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# Allosteric inhibition of *Dictyostelium discoideum* fructose-1,6-bisphosphatase by fructose 2,6-bisphosphate

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It has been found that the inhibition of *Dictyostelium discoideum* fructose-1,6-bisphosphatase by fructose 2,6-$P_2$ greatly diminished when the pH was raised to the range 8.5 – 9.5, which resulted in a marked decrease of the affinity for the inhibitor with no change in the $K_m$ for the substrate. This provides evidence for the involvement of an allosteric site for fructose 2,6-$P_2$. Moreover, the fact that excess substrate inhibition also decreased at the pH values for minimal fructose 2,6-$P_2$ inhibition, and was essentially abolished in the presence of fructose 2,6-$P_2$, strongly suggests that this inhibition takes place by binding of fructose 1,6-$P_2$ as a weak analogue of the physiological effector fructose 2,6-$P_2$.

Fructose-1,6-bisphosphatase; Fructose 2,6-bisphosphate; (*Dictyostelium discoideum*)

## 1. INTRODUCTION

Fructose-1,6-bisphosphatase plays an essential role in the regulation of gluconeogenesis. This enzyme is strongly inhibited by fructose 2,6-$P_2$, which enhances the allosteric inhibition produced by AMP [1,2], and is also inhibited by excess substrate [3]. The mechanism of inhibition of fructose-1,6-bisphosphatase by fructose 2,6-$P_2$ is still a matter of controversy, with some reports in favor of a purely competitive process through interaction with the catalytic site [2,4–9], while experiments from other laboratories point to an allosteric inhibition, suggesting a separate binding site for fructose 2,6-$P_2$ [1,10–13].

Recent work from this laboratory [14] has shown that the slime mold, *Dictyostelium discoideum*, exhibits a fructose-1,6-bisphosphatase activity inhibited by fructose 2,6-$P_2$ with the lowest $K_i$ value reported so far (~1 nM). This fructose-1,6-bisphosphatase is known to be insensitive to AMP, as well as markedly inhibited by excess substrate [14,15]. We have investigated the mechanism by which fructose 2,6-$P_2$ inhibits *D. discoideum* fructose-1,6-bisphosphatase. The results reported herein strongly support the occurrence of an allosteric site on fructose-1,6-bisphosphatase for fructose 2,6-$P_2$. Data are also presented suggesting that high concentrations of fructose 1,6-$P_2$ inhibit the enzyme activity by binding to the allosteric site for fructose 2,6-$P_2$.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Phosphoric esters, NADP, Pipes, phenylmethylsulfonyl fluoride, leupeptin and auxiliary enzymes were purchased from Sigma; triethanolamine from Fisher; Ches from Calbiochem. Other chemicals were from Merck.

### 2.2. *Enzyme preparation*

*D. discoideum*, strain AX-2, was grown axenically to a densi-

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ty of 2–4 × $10^6$ amoebae/ml on medium HL5 at 22°C [16]. Amoebae were harvested in the vegetative state and fructose-1,6-bisphosphatase activity was partially purified as described [14], except that in some experiments 2 mM phenylmethylsulfonyl fluoride and 2.5 μg/ml leupeptin were added to the buffer used for cell disruption, resuspension of precipitated material and dialysis to remove ammonium sulfate. The resulting preparation had a specific activity of 9 mU/mg.

### 2.3. *Assay for fructose-1,6-bisphosphatase*

Unless otherwise stated, the assay mixture contained 40 mM triethanolamine-HCl, pH 7.5, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 0.3 mM NADP, 1.2 units of glucose-6-P dehydrogenase, 1 unit of glucosephosphate isomerase and 10 μl of enzyme preparation in a total volume of 1 ml. After 3–4 min, the reaction was started by adding 5 μM fructose 1,6-$P_2$ and the change in absorbance at 340 minus 400 nm was measured at 25°C using a dual wavelength spectrophotometer (Shimadzu model UV 3000). Protein was determined by the method of Bradford [17].

