Thermal Stability of Microsomal Glucose-6-phosphatase*

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The thermal stability of glucose-6-phosphatase in rat liver microsomes was examined in untreated and cholate-treated microsomes. Activity of the enzyme was measured with both glucose-6-P and mannose-6-P as substrates. Heat treatment did not cause glucose-6-phosphatase activity to decline to zero with a single rate constant in untreated microsomes. Instead, heat treatment produced an enzyme with a small residual activity that was stable. The residual level of activity was not stimulated by addition of detergent. In untreated microsomes the energies of activation for the processes of decay were different for glucose-6-phosphatase and mannose-6-phosphatase activities, suggesting that the rate-limiting steps for the hydrolysis of these compounds were different. Treatment of microsomes with detergent increased the rate constants for the thermal decay of glucose-6-phosphatase by about 150 times, and, in contrast to untreated microsomes, glucose-6-phosphatase and mannose-6-phosphatase decayed to zero with a single rate constant in cholate-treated microsomes. Also, rate constants for thermal inactivation of glucose-6-phosphatase and mannose-6-phosphatase were the same in cholate-treated microsomes. Removal of cholate increased the stability of glucose-6-phosphatase but did not regenerate the form of the enzyme present in untreated microsomes. The data for the stability of glucose-6-phosphatase under different conditions provide evidence that the enzyme can exist in at least five different stable states that are enzymatically active.

Glucose-6-phosphatase is a critical enzyme because gluconeogenesis cannot proceed in its absence. The enzyme is interesting too because it is an integral component of microsomal membranes, and its activity and catalytic specificity are changed by treating microsomes with a variety of detergents, alkaline pH, or sonic energy (1–9). Activities with many sugar-P increase after adding detergents to microsomes, and the ratio of activities with different sugar-P changes. For example, whereas the ratio of activities with glucose-6-P and mannose 6 P is about 6:1 in intact microsomes, this ratio is about 1:1 in detergent-treated microsomes. Finally, there is a marked detergent-induced stimulation of the phosphotransferase function of the enzyme (see Ref. 4 for review). The most popular interpretation of these effects of detergent is that (i) glucose-6-phosphatase comprises a nonspecific catalytic unit, with its active site on the luminal side of the microsomal vesicle and two separate, trans-microsomal transport proteins that are specific for glucose-6-P or P/PP, respectively (1, 2, 4, 10–16). (ii) The putative transport proteins provide for access of substrates to the active site of the catalytic unit and for efflux of P. (iii) The barrier of the microsomal membrane and not the properties of the catalytic unit account for the constraint on maximal potential activity and the substrate specificity of the latter (1, 2, 4, 10–16).

Thus, it is proposed that detergents and other activators do not affect the catalytic unit directly, but influence its function by removing the barrier between substrates and the active site of the enzyme. To account for hydrolysis of mannose-6-P by enzyme in untreated microsomes it is proposed that mannose-6-P is hydrolyzed by enzyme in a small proportion of microsomes disrupted during preparation (2). We refer to this set of ideas as the compartmentation hypothesis. There is considerable indirect evidence in support of the compartmentation hypothesis, but all the ideas incorporated in it remain unproved. There is no direct evidence for the putative microsomal transport proteins or for the proposed topology of glucose-6-phosphatase in microsomal membranes (1, 2, 4, 10–16). There is evidence, on the other hand, that glucose-6-P does not enter the lumen of microsomes (17).

The compartmentation hypothesis is not a unique interpretation of the data relating to the regulation of glucose-6-phosphatase. The differences in the properties of the enzyme in untreated and detergent-treated microsomes can be explained as readily by the idea that treatments with detergent and other activators lead to changes in the conformation of glucose-6-phosphatase (6–9, 18–21), which has been proposed as an alternative to the compartmentation hypothesis. At present, however, there are insufficient data to choose between the several hypotheses for the regulation of glucose-6-phosphatase (see Ref. 21 for review).

