Fructose 1,6-Bisphosphate

KINETICS OF HYDROLYSIS CATALYZED BY RABBIT LIVER NEUTRAL FRUCTOSE-1,6-BISPHOSPHATASE WITH Mn**

Nicholas P. B. Dudman, Margaret M. deMaine, and Stephen J. Benkovic‡

From the Department of Chemistry, Davey Laboratory, The Pennsylvania State University, University Park, Pennsylvania 16802

(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 Mn** 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 [1-32P]Fru-1,6-P2. Data from experiments in which [Fru-1,6-P2] and [Mn**] 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 Mg** as cofactor (Marcus, C. J., Geller, A. M., and Bryne, W. L. (1973) J. Biol. Chem. 248, 8567-8573; Nimmo, H. G., and Tipton, K. F. (1975) Eur. J. Biochem. 58, 567-574). Fructose 6-phosphate is a competitive inhibitor with Ki ~ 70 μM at pH 8.5, and inorganic phosphate inhibits essentially competitively with Ki = 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-Cl buffer with Mn**. 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 8.5. 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-bisphosphatase 1-phosphohydrolase) in the presence of a required divalent metal ion, in this case Mn**. 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 reduced in the final step (1) is not sufficiently sensitive to measure rates at substrate concentrations below Km. We have used an assay in which the [15P]orthophosphate released from [1-32P]Fru-1,6-P2 is separated from unhydrolyzed Fru-1,6-P2 and counted, for many rate measurements. Secondly, solutions of MnCl2 in Tris buffers undergo atmospheric oxidation at alkaline pH, and thus assays at and above pH 8.5 have been carried out in deoxygenated solutions. 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], [MnFru-1,6-P2], and [MnFru-1,6-P2]-, at a constant level of free Mn** (rather than total Mn**), and in these respects it differed from the studies of Marcus et al. (3) and of Nimmo and Tipton (4). Consequently, effects arising from changes in the level of Mn** 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. Mn** was chosen in view of information which was already available concerning the binding of Mn** to fructose-1,6-bisphosphatase, and to Fru-1,6-P2 (4).

MATERIALS AND METHODS

Unlabeled tetrasodium d-Fru1,6-P4 disodium d-Fru6-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. [1-32P]D-Fru1,6-P (specific activity 110 Ci/mol), a gift of Dr. W. A. Frey and Dr. C. A. Capereili, was prepared starting with carrier-free [32P]H3PO4, and was diluted where necessary with unlabeled n-Fru-1,6-P4. The concentrations of stock solutions of Fru-1,6-P2 and Fru-6-P were monitored using the spectrophotometric assay described below for fructose-1,6-bisphosphatase. Tris base used in the preparation of buffers was either Trizma 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. Intrabray was provided by Yorktown Research, South Hackensack, N.J., and Chelex 100 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 Dace (5), 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-bisphosphatase, a gift of Dr., W. A. Frey and Dr. C. A. Capereili, 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 9), dialysis (90 mm Tris, pH 7.5), concentration by dialysis against saturated ammonium sulfate (pH 7.0), centrifugation, and dissolution of the precipitate in.

† N. P. B. Dudman and S. J. Benkovic, unpublished results.

‡ To whom correspondence should be addressed.
and dialyzed 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 was thawed and diluted to a concentration of 0.05 µg/ml in 50 mM Tris Cl 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 made underwent a slight loss of activity, although the ratio of activity at pH 7.5 to activity at pH 9.2, measured by the method of Tranin 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 90 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 Fr-P, 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% ([Mn+] = 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-Cl buffer. Preliminary experiments at pH 7.5 used a final concentration of 50 mM Tris and 39.96 mM Cl-. Buffers at higher pH were subsequently designed to contain 10.04 mM Tris base at 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 NaClO4 it was found that changes in ionic strength caused by these changes in the concentration of Tris and 10.04 mM protonated Tris above pH 8.1; Cl- concentration was always 39.96 mM. 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.