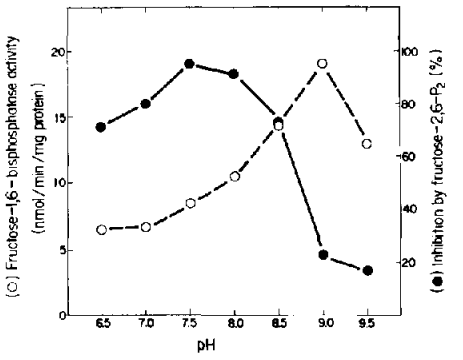


Fig.1. Effect of pH on *D. discoideum* fructose-1,6-bisphosphatase and inhibition by fructose 2,6-$P_2$. Fructose 1,6-$P_2$ was 5 μM. The effect of fructose 2,6-$P_2$ is expressed as percentage inhibition by 2.5 nM fructose 2,6-$P_2$ of the enzyme activity measured at the same pH but in the absence of the inhibitor. Buffers were: Pipes (pH 6.5), triethanolamine (pH 7.0–8.5), and Ches (pH 9 and 9.5).

## 3. RESULTS AND DISCUSSION

*D. discoideum* fructose-1,6-bisphosphatase is inhibited by low nanomolar concentrations of fructose 2,6-$P_2$ [14], and, like for the liver enzyme [1], this changes the substrate saturation curve from hyperbolic to sigmoidal. In the present work we have studied the pH dependence of this inhibition as compared to that of the enzymatic activity, to gain some insight into the nature of the interaction of fructose 2,6-$P_2$ with the enzyme.

As shown in fig.1, the catalytic activity exhibited a pH optimum at pH 9, while maximum inhibition by fructose 2,6-$P_2$ was obtained at pH 7.5. Similar results were observed even when protease inhibitors, like 2 mM phenylmethylsulfonyl fluoride and 2.5 μg/ml leupeptin were added to the buffers used in the enzyme preparation (data not shown). The effect of pH on fructose 2,6-$P_2$ inhibition was mediated by a great increase in $K_i$ values above pH 8.5, with no significant change in the $K_m$ under similar conditions (fig.2). This shows that the higher enzyme activity at pH 9 was not due to a change in $K_m$, and also indicates that the decrease in the affinity for the inhibitor with increasing pH did not imply a concomitant modification in the affinity for the substrate. Taken together, these results strongly suggest the involvement of specific amino acid residues in the interaction of fructose 2,6-$P_2$ with fructose-1,6-bisphosphatase which do not participate in the binding of fructose 1,6-$P_2$ at the catalytic site. Evidence for this conclusion has also been provided by Reyes et al. [13], who lately reported that treatment of pig kidney fructose-1,6-bisphosphatase with *N*-ethylmaleimide abolished fructose 2,6 $P_2$ inhibition, while enzymatic activity was retained. Besides, the existence of an allosteric fructose 2,6-$P_2$ site on the yeast enzyme has been suggested recently [18].

Studies of pH sensitivity have also been carried out in other laboratories with fructose-1,6-bisphosphatase from rat liver [4,7,10] and pig kidney [5]. Nevertheless, in contrast with the slime mold enzyme, the pH profiles for catalytic activity and sensitivity to fructose 2,6-$P_2$ inhibition were similar. This is consistent with but does not constitute substantial evidence in favor of the hypothesis that fructose 2,6-$P_2$ binds to the catalytic site. For the time being we can only speculate about a structural difference in the active site domain to account for our finding. A lack of unique interpretation can also be assigned to most other data reported on the mechanism of interaction of fructose 2,6-$P_2$, as pointed out by several authors [4,9,12,13].

Fig.3 shows that excess substrate inhibition, common to fructose-1,6-bisphosphatases from many origins, also decreased at the pH values at which the inhibitory action of fructose 2,6-$P_2$ was diminished. Furthermore, inhibition by high levels of fructose 1,6-$P_2$ was practically abolished at pH

