Evidence for the Common Identity of Glucose 6-Phosphatase, Inorganic Pyrophosphatase, and Pyrophosphate-Glucose Phosphotransferase*

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The glucose- and inorganic pyrophosphate-dependent formation of glucose 6-phosphate has been demonstrated in mouse (1) and rat (2) liver mitochondrial preparations. The mouse mitochondrial enzyme catalyzing this reaction appears to be very closely related to an inorganic pyrophosphatase activity found in such preparations (1). Recent studies have indicated that rat liver microsomes, which also contain an inorganic pyrophosphatase active at low pH (pH 4 to 6) (3, 4), are a considerably richer source of this phosphotransferase than are mitochondria. The common involvement of glucose 6-phosphate in the phosphotransferase and with another liver microsomal enzyme, glucose 6-phosphatase (5), as well as the acidic pH optima of both (4, 6), suggested that possibly the three enzymic activities might be interrelated. Interest in the problem was intensified by the finding that these activities could not be separated by repeated ammonium sulfate fractionations of deoxycholate-treated microsomes, and that all three activities were unstable to other fractionation procedures attempted. The results of investigations reported in this paper indicate that inorganic pyrophosphatase, pyrophosphate glucose phosphotransferase, and glucose 6-phosphatase activities may be due to one, common enzyme.

EXPERIMENTAL PROCEDURE

Materials—Sources of substrates were as follows: Na2HPO4, 10H2O, Fisher Scientific Company; D-mannose and D-glucose, Nutritional Biochemicals Corporation; and D-glucose 6-phosphate, Sigma Chemical Company. Type V glucose 6-phosphate dehydrogenase also was purchased from Sigma Chemical Company. The absence of glucose contamination in the mannose, as well as absence of phosphomannose isomerase or phosphomannose isomerase activity in glucose 6-phosphate dehydrogenase and phosphotransferase preparations, was verified by the complete lack of glucose 6-phosphate formation when mannose replaced glucose in the inorganic pyrophosphate (PPi)-sugar phosphotransferase reaction mixture. D-Mannose 6-phosphate was prepared by the method of Silen (7). Microsomes were isolated from homogenates of liver from young, adult (approximately 200 g) white rats (Simonsen Laboratories, White Bear Lake, Minnesota), as described by de Duve and Berthet (8), washed once, and suspended in 0.25 m sucrose solution. Details of ammonium sulfate fractionation of deoxycholate-treated microsomal suspensions are given under "Results." All preparations were stored at −15°C and were thawed at 0°C immediately before use.

Enzymic Assays—General methods for assay of inorganic pyrophosphatases (2), ATPase (reaction mixtures were supplemented with 4.5 mmoles of MgSO4) (9), and acid phosphatase (10) activities have been described. D-Glucose 6-phosphate replaced PPi in reaction mixtures in which hydrolysis of the sugar phosphate was studied. All incubations were carried out at 30°C in a vibrating, thermostatically regulated water bath. Phosphate substrates were adjusted to reaction mixture pH with HCl. P1 was measured by the method of Fiske and SubbaRow (11), and glucose 6-phosphate was assayed spectrophotometrically (12) after removal of the potassium perchlorate formed during neutralization of the perchloric acid used to terminate reactions. A 1:1 stoichiometric relationship between glucose 6-phosphate disappearance and P1 appearance was demonstrated with the glucose 6-phosphatase system.) Protein was determined as previously described (13). Other specific details are given in the legends of tables and figures.

RESULTS

Fractionation Studies—A 1.5- to 2-fold increase in specific activity of glucose 6-phosphatase, inorganic pyrophosphatase, and PPi-glucose phosphotransferase was achieved routinely by ammonium sulfate fractionation of deoxycholate-treated microsomes (Fig. 1 depicts a typical experiment). A partial separation of these activities from ATPase, Mg2+-stimulated inorganic pyrophosphatase (pH 7.4), and acid phosphatase also was achieved. The glucose 6-phosphatase to phosphotransferase ratio remained nearly constant (3.4 in deoxycholate-treated microsomes; 2.8 in Fraction I; 3.2 in Fraction II; 3.5 in Fraction III; and 3.2 in Fraction IV). Inorganic pyrophosphatase activity at
FIG. 1. Enzymic activity distribution following ammonium sulfate fractionation of deoxycholate-treated rat liver microsomes. Inorganic pyrophosphatase, glucose 6-phosphatase, and transferase reaction mixtures (pH 5.2) contained 60 µmoles of sodium acetate buffer, 7.5 µmoles of phosphate substrate, and 45 µmoles of n-glucose (transferase) per 1.5 ml. Other assay details are given in the text. Fractionations were carried out at 0° with solid ammonium sulfate (14). Specific activity is expressed as change in micromoles of substrate per 1.5 ml per minute per mg of protein. M + DOC indicates deoxycholate (0.3% w/v)-treated rat liver microsomes. Fractions I, II, III, and IV precipitated, respectively, from 0 to 30, 30 to 40, 40 to 50, and 50 to 90% saturated (calculated for 25°) ammonium sulfate solutions. Sucrose (0.25 m) was used as the suspending medium, inasmuch as the enzymic activities were more stable in this solution than in 0.1 m Tris, pH 7.4.

