Inhibition of Bovine Hepatic Fructose-1,6-diphosphatase by Substrate Analogs*

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Purified bovine hepatic fructose-1,6-diphosphatase, which exhibits maximal activity at neutral pH, is competitively inhibited by several analogs of its substrate, fructose 1,6-diphosphate. These include glucose 1,6-diphosphate ($K_1 = 9.4 \times 10^{-5}$ M), hexitol 1,6-diphosphate ($K_1 = 2.3 \times 10^{-4}$ M), 2,5-anhydro-D-mannitol 1,6-diphosphate ($K_1 = 3.3 \times 10^{-4}$ M), and 2,5-anhydro-D-glucitol 1,6-diphosphate ($K_1 = 5.5 \times 10^{-4}$ M). The $K_1$ values for both 2,5-anhydro-D-mannitol 1,6-diphosphate and 2,5-anhydro-D-glucitol 1,6-diphosphate are lower than the $K_1$ of $1.4 \times 10^{-6}$ M for fructose 1,6-diphosphate. Since 2,5-anhydro-D-mannitol 1,6-diphosphate is an analog of the $\beta$ anomer of fructose 1,6-diphosphate and 2,5-anhydro-D-glucitol 1,6-diphosphate is an analog of the $\alpha$ anomer, the lower $K_1$ for the mannitol analog may indicate that the $\beta$ anomer of fructose 1,6-diphosphate, which predominates in solution, is the true substrate. The substrate analog 1,5-pentanediol diphosphate inhibits slightly ($K_1 = 5 \times 10^{-3}$ M), but 1,4-cyclohexyldiol diphosphate does not. The $K_1$ for product inhibition by sodium phosphate is $9.4 \times 10^{-3}$ M. 2,5-Anhydro-D-mannitol 1,6-diphosphate and $\alpha$-D-glucose 1,6-diphosphate are substrates at pH 9.0, but not at pH 6.5.

Fructose-1,6-diphosphatase (EC 3.1.3.11 d-fructose-1,6-bisphosphate 1-phosphohydrolase) catalyzes the release of inorganic phosphate from fructose 1,6-diphosphate (Fru-1,6-P$_2$). In a continuing effort to elucidate the kinetic mechanism of fructose-1,6-diphosphatase, we have examined the effect of a series of substrate analogs on the enzyme from bovine liver. We have previously purified this enzyme to homogeneity in our laboratory (1-9), and determined that it is inhibited by high concentrations of its substrate, Fru-1,6-P$_2$, by high concentrations of its cofactor, Mg$^{2+}$, and by the allosteric modifier, AMP (4, 5). Fructose 1,6-diphosphate has been shown to exist in solution in four forms in the following percentages: $\beta$-furanose ($\sim 90$), $\alpha$-furanose ($\sim 10$), keto ($< 1.7$), and hydrated keto ($< 0.1$) (6). We have investigated the effects of several analogs of these four forms of the substrate, including 2,5-anhydro-D-glucitol 1,6-diphosphate (I), and 2,5-anhydro-D-mannitol 1,6-diphosphate (II), analogs of the $\alpha$ and $\beta$ anomers of fructose 1,6-diphosphate, respectively. Other cyclic analogs examined include $\alpha$-D-glucose 1,6-diphosphate (III) and 1,4-cyclohexylidion diphosphate (IV). We have also examined several analogs of the open chain form of fructose 1,6-diphosphate, including hexitol 1,6-diphosphate (V) (a mixture of $\alpha$-mannitol and $\alpha$-sorbitol, 1,6-diphosphates), 1,5-pentanediol diphosphate (VI), and phosphoenolpyruvate (VII), as well as inorganic phosphate (VIII). The effects of $\beta$-glycerophosphate on the bovine liver enzyme have been investigated previously (3).

Benkovic and co-workers (7-9) have investigated the anomer specificity of rabbit liver fructose-1,6-diphosphatase, and they have concluded that the rabbit liver enzyme uses the $\alpha$ anomer as its substrate.

Some of our results on the effects of substrate analogs on the bovine liver enzyme have been reported in a preliminary communication (10).

