Fructose 1,6-Bisphosphate

KINETICS OF HYDROLYSIS CATALYZED BY RABBIT LIVER NEUTRAL FRUCTOSE-1,6-BISPHOSPHATASE WITH Mn²⁺

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(Received for publication, April 18, 1977)

The kinetics of hydrolysis of d-fructose 1,6-bisphosphate (Fru-1,6-P₂) catalyzed by rabbit liver neutral fructose-1,6-bisphosphatase have been studied, using Mn²⁺ as cofactor. Some apparent Km values for Fru-1,6-P₂ lie well below 1 μM, and rates at substrate concentrations approaching Km were measured with a highly sensitive radioassay to follow the release of ³²P from [³²P]Fru-1,6-P₂. Data from experiments in which [Fru-1,6-P₂]₀ and [Mn²⁺] were separately varied were consistent with a kinetic model in which each enzyme subunit, essentially independently, binds first a structural metal ion, then Fru-1,6-P₂, and then a catalytic second metal ion after which hydrolysis occurs. This rapid equilibrium or steady state ordered model accounts for data collected at pH 7.5, 8.5, and 9.0, and also for results reported for the beef liver enzyme with Mg²⁺ as cofactor (Marcus, C. J., Geller, A. M., and Bryne, W. L. (1973) J. Biol. Chem. 248, 8567–8573; Nimmo, I. G., and Tipton, K. F. (1975) Eur. J. Biochem. 58, 567–574). Fructose 6-phosphate is a competitive inhibitor with Km = 70 μM at pH 8.5, and inorganic phosphate inhibits essentially competitively with Kᵦ = 2.7 mM at pH 7.5.

Fructose-1,6-bisphosphatase undergoes a reversible, pH-dependent change in activity in less than 15 min in Tris-HCl buffer with Mn²⁺. Compared with enzyme incubated at pH 6.89 and assayed over a range of pH values, enzyme incubated at pH 8.43 is about twice as active when assayed below pH 8.8. Above this pH the activities of the two enzyme preparations converge. Both enzyme forms have a peak of activity at pH 7.5.

This paper presents some simple kinetics of the hydrolysis of d-fructose 1,6-bisphosphate (Fru-1,6-P₂) catalyzed by rabbit liver fructose-1,6-bisphosphatase (EC 3.1.3.11, fructose-1,6-bisphosphate 1-phosphohydrolase) in the presence of a requisite divalent metal ion, in this case Mn²⁺. Despite the simplicity of the kinetics the measurement of the necessary rates has been made difficult by several factors. Firstly, apparent Km values for Fru-1,6-P₂ lie well below 1 μM, and thus the widely used spectrophotometric assay in which NADP⁺ is essentially competitively with Km = 2.7 μM at pH 7.5.

The kinetics of hydrolysis of n-fructose 1,6-bisphosphate (Fru-1,6-Pₙ) catalyzed by rabbit fructose-1,6-bisphosphatase, and to Fru-1,6-P₂, have been studied. Thirdly, rates have, under some conditions, been notably curved from the start. We have made an extensive study of this phenomenon, and initial rates reported here are believed to be reproducible and proportional to initial enzyme concentration.

The work which is reported here was developed by analyzing rates as a function of [free Fru-1,6-P₂], [Mn²⁺Fru-1,6-P₂], and [Mn²⁺Fru-1,6-P₂⁻], at a constant level of free Mn²⁺ (rather than total Mn²⁺), and in these respects it differed from the studies of Marcus et al. (9) and of Nimmo and Tipton (10). Consequently, effects arising from changes in the level of [Mn²⁺] or Fru-1,6-P₂ can be separated into simple substrate effects and "allosteric effects." In addition, the effect of pH on enzyme activity owing to ionizing groups on the protein could be more readily ascertained. Mn²⁺ was chosen in view of information which was already available concerning the binding of Mn²⁺ to fructose-1,6-bisphosphatase, and to Fru-1,6-P₂ (11).

MATERIALS AND METHODS

Unlabeled tetrasodium d-Fru-1,6-P₄, disodium d-Fru-6-P, NADP, NADPH, and bovine serum albumin were purchased from Sigma Chemical Co. Glucose-6-P dehydrogenase, type XI from Torula yeast (–350 units/ml, 25°C), and yeast phosphoglucose isomerase (2000 units/ml, 25°C) were obtained as crystalline suspensions in ammonium sulfate solution from Sigma. A mixture of the dehydrogenase (500 μl), isomerase (200 μl), and water (750 μl) was dialyzed against 50 mM Tris-Cl buffer, pH 7.5, 4°C, and the resulting clear solution was used in spectrophotometric assays. [³²P]Fru-1,6-P₂ (specific activity 110 Ci/mol), a gift of Dr. W. A. Frey and Dr. C. A. Capereelli, had been purified as previously described (12) with modifications (13). Unlabeled unlabeled n-Fru-1,6-P₄, disodium d-Fru-6-P, and MnCl₂ were purchased from Sigma, recrystallized from EtOH containing 0.005% EDTA, or ultrapure Tris base from Schwarz/Mann. NaCl and MnCl₂·4H₂O were purchased from Fisher Scientific Co., and MgCl₂·6H₂O was from J. T. Baker Chemical Co. Instrabray was provided by Yorktown Research, South Hackensack, N.J., and Chelex 109 by Bio-Rad Laboratories.