FIG. 2. Effect of pH on inorganic pyrophosphatase (circles), glucose 6-phosphatase (squares), and PPi-glucose phosphotransferase (triangles) activities. Acetate (unshaded symbols), histidine (shaded symbols), and glycylglycine (half-shaded symbols) buffers were employed. All reaction mixtures contained, in a volume of 1.5 ml, 60 µmoles of buffer, 0.14 unit4 of glucose 6-phosphatase activity, and 7.5 µmoles of PPi or glucose 6-phosphate. Trans- ferase reaction mixtures were supplemented with 270 µmoles of n-glucose. Activity (v) is expressed as change in micromoles of substrate per 1.5 ml of reaction mixture per 10 minutes. Assay mixtures were prepared in duplicate; activity was measured in one series and pH determined with a Beckman expanded scale meter in the second series.

FIG. 3 (left). Ammonium molybdate inhibition of inorganic pyrophosphatase (□), glucose 6-phosphatase (○), and PPi-glucose phosphotransferase (▲) activities. Assay mixtures and conditions were as described in the legend to Fig. 2 except that pH 5.2 (acetate buffer) was employed.

FIG. 4 (right). Thermal inactivation of inorganic pyrophosphatase (□), glucose 6-phosphatase (○), PPi-glucose phosphotransferase (▲), and nonspecific acid phosphatase (p-glycero-

pH 5.2 followed this same distribution pattern but was influenced by the presence of a second pyrophosphatase (pH 7.4). Total recovery of glucose 6-phosphatase activity in Fractions I to IV was 65% of that initially present in deoxycholate-treated microsomes. Further attempts at purification by fractional ethanol precipitation (14)—a process known to destroy microsomal glucose 6-phosphatase activity (6)—or by adsorption on and elution from calcium phosphate gel (15) resulted in complete loss of all three activities. The fraction precipitating between pH 5.2 and 7.4 contained 43% of the recovered glucose 6-phosphatase activity, was employed in subsequent studies. The resuspended ammonium sulfate precipitate initially remained in the supernatant fraction after 40 and 50% of ammonium sulfate saturation, which contained 43% of recovered glucose 6-phosphatase activity, was employed in subsequent studies. The resuspended ammonium sulfate precipitate initially remained in the supernatant fraction after

4One unit of activity is that amount of enzyme catalyzing the hydrolysis of 1 µmole of substrate (or formation of 1 µmole of sugar phosphate) per 1.5 ml of reaction mixtures per minute. Amount of enzyme employed in the various experiments is expressed in terms of glucose 6-phosphate hydrolysis at pH 6.0 under the other conditions specified in the legend to Fig. 2.
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Fig. 5 (left). Effects of PP$_i$ concentration (a) on the rate of PP$_i$ hydrolysis in the absence (O) and presence (●) of 2.5 μmoles of glucose 6-phosphate, and (b) on PP$_i$-glucose phosphotransferase activity in the presence of 270 μmoles of n-glucose (X). Incubations were for 10 minutes in citrate buffer at pH 6.0. Other details were as in Fig. 2. Glucose 6-phosphate formation in the transferase reaction and disappearance in the hydrolysis reaction were measured spectrophotometrically. PP$_i$ hydrolysis in the absence of glucose 6-phosphate was measured as one-half the change in Pi concentration. PP$_i$ hydrolysis in the presence of glucose 6-phosphate was calculated as (ΔP$_i$ - Δ glucose 6-phosphate)/2.

Fig. 6 (right). Effect of substrate concentration on rate of glucose 6-phosphate hydrolysis in the absence (O) and presence (●) of 9.0 μmoles of PP$_i$. Details were as in Fig. 2, except that citrate buffer, pH 6.0, was employed. In the absence of PP$_i$, glucose 6-phosphate hydrolysis was followed by Pi assay, while in the presence of PP$_i$, disappearance of the sugar phosphate was measured spectrophotometrically.