Purification of the catalytic unit of glucose-6-phosphatase and the transport proteins for glucose-6-P and P/PP would seem to be the most direct approach to unraveling the uncertainties of the regulation of glucose-6-phosphatase, but attempts to purify the enzyme have failed repeatedly (22–25). There is no assurance either that pure enzyme could be reconstituted with the properties of enzyme in microsomes; failure to purify the transport proteins will not disprove their existence. Experimental approaches other than purification are likely, for these reasons, to be important for understanding how glucose-6-phosphatase is regulated. Especially useful would be studies of glucose-6-phosphatase that can be interpreted independently of assumptions about the orientation of the active site of the catalytic unit. Changes in thermal stability provided Schultze et al. (19) with evidence that Triton had a direct effect on glucose-6-phosphatase. We have extended this approach by examining in detail the thermal stability of glucose-6-phosphatase in intact and detergent-treated microsomes.

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MATERIALS AND METHODS

Liver microsomes were prepared from 250- to 300-g male Wistar rats (Charles River) and kept at -20 °C until use. Initial rates of glucose-6-phosphatase were measured in 50 mM Pipes, pH 6.1 (9). Since the activity of the enzyme decayed with a significant rate for assays at T > 37 °C, production of P_i, at this and higher temperatures, was measured serially in at least five different intervals. The decline in P_i produced per interval, as a function of time, fit a first order process for decay. Initial rates of activity were determined by extrapolation of the decay curve to zero time. All studies of heat-induced inactivation were carried out in 50 mM Pipes, pH 6.1. Rates of thermal inactivation in the absence of substrates were measured by heating microsomes in Pipes, pH 6.1, at each temperature and removing aliquots at seven sequential time points for assay of remaining hexose-6-phosphatase activities. These assays were carried out as outlined above and in the legends to figures. Activities as a function of time of treatment at elevated temperature fit a first order process of decay, as described under "Results." Repetitive measurements of rate constants for thermal inactivation deviated from each other by less than 10% when the precision of the method was tested.

Cholate was removed from microsomes by chromatography on Bio-Beads, at 0 °C, as described in Ref. 20. A column (1.0 × 15 cm) of Bio-Beads (7.5 g Bio-Beads) in 50 mM Tris, pH 8.0, was used. The column was equilibrated at a slightly alkaline pH to facilitate removal of cholate, which precipitates at acid pH. A tracer amount of [14C] cholate was added to microsomes in these experiments to quantify the cholate removed. Microsomes (10.2 mg of protein containing 4.8 mg of cholate) were added to the column and eluted in Tris as above. No detectable cholate was associated with microsomal protein after passage over Bio-Beads. Adjustment of pH to 8.0 did not increase the extent of cholate-induced activation of glucose-6-phosphatase.

Protein was measured by the biuret method (27) and inorganic phosphate by the method of Sumner (28). Details of each experiment are given in the figure legends.

RESULTS

Effect of Temperature on the Activity of Glucose-6-phosphatase—Microsomal glucose-6-phosphatase has a property referred to as "latency," i.e. several treatments (cholate, other detergents, sonic energy) activate the enzyme and change the ratio of activities measured with glucose-6-P and mannose-6-P. It was necessary, therefore, to determine whether treatment of microsomes at relatively high temperatures activated glucose-6-phosphatase beyond what would be expected from the thermal effects on the rate of the reaction catalyzed by enzyme in the so called "latent state." The data in Fig. 1 illustrate the effects of temperature on the catalytic activity of glucose-6-phosphatase with glucose-6-P and mannose-6-P as substrates across the temperature range 27-50 °C. The important features of the data are (i) that plots of log v versus 1/K were linear and without change in slope, as a function of temperature, with either glucose-6-P or mannose-6-P as substrates, and (ii) that the large difference in activities for glucose-6-phosphatase and mannose-6-phosphatase that is characteristic of enzyme in the latent state was preserved at all temperatures. Note in this regard that the scales in Fig. 1 are different for activities with glucose-6-P and mannose-6-P. The data in Fig. 1 indicate that heating did not activate glucose-6-phosphatase.