Measurements of pH were made on a Radiometer pH meter 22r with scale expander PHA630Pa at 25°C, standardized according to Data (14), and are accurate to ±0.01 pH unit. EPR measurements were made with a Varian E-line EPR spectrometer at 25°C, using a single silica capillary for all readings.

Fructose-1,6-bisphosphate, a gift of Dr. W. A. Frey and Dr. C. A. Capereelli, had been purified as previously described (6) with modifications (7). Unless used for pH-rate profile studies the enzyme was further purified by isoelectric focusing (pH 3.5 to 10), dialysis (90 mm Tris, pH 7.5), concentration by dialysis against saturated ammonium sulfate (pH 7.0), centrifugation, and dissolution of the precipitate in...
Fructose-1,6-bisphosphatase Kinetics with Mn$^{2+}$

and dialyze against 50 mM Tris buffer, pH 7.5. The enzyme was finally passed through a column of Chelex 100 in the same buffer and stored frozen at either -20°C or -180°C, at a concentration greater than 1 mg/ml. This enzyme preparation showed a single band of protein both after polyacrylamide gel electrophoresis at pH 9.5, and after sodium dodecyl sulfate electrophoresis. For rate measurements this enzyme solution was dialyzed and diluted to the final volume of the assay (2 ml) with 50 mM Tris-CI buffer, pH 7.5, containing 10 μg/ml of bovine serum albumin. This very dilute fructose-1,6-bisphosphatase was prepared freshly each day; identical assays at the start and finish of a series of assays showed that the enzyme was stable over this period. The stock frozen fructose-1,6-bisphosphatase from which these dilutions were undertaken suffered a small loss of activity, although the ratio of activity at pH 7.5 to activity at pH 9.2, measured by the method of Tranelli et al. (8), remained at 2.70, and sodium dodecyl sulfate electrophoresis confirmed that no change from the "neutral" to the "alkaline" form (7) of fructose-1,6-bisphosphatase had occurred.

Enzyme to be used in the pH-rate profile study was not taken through isoelectric focusing and the subsequent steps. The enzyme which was stored at -180°C at 7 mg/ml in 50 mM Tris buffer, pH 7.5, was thawed and used at once as described under "Results."

All fructose-1,6-bisphosphatase solutions were prepared and stored in polypropylene test tubes to minimize contamination by adventitious metal ions.

Fructose-1,6-Bisphosphatase Assays—Studies to be reported elsewhere showed that assays of fructose-1,6-bisphosphatase, particularly below pH 8, were often curved from the start. However, if the assays were initiated by addition of Fru-1,6-P₂, and if the rate was obtained from the earliest part of the curve, the rates were linear, reproducible, and proportional to the initial concentrations of fructose-1,6-bisphosphatase. Under these conditions EDTA, which has frequently been reported to activate fructose-1,6-bisphosphatase (9) was found to activate the enzyme only about 12% ([Mn²⁺] = 0.5 mM, [EDTA] = 0.1 mM) at pH 7.5 and was entirely omitted from the assays reported here.

All assays were performed at 25.0 ± 0.1°C, using only Tris-CI buffer. Preliminary experiments at pH 7.5 used a final concentration of 50 mM Tris and 30.96 mM Cl⁻. Buffers at higher pH were subsequently designed to contain 10.04 mM Tris base and below pH 8.1, and 10.04 mM protonated Tris above pH 8.1; Cl⁻ concentration was maintained at 39.96 mM with added NaCl. In buffers at pH values below 7.5 the total concentration of Tris was adjusted so that when the solution of the Tris base was brought to the desired pH with HCl, the final Cl⁻ concentration was 39.96 mM. By assaying fructose-1,6-bisphosphatase in the presence of NaClO₃ it was found that changes in ionic strength caused by these changes in the concentration of Tris had no effect on the rates.

Spectrophotometric assays of fructose-1,6-bisphosphatase were done with a Cary 219 recording spectrophotometer using the 0.0 to 0.02 absorbance capacity or with a Gilford 240 using the 0 to 0.1 absorbance capacity. In these assays, except as noted in the pH-rate profile study, fructose-1,6-bisphosphatase (~50 ng) was equilibrated for 15 min in the thermostatted cell compartment of the spectrophotometer, in a cuvette containing buffer and MnCl₂ (total volume ~0.96 ml). Then NADP (10 mM, 20 μl) and auxiliary enzymes (15 μl of the diazyl glucose-phosphate dehydrogenase and fructose-1,6-bisphosphate dehydrogenase solution) were added, and the assay was started by adding Fru-1,6-P₂ which brought the volume of the assay to 1 ml. The recorder was started 15 s after addition of Fru-1,6-P₂, and the rate was obtained from the increasing absorbance at 340 nm, using ε₅ = 6220 M⁻¹ cm⁻¹ (10). Alternatively, the rate of NADPH formation was monitored by the increase in fluorescence excitation at 340 nm on an Amino-Bozman spectrophotofluorometer.

