Biphasic effect of fructose 2,6-bisphosphate on the liver fructose-1,6-bisphosphatase: mechanistic and physiological implications

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Fructose 2,6-bisphosphate has been claimed to be both a substrate analogue and an allosteric inhibitor of fructose-1,6-bisphosphatase. The results reported here show that fructose 2,6-bisphosphate can be both an inhibitor and an activator of the enzyme, depending on the substrate concentration. This biphasic behaviour at saturating concentrations of substrate can only be due to an allosteric effect. In addition to the mechanistic implication it is possible that this finding may have physiological meaning.

Fructose-1,6-bisphosphatase *Fructose 2,6-bisphosphate* *Fructose 1,6-bisphosphate* *AMP* *Liver metabolism* *Allosteric modulation*

1. INTRODUCTION

The interconversion of fructose 6-phosphate (F6P) and fructose 1,6-bisphosphate (F-1,6-P_2) is catalyzed by two antagonistic enzymes, 6-phosphofructokinase and fructose-1,6-bisphosphatase (FBPase) that are both subject to multimodulation by a variety of effectors whose action on one enzyme is usually opposite to that on the other [1]. Fructose-2,6-P_2 (F-2,6-P_2) has been shown to be the most potent of such effectors as regards activation of the phosphofructokinase and to have an inhibitory effect on FBPase [2–8]. The mechanism of the inhibitory action has been the subject of controversy, with one group of investigators providing evidence for an allosteric type of interaction [3,5,6] while others claimed it to be competitive inhibition [7–13]. In the course of our studies on the action of hexose bisphosphates on key glycolytic enzymes we were surprised to find that F-2,6-P_2 is an inhibitor of FBPase only at low concentrations of the substrate, while becoming an activator at higher concentrations. Furthermore, at fixed substrate concentrations, the effector may be either positive or negative, depending on its own concentration. These effects are enhanced by certain commonly used buffers. Here we report our observations in this regard, and discuss the possible physiological role of this phenomenon.

2. MATERIALS AND METHODS

2.1. *Chemicals and enzymes*

Hepes (*N*-2-hydroxethylpiperazine-*N'*-2-ethanesulphonic acid), Pipes (piperazine-*N,N'*-bis-(2-ethanesulphonic acid)), EGTA, F-1,6-P_2, F-2,6-P_2, NADP, AMP and the auxiliary enzymes were purchased from Sigma (St. Louis, MO); imidazole was from Scharlau (Barcelona); Sephadex G-25 was from Pharmacia (Uppsala). Other chemicals were purchased from Carlo Erba (Milano).

F-1,6-P_2 was treated at pH 2 (with 0.1 N HCl)

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for 10 min at room temperature and then adjusted to pH 7 (with 0.5 N KOH) to prevent a possible contamination of F-2,6-P_2.

2.2. *Partial purification of fructose-1,6-bisphosphatase*

The enzyme was partially purified from rat liver as in [14], but instead of the gel fractionation steps, the enzyme was precipitated with ammonium sulphate, and the 35 to 60% fractions were collected. The enzyme was desalted through Sephadex G-25 prior to use. The specific activity of this preparation was 30 μmol F-1,6-P_2 hydrolyzed $\cdot$ $min^{-1} \cdot$ mg $protein^{-1}$. The protein was measured as in [15]. Aldolase content was lower than 0.4%.

2.3. *Assay of fructose-1,6-bisphosphatase*

The enzymatic activity was measured spectrophotometrically. The standard assay contained, in final concentrations: 50 mM buffer at pH 7.2 (Pipes, Hepes or imidazole), 100 mM KCl, 1 mM $MgSO_4$, 1 mM NH_4Cl, 5 mM EGTA, 0.5 mM NADP, 5 μg glucose-6-phosphate dehydrogenase and 20 μg glucose-phosphate isomerase, both desalted through Sephadex G-25, in a final volume of 1 ml. F-1,6-P_2 and AMP were added at the concentrations indicated on the bottom of the figures; when the assay was performed without buffer, the reaction mixture was brought to pH 7.2 (with 0.5 N KOH) and the pH was maintained mainly by the low buffer capacity of EGTA. FBPase was incubated with this assay mixture for 5 min at 37°C and the reaction was initiated by the addition of 10–40 μl F-1,6-P_2 to obtain the concentration as indicated in each figure legend.

3. RESULTS

3.1. *Effect of F-2,6-P_2 at different substrate concentrations*

The effect of 1 μM F-2,6-P_2 on the activity of FBPase was measured at different concentrations of F-1,6-P_2. As can be seen in fig.1, 1 μM F-2,6-P_2 inhibits the enzyme activity within the range 5-20 μM F-1,6-P_2. As the concentration of F-1,6-P_2 increases, F-2,6-P_2 becomes an activator, and the positive effect is observed at all higher concentrations tested. This activatory behaviour of

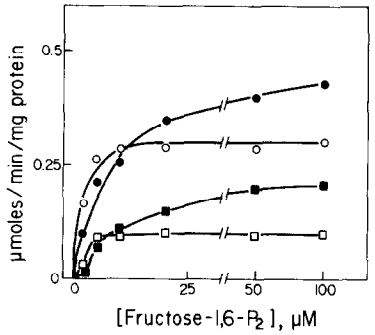

Fig. 1. Effect of 1 μM F-2,6-P_2 on FBPase activity at different concentrations of substrate. (○,□) control; (●,■) with F-2,6-P_2; (○,●) Hepes; (□,■) Pipes; for more details see section 2.