Fig. 7 (left). Effect of n-mannose concentration on the glucose 6-phosphate-mannose phosphotransferase reaction. Assay mixtures, pH 6.0, contained, in 1.5 ml, 60 μmoles of citrate buffer, 7.5 μmoles of glucose 6-phosphate, 0.14 unit of enzyme, and indicated concentrations of n-mannose. Changes in glucose 6-phosphate concentrations were measured spectrophotometrically, and Pi concentration was determined colorimetrically. Mannose phosphophate formation was calculated as the difference between glucose 6-phosphate disappearance and Pi appearance in the presence of mannose. Activity is expressed as in Fig. 2. K$_{mannose}$ determined with 30 μmoles of glucose 6-phosphate agreed within experimental error with that calculated from the data obtained in this experiment.

Fig. 8 (right). Mannose inhibition of the PP$_i$-glucose phosphotransferase reaction. Assay mixtures, pH 6.0, contained, in 1.5 ml., 60 μmoles of citrate buffer, 15 μmoles of sodium pyrophosphate, 0.14 unit of enzyme, indicated amounts of n-glucose, and no (O) or 270 μmoles (●) of n-mannose. Glucose 6-phosphate formation was measured spectrophotometrically. Activity is as expressed in Fig. 2.

Fig. 5 (left). Effects of PP$_i$ concentration (a) on the rate of PP$_i$ hydrolysis in the absence (O) and presence (●) of 2.5 μmoles of glucose 6-phosphate, and (b) on PP$_i$-glucose phosphotransferase activity in the presence of 270 μmoles of n-glucose (X). Incubations were for 10 minutes in citrate buffer at pH 6.0. Other details were as in Fig. 2. Glucose 6-phosphate formation in the transferase reaction and disappearance in the hydrolysis reaction were measured spectrophotometrically. PP$_i$ hydrolysis in the absence of glucose 6-phosphate was measured as one-half the change in Pi concentration. PP$_i$ hydrolysis in the presence of glucose 6-phosphate was calculated as (ΔP$_i$ - Δ glucose 6-phosphate)/2.

Fig. 6 (right). Effect of substrate concentration on rate of glucose 6-phosphate hydrolysis in the absence (O) and presence (●) of 9.0 μmoles of PP$_i$. Details were as in Fig. 2, except that citrate buffer, pH 6.0, was employed. In the absence of PP$_i$, glucose 6-phosphate hydrolysis was followed by Pi assay, while in the presence of PP$_i$, disappearance of the sugar phosphate was measured spectrophotometrically.

Effect-The pH optima obtained with our preparation (Fig. 2) agree with reported values for rat liver microsomal glucose 6-phosphatase (pH 6.5) (6), whereas observed optima for the inorganic pyrophosphatase and PP$_i$-glucose phosphotransferase systems (pH 5.7) are slightly higher than values (pH 5.3 and 5.2, respectively) (1, 16) reported for mouse mitochondrial activities. On the basis of the overlapping of activities (Fig. 2), pH 5.2 and 6.0 were chosen for comparative studies of the three activities.

Molybdate Inhibition—The sensitivity of PP$_i$-glucose phosphotransferase to low concentrations of ammonium molybdate (1) also was exhibited by inorganic pyrophosphatase and glucose
Assay mixtures, pH 6.0, contained 60 μmoles of sodium citrate buffer, 0.14 unit of enzyme (see footnote 3), and indicated phosphate compounds in 1.5 ml of solution. Incubations were carried out for 10 minutes at 30°. Activity was based on Pi assays where phosphate disappearance was measured spectrophotometrically in the combined substrate experiment. Calculated values were determined by substituting kinetic parameters determined from substrates were incubated individually; in addition, glucose 6-phosphate disappearance was measured spectrophotometrically in the combined substrate experiment. Calculated values were determined by substituting kinetic parameters determined from the data given in Figs. 5 and 6 (recorded in Table II) into Equations 1, 2, and 3.

**Table I**

**Rate of hydrolysis of PPi and glucose 6-phosphate, individually and combined**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration</th>
<th>Experimental values</th>
<th>Calculated values</th>
<th>Glucose 6-P utilized</th>
<th>Glucose 6-P found</th>
<th>PPi utilized</th>
<th>PPi found</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPi</td>
<td>7.5</td>
<td>1.12</td>
<td>2.24</td>
<td>1.19</td>
<td>2.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Glucose 6-phosphate</td>
<td>7.5</td>
<td>1.34</td>
<td>1.40</td>
<td>1.40</td>
<td>1.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPi + d-glucone 6-phosphate</td>
<td>7.5</td>
<td>0.00</td>
<td>0.19</td>
<td>1.07</td>
<td>0.54</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Change in PPi = (Pi found - glucose 6-phosphate utilized)/2.