The Thermal Stability of Glucose-6-phosphatase in Intact Microsomes—The data in Fig. 2 show the time course for the temperature-induced loss of activity at 47.5 °C. Microsomes were treated at 47.5 °C for the indicated times, in the absence of substrate. Aliquots were removed and assayed at 20 °C with glucose-6-P (Fig. 2A) or mannose-6-P (Fig. 2B). The data are important for two reasons. First, glucose-6-phosphatase and mannose-6-phosphatase did not become inactivated to zero with a single rate constant with either substrate. Indeed, the enzyme appeared to be thermally transformed to a new form with a small but measurable activity that was quite stable at 47.5 °C. The alternative possibility that microsomes contained two enzymes with different thermal stabilities was excluded by data presented below. The stable level of residual activity, as a percent of the initial activity, was greater with mannose-6-P as substrate than with glucose-6-P, and the ratio of activities glucose-6-phosphatase/mannose-6-phosphatase approached 1 in some preparations after heat treatment of microsomes (see Table I). The second important feature of the data in Fig. 2 was that activities measured with glucose-6-P and mannose-6-P decayed at different rates. The effect of temperature on rates of inactivation was limited to the duration the enzyme was heated. For example, if microsomes were treated at 47.5 °C for several minutes and...
The effect of cholate on the activity of partially inactivated glucose-6-phosphatase

Microsomes (30 mg protein/ml) were kept on ice or treated at 45.5 °C for 1.5 or 11 min in 50 mM Pipes, pH 6.1, and then placed on ice. Microsomes were then titrated with increasing concentrations of cholate (at 0°C) over the range of protein/cholate (w/w) of 10:1 to 1:1. Initial rates of activity for hexose-6-phosphatase were measured as shown under "Materials and Methods" at 5 mM glucose-6-P or mannose-6-P. Protein concentrations in assays were 0.15 mg/ml for microsomes not treated with heat and 0.3 mg/ml for heat-treated microsomes. The data below are for concentrations of cholate that gave a maximally activated enzyme, which were protein/cholate of 5:1 for microsomes heated for 1.5 min and 2:1 for control microsomes. No amount of cholate activated microsomes heat-treated for 11 min. The data shown for these microsomes are for a protein/cholate of 5:1. Activities are nmol/min/mg protein.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cholate</th>
<th>Glucose-6-P Activity</th>
<th>Mannose-6-P Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>83</td>
<td>14</td>
</tr>
<tr>
<td>45.5°C for 1.5 min</td>
<td>+</td>
<td>144</td>
<td>137</td>
</tr>
<tr>
<td>45.5°C for 11 min</td>
<td>-</td>
<td>26</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>42</td>
<td>41</td>
</tr>
</tbody>
</table>

The rates of inactivation as a function of temperature were independent of pH in the range of 6.0 to 7.0, which were the limits of the range tested.

Temperature Dependence of the Rate of Inactivation of Glucose-6-phosphatase—Rate constants for inactivation were estimated by assuming that residual activity at any temperature was due to the process $E_1 \rightarrow E_2$. Since the amount of $E_2$ at $t = \infty$ was small as compared with $E_1$ at $t = 0$, the residual activity of $E_2$ was subtracted from activity at each time point to give the amount of $E_1$ remaining. The percent of $E_1$ remaining at each time point in the course of the decay to $E_2$ fit a first order plot (Fig. 3). The temperature dependence of the inactivation of glucose-6-phosphatase at pH 6.1 is displayed as an Arrhenius plot in Fig. 4. Data are shown with either glucose-6-P or mannose-6-P as substrate. These data confirm the result in Fig. 2 showing different thermal stabilities of glucose-6-phosphatase and mannose-6-phosphatase activities.

Does Heating Inactivate the Putative Microsomal Transport Protein for Glucose-6-P?—A possible explanation for the thermal inactivation of glucose-6-phosphatase is that decay of activity reflected inactivation of the putative microsomal transport protein for glucose-6-P, separately or in addition to direct effects of temperature on the enzyme. Assuming that the transport protein exists and that the active site of glucose-6-phosphatase is on the luminal side of the microsomal vesicles, this explanation can be valid only if the rate of inactivation of the transport protein exceeds that for the enzyme. The idea that a transport protein becomes inactivated at a faster rate than the enzyme can be tested by measuring the ratio of enzyme activities in heat-treated microsomes in the presence and absence of detergent. Microsomes were treated at 45.5 °C for 1.5 min, which caused a 70% inactivation of glucose-6-phosphatase. These microsomes were placed on ice and titrated with cholate (Table I). The ratios of activities in the presence and absence of cholate were the same for microsomes not treated with heat and for microsomes treated with sufficient heat (at 45.5 °C) to cause partial inactivation of glucose-6-phosphatase. The ratio of activities of glucose-6-phosphatase in heat-treated microsomes with and without added cholate was 1.62. This ratio was 1.74 for microsomes not treated at 45.5 °C before addition of detergent. The expected result for inactivation of a putative carrier protein for glucose-6-P would be greater cholate-induced activation of...
enzyme that was apparently partially inactivated as compared with cholate-induced activation of glucose-6-phosphatase in microsomes that were not heat-treated.