In assays to be reported elsewhere, a final concentration of 250 µM NADP (10 µM, 20 µM) and auxiliary enzymes (15 µl of the dialyzed and concentrated fructose-1,6-bisphosphatase) was equilibrated for 15 min 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 calculated from the above two measurements by simple proportion.

Spectroscopic rates. The integrated area of the Mn2+ signal of the chloride complex had the Mn2+ signal of the chloride complex been negligible. Spectra of Mn2Cl2 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 Fr-1,6-P2 and Mn2+—Fructose-1,6-bisphosphatase was assayed at pH 7.5 with several concentrations of Fr-1,6-P2 and Mn2+ at different concentrations of free Mn2+. Selected results are illustrated as 1/θA against 1/[Total Fr-1,6-P2], in Fig. 1; at [MnCl2] = 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 [MnCl2] 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 Fr-1,6-P2 to the enzyme (4), a result which we are further investigating.

Downloaded from http://www.jbc.org/ by guest on October 31, 2017
Fructose-1,6-bisphosphatase Kinetics with Mn$^{2+}$

TABLE I

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

* Dissociation constants used in Equation 1.

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 (Δ), 52 μM (○), and 9.9 μM (●).

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$^2$-1. 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 0.990 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 ≤ 8.18 but fell by almost 50% between 8.18 and pH 6.89 (Fig. 3). Further measurements showed firstly that any change

![Fig. 1. Selected plots of 1/ν₀ versus 1/(total Fru-1,6-P$_2$)$_0$ at pH 7.5 and for [Mn$^{2+}$] = 0.50 mm (△); 1.6 mm (●); 2.6 mm (○); 5.2 mm (●); and 9.9 mm (●).](http://www.jbc.org/)

![Fig. 2. Plots of 1/ν₀ against 1/(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 (△); 72 μM (○); 220 μM (○).](http://www.jbc.org/)

![Fig. 3. Specific activity of fructose-1,6-bisphosphatase at pH 7.5 and 8.5 at various [Mn$^{2+}$].](http://www.jbc.org/)

![Fig. 4. Table II. Values for slope of double reciprocal plots as in Fig. 1 at the indicated [Mn$^{2+}$].](http://www.jbc.org/)

**TABLE II**

<table>
<thead>
<tr>
<th>Slope pM mg/unit</th>
<th>[Mn$^{2+}$] (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.109*</td>
<td>0.036</td>
</tr>
<tr>
<td>0.049</td>
<td>0.072</td>
</tr>
<tr>
<td>0.080</td>
<td>0.145</td>
</tr>
<tr>
<td>0.029</td>
<td>0.050</td>
</tr>
<tr>
<td>0.030</td>
<td>1.60</td>
</tr>
<tr>
<td>0.068</td>
<td>2.60</td>
</tr>
<tr>
<td>0.127</td>
<td>5.20</td>
</tr>
<tr>
<td>0.350*</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>
</tr>
</tbody>
</table>

* pH = 7.5.

**TABLE III**

<table>
<thead>
<tr>
<th>pH</th>
<th>[Mn$^{2+}$] (μM)</th>
<th>$K_m$ for total Fru-1,6-P$_2$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>9.9</td>
<td>173</td>
</tr>
<tr>
<td>8.0</td>
<td>5.2</td>
<td>363</td>
</tr>
<tr>
<td>1.6</td>
<td>2.6</td>
<td>170</td>
</tr>
<tr>
<td>1.6</td>
<td>1.5</td>
<td>107</td>
</tr>
<tr>
<td>0.30</td>
<td>0.50</td>
<td>375</td>
</tr>
<tr>
<td>8.5</td>
<td>0.072</td>
<td>261</td>
</tr>
<tr>
<td>0.036</td>
<td>0.072</td>
<td>332</td>
</tr>
<tr>
<td>9.0</td>
<td>0.22</td>
<td>731</td>
</tr>
<tr>
<td>0.072</td>
<td>0.072</td>
<td>1100</td>
</tr>
<tr>
<td>0.036</td>
<td>0.036</td>
<td>1500</td>
</tr>
</tbody>
</table>
inhibition which was essentially competitive. In experiments and 0.892 estimated from the data obtained in radioassays. Using this was found to inhibit competitively, with values for cuvettes at 25°C containing all the assay components at each pH (Fig. 4).