In radioassays, fructose-1,6-bisphosphatase (~2 nm) was equilibrated at 25°C with buffer containing MnCl₂ and bovine serum albumin² (total volume ~1.96 ml). After 10 min, the reaction was started by adding Fru-1,6-P₂, bringing the volume of the assay to 2.0 ml. At intervals of 5, 10, and 15 min after adding Fru-1,6-P₂, aliquots (400 μl) of the reaction mixture were quenched with 50 μl of 70% HClO₄, and then each aliquot was treated with 400 μl of 0.1 M sodium ammonium molybdate (2.0 g of ammonium molybdate and 5.56 ml of concentrated H₂SO₄ made to 100 ml with H₂O (11), and 400 μl of water-saturated isobutyl alcohol/benzene (1:1 by volume), then vigorously shaken for 60 s and centrifuged to separate the layers. In this extraction procedure any P₁ in the original assay mixture was extracted into the upper layer, 250 μl of which were removed, added to 10 ml of Instabray solution, and counted with a Packard Tri-Carb scintillation counting system. The radioactivity level was established by extracting and counting an assay in which unlabeled Fru-1,6-P₂ (final concentration 4 μM) was substituted for [1-³²P]Fru-1,6-P₂. A nonenzyme control was obtained by preparing an assay with 4.42 μM [1-³²P]Fru-1,6-P₂ and no fructose-1,6-bisphosphatase, allowing it to remain for 15 min at 25°C, and then extracting and counting. Counts for nonenzymic controls at lower Fru-1,6-P₂ concentrations were calculated from the above two measurements by simple proportions. All enzyme reaction rates obtained by radioassay appeared to be linear for 15 min. The rates were calculated by comparison of counts released, with counts obtained from total hydrolysis of 4.42 μM Fru-1,6-P₂ as follows. An assay was prepared containing 4.42 μM [1-³²P]Fru-1,6-P₂ and also sufficient fructose-1,6-bisphosphatase so that all the substrate was hydrolyzed in less than 10 min. After 15 min the assay was quenched, extracted, and subsequently counted as a standard every time an enzyme assay was performed. Vₘₚ values from radioassays at pH 7.5 and 8.5 are fully consistent with figures from spectrophotometric assays and with the pH rate profile mentioned under "Results," which was done with a spectrophotometric assay.

Concentration of Free Mn²⁺ in Fructose-1,6-bisphosphatase Assays—As detailed in Table I, Mn²⁺ was assumed to complex with Cl⁻, Fru-1,6-P₂, P₁, Fru-6-P, and NADP. Dissociation constants quoted in Table I were taken to be independent of pH from pH 7.5 to pH 9.0. For each fructose-1,6-bisphosphatase assay a concentration of Fru-1,6-P₂ and a concentration of free Mn²⁺ were chosen, the final concentration of NADP in all spectrophotometric assays was 0.90 mM, and the concentration of Cl⁻ derived from buffer was always 39.96 mM. In each assay the total concentration of Mn²⁺ (added as MnCl₂) was calculated by computer, from Equation 1.

\[ [\text{Mn}]_{\text{total}} = [\text{Mn}]_{\text{free}} + [\text{Mn}]_{\text{complex}} = [\text{Mn}]_{\text{free}} + K_{\text{MnCl}} [\text{Cl}^-] / [\text{Mn}]_{\text{free}} \]

\[ K_{\text{MnCl}} = \frac{[\text{Mn}]_{\text{complex}}}{[\text{Mn}]_{\text{free}} [\text{Cl}^-]} \]

\[ [\text{NADP}] [\text{KADP}] = 2 [\text{Mn}]_{\text{free}} \]

\[ [\text{Fru-6-P}] [\text{P}] + K_{\text{Fru-6-P}} + \frac{[\text{Mn}]_{\text{free}}}{[\text{Mn}]_{\text{free}} + K_{\text{Fru-6-P}}} \]

Fru-6-P and P₁ were used only as inhibitors and the final two parts of the equation disappear if these two compounds are absent from the assays.

An attempt was made to measure the concentration of free Mn²⁺ in various assay solutions by EPR. The integrated peak heights of the Mn²⁺ sextet at 9.1 GHz from spectra of the assay solutions were compared with values for standard solutions of MnCl₂. The peak heights of the assay solutions were about 7% greater than expected had the Mn²⁺ signal of the chloride complex been negligible. Spectra of MnCl₂ run in the presence of NaCl confirmed that the additional Cl⁻ did not reduce the Mn²⁺ signal as much as expected, and quantitatively this observation accounted for the discrepancy of ~7% between the observed and calculated values.

RESULTS

Dependence of Fructose-1,6-bisphosphatase Rates on Concentrations of Fru-1,6-P₂ and Mn²⁺—Fructose-1,6-bisphosphatase was assayed at pH 7.5 with several concentrations of Fru-1,6-P₂ at different concentrations of free Mn²⁺. Selected results are illustrated as 1/V₀ against 1/[Total Fru-1,6-P₂] in Fig. 1; at [Mn²⁺] = 0.50, 1.6, 2.6, and 5.2 mM the linear plots showed a common intercept on the ordinate. Similar assays at pH 8.5 and 9.0 are shown in Fig. 2. Values of the slopes at various [Mn²⁺] at pH 7.5 and 9.0 are listed in Table II. It is noteworthy that there is no suggestion of cooperative effects as reported in an earlier study of the equilibrium binding of Fru-1,6-P₂ to the enzyme (4), a result which we are further investigating.