The mannose-6-phosphatase activity was also activated by cholate treatment of partially inactivated enzyme (Table I). In contrast to the results with glucose-6-P, however, the extent of cholate-induced activation of mannose-6-phosphatase activity was less for partially heat-inactivated enzyme (5-fold increase in activity after addition of cholate) than for the intact, untreated enzyme (10-fold increase with cholate treatment). This is the expected result if mannose-6-P and glucose-6-P are hydrolyzed by the same enzyme in cholate-treated microsomes. Thus, mannose-6-phosphatase activity decayed at a slower rate than did glucose-6-phosphatase activity (Fig. 2), but activity with mannose-6-P does not exceed that for glucose-6-P after treatment of microsomes with detergent (2, 3, 5, 14; Table I). Cholate had no effect on glucose-6-phosphatase or mannose-6-phosphatase after the stable level of residual activity was reached (Table I). These results again make it unlikely that the data in Figs. 2 and 3 reflect the inactivation of a transport protein for glucose-6-P.

The Thermal Stability of Glucose-6-phosphatase in Cholate-treated Microsomes—As compared with control microsomes, the activity of glucose-6-phosphatase in cholate-treated microsomes decayed completely to zero with a single rate constant (Fig. 5). The rate constants for inactivation of activity with glucose-6-P in cholate-treated microsomes, as a function of temperature, are shown in Fig. 6 as an Arrhenius plot. Microsomes in the experiments in Fig. 6 were treated with an amount of cholate that activated glucose-6-phosphatase maximally. Comparison of Fig. 6 with Fig. 4 shows that treatment with cholate decreased the thermal stability of glucose-6-phosphatase by as much as 150-fold. Another difference between untreated and cholate-treated microsomes was that whereas the rate of inactivation of glucose-6-phosphatase activity differed from that for mannose-6-phosphatase in untreated microsomes, these rates were the same after treatment of microsomes with cholate (Fig. 7).

Thermodynamic Parameters for Thermal Inactivation of Glucose-6-phosphatase and for Stabilization by Glucose-6-P—The thermodynamic parameters for the inactivation of glucose-6-phosphatase under variable conditions are given in Table II. The most important aspect of these data are the comparisons of the thermodynamic parameters for inactivation of untreated as compared with cholate-treated enzyme and the effect of glucose-6-P in stabilizing untreated as compared with cholate-treated enzyme. The data for untreated microsomes and cholate-treated microsomes show the typical large activation enthalpies and entropies for protein denaturation with relatively small values for $\Delta G^0$. Addition of cholate led to a large decrease in $\Delta H^0$ for inactivation of glucose-6-phosphatase, but $\Delta S^0$ also was much less for cholate-treated enzyme than for untreated enzyme. These data imply that the pathways by which untreated enzyme and cholate-treated enzyme decayed were different.

In order to determine the extent to which glucose-6-P stabilized glucose-6-phosphatase against thermal inactivation, rates of inactivation were measured for untreated and...
TABLE II
Thermodynamic parameters for the thermal inactivation of different forms of glucose-6-phosphatase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>( \Delta H^\ddagger ) (kcal/mol)</th>
<th>( \Delta S^\ddagger ) (cal/K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>96.480</td>
<td>235</td>
</tr>
<tr>
<td>Cholate</td>
<td>43.600</td>
<td>76</td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>68.458</td>
<td>141</td>
</tr>
<tr>
<td>Cholate + glucose-6-P</td>
<td>59.164</td>
<td>112</td>
</tr>
<tr>
<td>Cholate-stripped</td>
<td>39.950</td>
<td>51</td>
</tr>
</tbody>
</table>

\( \Delta H^\ddagger \) and \( \Delta S^\ddagger \) were calculated from the data in Figs. 4, 6, and 8 as follows. \( \Delta H^\ddagger = E_a - RT \), where \( E_a \) is the Arrhenius activation energy. \( \Delta S^\ddagger \) was calculated from the expression \( \Delta S^\ddagger = R \ln (N_{ih}X/RT) \), where \( X = k/e^{-\Delta H^\ddagger/RT} \). \( k \) is the measured rate constant for thermal inactivation at \( T \) (41).

