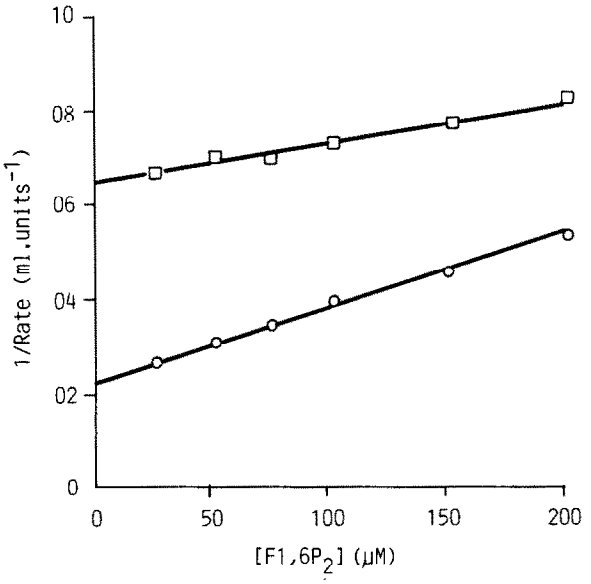


Fig.2. Dixon plots for the inhibition of control and NEM-treated F1,6P₂ase by high levels of F1,6P₂. Control F1,6P₂ase (○), F1,6P₂ase modified with NEM as described in the text (□). The fluorimetric assay was used and the free Mg^{2+} concentration was 2 mM.

Table 1

The protection of rat liver F1,6P₂ase against inactivation by NEM

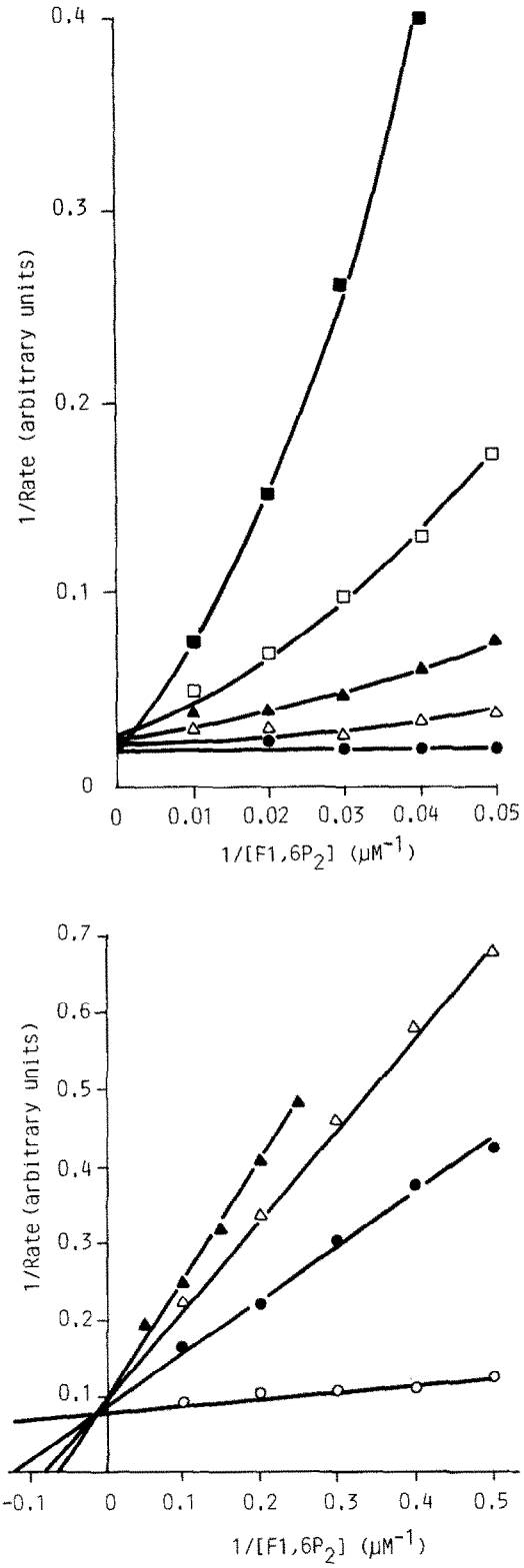
Addition	Activity remaining (%)
None	55
0.2 mM F1,6P₂	58
5 mM F1,6P₂	94
0.2 mM AMP	50
0.2 mM AMP + 0.2 mM F1,6P₂	54
5 mM Fructose 6-phosphate	65
5 mM P_i	61
4 μM F2,6P₂	87
10 μM F2,6P₂	98
100 μM F2,6P₂	102

F1,6P₂ase was incubated with NEM for 20 min as in section 2 with the indicated additions. Activity is expressed as a percentage of the value at zero time. No activity was lost in the absence of NEM

F1,6P₂; it is not the catalytic site. Neither AMP, in the presence or absence of F1,6P₂, nor fructose 6-phosphate nor P_i afforded any significant protection against NEM.