Two Tris-HCl buffer at either pH 8.43 or pH 6.89, each containing the MnFru-6-P complex, the and 75.8 pm with Pi, the value, an average of 64.6 & 0.4 mM. If the species actually inhibiting were the MnPi complex the value, an average would fall to 22 ± 3 μM.

which was caused by putting the native enzyme (at pH 7.5) into buffer at a different pH was apparently complete in 15 min, and secondly that such a change in activity was fully reversible in 15 min by returning the enzyme to pH 7.5. Two pH-rate profiles were prepared by incubating the enzyme in Tris-HCl buffer at either pH 8.43 or pH 6.89, each containing 50 μM MnCl₂, for at least 15 min at 25°C and by adding 10-μl aliquots of one of these enzyme solutions (98.2 μg/ml) to cuvettes at 25°C containing all the assay components at each pH (Fig. 4).

**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 0.760 and 0.892 μM. Inorganic phosphate at 5 and 10 mM produced inhibition which was essentially competitive. In experiments with Pᵢ, the Kᵢ for Fru-1,6-P₂ in the absence of Pᵢ was estimated from the data obtained in radioassays. Using this value, an average Kᵢ for Pᵢ (assuming competitive inhibition) was 2.7 ± 0.4 mM. If the species actually inhibiting were the MnPᵢ complex the Kᵢ would fall to 22 ± 3 μM.

**DISCUSSION**

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 Mg²⁺ at pH 6.5 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 [MnFru₂⁺] ≥ 0.5 mM (pH 7.5) or 72 μM (pH 9.0) the plots of 1/V₀ against 1/([total Fru-1,6-P₂]) have a common intercept on the ordinate. Below this concentration the value of the intercept increases as [MnFru₂⁺] decreases; (ii) at [MnFru₂⁺] > 500 μM the slopes of these plots increase linearly with increasing [MnFru₂⁺], while below this value the slopes increase as [MnFru₂⁺] decreases. Data collection over a wide range of [MnFru₂⁺] is experimentally restricted. At pH ≥ 8.5 and [MnFru₂⁺] ≥ 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/V₀ 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 [MgFru₂⁺] at pH 7.2 and 9.5. Likewise, the slopes decreased as [MgFru₂⁺] 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 [MgFru₂⁺]. The [MgFru₂⁺] levels ranged from 0.17 to 1.0 mM. Marcus et al. (2) also reported that plots of 1/V₀ against 1/([total Fru-1,6-P₂]) for the bovine liver enzyme at [MgFru₂⁺] ≥ 4.9 mM were linear but with a common intercept on the ordinate; at [MgFru₂⁺] = 0.4 mM the intercept on the ordinate is larger. For [MgFru₂⁺] ranging from 4.9 to 49.9 mM the slopes increase with increasing [MgFru₂⁺].

Thus it seems (a) that below a certain concentration of M²⁺ the double reciprocal plots have an increasing intercept on the ordinate with decreasing [M²⁺]. Above this concentration the plots intercept on the ordinate. (b) Above a second concentration of M²⁺ 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 [M²⁺]. Below this second concentration of M²⁺ the slopes increase with decreasing [M²⁺]. The double reciprocal plot at pH 7.5, 9.9 mM Mn²⁺, reproduced a higher intercept and lower slope than expected from these generalizations (see Fig. 1). It is quite reasonable that this very high [MnFru₂⁺] (about 10² 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 Mn²⁺ levels > 5 mM (up to 100 mM) could be correlated by binding of FM₂ 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 [M²⁺] increases. A previous study (4) has shown that, at the concentrations of free Mn²⁺ used in the present work, fructose-1,6-bisphosphatase will have at least one Mn²⁺ 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.
in the following kinetic schemes, and E represents one active subunit of enzyme, F represents Fru-1,6-P_2, 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-P_2. 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:

\[
E + M \rightleftharpoons EM
\]

\[
F + M \rightleftharpoons FM
\]

\[
EM + FM \rightleftharpoons EFM + M + P
\]

Assuming rapid equilibrium binding

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

but

\[
[F] = [F] + [FM] + [FM^2]
\]

so

\[
[FM] = \frac{[F][FM][M]}{K_f K_\text{FM} + [FM][M] + [M]^2}
\]

and (5) becomes

\[
\frac{1}{v_o} = \frac{K_a(K_r + [M])(K_r K_{\text{FM}} + K_{\text{FM}}[M] + [M]^2)}{V[M]^2[F]M}
\]

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] = \( \sqrt{K_F K_{\text{FM}}} \). This situation occurs only if Fru-1,6-P_2 binds two metal ions.

2. Random equilibrium:

\[
E + M \rightleftharpoons EM
\]

\[
EMF \rightleftharpoons K_{\text{FM}} EFM \rightleftharpoons EFM + P + \text{Fru-6-P}
\]

Here using rapid equilibrium assumptions

\[
\frac{1}{v_o} = \frac{K_a(K_r + [M])(K_r K_F + K_F[M] + [M]^2)}{K_v M[F]} + \frac{K_a + [M]}{V[M]}
\]

or

\[
\frac{1}{v_o} = \frac{K_aK_r K_F + K_F[M] + [M]^2)}{K_v M[F]K_{\text{FM}}} + \frac{K_a + [M]}{V[M]}
\]

3. The question of whether the free anion and/or the Mn^{2+} complex of Fru-6-P and of P, inhibits arises, and our data, gathered at only one concentration of free Mn^{2+}, do not discriminate. Binding studies at present in progress in this laboratory are hoped to be able to settle this question.

4. Inclusion of an abortive EFM complex introduces the required dependence upon [M] into the intercept term. However, given the experimental protocol of preincubation of the enzyme with M before addition of F and the [M] employed, the formation of EFM should be negligible.

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_{fr}. Our data (Figs. 1 and 2) show that the intercept is independent of [M_{total}] 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 [M_{total}] decreases as predicted in Equation 11. Data from Marcus et al. (2) show that at pH 6.5 the intercept is independent of [Mg^{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 [Mg^{2+}] the plot of intercept against 1/[Mg^{2+}] is linear and would pass through the origin if extrapolated. At higher values of [Mg^{2+}] the value of K_{fr} 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/[Mg^{2+}] on the abscissa.

Both studies of the beef enzyme would place K_{fr} at \( \sim 1 \) mM (2, 3).

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

\[
slope = \frac{K_s K_a K_{\text{FM}} + [M]^2}{K_v M K_{\text{FM}}}
\]

At high [M]

\[
slope = \frac{K_s K_a}{K_v M K_{\text{FM}}}
\]

and at low [M]

\[
slope = \frac{K_s}{K_{\text{FM}} V[M]}
\]

For the rabbit liver enzyme with Mn^{2+}, \( K_s \gg [M] \gg K_c \), since EPR measurements (4) 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 \( K_s \). No sign of further binding of Mn^{2+} ions to the enzyme was observed which would indicate \( K_c > 10^{-5} \) M.