² Bovine serum albumin at 10 μg/ml was used to prevent loss of fructose-1,6-bisphosphatase activity at very low [enzyme]. Higher concentrations of bovine serum albumin resulted in incomplete recovery of radioactivity in radioassays, probably because of co-precipitation of P₁ and Fru-1,6-P₂ with albumin in the HClO₄ step.
Fructose-1,6-bisphosphatase Kinetics with Mn$^{2+}$

TABLE I

<table>
<thead>
<tr>
<th>Anion</th>
<th>Complex formed</th>
<th>$K_m$ $^\text{a}$</th>
<th>Reference</th>
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<tr>
<td>Cl$^-$</td>
<td>MnCl$^+$</td>
<td>2.70 ($K_1$)</td>
<td>12</td>
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<tr>
<td>Fru-1,6-P$_2$</td>
<td>MnFru-1,6-P$_2$</td>
<td>0.476 ($K_r$)</td>
<td>4</td>
</tr>
<tr>
<td>MnFru-1,6-P$_2$</td>
<td>MnFru-1,6-P$_2$</td>
<td>0.476 ($K_m$)</td>
<td>4</td>
</tr>
<tr>
<td>NADP</td>
<td>MnNADP</td>
<td>0.781 ($K_{NADP}$)</td>
<td>13</td>
</tr>
<tr>
<td>MnNADP</td>
<td>MnNADP</td>
<td>10 ($K_{NADP}$)</td>
<td>b</td>
</tr>
<tr>
<td>Fru-6-P</td>
<td>MnFru-6-P</td>
<td>3.23 ($K_{Fru}$)</td>
<td>c</td>
</tr>
<tr>
<td>HPO$_4^{2-}$</td>
<td>MnHPO$_4$</td>
<td>4.6 ($K_r$)</td>
<td>14</td>
</tr>
<tr>
<td>Fru-1,6-P$_2$</td>
<td>MgFru-1,6-P$_2$</td>
<td>4.0 ($K_r$)</td>
<td>3, 15</td>
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<tr>
<td>MgFru-1,6-P$_2$</td>
<td>MgFru-1,6-P$_2$</td>
<td>4.0 ($K_{Mg}$)</td>
<td>3, 15</td>
</tr>
</tbody>
</table>

$^a$ Dissociation constants used in Equation 1.
$^b$ Personal communication from J. J. Villafranca.
$^c$ W. A. Frey, unpublished measurement.

Assays at pH 8.5 and above in Tris-Cl buffer were complicated by atmospheric oxidation (presumably of Mn$^{2+}$ to Mn$^{3+}$) dependent on [OH$^-$] and [buffer] with a rapidly increasing rate above pH 8.8. Thus above pH 9.0 difficulty in measuring rates was experienced even after solutions had been prepared under N$_2$ and mixed in a N$_2$-filled glove bag, because of residual traces of O$_2$.

Values of $K_m$ for total Fru-1,6-P$_2$ at pH 7.5, 8.5, and 9.0 and several different concentrations of Mn$^{2+}$ are set out in Table III. The following conditions were chosen for the pH-rate profile measurements described below: [Mn$^{2+}$] = 36 µM (A); 1.6 mM (X); 2.6 mM (O); 5.2 mM (C); and 9.9 mM (C).

Dependence of Fructose-1,6-bisphosphatase Activity on pH—Preliminary rate measurements indicated the presence of a more active form of fructose-1,6-bisphosphatase at higher pH and a less active form at lower pH, in general agreement with a previous report (16). The pH dependence of the transition between the two forms of the enzyme was established as follows. Fructose-1,6-bisphosphatase (103 µg/ml) was incubated for 15 min at 25°C in each of a series of buffers with pH values from 6.89 to 9.19 containing 50 µM MnCl$_2$. After this a 10-µl aliquot of enzyme solution was assayed by adding it to a cuvette at 25°C containing all the necessary components for a spectrophotometric assay at pH 7.5 in 0.99 ml ([Mn$^{2+}$] = 36 µM, [total Fru-1,6-P$_2$]$_0$ = 10 µM). The fructose-1,6-bisphosphatase activity was approximately constant at pH 7.18 but fell by almost 50% between 8.18 and pH 6.89 (Fig. 3). Further measurements showed firstly that any change

![Figure 1](http://www.jbc.org/)  
Fig. 1. Selected plots of $1/U_0$ versus $1/([\text{total Fru-1,6-P}_2]_0$) at pH 7.5 and for [Mn$^{2+}$] = 0.50 mM (A); 1.6 mM (X); 2.6 mM (O); 5.2 mM (C); and 9.9 mM (C).

![Figure 2](http://www.jbc.org/)  
Fig. 2. Plots of $1/U_0$ against $1/([\text{total Fru-1,6-P}_2]_0$ for fructose-1,6-bisphosphatase, pH 8.5 (A) and pH 9.0 (B). [Mn$^{2+}$] = 36 µM (A); 72 µM (C); 220 µM (O).