A sample of rat liver F1,6P₂ase was treated with NEM in the absence of any protecting agents. The reaction was terminated by the addition of excess dithiothreitol after 30 min; the enzyme activity remained constant after this treatment. The inhibition of control and NEM-treated F1,6P₂ases by F1,6P₂ and F2,6P₂ was then examined. Fig.2 shows Dixon plots for the inhibition by high levels of F1,6P₂. The values of K_i for F1,6P₂ were 150 μM and 850 μM for the control and NEM-treated samples, respectively. This shows that the reaction of F1,6P₂ase with NEM caused a very considerable reduction in the affinity of the enzyme for F1,6P₂ as an inhibitor.

The inhibition by F2,6P₂ is expressed in fig.3 as double reciprocal plots of velocity against F1,6P₂ concentration at different levels of F2,6P₂. For the control F1,6P₂ase F2,6P₂ clearly induces co-operativity in the response to F1,6P₂ (fig.3a); the response to F2,6P₂ is itself co-operative [14]. These



results agree with those in [5]. For the NEM-treated enzyme F2,6P$_2$ is a linear competitive inhibitor with respect to F1,6P$_2$ and the enzyme responds hyperbolically to F1,6P$_2$ (fig.3b). Slope replots of the data in fig.3b are linear (not shown), indicating that the NEM-treated enzyme does not respond co-operatively to F2,6P$_2$. The K_m of the NEM-treated enzyme for F1,6P$_2$ is 1.4 μM at 2.0 mM free Mg^{2+} ions (fig.3b); this is similar to the value for the control enzyme [14].

The simplest interpretation of these data involves the assumptions that F2,6P$_2$ can interact at two sites on F1,6P$_2$ase, the catalytic site and an allosteric site, and that high substrate inhibition is caused by low affinity binding of F1,6P$_2$ to the allosteric site. We assume that NEM reacts preferentially with the one particularly reactive thiol group per subunit; the protection data suggest that this group is adjacent to or part of the allosteric site. The reaction of this thiol group with NEM seems to prevent binding of F2,6P$_2$ to the allosteric site completely and to reduce the affinity of this site for F1,6P$_2$ considerably.

These ideas are compatible with much previous work on F1,6P$_2$ases and go some way towards resolving the controversy concerning the mechanism of action of F2,6P$_2$. The interaction of F2,6P$_2$ with an allosteric site is supported by a number of observations. The effects of F2,6P$_2$ and AMP are synergistic [2–6,14], F2,6P$_2$ induces co-operativity in the response to F1,6P$_2$ [5,6,14] and F2,6P$_2$ gives sigmoid inhibition of F1,6P$_2$ase [5,14]. None of these effects would be expected of a simple competitive inhibitor. Moreover, F2,6P$_2$ can protect the AMP site of rat liver F1,6P$_2$ase against modification by acetylimidazole [6] and can protect the rabbit liver enzyme against proteolysis [3] whereas levels of F1,6P$_2$ sufficient to saturate the catalytic site but not to cause high substrate inhibition do not have these effects. Our observation that NEM both reduces the K_i for

Fig.3. Inhibition of F1,6P$_2$ase by F2,6P$_2$. The fluorimetric assay was used and the free Mg^{2+} concentration was 2 mM. (a) Control F1,6P$_2$ase. The F2,6P$_2$ concentrations were 0 (●), 1.5 μM (△), 2.9 μM (▲), 5.9 μM (□) and 11.8 μM (■); (b) F1,6P$_2$ase modified with NEM as described in the text. The F2,6P$_2$ concentrations were 0 (○), 1.2 μM (●), 2.4 μM (△) and 3.5 μM (▲).

$F1,6P_2$ as an inhibitor and eliminates the sigmoid component of the inhibition by $F2,6P_2$ strongly suggests that the allosteric site for $F2,6P_2$ can also bind $F1,6P_2$ with low affinity and that this is responsible for the high substrate inhibition of the enzyme. In agreement with this, high levels of $F1,6P_2$ cause little or no additional inhibition if $F2,6P_2$ is present [5,14]. The fact that $F2,6P_2$ is a simple linear competitive inhibitor of NEM-treated $F1,6P_2$ase with respect to $F1,6P_2$ indicates that $F2,6P_2$ can also bind to the catalytic site of the enzyme. This is supported by the observations that $F2,6P_2$ can protect the enzyme against inactivation by acetylimidazole [4,6] and pyridoxal phosphate [3].

Our suggestion that $F2,6P_2$ can interact with $F1,6P_2$ase at two distinct sites could be tested directly by binding studies. However, our model for the allosteric properties of rat liver $F1,6P_2$ase [14] indicates that binding of more than one molecule of $F2,6P_2$ per subunit might be detectable only at high concentrations of $F2,6P_2$. Ultimately, crystallographic studies may be required to resolve this problem.

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