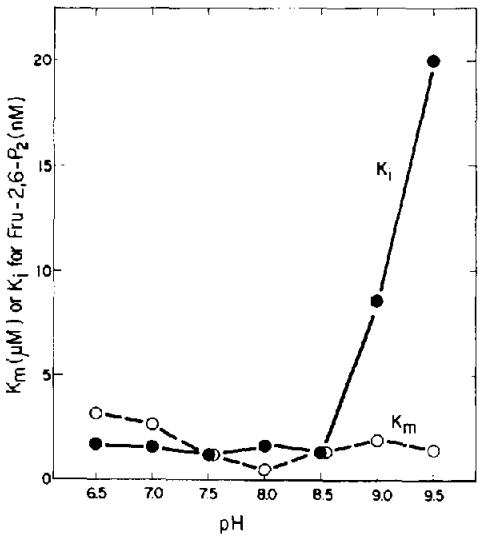


Fig.2. Effect of pH on the $K_m$ of *D. discoideum* fructose-1,6-bisphosphatase and the $K_i$ for fructose 2,6-$P_2$. $K_i$ is defined as the concentration of fructose 2,6-$P_2$ that gave 50% inhibition. The assay conditions were as described in fig.1.

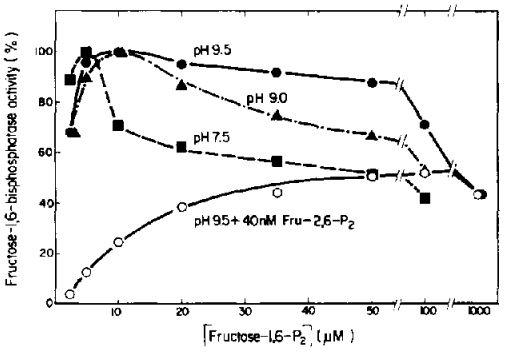


Fig.3. Effect of selected pH values and fructose 2,6-$P_2$ on excess substrate inhibition of *D. discoideum* fructose-1,6-bisphosphatase. 100% activity is represented by the highest velocity measured at each pH value.

9.5 if a highly inhibitory concentration of fructose 2,6-$P_2$ was added to the assay. Excess substrate inhibition was equally observed at pH 7.5 if fructose 1,6-$P_2$ was treated at pH below 2 at 30°C for 30 min and neutralized with 5 N KOH prior to use, to discard a possible contamination by fructose 2,6-$P_2$. Thus, in contrast with the liver enzyme [3], this fructose-1,6-bisphosphatase does not exhibit maximum substrate inhibition at the optimum pH for activity. Hence, our data suggest that fructose 1,6-$P_2$ inhibits the slime mold enzyme not by competitive binding at the catalytic site, but by acting at a separate site. The fact that fructose 1,6-$P_2$ inhibition can be relieved by fructose 2,6-$P_2$ indicates that high levels of substrate inhibit the enzyme by low affinity interaction with the specific site for fructose 2,6-$P_2$. This is in agreement with the proposal for the liver [11] and kidney enzyme [13], after the observation of an intense reduction in fructose 1,6-$P_2$ inhibition by treatment with *N*-ethylmaleimide, which suppresses fructose 2,6-$P_2$ inhibition [13].

With respect to the location of the specific fructose 2,6-$P_2$ binding site, the lack of coincidence found in pH sensitivity of fructose 2,6-$P_2$ inhibition and catalytic activity indicates that fructose 2,6-$P_2$ interacts at an allosteric site. Nevertheless, this result does not exclude the possibility of partial overlapping with the catalytic site. However, the observations in favor of the interaction of fructose 1,6-$P_2$ at high concentrations with the fructose 2,6-$P_2$ binding site make unlikely the latter possibility, and suggest that fructose 2,6-$P_2$ inhibits fructose-1,6-bisphosphatase by acting at a separate site.

After completion of this work, Liu and Fromm [19] have reported that rabbit liver fructose-1,6-bisphosphatase incubated with *N*-ethylmaleimide behaves differently from a quantitative point of view, as compared to the pig kidney enzyme shown by Reyes et al. [13]. However, Liu and Fromm observed a great decrease of fructose 2,6-$P_2$ inhibition with no substantial change in $K_m$, which in spite of their interpretation is, of course, fully compatible with our conclusion as outlined above. Their emphasis of the similarity between the two bisphosphorylated fructoses is in fact crucial for our conclusion of an allosteric site for fructose 2,6-$P_2$ that also accounts for the excess substrate inhibition.

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