EXPERIMENTAL PROCEDURE

Materials

1,5-Pentanediol diphosphate, 1,4-cyclohexylidion diphosphate, and 2,5-anhydro-D-mannitol 1,6-diphosphate (tetracyclohexylammonium salts) were the generous gifts of Dr. Robert Barker, Michigan State University. 2,5-Anhydro-D-glucitol 1,6-diphosphate, tetracyclohexylammonium salt, was a gift from Dr. Gary Gray, University of Minnesota. $\alpha$-D-Glucose 1,6-diphosphate, potassium salt; NADP$^+$, sodium salt; pyruvate kinase, alkaline phosphatase; NADH, sodium salt; and tris(hydroxymethyl)aminomethane, were purchased from

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Methods

Enzyme Purification—Fructose-1,6-diphosphatase was prepared from bovine liver as described previously (1) or by the modified procedure of Arneson (11, 12).

Enzyme Assays—The procedure used in the phosphate release assay was that of Byrne et al. (1). The standard reaction mixture contained 0.025 M Tris/0.025 M histidine, pH 6.5/5 mM MgSO₄/0.1 mM EDTA/4.2 mM Fru-1,6-P₄, and enzyme in a final volume of 2.0 ml at 37°C. In the coupled spectrophotometric assay, as described previously (5), the standard assay mixture contained 0.025 M Tris/0.025 M histidine, pH 6.5/0.1 mM EDTA/5.0 mM MgSO₄/0.19 mM NADP⁺/8.4 µM Fru-1,6-P₄, and phosphoglucone isomerase and glucose-6-phosphate dehydrogenase in excess, as determined for each experiment, in a final volume of 1.0 ml at 35°C. The reaction was initiated by the addition of fructose-1,6-diphosphatase.

Assays of Analogs, Protein, and Other Reagents—The concentrations of Fru-1,6-P₄ and the substrate analogs were determined by the phosphate procedure of Ames and Dubin (13). Protein, Mg²⁺, and NADP⁺ were determined as described previously (5).

Hexitol 1,6-diphosphate was prepared by the method of Ginsburg and Mehler (14), with a single modification; at the final step an ether extraction was performed. Hexitol 1,6-diphosphate concentration was measured by following the conversion of NADP⁺ to NADPH in the presence of glucose-6-phosphate dehydrogenase and NADH was employed, and the decrease in absorbance at 340 nm was followed. The assay contained 0.025 M Tris/0.025 M histidine (pH 7.5 or pH 9.0)/0.1 mM EDTA/5 mM MgSO₄/1 mM phosphoenolpyruvate/0.21 mM NADH/0.2 unit of lactate dehydrogenase in a volume of 1.0 ml at 35°C. The control contained 100 µM KCN/0.7 mg/ml of ADP/0.1 unit of pyruvate kinase (15).

RESULTS

Inhibition by 2,5-Anhydro-D-glucitol 1,6-Diphosphate (I) and 2,5-Anhydro-D-mannitol 1,6-Diphosphate (II)—The effects of I, the analog of the α anomer of Fru-1,6-P₄, and II, the analog of the β anomer, on fructose-1,6-diphosphatase activity were examined at both pH 6.5 and pH 9.0. The bovine enzyme exhibits maximal activity at pH 6.5. At high substrate concentrations (~5 mM) in the range in which there is significant substrate inhibition, a second pH optimum is observed at pH 9.0 (4, 16). The enzyme frequently displays different properties at pH 6.5 and pH 9.0. Therefore, we routinely study the effects of both pH 6.5 and pH 9.0. Both Analogs I and II were found to be extremely potent competitive inhibitors at both pH 6.5 and pH 9.0. Lineweaver-Burk plots of inhibition by Analog II at pH 6.5 (Fig. 1) and pH 9.0 (not shown) were used to calculate Kᵢ values for Analog II of 3.8 × 10⁻⁴ M at pH 6.5 and 2.5 × 10⁻⁴ M at pH 9.0, compared to a Kᵢ for Fru-1,6-P₄ of 1.4 µM at pH 6.5 and 4.4 µM at pH 9.0 (Table I). Using the phosphate release assay, Analog II was found to be a substrate at pH 9.0, but not at pH 6.5. At pH 9.0 it was hydrolyzed at 1.1% the rate of the same concentration of Fru-1,6-P₄ under identical conditions.

Similar results were found for Analog I, although it was not quite as effective an inhibitor as Analog II. At pH 6.5 the Kᵢ for Analog I was calculated to be 5.5 × 10⁻⁷ M from the data shown in Fig. 2. Similarly, at pH 9.0 the Kᵢ was determined to be 4.7 × 10⁻⁴ M. The inhibition constants for both Analogs I and II at both pH 6.5 and pH 9.0 are compared with the Kᵢ values for Fru-1,6-P₄ in Table I.