Cholate treatment was as in Fig. 5; removal of cholate is described under "Materials and Methods." Data for inactivation fit first order curves for each preparation at each temperature studied.

Cholate-treated microsomes in the presence of 20 mM glucose-6-P. A high concentration of substrate was used so that the concentration of glucose-6-P was essentially constant over the course of each experiment. The thermal stabilities of untreated and cholate-treated enzymes, in the presence of 20 mM glucose-6-P, are shown in Fig. 8. As compared with thermal inactivation of enzyme in the absence of substrate, glucose-6-P stabilized glucose-6-phosphatase against thermal inactivation (compare Figs. 4 and 8). On the other hand, the activity of glucose-6-phosphatase decayed to zero with a single rate constant in the presence of 20 mM glucose 6-P (see Fig. 9). Also, despite the destabilization of the enzyme by cholate, the cholate-treated enzyme was at least as stable as the untreated enzyme in the presence of 20 mM glucose-6-P (Fig. 8).

\( \Delta H^\ddagger \) for thermal inactivation of enzyme in the presence of glucose-6-P, but not treated with cholate, was smaller than in the absence of substrate. \( \Delta S^\ddagger \) for inactivation of enzyme-glucone-6-P was also smaller than for enzyme in the absence of substrate. Binding of glucose-6-P appeared, therefore, to alter the basic mechanism for thermal inactivation of glucose-6-phosphatase. In addition, \( \Delta H^\ddagger \) for inactivation of cholate-treated enzyme was greater for the complex E-glucose-6-P (or cholate-treated enzyme) than for cholate-treated enzyme in the absence of glucose-6-P, and an increase in \( \Delta H^\ddagger \) for inactivation of the complex E-glucose-6-P versus enzyme alone was the thermodynamic basis for stabilization of cholate-treated enzyme by glucose-6-P. Thus, \( \Delta S^\ddagger \) for inactivation of cholate-treated enzyme was greater for enzyme-glucose-6-P than for substrate-free enzyme. The mechanism for substrate-induced stabilization of cholate-treated enzyme appeared, therefore, to be different from that for enzyme not treated with cholate, suggesting that glucose-6-P interacted with untreated and cholate-treated enzyme in fundamentally different ways. Indeed, by using the ratios for rates of inactivation in the presence and absence of glucose-6-P to estimate the stabilization energy due to enzyme-glucose-6-P interactions, we calculate that glucose-6-P, at 46 °C, provided about 2100 cal/mol to stabilize the untreated enzyme and 4100 cal/mol to stabilize the cholate-treated enzyme.

The Pathway for Thermal Inactivation of Glucose-6-phosphatase in Untreated Microsomes—The decay of glucose-6-phosphatase activity to zero with a single rate constant, in the presence of 20 mM glucose-6-P (Fig. 9), provided an experimental approach for distinguishing between the decay of a single form of enzyme to a less active form versus the
presence in microsomes of two forms of glucose-6-phosphatase with different rates for hydrolysis of substrates, specificities for glucose-6-P and mannose-6-P, and thermal stabilities. We will consider for this purpose that microsomes contain two glucose-6-phosphatases \( E_1 \) and \( E' \), and that \( E' \) has less activity but is more stable than \( E_1 \). The data in Fig. 9 predict that \( E_1 \)-glucose-6-P and \( E' \)-glucose-6-P, if the latter exists, will decay to zero activity with nearly the same rate constants, taking into account the problem of detecting the rate of decay of a small amount of \( E' \)-glucose-6-P in the presence of a larger amount of \( E_1 \)-glucose-6-P. To examine this question, we prepared microsomes that contained only the residual stable activity of glucose-6-phosphatase, which we refer to as \( E \). This was done by heat inactivation of glucose-6-phosphatase until the stable residual level of activity was reached. Microsomes were treated at \( 53.5 \, ^\circ \text{C} \) for 11 min, assaying aliquots for activity during this interval to ensure that the activity of glucose-6-phosphatase had decayed to the stable residual level. This residual activity was 7% of starting activity, which we attribute to the \( E' \) form of the enzyme. Microsomes containing only the \( E' \) form of the enzyme then were mixed with 20 mM glucose-6-P. The half-time for inactivation of residual enzyme (\( E' \)) in the presence of 20 mM glucose-6-P at \( 53.5 \, ^\circ \text{C} \) was 28 min. These data are shown in the open squares in Fig. 9. The rate of inactivation, to zero activity, of \( E_1 \)-glucose-6-P in untreated microsomes in the presence of 20 mM glucose-6-P was 2.2 min at \( 53.0 \, ^\circ \text{C} \), as shown by the closed circles in Fig. 9. The data in open circles in Fig. 9 indicate inactivation of enzyme in untreated microsomes in the absence of substrates. The data in Fig. 9 show that the rates of inactivation for \( E_1 \)-glucose-6-P and the proposed \( E' \)-glucose-6-P are sufficiently different so that inactivation of glucose-6-phosphatase to zero activity in the presence of 20 mM glucose-6-P would not fit a single rate constant if activity were due to two preexisting forms of the enzyme with differing stabilities. We think it is reasonable to conclude, therefore, that the decay of glucose-6-phosphatase in the absence of substrate is described by the expression:

\[ E_1 \rightarrow E_2 \rightarrow E_3 \]

Reversal of the Effects of Cholate—In order to examine further the effects of cholate on the stability of glucose-6-phosphatase, cholate was removed from the microsomes by treatment with Bio-Beads (26). We refer to enzyme treated in this way as the cholate-stripped enzyme. The specific activities of glucose-6-phosphatase in untreated, cholate-treated, and cholate-stripped microsomes are shown in Table III. An interesting result from these measurements was that removal of cholate from microsomes was associated with a further 2-fold increase in activity with either glucose-6-P or mannose-6-P so that the specific activity of mannose-6-phosphatase was more than 20-fold greater for cholate-stripped as compared with untreated microsomes. Since 91% of added protein was recovered after chromatography of cholate-treated microsomes on Bio-Beads, only a small part of this large increase in activity can be attributed to selective enrichment of glucose-6-phosphatase in the microsomes.

There are two possible mechanisms for the results in Table III. The first is that cholate not only activated glucose-6-phosphatase but that it also inhibited the enzyme by binding at specific sites on it, i.e., in the classical sense of noncompetitive or competitive inhibition. The second is that glucose-6-phosphatase has many available stable and enzymatically active conformations and that removal of cholate led to a change in the functional state of the enzyme. Obviously, these mechanisms are not mutually exclusive. Data for the thermal stability of glucose-6-phosphatase in cholate-stripped microsomes suggest that the latter mechanism applies, however. For example, as shown in Fig. 10, removal of cholate from microsomes produced a form of the enzyme that was different from either the untreated or the cholate-treated enzymes. The enzyme in cholate-stripped microsomes became inactivated with a \( t_{1/2} \) of 6.5 min (at \( 34 \, ^\circ \text{C} \)), but only until activity reached about 75% of starting activity. The rate of inactivation then fell, suggesting a process of conversion of an active to a somewhat less active form of glucose-6-phosphatase. Removal of cholate stabilized glucose-6-phosphatase as compared with stability in the presence of cholate, but did not return the enzyme to its original state. Enzyme in cholate-stripped microsomes was not as stable as glucose-6-phosphatase in untreated microsomes. In addition, the pathway for inactivation in untreated microsomes was different from that in cholate-stripped microsomes, as is apparent from the differences in the levels of the stable residual activities after thermal inactivation of untreated and cholate-stripped enzyme in the absence of glucose-6-P (compare Figs. 2 and 10). Verification that removal of cholate altered the functional state of the enzyme does not exclude the possibility that cholate bound to glucose-6-phosphatase at a specific site to cause inhibition, but the mechanism validated by the data for thermal inactivation of cholate-stripped enzyme makes it impossible to establish the kinetic mechanism of cholate-induced inhibition.

