

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

D.W. Meek* and H.G. Nimmo⁺

Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland

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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.

<i>Fructose 1,6-bisphosphatase</i>	<i>Fructose 2,6-bisphosphate</i>	<i>N-Ethylmaleimide</i>
<i>High substrate inhibition</i>	<i>Allosteric site</i>	

1. INTRODUCTION

Fructose 2,6-bisphosphate (F2,6P₂) 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 (F1,6P₂ase) and its level could therefore control glycolysis and gluconeogenesis at the level of the fructose 6-phosphate-fructose 1,6-bisphosphate (F1,6P₂) cycle [1].

There has been considerable controversy regarding the mechanism of the inhibition of liver F1,6P₂ases by F2,6P₂. In [2] it was reported that F2,6P₂ was a simple competitive inhibitor of rat liver F1,6P₂ase with respect to F1,6P₂ and they concluded that F2,6P₂ interacted with the enzyme at the catalytic site. Similar conclusions have been drawn from kinetic and chemical modification data for the F1,6P₂ases from rabbit and pig liver

[3,4]. However, authors in [5] argued that the inhibition of rat liver F1,6P₂ase by F2,6P₂ was more complex than this. They showed that in kinetic experiments F2,6P₂ reduced the affinity of the enzyme for F1,6P₂ and also induced co-operativity in the response to F1,6P₂. There is also general agreement that the inhibitions of liver F1,6P₂ases by AMP and F2,6P₂ are synergistic [2–6]. Moreover, F2,6P₂ but not F1,6P₂ can protect the AMP-binding properties of rat liver F1,6P₂ase against modification by acetylimidazole [6]. These 3 findings are consistent with the idea that F2,6P₂ interacts with an allosteric site on F1,6P₂ase distinct from both the catalytic site and the AMP site.

We report here observations that suggest that F2,6P₂ can interact with two distinct sites on rat liver F1,6P₂ase and we propose that high substrate inhibition of liver F1,6P₂ases results from binding of F1,6P₂ to the allosteric site for F2,6P₂.

2. MATERIALS AND METHODS

Glucose 6-phosphate dehydrogenase, phosphoglucose isomerase, NADP⁺, F1,6P₂ and

* Present address: Department of Molecular Biology, University of Edinburgh, Scotland

⁺ To whom correspondence should be addressed

triethanolamine hydrochloride were obtained from Boehringer (London). *N*-Ethylmaleimide (NEM) was obtained from Aldrich (Gillingham). 5,5'-Dithio-bis-(2-nitrobenzoate) (Nbs₂) was obtained from Sigma (London). F₂,6P₂ was prepared using a minor modification of the method in [7]; during the hydrolysis of F₁,6P₂ by F₁,6P₂ase, 10 mM MgCl₂ was used instead of 0.5 mM MnCl₂. All other chemicals were of the highest available purity.

F₁,6P₂ase was purified to homogeneity from rat liver by a method involving fractionation with (NH₄)₂SO₄ 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 F₁,6P₂ase using the standard assay.

F₁,6P₂ase 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 Mg²⁺, F₁,6P₂ and F₂,6P₂ were as stated in the text. The reaction was initiated by the addition of F₁,6P₂ase 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 Mg²⁺-F₁,6P₂ complex was taken to be 250 M⁻¹ [10] and that for the Mg²⁺-F₂,6P₂ 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 F₁,6P₂ases are inhibited by high levels of F₁,6P₂ (e.g., [10,13]). Treatment of an ox liver F₁,6P₂ase 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 F₁,6P₂, but not non-inhibitory levels, protected the enzyme against thiol group reagents [10]. These observations suggest that the inhibition of liver F₁,6P₂ases by high levels of F₁,6P₂ may be mediated by low affinity binding of F₁,6P₂ to a site distinct from the catalytic site; this site could be an allosteric F₂,6P₂ site.

Titration of the thiol groups of rat liver F₁,6P₂ase with a large excess of Nbs₂ 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 F₁,6P₂ase with Nbs₂ 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 Nbs₂ had been terminated by the addition of excess dithiothreitol and so the kinetic properties of Nbs₂-modified F₁,6P₂ase were not examined. These results show that rat liver F₁,6P₂ase resembles other F₁,6P₂ases in containing one particularly reactive thiol group per subunit.