TABLE II

<table>
<thead>
<tr>
<th>Slope µM mg/unit</th>
<th>[Mn$^{2+}$] $^a$</th>
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<tbody>
<tr>
<td>0.109 $^a$</td>
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<tr>
<td>0.092</td>
<td>0.072</td>
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<tr>
<td>0.060</td>
<td>0.145</td>
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<tr>
<td>0.029</td>
<td>0.50</td>
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<tr>
<td>0.042</td>
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<tr>
<td>0.068</td>
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<tr>
<td>0.127</td>
<td>5.20</td>
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<tr>
<td>0.350 $^a$</td>
<td>0.036</td>
</tr>
<tr>
<td>0.225</td>
<td>0.072</td>
</tr>
<tr>
<td>0.175</td>
<td>0.220</td>
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$^a$ pH = 7.5.

TABLE III

<table>
<thead>
<tr>
<th>pH</th>
<th>[Mn$^{2+}$] $^a$</th>
<th>$K_m$ for total Fru-1,6-P$_2$ $^a$</th>
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<tr>
<td>7.5</td>
<td>9.9</td>
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<tr>
<td>5.2</td>
<td>363</td>
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</tr>
<tr>
<td>2.6</td>
<td>170</td>
<td></td>
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<tr>
<td>1.6</td>
<td>107</td>
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<tr>
<td>0.50</td>
<td>37.5</td>
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<tr>
<td>0.072</td>
<td>261</td>
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<tr>
<td>0.036</td>
<td>332</td>
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<tr>
<td>0.072</td>
<td>500</td>
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<tr>
<td>2.22</td>
<td>731</td>
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<tr>
<td>0.22</td>
<td>1100</td>
<td></td>
</tr>
<tr>
<td>0.072</td>
<td>1500</td>
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</tbody>
</table>

$^a$ pH = 9.0.
The results reported here will be considered in conjunction with those reported by Marcus et al. (2) for the hydrolysis of Fru-1,6-P₂ by bovine liver fructose-1,6-bisphosphatase with Mn²⁺ at pH 7.5 and with those of Nimmo and Tipton (3) for hydrolysis of Fru-1,6-P₂ by the bovine liver enzyme with Mg²⁺ at pH 7.2 and 9.5.

Our results from studies in which concentrations of Fru-1,6-P₂ and Mn²⁺ were varied at pH 7.5, 8.5, and 9.0 can be generalized as follows. (i) at [Mn⁺] ≥ 0.5 μM (pH 7.5) or 72 μM (pH 9.0) the plots of 1/υ₀ against 1/[total Fru-1,6-P₂] have a common intercept on the ordinate. Below this concentration the value of the intercept increases as [Mn⁺] decreases; (ii) at [Mn⁺] > 500 μM the slopes of these plots increase linearly with increasing [Mn⁺], while below this value the slopes increase as [Mn⁺] decreases. Data collection over a wide range of [Mn⁺] is experimentally restricted. At pH ≥ 8.5 and [Mn⁺] ≥ 220 μM, an additional difficulty is encountered owing to rapid oxidation of the metal ion which kinetically exhibits a greater than first order dependence on the latter's concentration.

Nimmo and Tipton (3) found that plots of 1/υ₀ against 1/[total Fru-1,6-P₂] for the bovine liver enzyme were linear with the value of the intercept on the ordinate increasing with decreasing [Mg⁺] at pH 7.2 and 9.5. Likewise, the slopes decreased as [Mg⁺] increased at pH 7.2 and 9.5. At pH 7.2, the change in slope could not be described in terms of either a direct or reciprocal dependence on [Mg⁺]. The [Mg⁺] levels ranged from 0.17 to 1.0 mM. Marcus et al. (2) also reported that plots of 1/υ₀ against 1/[total Fru-1,6-P₂] for the bovine liver enzyme at [Mg⁺] ≥ 4.9 mM were linear but with a common intercept on the ordinate; at [Mg⁺] = 0.4 mM the intercept on the ordinate is larger. For [Mg⁺] ranging from 4.9 to 49.9 mM the slopes increased with increasing [Mg⁺].

Thus it seems (a) that below a certain concentration of M⁺ the double reciprocal plots have an increasing intercept on the ordinate with decreasing [M⁺]. Above this concentration the plots intercept on the ordinate. (b) Above a second concentration of M⁺ which may or may not be the same as in (a), depending on the nature of the metal ion or the pH, or both, the slopes increase with increasing [M⁺]. Below this second concentration of M⁺ the slopes increase with decreasing [M⁺]. The double reciprocal plot at pH 7.5, 9.9 mM Mn⁺, reproduced a higher intercept and lower slope than expected from these generalizations (see Fig. 1). It is quite reasonable that this very high [Mn⁺] (about 10⁻² higher than physiological levels, as noted below) could have effects on the enzyme function unrelated to the simple kinetic schemes to be discussed. Earlier we demonstrated that the fall off in enzymic activity at Mn⁺ levels > 5 mM (up to 100 mM) could be correlated by binding of FM to an allosteric site on the enzyme (4).