### Table III

Activity of glucose-6-phosphatase after removal of cholate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucose-6-P (nmol/min/mg protein)</th>
<th>Mannose-6-P (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>120</td>
<td>23</td>
</tr>
<tr>
<td>Cholate</td>
<td>232</td>
<td>210</td>
</tr>
<tr>
<td>Cholate-stripped</td>
<td>561</td>
<td>465</td>
</tr>
</tbody>
</table>

**Fig. 10. Time course for the thermal inactivation of cholate-stripped glucose-6-phosphatase (C).** Cholate was removed from microsomes as described under “Materials and Methods.” The cholate-stripped microsomes (final concentration of 1.5 mg protein/ml) were then mixed with 50 mM Pipes, pH 6.1, at \( 34 \, ^\circ \text{C} \). Aliquots were removed and assayed for glucose-6-phosphatase activity at \( 23 \, ^\circ \text{C} \) at the indicated times. Protein concentration in the assays was 0.3 mg/ml. The data are plotted as percent of original activity remaining at each time point.
Glucose-6-P stabilized cholate-stripped glucose-6-phosphatase. However, the cholate-stripped enzyme was less stable to thermal inactivation in the presence of glucose-6-P than either the untreated or cholate-treated enzyme (Fig. 8). As for the untreated enzyme, the activity of cholate-stripped enzyme decayed to zero with a single rate constant in the presence of 20 mM glucose-6-P (data not shown). The thermodynamic parameters in Table II for the cholate-stripped enzyme also show that removal of cholate did not return the enzyme to its original state.

Thermotropic Properties of Nonspecific Acid Phosphatase in Microsomes—Microsomes are believed to contain nonspecific acid phosphatase in addition to glucose-6-phosphatase (29, 30). Since these enzymes have not been purified, their substrate specificities and values of $k_{on}$ with different substrates are unknown. As a result, there is no way to estimate the extent of hydrolysis of a sugar-P by each of these enzymes in microsomes. Nevertheless, the question arises as to whether the hydrolysis of glucose-6-P and/or mannose-6-P by acid phosphatase (or any other nonspecific phosphatase) could confound the interpretation of the data for the thermal inactivation of glucose-6-phosphatase. To address this problem we characterized the thermotropic properties in microsomes of the activity that catalyzes the hydrolysis of glycerol-2-P, which is believed to be a substrate of the acid phosphatase (29, 30). These studies were carried out using 5 mM glycerol-2-P and Pipes, pH 6.1. The key findings in these experiments were that the rate of hydrolysis of glycerol-2-P was insensitive to treatment at 47 °C (the highest temperature studied) in either untreated or cholate-treated microsomes and was insensitive as well to activation by treatment with cholate. These data exclude that the thermal decay of glucose-6-phosphatase and mannose-6-phosphatase activities. under any of the experimental conditions in this work, reflect combined activities of glucose-6-phosphatase and acid phosphatase. They also exclude that the residual stable levels of the glucose-6 and mannose-6-phosphatases are due to acid phosphatase in microsomes and that glycerol-2-P was hydrolyzed by glucose-6-phosphatase.

**DISCUSSION**

Evidence That Glucose-6-phosphatase Has Several Stable Enzymatically Active States—The differing patterns of inactivation of glucose-6-phosphatase under different conditions and the thermodynamic parameters for thermal inactivation indicate that there are several stable and active states of the enzyme. We refer to states of glucose-6-phosphatase because there is no independent evidence that changes in function were associated with changes in conformation and because conformational changes may not be required to account for the differences in the pathways for thermal inactivation of untreated and cholate-treated enzyme. For example, changes in the apolar environment of a membrane-bound enzyme may not affect the conformation of the enzyme but still influence function by determining in part how energy added to the enzyme in the form of heat or binding of a ligand will be relaxed (31–34). Nonspecific effects of the environment on the functional state of water-soluble proteins seems to apply too in the case of thermal stabilization by glycerol (35, 36). The different functional states of glucose-6-phosphatase apparent from the stability data hence may not be due to changes in conformation. Nevertheless, the data do show that five different states of the enzyme can be identified: enzyme in untreated microsomes ($E_i$); enzyme produced by the thermal decay of $E_i$ in the absence of glucose-6-P ($E_o$), enzyme in cholate-treated microsomes ($E_i$); enzyme in cholate-stripped microsomes ($E_i$); and enzyme ($E_o$) produced by thermal decay of $E_o$ in the absence of glucose-6-P. The conclusion that these enzymes are different functional states of glucose-6-phosphatases does not rest on differences in their specific activities or assumptions about the causes for differences in specific activities.

The different states of glucose-6-phosphatase cannot be interconverted freely since it appears that the barriers between certain states must be high. For example, removal of cholate did not regenerate $E_i$. What seems most important in the present state of knowledge of glucose-6-phosphatase, however, is that thermal energy led to interconversion between states and that the environment of the enzyme, as reflected by the presence or absence of cholate and/or glucose-6-P, determined the pathway by which the input of thermal energy was relaxed.