**Table II**

**Compilation of Michaelis constants calculated from data in Figs. 5 to 8**

<table>
<thead>
<tr>
<th>System studied</th>
<th>Data given in Fig No.</th>
<th>Michaelis constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPi hydrolysis</td>
<td>5</td>
<td>$K_{PPi}$: $3.45 \times 10^{-2}$</td>
</tr>
<tr>
<td>PPi glucose phosphotransferase</td>
<td>5</td>
<td>$K_{PPi}$: $3.45 \times 10^{-2}$</td>
</tr>
<tr>
<td>PPi inhibition of glucose 6-phosphate hydrolysis</td>
<td>6</td>
<td>$K_{PPi}$: $3.22 \times 10^{-2}$</td>
</tr>
<tr>
<td>d-Glucose 6-phosphate hydrolysis*</td>
<td>6</td>
<td>$K_{glucose-6-P}$: $1.75 \times 10^{-2}$</td>
</tr>
<tr>
<td>d-Glucose 6-phosphate in inhibition of PPi hydrolysis</td>
<td>5</td>
<td>$K_{glucose-6-P}$: $1.80 \times 10^{-2}$</td>
</tr>
<tr>
<td>d-Glucose 6-phosphate-n-mannose phosphotransferase</td>
<td>7</td>
<td>$K_{mannose}$: 0.10</td>
</tr>
<tr>
<td>d-Mannose inhibition of PPi-d-glucone 6-phosphate phosphotransferase</td>
<td>8</td>
<td>$K_{mannose}$: 0.12</td>
</tr>
</tbody>
</table>

* Maximal initial reaction velocities for PPi (Fig. 5) and glucose 6-phosphate (Fig. 6) hydrolyses were 1.85 and 1.79 rmoles per 10 minutes per 1.5 ml of reaction mixture, respectively.

6-phosphatase at pH 5.2 (Fig. 3). All three activities were inhibited parallelly by this compound at concentrations 0.0002 to 0.02 of those of phosphate substrates. Molybdate probably interacted with an active enzymic site, as it also has been shown to duplicate the protection by PPi of inorganic pyrophosphatase and phosphotransferase against heat inactivation (1).

**Partial Thermal Inactivation**—Lability to mild heating in the absence of substrate is a characteristic of inorganic pyrophosphatase and PPi-glucose phosphotransferase (1, 16). Glucose 6-phosphatase also was found to be similarly unstable to such treatment (Fig. 4). Inactivation of all three activities due to heating at 10, 20, 25, 30, and 35° for 5 minutes in the absence of substrates was identical. Acid phosphatase in the preparation was unaffected by this treatment.

**Kinetic Studies**—Experimental details and results of kinetic studies are given in Figs. 5 to 8 and in Table I. Data are presented as conventional double reciprocal plots (17). Michaelis constants calculated (as described by Dixon and Webb (18)) from the data in Figs. 5 to 8 are compiled in Table II. Activity measurements were carried out with enzyme concentrations and incubation periods, established on the basis of preliminary experiments, such that initial reaction velocities were determined in all instances.

When two compounds may act as alternate substrates for the same enzyme, each will behave as a competitive inhibitor for the reaction involving the other (19). Glucose 6-phosphate acted as a competitive inhibitor of PPi hydrolysis (Fig. 5), and PPi behaved similarly toward hydrolysis of the sugar phosphate (Fig. 6), suggesting that the same enzyme was involved in both reactions. Rate Equations 1 and 2 have been shown to apply to such a situation (19). $K_{PPi}$ calculated from PPi inhibition of glucose 6-phosphate hydrolysis (Fig. 6) was identical with the Michaelis constant obtained for the hydrolysis of PPi (Fig. 5), while values for $K_{glucose-6-P}$ calculated from inhibition of PPi hydrolysis (Fig. 5) and determined for the hydrolysis of the sugar compound (Fig. 6), also agreed as predicted by Equations 1 and 2. Table I contains the results of an experiment in which the rates of hydrolysis of PPi and glucose 6-phosphate, individually and together, were determined. Also included in this table are values obtained when kinetic parameters calculated from data in Figs. 5 and 6 (see Table II) were substituted into Equations 1, 2, and 3. Good agreement was found between experimental and calculated values.