F-2,6-P_2 is relatively greater when Pipes, rather than Hepes, buffer is used.

3.2. *The effect of varying F-2,6-P_2 concentration*

To determine whether or not the biphasic effect of F-2,6-P_2 was dependent on the effector concentration at a fixed F-1,6-P_2 concentration, the activity of the enzyme was measured at 20 and 200 μM substrate. As can be seen in fig.2A a clear activation effect is seen at low F-2,6-P_2 that falls off rapidly as the concentration of the effector reaches 5 μM and above. Note that the extent, but not the quality, of the effect depends on the buffer used. Thus, maximal activations were obtained with 1 μM F-2,6-P_2. However, while in Pipes the activation was almost 2-fold, in Hepes and in imidazole only a 50% increase in activity was observed. Fur-

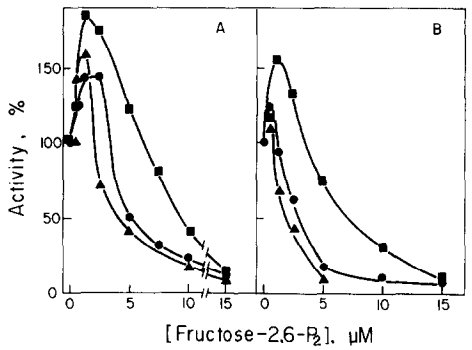

Fig. 2. Effect of F-2,6-P_2 and AMP on rat liver FBPase at 20 μM F-1,6-P_2. (A) Without AMP, (B) with 10 μM AMP. Buffers: (■) Pipes; (●) Hepes; (▲) imidazole.

thermore, at 5 μM F-2,6-P_2 a moderate activation could be seen in Pipes, while 50% inhibition was apparent in both Hepes and imidazole. The inhibition becomes more pronounced reaching about 90%, when F-2,6-P_2 concentration approaches that of F-1,6-P_2. In fig.2B it can be seen that 10 μM AMP displaces all curves downwards and to the left without changing their slope. It is thus observed that the activatory effect at low F-2,6-P_2 concentrations is smaller and that the inhibitory effect of the metabolite is quite pronounced at lower concentrations of F-2,6-P_2. Qualitatively similar results were obtained at 200 μM F-1,6-P_2, although the inhibition by F-2,6-P_2 was smaller than at 20 μM and AMP enhanced the effect of F-2,6-P_2 in all buffers used (not shown).

4. DISCUSSION

Authors in [5] and in [7] independently reported in 1981 that F-2,6-P_2 is an inhibitor of FBPase. Following these initial reports the first group has obtained evidence suggesting that the effect is allosteric [5,6], while the second group has published results concluding that F-2,6-P_2 behaves as a competitive inhibitor substrate analogue [7–13]. That the effect is allosteric is suggested by the fact that F-2,6-P_2 seems to change the kinetics of the enzyme toward its substrate from hyperbolic to sigmoid [5–12], that its effect is synergistic with the allosteric inhibitor AMP [5,8], and that different physical conditions and chemical reagents affect F-2,6-P_2 and AMP actions in parallel. In apparent contraposition to these observations it has been shown that the inhibitory effect is larger at low than at high concentrations of substrate [3,5,6,12], that treatments that affect AMP binding do not necessarily affect F-2,6-P_2 action [12], and that product analogues thought to act at the active site give kinetic plots similar to those obtained with F-2,6-P_2 [10]; these results have been interpreted to mean that F-2,6-P_2 acts as a substrate analogue. However, as pointed out in [8] the available data could also be interpreted to mean that F-2,6-P_2 acts both at the active and at an allosteric site. Binding studies seem to confirm that F-2,6-P_2 binds at a site which is the same or close to the active site, as shown by the fact that F-2,6-P_2 binding is competitively inhibited by F-1,6-P_2, F-6-P and P_i, while AMP does not affect F-2,6-P_2 binding [13]. Nevertheless, more recently it has been reported that rat liver FBPase has two binding sites for F-2,6-P_2, the catalytic site and an allosteric site [16].

Our finding that F-2,6-P_2 can activate FBPase at high concentrations of substrate authenticates the allosteric nature of the stronger inhibition observed at more physiological concentrations of substrate. An isosteric analogue could not exhibit the biphasic behaviour reported here.

Although our results indicate that the main effect of F-2,6-P_2 is allosteric, they do not preclude that when the concentration of the effector approaches or surpasses that of the substrate, competition for the active site might contribute to the overall effect. The fact that the activation at relatively low F-1,6-P_2 concentrations had not been previously observed might be due to the relative concentrations of F-1,6-P_2 and F-2,6-P_2 and by the buffers used by other authors.

While the activation of F-2,6-P_2 might not have physiological significance under most circumstances, it should be pointed out that at 20 μM F-1,6-P_2, 1–3 μM F-2,6-P_2 causes an activation rather than inhibition of FBPase in all buffers tested. The levels of F-1,6-P_2 in rat liver are in the range 5–32 nmol/g [17], the lowest value corresponding to starved animals. On the other hand, the levels of F-2,6-P_2 also change with the nutritional state from about 1 to 9 nmol/g cells [18]. Our findings and the physiological concentration of the bisphosphates may correlate in the sense that under gluconeogenic conditions, F-2,6-P_2 will either not inhibit or may actually cause a slight activation of the FBPase.

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