Evidence from Thermal Stability That the Specific Activity and Catalytic Specificity of Glucose-6-phosphatase Are Regulated Dynamically—The stability data suggest that detergent changed the manner in which glucose-6-phosphatase interacted with glucose-6-P, which must be an effect independent of the question of access of substrate to the active site of the enzyme. The stability data also suggest that the ratio of activities with glucose-6-P and mannose-6-P is not an immutable property of the catalytic unit of glucose-6-phosphatase but is subject to dynamic regulation. For example, during the thermal inactivation of the enzyme, the ratio of activities with glucose-6-P and mannose-6-P changed. In addition, the rate constants for thermal inactivation of glucose-6-phosphatase were different with glucose-6-P and mannose-6-P as substrates. As shown above (Fig. 9), these results cannot be attributed to two forms of glucose-6-phosphatase in untreated microsomes nor can they be attributed to a change in the access of substrate to the enzyme. Thus, the extent of detergent-induced activation of enzyme was the same before and after partial thermal inactivation of glucose-6-phosphatase (Table I). Moreover, under the conditions used to measure thermal inactivation, any limitation on the access of substrate to the enzyme was constant for each measurement of activity. If this were not so, then the Arrhenius plots in Fig. 1 would not have been linear. Therefore, the only variable in the measurement of stability was the amount of active enzyme ($E_i$) remaining after different periods of treatment at a given temperature, and activity at each point in a decay curve (Fig. 2, for example) can be expressed as $v = k[E_i]$, where $k$ includes terms for any factor limiting access of substrate as well as the binding constant for enzyme-substrate interaction. The rate constant for thermal inactivation of the enzyme will be independent of $k$.

Proposed Explanation of the Differential Stability of Glucose-6-phosphatase and Mannose-6-phosphatase—The data presented above and elsewhere (1–16) require that we be able to explain differences in rates of hydrolysis of glucose-6-P and mannose-6-P in untreated microsomes, changes in these rates after treatment of microsomes with cholate and other agents, differences in the thermal stability measured with these substrates, and changes in stability after treatment of microsomes with cholate. All these events can be explained as follows.

The hydrolysis of sugar-P catalyzed by glucose-6-phosphatase proceeds according to reactions (1) and (2) (3, 37–39).

1. **Sugar-P + enzyme → enzyme-P + sugar**

2. **Enzyme P + H₂O → enzyme + P**

We propose (i) that hydrolysis of mannose-6-P in untreated microsomes is limited by reaction 1 while hydrolysis of glucose-6-P is limited by reaction 2, and (ii) that treatment with...
cholate increases the rate of reaction 1 with mannose-6-P sufficiently so that mannose 6-phosphatase activity is limited in cholate-treated microsomes by the rate of reaction 2, which also increases as compared with untreated microsomes. These changes can account for the limitation on the rate of hydrolysis of mannose-6-P in untreated microsomes versus cholate-treated microsomes and why glucose-6-phosphatase and mannose-6-phosphatase activities become equal after cholate is added to microsomes. Obviously, i and ii do not comprise a unique explanation for these observations. However, they do account for experimental observations that are not explained by other proposals for the regulation of glucose-6-phosphatase. Most important, i and ii account for the different thermal stabilities of glucose-6-phosphatase and mannose-6-phosphatase in microsomes not treated with cholate, and they predict that the thermal stabilities of these two activities will be the same for cholate-treated microsomes, which were observed. The effect of cholate on the energetics of enzyme-substrate interactions evident from the data in Table II is compatible with a fundamental change in the active site region of the enzyme, which supports the idea that cholate changes the rates of reactions 1 and 2. Stopped-flow studies have shown that the rate of reaction 1 with glucose-6-P exceeds that of reaction 2 by at least 10-fold and that the rate of reaction 1 with glucose-6-P does not change with cholate treatment (18).

Ness et al. (40) have reported recently that radiation inactivation analysis of glucose-6-phosphatase in untreated and detergent-treated microsomes yielded the same target sizes for all activities of the enzyme whether or not microsomes had been treated with detergent. They point out that this result is contrary to the expectations of the compartmentation hypothesis. Thus, there is increasing evidence supporting the idea that glucose-6-phosphatase is regulated dynamically by its membrane environment.

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