Effect of Glucose 1,6-Diphosphate—Hydrolysis of Glc-1,6-P₄ was measured using the coupled assay in the absence of Fru-1,6-P₄ and phosphoglucone isomerase. In this way Glc-6-P production could be measured by following the conversion of NADPH to NADP⁺ in the presence of glucose-6-phosphate dehydrogenase and NADH was employed, and the decrease in absorbance at 340 nm was followed. The assay contained 0.025 M Tris/0.025 M histidine, pH 6.5/5.0 mM MgSO₄/0.1 mM EDTA/0.19 mM NADP⁺/0.12 µM, 0.24 µM, or 0.60 µM Analog II, varied concentrations of Fru-1,6-P₄, excess glucose-6-phosphate dehydrogenase and phosphoglucone isomerase, and 0.015 unit of fructose-1,6-diphosphatase in a final volume of 1.0 ml at 35°C.

Fig. 1 (left). Double reciprocal plot of inhibition of fructose-1,6-diphosphatase activity by 2,5-anhydro-D-mannitol 1,6-diphosphate (II) at pH 6.5. The coupled assay was performed as described under "Methods." The assay contained 0.025 M Tris/0.025 M histidine, pH 6.5, 5.0 mM MgSO₄, 0.1 mM EDTA, 0.19 mM NADP⁺, 0.12 µM, 0.24 µM, or 0.60 µM Analog II, varied concentrations of Fru-1,6-P₄, excess glucose-6-phosphate dehydrogenase and phosphoglucone isomerase, and 0.015 unit of fructose-1,6-diphosphatase in a final volume of 1.0 ml at 35°C.

Fig. 2 (right). Double reciprocal plot of inhibition of fructose-1,6-diphosphatase activity by 2,5-anhydro-D-glucitol 1,6-diphosphate (I) at pH 6.5. The conditions of the coupled assay were the same as those described in Fig. 1, except that all assays were run in the presence of either 0, 1.2 µM, 6.0 µM, or 8.4 µM Analog I and varied concentrations of Fru-1,6-P₄.
Rate of phosphoenolpyruvate hydrolysis at pH 7.4 by the rabbit liver enzyme to be 2 to 4% of the rate of Fru-1,6-P₂ hydrolysis at pH 6.5. Kirtley and Dix (17) estimate the rate of Fru-1,6-P₂ hydrolysis at pH 6.5 and a Fru-1,6-P₂ concentration of 8.4 mM to be 10⁻¹⁰ M ± 0.3. The extremely low Kᵢ values for Analogs I and II indicate they may be bound much more tightly than the substrate, Fru-1,6-P₂. Space-filling models of the α and β anomers of Fru-1,6-P₂ and of Analogs I and II have been constructed. The models indicate differences in freedom of rotation of the phosphate group on the anomeric carbon: (a) the -PO₂H₂ grouping has freedom of rotation in all four forms; (b) the -PO₂H₂ grouping has some rotation in both anomers of Fru-1,6-P₂ but slightly more rotation is possible in both Analogs I and II; and (c) the -CH₂PO₂H₂ grouping has essentially free rotation in the β anomer of Fru-1,6-P₂ and in Analog II, but restricted rotation in the α anomer of Fru-1,6-P₂ and in Analog I. This suggests that the positioning of the phosphate group on carbon 1 is important in substrate binding, and that the fructose-1,6-diphosphatase reaction may require a specific orientation of the phosphate group which can be more easily realized by the analogs than by Fru-1,6-P₂. The molecular models further suggest that the reason Analog II has a lower Kᵢ than Analog I is because it has less restricted rotation as well as a different orientation of the -CH₂PO₂H₂ grouping.