Several kinetic schemes have been considered to account for these results. These schemes assume that fructose-1,6-bisphosphatase from beef and rabbit liver use the same catalytic processes. Fru-1,6-P₂ is assumed to bind two metal ions with dissociation constants which are very similar (4). This assumption is necessary in all schemes and, as will be seen, for the value of the slope term going through a minimum as [M⁺] increases. A previous study (4) has shown that, at the concentrations of free Mn⁺ used in the present work, fructose-1,6-bisphosphatase will have at least one Mn⁺ bound per subunit. Furthermore, the linearity of the above reciprocal plots suggests the four subunits of the enzyme are functioning independently, at least under these conditions. Thus the binding of this "structural" metal ion is taken into consideration.

**DISCUSSION**

**Inhibitor Studies**—At pH 8.5, Fru-6-P at 0.1 and 0.25 mM was found to inhibit competitively, with values for Kᵢ of 64.6 and 75.8 μM, respectively. If the actual inhibitor species were the MnFru-6-P complex, the Kᵢ values would become 76.0 and 892 μM. Inorganic phosphate at 5 and 10 mM produced inhibition which was essentially competitive. In experiments and 0.892 estimated from the data obtained in radioassays. Using this value, an average Kᵢ for P₀ (assuming competitive inhibition) was 2.7 ± 0.4 mM. If the species actually inhibiting were the MnP complex the Kᵢ would fall to 22 ± 3 μM.

**FIG. 3.** Specific activity of fructose-1,6-bisphosphatase incubated at different pH values and assayed at pH 7.5.

**FIG. 4.** pH rate profile of fructose-1,6-bisphosphatase incubated at pH 6.89 (Δ) or pH 8.43 (○).
in the following kinetic schemes, and E represents one active subunit of enzyme, F represents Fru-1,6-P₂, and M represents a single divalent metal ion. Our experiments showed that Fru-6-P and P, are inhibitors, both apparently competitive with respect to Fru-1,6-P₂. This finding is consistent with a rapid equilibrium random release of products, though such a conclusion is not demanded. Since any order in which products may be released has therefore not been established, this detail is omitted from the following kinetic schemes.

1. Fructose-1,6-bisphosphatase binds with FM directly and hydrolyzes:

\[
E + M \rightleftharpoons EM + F + M \rightleftharpoons FM, \quad E + FM \rightleftharpoons EMF
\]

Assuming rapid equilibrium binding

\[
\frac{1}{v_o} = \frac{K_a(K_a + [M])}{V} + \frac{K_a + [M]}{V[F][M]}
\]

From Equation 8 it can be seen that this scheme does not account for the dependence of the intercept of a double reciprocal plot upon [M] at low values of [M] and consequently is discarded. It is of interest that Equation 8 accounts for the observed dependence of the slopes of double reciprocal plots upon [M] because [FM] is at a maximum when [M] = \(\sqrt{K_F K_{FM}}\). This situation occurs only if Fru-1,6-P₂ binds two metal ions.

2. Random equilibrium:

\[
E + M \rightleftharpoons EM + F + M \rightleftharpoons EMF \rightleftharpoons EMF \rightleftharpoons E + M + F + M \rightleftharpoons E + M + F + M
\]

Here using rapid equilibrium assumptions

\[
\frac{1}{v_o} = \frac{K_a(K_a + [M])}{K_a V[M][F]} + \frac{K_a + [M]}{V[M]}
\]

or

\[
\frac{1}{v_o} = \frac{K_a(K_a + [M])}{K_a V[M][F]} + \frac{K_a + [M]}{V[M]}
\]

For the rabbit liver enzyme with \(Mn^{2+}\), \(K_a \gg [M] \gg K_c\), since EPR measurements have shown that the enzyme binds allosterically to four \(Mn^{2+}\) ions per enzyme tetramer in the absence of substrate with the approximate dissociation constants being 0.3 to 15 \(\mu M\), corresponding to \(K_c\). No sign of further binding of \(Mn^{2+}\) ions to the enzyme was observed which would indicate \(K_c > 10^{5} \mu M\).

In Fig. 5 is plotted the term \((K_a V + K_{FM}[M] + [M]^2)/K_a K_{FM}[M]\) as a function of [M], in order to illustrate the dependence of the slope values on [M] since the remaining factor, \(K_a/V\), is constant at a fixed pH and enzyme concentration. The theoretical line charts the reciprocal fraction of (F)×[M] for the Mn^{2+} assay at pH 7.5. It is notable that all slopes increase at \([Mn^{2+}] > 0.5 \mu M\), while slopes below that concentration increase sharply with decreasing \([Mn^{2+}]\). The slope term has a maximum value at [M] = \(\sqrt{K_F K_{FM}}\) which for \([Mn^{2+}]\) = 0.48 mm, the slope data at pH 9.0 (Table II) decrease in magnitude with increasing \([Mn^{2+}]\) consistent with the \([Mn^{2+}]\) < 0.5 mm. No marked change in the magnitude of \(K_a\) or \(K_{FM}\) is anticipated since the phosphoryl moiety is largely dissociated at both pH values. The fact that the slope term can depend on [1/[M]] or [M] accounts for the intermediate behavior observed by Nimmo and Tipton in replots of slope against 1/[M] (3). Moreover their slope replot at pH 9.5 which, though linear, extrapolates to a negative value of 1/[M] on the abscissa.