$$V_{PPi} = \frac{V_{PPi}}{1 + \frac{K_{PPi}}{[PPi]} \left(1 + \frac{[\text{glucose 6-phosphate}]}{K_{glucose-6-P}}\right)}$$

(1)
The involvement of a phosphoenzyme intermediate has been suggested for mouse mitochondrial inorganic pyrophosphatase and phosphotransferase (1); evidence has been presented for the catalysis of the glucose 6-phosphatase, inorganic pyrophosphatase, and phosphotransferase reactions by one enzyme. The probability of obtaining such a combination of enzyme activities might by chance equal the Michaelis constant for the sugar phosphate in its own hydrolysis, especially if they were associated in a common particulate body. The inhibition constant for glucose 6-phosphate as an inhibitor of inorganic pyrophosphatase might be expected if the scheme in Equation 4 is correct, since the rate-limiting step in the steady state kinetic treatment of the system is assumed to be the hydrolysis of $E-Pi$, which is common both to inorganic pyrophosphatase and glucose 6-phosphatase. With saturating levels of PPi or glucose 6-phosphate, the rates of hydrolysis ($v = k [E-Pi]$), where $k$ is the rate constant for the reaction $E-Pi + H_2O \rightarrow E + Pi$) would be equal. Further studies on the mechanisms of the transferase reactions are being carried out by kinetic and isotopic exchange methods.

The PPi-sugar phosphotransferase reactions may afford a mechanism for the conservation of phosphoanhydride bond energy of PPi in microsomes, for at high sugar concentrations the rate of transferase activity is approximately one-half that of the hydrolysis reaction in the absence of sugar (Figs. 2 and 5). However, the high sugar concentration requirement makes the physiological significance of such a process speculative.

The PPi-sugar phosphotransferase reactions may afford a mechanism for the conservation of phosphoanhydride bond energy of PPi in microsomes, for at high sugar concentrations the rate of transferase activity is approximately one-half that of the hydrolysis reaction in the absence of sugar (Figs. 2 and 5). However, the high sugar concentration requirement makes the physiological significance of such a process speculative.

**SUMMARY**

Inorganic pyrophosphatase, glucose 6-phosphatase, and inorganic pyrophosphatase (PPi)-glucose phosphotransferase activities could not be separated from one another by ammonium molybdate fractionation of deoxycholate-treated rat liver microsomes. All three activities were affected in a parallel fashion by molybdate concentrations as low as $10^{-5} M$, and by mild heating in absence of substrate. Excellent agreement was observed among Michaelis constants determined as follows. $K_{PPi}$ was determined for the hydrolysis and PPi-glucose phosphotransferase reactions catalyzed by the same enzyme. The excellent agreement of $V$ for PPi and glucose 6-phosphate hydrolysis (see Figs. 5 and 6, and Table II), is consistent with the catalysis of phosphotransferase and hydrolysis reactions by one enzyme.

The results of each of the experiments described, taken individually, could be explained on a basis other than the involvement of a single enzyme. For example, it is possible that three enzymes might show identical susceptibility to thermal inactivation, especially if they were associated in a common particulate body. The inhibition constant for glucose 6-phosphate as an inhibitor of inorganic pyrophosphatase might be expected if the scheme in Equation 4 is correct, since the rate-limiting step in the steady state kinetic treatment of the system is assumed to be the hydrolysis of $E-Pi$, which is common both to inorganic pyrophosphatase and glucose 6-phosphatase. With saturating levels of PPi or glucose 6-phosphate, the rates of hydrolysis ($v = k [E-Pi]$), where $k$ is the rate constant for the reaction $E-Pi + H_2O \rightarrow E + Pi$) would be equal. Further studies on the mechanisms of the transferase reactions are being carried out by kinetic and isotopic exchange methods.

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determined directly for pyrophosphate hydrolysis and for the pyrophosphate-glucose phosphotransferase reaction, and calculated from pyrophosphate inhibition of glucose 6-phosphatase activity; $K_{\text{glucose}}$ was determined directly for the hydrolysis reaction and from glucose 6-phosphate inhibition of the pyrophosphatase reaction; and $K_{\text{mannose}}$ was determined directly for the glucose 6-phosphate + mannose phosphotransferase reaction and calculated from data for mannose inhibition of the PP$_i$-glucose phosphotransferase reaction. Observed rates of hydrolysis of PP$_i$ and glucose 6-phosphate, when incubated with enzyme individually and together, agree closely with theoretically calculated values based on the assumption of the involvement of a single enzyme. It is concluded that all the reactions are catalyzed by one enzyme.

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