The results in fig.1 show that the addition of NEM to rat liver F₁,6P₂ase 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. F₂,6P₂ (100 μM) afforded complete protection against inactivation, and F₁,6P₂ (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 F₂,6P₂ gave considerable protection and 10 μM F₂,6P₂ gave complete protection, 200 μM F₁,6P₂ gave no protection; this concentration is about 100-times the *K_m* of the enzyme for F₁,6P₂ [14]. These results show that the target for modification by NEM is a high affinity binding site for F₂,6P₂ and a low affinity binding site for

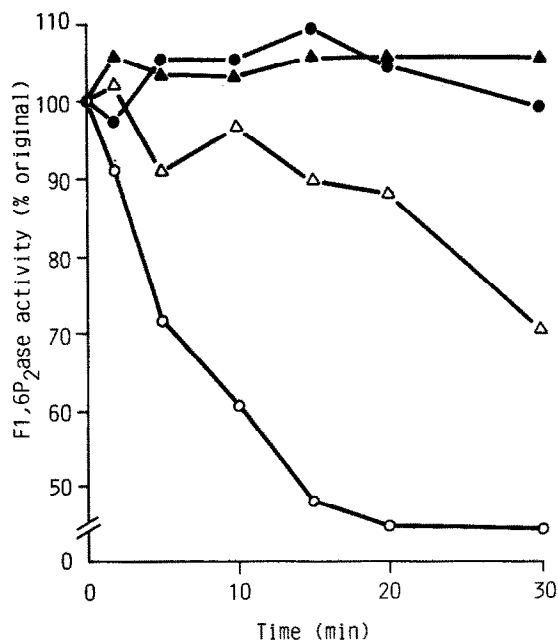


Fig.1. The effect of NEM on the activity of rat liver F1,6P₂ase. F1,6P₂ase was incubated as described in section 2. The standard assay for F1,6P₂ase was used. Symbols represent: no NEM (●), NEM with no other additions (○), NEM plus 5 mM F1,6P₂ (Δ), NEM plus 100 μM F2,6P₂ (▲).

Table 1

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

Addition	Activity remaining (%)
None	55
0.2 mM F1,6P ₂	58
5 mM F1,6P ₂	94
0.2 mM AMP	50
0.2 mM AMP + 0.2 mM F1,6P ₂	54
5 mM Fructose 6-phosphate	65
5 mM P _i	61
4 μM F2,6P ₂	87
10 μM F2,6P ₂	98
100 μM F2,6P ₂	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

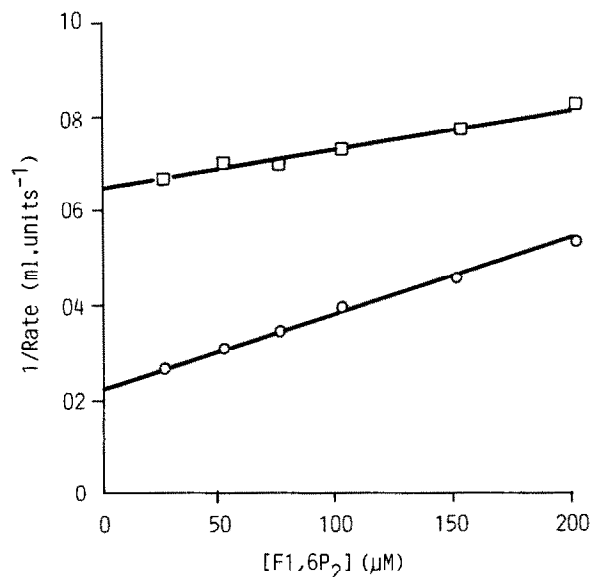
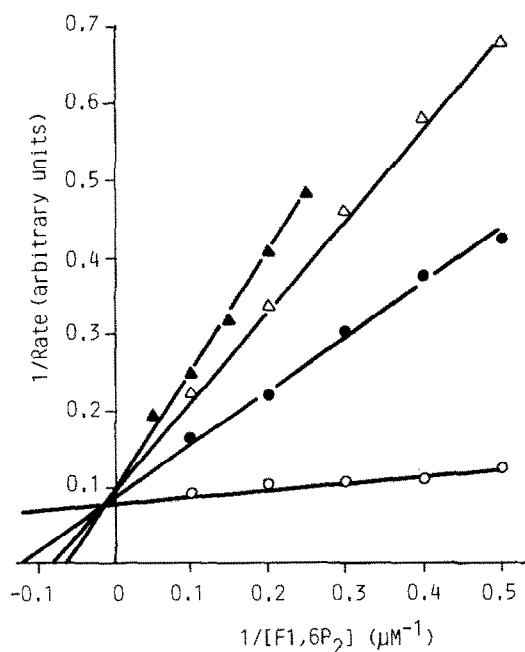
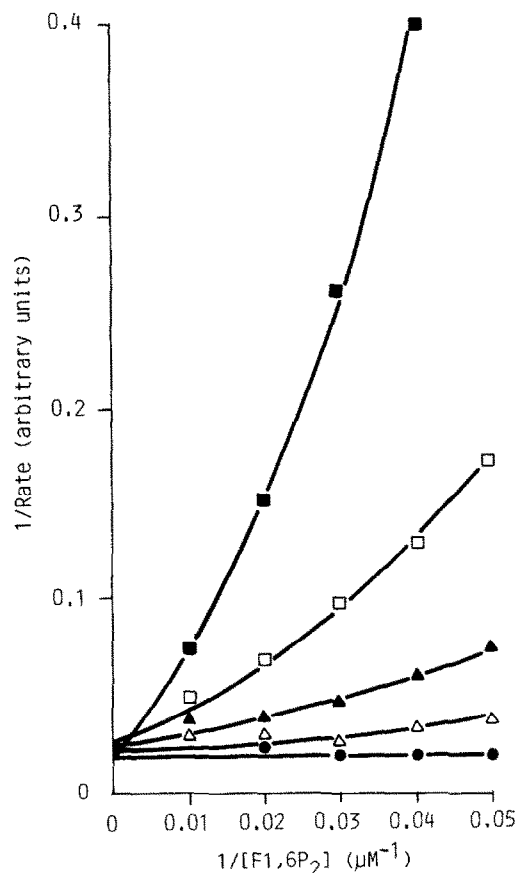