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Qualitatively, Equation 11 accounts for the constancy of the double reciprocal intercept at high [M] and for the increase in the intercept as [M] approaches and becomes less than \(K_a\). Our data (Figs. 1 and 2) show that the intercept is independent of \([Mn^{2+}]\) down to a value of 500 \(\mu M\) (pH 7.5) and 72 \(\mu M\) (pH 9.0). Below this value the intercept increases as \([Mn^{2+}]\) decreases as predicted in Equation 11. Data from Marcus et al. (2) show that at pH 6.5 the intercept is independent of \([Mn^{2+}]\) down to a value of 4.9 mm, below which the intercept increases. The intercept in the work of Nimmo and Tipton (3) at pH 7.5 conforms with the behavior expected in that at low values of \([Mn^{2+}]\) the plot of intercept against 1/[M] is linear and would pass through the origin if extrapolated. At higher values of \([Mn^{2+}]\) the value of \(K_a\) is approached, and the plot deviates upward accordingly. We have no explanation for their intercept replot at pH 9.5 which, though linear, extrapolates to a negative value of 1/[M] on the abscissa.

Both studies of the beef enzyme would place \(K_a\) at ~1 mm (2, 3).

Equation 11 also accounts for the dependence of slope on [M] if \(K_a \gg [M] \gg K_c\). Consequently the slope term of Equation 11 reduces to

\[
slope = \frac{K_a K_{FM} + K_{FM}[M] + [M]^2}{V[M]} \cdot \frac{V[M]}{K_a K_{FM}}
\]

At high [M]

\[
slope = \frac{K_a}{V[M]}
\]

and at low [M]

\[
slope = \frac{K_c}{V[M]}
\]

For the rabbit liver enzyme with \(Mn^{2+}\), \(K_a \gg [M] \gg K_c\), since EPR measurements have shown that the enzyme binds allosterically to four \(Mn^{2+}\) ions per enzyme tetramer in the absence of substrate with the approximate dissociation constants being 0.3 to 15 \(\mu M\), corresponding to \(K_c\). No sign of further binding of \(Mn^{2+}\) ions to the enzyme was observed which would indicate \(K_c > 10^{5} \mu M\).
3. Rapid equilibrium ordered.

This mechanism is simply a limiting case of the random sequence in which one pathway totally dominates. Consequently two versions are possible, the first involving the ordered binding to EM of M followed by F, and the second the converse. Again using rapid equilibrium assumptions and the above designations, the respective rate relationships are:

$$ \frac{1}{v} = \frac{K_{s1}K_{s2}[M]K_{s3}[M]+}[M]^2]}{V[M][F]K_{s1}K_{s2} + 1} $$

(15)

$$ \frac{1}{v} = \frac{K_{s1}K_{s2}K_{s3} + [M]K_{s4}K_{s5} + [M]^2]}{V[M][F]K_{s1}K_{s2} + 1} + \frac{K_{s6}[M]}{V[M]} $$

(16)

The intercept term of Equation 15 is completely independent of [M] which argues against this scheme. On the other hand, Equation 16 seems fully consistent with the experimental data, as follows: (i) the slope reduces to Equations 13 and 14 under conditions of high and low [M], respectively, and (ii) the intercept term is identical with that in Equation 11.

Thus, of the kinetic schemes discussed, the two which appear to be consistent with the data are the random equilibrium and the rapid equilibrium ordered with F binding before the catalytic metal ion. It is important to point out that the latter scheme can be treated employing a steady state assumption for the intermediate EMF and EMFM species which results in an analogous kinetic equation predicting identical behavior for $v_0$ as a function of [F] or [M] although obviously the numerical evaluation would involve partition coefficients rather than equilibrium constants. In the absence of evidence for the formation of an EMF species, we will assume for a working hypothesis the rapid equilibrium or steady state ordered scheme since it adequately describes with the fewest assumptions all presently available data. This explanation implicates the presence of a binding site for the catalytic metal ion with greater affinity than derived from cationization to the phosphoryl moiety. It also infers that the rate inhibition observed at very high Mn$^{2+}$ might be associated with the formation of less reactive MEFM$^2$ complexes.

On this basis some values for the various constants can be calculated. From Fig. 5, $K_{s1}K_{s2} = 15.2 \mu M^2$ (pH 7.5) and if $K_{s1}$ is taken to be 30 $\mu M$, $K_{s2} = 0.5 \mu M$ for the rabbit liver enzyme with Mn$^{2+}$. The presumed value for $K_{s1}$ based on the assumption the $K_{s1} \approx 0.2 \mu M$ when the intercept term becomes sensitive to [M], can be compared with the measured binding constants for the second four Mn$^{2+}$ ions to rabbit liver fructose-1,6-bisphosphatase in the presence of the competitive inhibitor ($\alpha + \beta$)methyl-1,6-fructofuranoside-1,6-P$_2$; the values are 12.5 to 200 $\mu M$ (4) and are consistent with the assumed value of 30 $\mu M$. For the bovine enzyme the value of $K_{s1}$ calculated from the reciprocal of the intercepts against 1/[EM$^{2+}$] from Fig. 1 in Ref. 3, is 0.64 mm (pH 7.5). Both values for $K_{s1}$ indicate that the binding of M to EMF is greater than to free F (EMF), by a factor of six for Mg$^{2+}$ and sixteen for Mn$^{2+}$.