In Fig. 5 is plotted the term \( (K_s K_F + K_{\text{FM}}[M] + [M]^2)/K_v K_{\text{FM}}[M] \) as a function of [M], in order to illustrate the dependence of the slope values on [M] since the remaining factor, \( K_v/V \), is constant at a fixed pH and enzyme concentration. The theoretical line charts the reciprocal fraction of [F] \times [M]^{-1} for the Mn^{2+} assay at pH 7.5. It is notable that all slopes increase at [M_{total}] > 0.5 mM, while slopes below that concentration increase sharply with decreasing [M_{total}].

One can readily show that the slope term has a minimum value at [M] = \( \sqrt{K_s K_{\text{FM}}} \) which for [Mn^{2+}] \( \approx 0.48 \) mM. The slope data at pH 9.0 (Table II) decrease in magnitude with increasing [M_{total}] consistent with the [Mn^{2+}] < 0.5 mM. No marked change in the magnitude of K_{fr} 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/[Mg^{2+}] (3). Moreover their slope values change with [Mg^{2+}] in accord with Equation 12 reaching a minimal value at [Mg^{2+}] \approx 4.0 mM. When behavior of the slope is considered the data of Marcus et al. (2) also is anticipated since their experiments were conducted at [Mg^{2+}] > 4.0 mM. Thus the random equilibrium scheme can account for all major aspects of the fructose-1,6-bisphosphatase kinetics which have been examined.

5 The value of \( K_s \) for Mg^{2+} binding to the beef liver enzyme is unknown.
Fructose-1,6-bisphosphatase Kinetics with Mn$^{2+}$

5717

![Graph](image)

**Fig. 3.** Plot of experimental slope values (Table II) multiplied by the factor $V/K_1K_m$ (see Equation 12) as a function of [Mn$^{2+}$], $V/K_1K_m = 210 \times 10^2$ units/mg (mm)$^2$ at pH 7.5 where $V = 3.2$ units/mg. The solid line is generated from the ordinate expression with values for $K_p$ and $K_{FM}$ (Table I).

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:

$$\frac{1}{v_o} = \frac{V[M][F]K_{KM}}{K_{KM} + [M] + [F]}$$

and

$$\frac{1}{v_o} = \frac{K_{KM}K_r + [M][F]K_{KM} + [M]}{V[M][F]K_{KM}}$$

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 $v_o$ 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 EM$_2$ 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 chelation 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, $K_aK_n = 15.2$ (mm)$^3$ (pH 7.5) and if $K_a$ is taken to be 30 mm, $K_n = 0.5$ mm for the rabbit liver enzyme with Mn$^{2+}$. The presumed value for $K_{KM}$ based on the assumption the $K_a = 0.2$ [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-D-fructofuranoside-1,6-P$_2$; the values are 12.5 to 200 mm (4) and are consistent with the assumed value of 30 mm. For the bovine enzyme the value of $K_{KM}$ calculated from the replots of the intercepts against $1/[Mg^{2+}]$ from Fig. 1 in Ref. 3, is 0.64 mm (pH 7.5). Both values for $K_{KM}$ indicate that the binding of M to EMF is greater than to free F$(K_F)$, by a factor of six for Mg$^{2+}$ and sixteen for Mn$^{2+}$.

Let us now compare the values obtained for $K_{KM}$ 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 mmol/kg of moist liver. The level of free Mn$^{2+}$ in cytoplasm should be much lower than 90 mm because of its binding to proteins and phosphate-containing compounds *inter alia*. By analogy, the concentration of total Mn$^{2+}$ in liver cells was reported as 10.4 mm (18) while that of free Mg$^{2+}$ was measured at 0.6 to 1.3 mm. The concentration of free Mn$^{2+}$ in rabbit livers to be about 9 mm (assuming a parallel to the Mg$^{2+}$ partitioning), this is close to the estimated value of $K_{KM}$ 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 mm 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 mm. 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 estimations 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 HPrf-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 $\leq 8$. 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 $K_a$ as the pH falls, leading to an increase in both the slope and...
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 $K_e$ 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.

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

N P Dudman, M M deMaine and S J Benkovic