**Table 1**

<table>
<thead>
<tr>
<th>Substrate or analog</th>
<th>pH 6.5</th>
<th>pH 9.0</th>
<th>pH 6.5</th>
<th>pH 9.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose-1,6-diphosphate</td>
<td>Yes</td>
<td>Yes</td>
<td>Kᵢ = 1.4 x 10⁻³ M ± 0.2</td>
<td>Kᵢ = 4.4 x 10⁻⁴ M ± 0.3</td>
</tr>
<tr>
<td>2,5-Anhydro-d-mannitol 1,6-diphosphate (II)</td>
<td>No</td>
<td>Yes</td>
<td>Kᵢ = 3.3 x 10⁻⁴ M ± 0.2</td>
<td>Kᵢ = 2.5 x 10⁻⁴ M ± 0.5</td>
</tr>
<tr>
<td>2,5-Anhydro-d-glucitol 1,6-diphosphate (I)</td>
<td>No</td>
<td>No</td>
<td>Kᵢ = 5.5 x 10⁻⁷ M ± 0.7</td>
<td>Kᵢ = 4.7 x 10⁻⁸ M ± 0.1</td>
</tr>
<tr>
<td>Glucose 1,6-diphosphate (III)</td>
<td>No</td>
<td>Yes</td>
<td>Kᵢ = 9.4 x 10⁻⁷ M ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Hexitol 1,6-diphosphate (V)</td>
<td>No</td>
<td>No</td>
<td>Kᵢ = 2.3 x 10⁻⁷ M ± 0.3</td>
<td></td>
</tr>
<tr>
<td>1,5-Pentanediol diphosphate (VI)</td>
<td>No</td>
<td>No</td>
<td>Kᵢ = 0.9 x 10⁻⁷ M ± 0.4</td>
<td></td>
</tr>
<tr>
<td>1,4-Cyclohexyldiol diphosphate (IV)</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Phosphoenolpyruvate (VII)</td>
<td>No</td>
<td>No</td>
<td></td>
<td></td>
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<tr>
<td>Sodium phosphate (VIII)</td>
<td>No</td>
<td>No</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* a The standard phosphate release assay was used unless otherwise specified.
* b The coupled assay conditions were those described under “Methods,” except that variable amounts of analog were added. Errors are calculated as the standard deviation of replicate determinations.
* c A coupled assay was used as described under “Methods.”
* d Determined at 8.4 μM Fru-1,6-P₂.
* e A coupled assay was used at pH 7.5, see “Methods.”

**Discussion**

The extremely low Kᵢ values for Analogs I and II indicate they may be bound much more tightly than the substrate, Fru-1,6-P₂. Space-filling models of the α and β anomers of Fru-1,6-P₂ and of Analogs I and II have been constructed. The models indicate differences in freedom of rotation of the phosphate group on the anomeric carbon: (a) the -PO₂H₂ grouping has freedom of rotation in all four forms; (b) the -PO₂H₂ grouping has some rotation in both anomers of Fru-1,6-P₂ but slightly more rotation is possible in both Analogs I and II; and (c) the -CH₂PO₂H₂ grouping has essentially free rotation in the β anomer of Fru-1,6-P₂ and in Analog II, but restricted rotation in the α anomer of Fru-1,6-P₂ and in Analog I. This suggests that the positioning of the phosphate group on carbon 1 is important in substrate binding, and that the fructose-1,6-diphosphatase reaction may require a specific orientation of the phosphate group which can be more easily realized by the analogs than by Fru-1,6-P₂. The molecular models further suggest that the reason Analog II has a lower Kᵢ than Analog I is because it has less restricted rotation as well as a different orientation of the -CH₂PO₂H₂ grouping.
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compared to the $\beta$ anomer (6, 18), it would therefore act as an inhibitor present in the substrate. This assumption would predict that substrate inhibition would be slight at low substrate concentrations, increase with increasing substrate concentration as the amount of $\alpha$ anomer increases, and level off when the substrate concentration becomes high enough due to anomerization of $\alpha \rightarrow \beta$ or hydrolysis of the $\alpha$ anomer which would prevent 100% inhibition. Studies of substrate inhibition (5) have shown that this is the case: substrate inhibition is negligible at Fru-1,6-P\textsubscript{2} concentrations below 10 $\mu$M, then increases as the Fru-1,6-P\textsubscript{2} concentration is raised above 10 $\mu$M (note that at 10 $\mu$M Fru-1,6-P\textsubscript{2} the concentration of $\alpha$ anomer would be approximately 1 to 2 $\mu$M which is the $K_m$ of the enzyme for Fru-1,6-P\textsubscript{2} and finally levels off as the concentration of Fru-1,6-P\textsubscript{2} approaches 5 mM. Extrapolation of data at pH 6.5 and 5 mM Mg\textsuperscript{2+} indicates a maximum inhibition of 50%.

Although the cyclic compounds bind more tightly to the active site, the ring form is not a necessity for binding, since Analogs V and VI and $\beta$-glycerophosphate (3) inhibit. Also, a ring oxygen is not essential for weak binding and neither are free hydroxyl groups, since Analog VI inhibits slightly. Analogs with pentose rings appear to bind more tightly than those with hexose rings. Another possible conclusion is that compounds with the phosphate groups trans to one another ($\beta$-Fru-1,6-P\textsubscript{2}, Analogs II and III) may bind better than their anomers with the phosphate groups cis ($\alpha$-Fru-1,6-P\textsubscript{2}, and Analog I). It would be of interest to evaluate $\beta$-d-glucose 1,6-diphosphate in this regard.

