Regulation of Liver Phosphorylase Phosphatase by Glutathione Disulfide

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The mechanism of inactivation of rabbit liver phosphorylase phosphatase by glutathione disulfide (GSSG) was investigated. The catalytic subunit of phosphorylase phosphatase was inactivated by GSSG and other disulfides. Inactivation by GSSG was a concentration-dependent process and resulted in the formation of an inactive, stable enzyme species. The inactivated enzyme could be reactivated by addition of various sulfhydryl compounds, including glutathione (GSH). Homogeneous phosphorylase phosphatase contains, per mol of catalytic subunit ($M_r = 33,000$), two sulfhydryl groups, one of which reacted with GSSG to form inactive enzyme.

Binding studies with [glycine-2-$^3$H]GSSG revealed simultaneous incorporation of $^3$H radioactivity into the catalytic subunit and stoichiometric loss of catalytic activity. Treatment of the $^3$H-labeled enzyme with GSH resulted in the formation of an inactive enzyme. The results suggest that inactivation of phosphorylase phosphatase by GSSG results from the formation of a mixed disulfide between GSSG and one of the two sulfhydryl groups in the catalytic subunit.

Recently, protein inhibitors of phosphorylase phosphatase have been isolated from liver and muscle extracts and were suggested to be involved in regulation of the phosphatase (1–5). In a previous study, we found, in the liver extract, another factor that can inactivate liver phosphorylase phosphatase. By isolation, this factor was identified as glutathione disulfide (GSSG) (6). The GSSG-inactivated phosphatase was reactivated by incubation with sulfhydryl compounds such as GSH and 2-mercaptoethanol.

These results suggest that the regulation of phosphorylase phosphatase activity involves a sulfhydryl-disulfide exchange reaction between sulfhydryl groups of the phosphatase and GSSG, similar to that previously described for liver glycogen synthetase (7, 8) and fructose diphosphatase (9). We now report that GSSG inactivates liver phosphorylase phosphatase by forming a mixed disulfide with one of the two sulfhydryl groups contained in the catalytic subunit.

**EXPERIMENTAL PROCEDURES**

**Preparation of Liver Phosphorylase Phosphatase Catalytic Subunit.—**Homogeneous phosphorylase phosphatase catalytic subunit

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RESULTS

Inactivation of Phosphorylase Phosphatase Catalytic Subunit by Disulfides and Reactivation by Sulfhydryl Compounds—Of the disulfide compounds listed in Table II, GSSG was purified from rabbit liver by means of ethanol precipitation according to a slight modification of the method of Brandt et al. (10).

Briefly, the procedure involved preparation of crude liver extract with 50 mM imidazole (pH 7.45), 5 mM EDTA, 0.5 mM dithiothreitol, acid precipitation at pH 5.8, ammonium sulfate precipitation, ethanol precipitation, diaminohexane-Sepharose 4B column chromatography, Sephadex G-200 column chromatography, and finally DE52 column chromatography. The overall purification of the phosphatase is summarized in Table I. The final preparation of phosphatase catalytic subunit was approximately 21,000-fold-purified and had a specific activity of 2,300 units/mg of protein. Sodium dodecyl sulfate-disc gel electrophoresis of the purified enzyme revealed a single component with an apparent molecular weight of 33,000.

**Assay of Phosphorylase Phosphatase Activity—**Phosphorylase phosphatase activity was assayed by measuring the liberation of radioactive P, from ["P"]phosphorylase a as described previously (6). The standard assay mixture contained approximately 30 pmol of phosphorylase a, 5 mM theophylline, 70 mM Tris (pH 7.4), and enzyme preparation in a total volume of 0.3 ml. One unit of phosphorylase phosphatase was defined as the amount liberating 1 nmol of P, from phosphorylase a/min.

Inactivation and Reactivation of Phosphorylase Phosphatase Catalytic Subunit—Inactivation of phosphorylase phosphatase was performed as described previously (6). After a 30-min incubation at 30°C with GSSG or other disulfides in 40 mM Tris (pH 7.4), the phosphatase preparation was passed through a small column (1 × 11 cm) of Sephadex G-25 to remove the reactants. In some experiments, the phosphatase preparation was dialyzed against 50 mM imidazole, 5 mM EDTA (pH 7.45), instead of passing through the Sephadex G-25 column. Reactivation of the inactivated phosphatase was carried out by incubation with sulfhydryl compounds added in the assay mixture for 10 min at 37°C.

**Determination of Sulfhydryl Groups—**Sulfhydryl groups were measured spectrophotometrically by titration with DTNB. Phosphorylase phosphatase catalytic subunit was treated with 50 mM 2-mercaptoethanol for 30 min at room temperature and dialyzed for 36 h against three changes of 50 mM imidazole, 5 mM EDTA (pH 7.45) at 4°C. The sample was made in 2% sodium dodecyl sulfate followed by the addition of an excess of DTNB. The absorbance at 412 nm was monitored and the number of sulfhydryl groups was calculated based on a molar extinction coefficient of 13,600 M$^{-1}$ cm$^{-1}$ (11). In some cases, sulfhydryl groups were also determined in the absence of sodium dodecyl sulfate. Protein was determined by the method of Lowry et al. (12).