Let us now compare the values obtained for Mn$^{2+}$ with the appropriate physiological concentrations. The amount of manganese reported in rabbit liver is 0.3 mmol/kg of dried liver (17), corresponding to about 90 $\mu M$ of moist liver. The level of free Mn$^{2+}$ in cytoplasm should be much lower than 90 $\mu M$ because of its binding to proteins and phosphate-containing compounds *inter alia*. By analogy, the concentration of total Mg$^{2+}$ in liver cells was reported as 10.4 nm (18) while that of free Mg$^{2+}$ was measured at 0.6 to 1.3 mm. The concentration of free Mn$^{2+}$ is very close to $K_{s1}$ and also to $K_{s2}$ for the beef liver enzyme indicating that slight changes in the physiological concentration of free Mg$^{2+}$ may have a major effect on the kinetic behavior of the enzyme. Taking the concentration of free Mn$^{2+}$ in rabbit livers to be about 9 $\mu M$ (assuming a parallel to the Mg$^{2+}$ partitioning), this is close to the estimated value of $K_{s1}$ again. These calculations suggest that the enzyme, whether it is reacting with Mg$^{2+}$ or Mn$^{2+}$ as cofactor, will be very sensitive to slight changes in metal ion concentration.

Various estimates of Fru-1,6-P$_2$ in livers have been made (19-22). Values range from about 10 $\mu M$ in the cytosol of gluconeogenic rat livers (19) to above 50 nmol/g in other unstarved livers (20-22). The concentration of fructose-1,6-bisphosphatase active sites in liver is estimated to be at least 10 $\mu M$. Thus, there is a strong possibility that, especially under conditions of gluconeogenesis, the concentration of Fru-1,6-P$_2$ in liver cells will be of the same order as that of enzyme-active sites. This will mean that the assumption [F] $\gg$ [E] will no longer be tenable and that the kinetics of fructose-1,6-bisphosphatase under physiological conditions will become more complex than here described. In this context Achs et al. (19) constructed a kinetic model for gluconeogenesis but encountered difficulty in matching the low measured rate of glucose production with the higher value of fructose-1,6-bisphosphatase activity, which ought to have been rate limiting. In their study the rate equations were set up assuming [S] $\gg$ [E]. However, the estimates noted above suggest that the substrate concentrations may limit flux through fructose-1,6-bisphosphatase in gluconeogenesis, and that if this is taken into consideration, fructose-1,6-bisphosphatase may become the bottleneck for gluconeogenesis that was expected.

It is striking that fructose-1,6-bisphosphatase undergoes a reversible activation as the pH increases (16), an effect which is controlled by a group of pK$_a$ ~ 7.6, and which is apparently complete in 15 min. Because of this transition the pH-rate profile was determined with enzyme preincubated at pH 6.89 and 8.43, at the two extremes of activity noted in Fig. 4. The pH-activity profiles for the “two forms” both show a peak at pH 7.5. A part of the fall in activity below pH 7.4 is attributed to protonation of tetraanionic Fru-1,6-P$_2$ (with a pK$_a$ ~ 7.0 (15)). It can be shown that the effect of the equilibria of $H^+$ with Fru-1,6-P$_2$ and of Mn$^{2+}$ with HPrU-1,6-P$_2$ in Equation 12 is an expanded slope term; thus, the slope of the double reciprocal plot at pH 7 should be approximately twice that at pH $\geq 8$. However, a much larger factor in the slope term would be required to account for the difference in activity between pH 6.9 and 7.4, and it seems reasonable to suggest that this difference is caused by a modest increase in $K_{s1}$ as the pH falls, leading to an increase in both the slope and...
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intercept terms of Equation 11. In physical terms this could be due to protonation of the EMF complex, making association with the catalytic M less favorable. At pH 9.2, the activity of the enzyme becomes independent of the incubation conditions suggesting that the transformation between the two forms is base catalyzed, or, alternatively, that both forms have the same specific activity. Under physiological conditions both the pH-rate profile effect and the preincubation effect have the same consequence. When the pH of the tissues is decreased, as happens after intense physical activity, for example, the activity of fructose-1,6-bisphosphatase is severely reduced, possibly leading to a throttling back of gluconeogenesis (19). Increasing the pH above 7.5, while of limited physiological importance, would hardly affect the activity of the enzyme because the fall of activity observed in the pH-rate profile in the pH 7.5 to 7.9 region is accompanied by a rise of similar magnitude in the preincubation profile up to pH 8.2.

In conclusion, various effects of metal ion concentration on the catalysis of fructose-1,6-bisphosphatase have been determined. It is of interest that in each of the kinetic schemes described, the equilibrium between E and M defined by $K_r$ only affects the slope of the double reciprocal plots, and not the intercept term. Inhibition by high [Fru-1,6-P$_2$] which has been reported elsewhere (3, 4, 23) was not encountered here, due to the low [Fru-1,6-P$_2$] employed. Such inhibitory effects were not included in the scheme.

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Fructose 1,6-bisphosphate. Kinetics of hydrolysis catalyzed by rabbit liver neutral fructose-1,6-bisphosphatase with Mn2+.

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