The bovine enzyme can hydrolyze either cyclic or acyclic compounds at pH 9.0, since it can hydrolyze Analogs II and III as well as $\beta$-glycerophosphate (3). Analog II may not be hydrolyzed at pH 6.5 because the hydroxyl group at C-2 is necessary for catalysis; perhaps to stabilize an intermediate by hydrogen bonding, a possibility first suggested by Benkovic and co-workers (9, 19) who found that $\beta$-methyl-d-fructofuranoside 1,6-diphosphate (in which the hydroxyl group at C-2 is replaced by $\text{OCH}_3$) is an inhibitor and not a substrate of rabbit liver fructose-1,6-diphosphatase. This compound, like Analog II would also be unable to hydrogen bond. Another possibility would be that the C-2 hydroxyl group is needed so that the ring can open during catalysis, i.e., a free keto group is needed for hydrolysis. However, it should be pointed out that as mentioned above, Analog II, in which the ring cannot open, is a substrate although a very poor one, at pH 9.0 but not at pH 6.5. In addition Glc-1,6-P\textsubscript{2} which cannot open, is also a substrate at pH 9.0 but not at pH 6.5. This leads to the conclusion that either ring opening or a free keto group is not essential at either pH 6.5 or pH 9.0, or that the mechanism of hydrolysis as well as substrate specificity differ at pH 6.5 and pH 9.0.

The bovine liver enzyme and the rabbit liver enzyme appear to differ in a number of respects. The $K_i$ values determined for Analogs I and II for the rabbit liver enzyme are about 100-fold higher than those observed for the bovine enzyme, and the rabbit enzyme exhibits a lower $K_i$ for Analog I than II (9); whereas the bovine enzyme has a lower $K_i$ for Analog II than I. The bovine enzyme can hydrolyze Analogs II and III and $\beta$-glycerophosphate at pH 9.0; compounds which are not hydrolyzed by the rabbit liver enzyme (9). The bovine enzyme is inhibited by hexitol 1,6-diphosphate to a much greater extent than by inorganic phosphate, as contrasted with the rabbit liver enzyme, which has essentially the same $K_i$ values for both compounds (20). These differences may reflect previ-ously observed differences in the catalytic mechanisms of the two enzymes (5, 21).

The effects of $\alpha$-d-glucose 1,6-diphosphate on fructose-1,6-diphosphatase are of interest since phosphoglucomutase can catalyze the conversion of fructose 1,6-diphosphate to glucose 1,6-diphosphate by transfer of a phosphate group from Fru-1,6-P\textsubscript{2} to Glc-6-P, and the concentration of Glc-1,6-P\textsubscript{2} in liver is approximately 14 $\mu$mol/kg (22, 23). Assuming 1.0 $\mu$g of tissue is equivalent to a 0.7-$\mu$l volume, this would mean the concentration of Glc-1,6-P\textsubscript{2} in liver is about 20 $\mu$M. The $K_i$ for Glc-1,6-P\textsubscript{2} determined here was 9.4 $\times$ 10$^{-3}$ $\mu$M. It is therefore possible that inhibition of fructose 1,6-diphosphatase by Glc-1,6-P\textsubscript{2} might have physiological significance, perhaps in regulation of gluconeogenesis and glycolysis in liver.

Acknowledgments—I am grateful to Dr. Robert Barker, Michigan State University, for generously providing the samples of 1,5-pentanediol diphosphate, 1,4-cyclohexyldiol diphosphate, and 2,5-anhydro-d-mannitol 1,6-diphosphate; to Dr. Gary Gray, University of Minnesota, for his gift of the 2,5-anhydro-d-glucitol 1,6-diphosphate; and to Dr. Richard Arneson and Dr. Richard Lane for preparing the hexitol 1,6-diphosphate. I also wish to acknowledge the helpful advice of Dr. Arthur M. Geller and Dr. William L. Byrne.

REFERENCES
15. Biochemica Catalog (1968) Boehringer C. F., and Soehne GmbH Mannheim, Germany
Inhibition of bovine hepatic fructose-1,6-diphosphatase by substrate analogs.
C.J. Marcus


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