Fig.2. Dixon plots for the inhibition of control and NEM-treated F1,6P₂ase by high levels of F1,6P₂. Control F1,6P₂ase (○), F1,6P₂ase modified with NEM as described in the text (□). The fluorimetric assay was used and the free Mg²⁺ concentration was 2 mM.

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 F2,6P₂ is a linear competitive inhibitor with respect to F1,6P₂ and the enzyme responds hyperbolically to F1,6P₂ (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 F2,6P₂. The K_m of the NEM-treated enzyme for F1,6P₂ is 1.4 μ M at 2.0 mM free Mg²⁺ ions (fig.3b); this is similar to the value for the control enzyme [14].

The simplest interpretation of these data involves the assumptions that F2,6P₂ can interact at two sites on F1,6P₂ase, the catalytic site and an allosteric site, and that high substrate inhibition is caused by low affinity binding of F1,6P₂ 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 F2,6P₂ to the allosteric site completely and to reduce the affinity of this site for F1,6P₂ considerably.

These ideas are compatible with much previous work on F1,6P₂ases and go some way towards resolving the controversy concerning the mechanism of action of F2,6P₂. The interaction of F2,6P₂ with an allosteric site is supported by a number of observations. The effects of F2,6P₂ and AMP are synergistic [2-6,14], F2,6P₂ induces co-operativity in the response to F1,6P₂ [5,6,14] and F2,6P₂ gives sigmoid inhibition of F1,6P₂ase [5,14]. None of these effects would be expected of a simple competitive inhibitor. Moreover, F2,6P₂ can protect the AMP site of rat liver F1,6P₂ase against modification by acetylhydrazide [6] and can protect the rabbit liver enzyme against proteolysis [3] whereas levels of F1,6P₂ 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 F1,6P₂ase by F2,6P₂. The fluorimetric assay was used and the free Mg²⁺ concentration was 2 mM. (a) Control F1,6P₂ase. The F2,6P₂ concentrations were 0 μ M ($\bullet$), 1.5 μ M (Δ), 2.9 μ M ($\blacktriangle$), 5.9 μ M ($\square$) and 11.8 μ M ($\blacksquare$); (b) F1,6P₂ase modified with NEM as described in the text. The F2,6P₂ concentrations were 0 μ M ($\circ$), 1.2 μ M ($\bullet$), 2.4 μ M (Δ) and 3.5 μ M ($\blacktriangle$).

F1,6P₂ as an inhibitor and eliminates the sigmoid component of the inhibition by F2,6P₂ strongly suggests that the allosteric site for F2,6P₂ can also bind F1,6P₂ with low affinity and that this is responsible for the high substrate inhibition of the enzyme. In agreement with this, high levels of F1,6P₂ cause little or no additional inhibition if F2,6P₂ is present [5,14]. The fact that F2,6P₂ is a simple linear competitive inhibitor of NEM-treated F1,6P₂ase with respect to F1,6P₂ indicates that F2,6P₂ can also bind to the catalytic site of the enzyme. This is supported by the observations that F2,6P₂ can protect the enzyme against inactivation by acetylimidazole [4,6] and pyridoxal phosphate [3].

Our suggestion that F2,6P₂ can interact with F1,6P₂ase at two distinct sites could be tested directly by binding studies. However, our model for the allosteric properties of rat liver F1,6P₂ase [14] indicates that binding of more than one molecule of F2,6P₂ per subunit might be detectable only at high concentrations of F2,6P₂. Ultimately, crystallographic studies may be required to resolve this problem.

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