**Preparation of [glycine-2-$^3$H]GSSG—**[Glycine-2-$^3$H]GHSH (100 μCi; specific activity, 5 Ci/mmol; obtained from New England Nuclear) was dissolved in 0.01 M sodium phosphate buffer (pH 7.5). After the addition of 0.5 μmol of CuCl$_2$, the solution was bubbled with oxygen for 2 h at 25°C (7). [Glycine-2-$^3$H]GSSG was separated from unreacted ["H]GHSH, CuCl$_2$, and other impurities containing H on a column of Sephadex G-10 equilibrated with 0.3 M acetic acid.

1. The abbreviation used is: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).
TABLE I
Purification of the catalytic subunit of phosphorylase phosphatase from rabbit liver

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Specific activity</th>
<th>Recovery</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>0.110</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Acid supernatant</td>
<td>0.226</td>
<td>75</td>
<td>2</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation</td>
<td>3.72</td>
<td>513</td>
<td>33.8</td>
</tr>
<tr>
<td>Ethanol precipitation</td>
<td>8.09</td>
<td>251</td>
<td>73.5</td>
</tr>
<tr>
<td>Diaminohexane-Sepharose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First column</td>
<td>207.1</td>
<td>133</td>
<td>1,883</td>
</tr>
<tr>
<td>Second column</td>
<td>701.3</td>
<td>80</td>
<td>6,375</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>1,215</td>
<td>15</td>
<td>11,045</td>
</tr>
<tr>
<td>DE52 column</td>
<td>2,314</td>
<td>7</td>
<td>21,036</td>
</tr>
</tbody>
</table>

TABLE II
Inactivation of phosphorylase phosphatase by disulfides and its reactivation by 2-mercaptoethanol

For inactivation, phosphorylase phosphatase catalytic subunit (5 units) was incubated for 30 min at 36°C with 10 mM of various disulfides in a total volume of 0.42 ml. After passage through a Sephadex G-25 column, the enzyme preparation was assayed for phosphorylase phosphatase activity. For reactivation of the enzyme, aliquots of the inactivated enzyme preparation were preincubated for 10 min at 37°C in the assay mixture with 50 mM 2-mercaptoethanol, and then phosphorylase activity was assayed. The phosphorylase activity of the control preparation, which was similarly treated but without disulfide, was taken as 100, and all other enzyme activities are shown relative to this value.

<table>
<thead>
<tr>
<th>Disulfides</th>
<th>Phosphatase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inactivation</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>GSSG</td>
<td>26</td>
</tr>
<tr>
<td>Cystine</td>
<td>40</td>
</tr>
<tr>
<td>Homocystine</td>
<td>37</td>
</tr>
<tr>
<td>Cystamine</td>
<td>12</td>
</tr>
<tr>
<td>Lipoyic acid</td>
<td>82</td>
</tr>
</tbody>
</table>

Fig. 1. Reactivation of GSSG-inactivated phosphorylase phosphatase by thiol compounds. Phosphorylase phosphatase catalytic subunit was inactivated with 20 mM of GSSG as described in the legend to Table II. Aliquots of the inactivated enzyme preparation were assayed for phosphorylase phosphatase activity after 10-min preincubation with various concentrations of thiol compounds added in the standard assay mixture. DTT, dithiothreitol.

had a potent inactivating effect on the catalytic subunit of liver phosphorylase phosphatase, and its effect was completely reversed by incubation with 2-mercaptoethanol. Cystine and homocystine also caused reversible inactivation of the catalytic subunit. Cystamine caused a strong inactivation of the phosphatase, but cystamine-inactivated enzyme could not be reactivated by 2-mercaptoethanol. A similar effect of cystamine on muscle phosphorylase phosphatase has been reported (13). Lipoic acid, a ring disulfide compound, had little effect in phosphatase inactivation.

Fig. 2. Titration of phosphorylase phosphatase catalytic subunit with DTNB. Phosphorylase phosphatase catalytic subunit was treated previously with 50 mM 2-mercaptoethanol for 30 min at room temperature and dialyzed against three changes of 50 mM imidazole, 5 mM EDTA buffer (pH 7.45). The enzyme solution (0.5 ml; 200 units) was mixed with 0.25 ml of 0.25 M sodium phosphate buffer (pH 8.5) with (O) and without (□) 6% sodium dodecyl sulfate (SDS) and 0.1% EDTA. To this solution, 25 µl of 10 mM DTNB was added 10 min later. The absorbance at 412 nm was followed as a function of time and the value was converted to sulfhydryl equivalents as described under "Experimental Procedures". The molecular weight of the catalytic subunit was taken as 33,000 for calculation.
the concentration of 5 to 10 mM; however, higher concentrations resulted in somewhat less reactivation, probably due to its easily oxidizable nature. In our previous study using partially purified phosphorylase phosphatase, maximal reactivation of the GSSG-inactivated enzyme was achieved with both thiol and Mn$^{2+}$ (6). However, in the present experiment using homogeneous catalytic subunit of the phosphatase, reactivation by thiol did not require Mn$^{2+}$. Instead, the reactivating effect of the thiols was reduced by about 10% in the presence of Mn$^{2+}$.

**Sulfhydryl Content of the Catalytic Subunit and Its Relation to Catalytic Activity**—The titration curve for sulfhydryl content in the phosphatase catalytic subunit is shown in Fig. 2. Titration with excess DTNB in the absence of sodium dodecyl sulfate gave 1 mol of sulfhydryl group/mol of catalytic subunit. However, in the presence of sodium dodecyl sulfate, 2 mol of sulfhydryl groups/mol of catalytic subunit were detected. To determine whether these two sulfhydryl groups are both involved in inactivation of phosphorylase phosphatase, the relationship between the content of sulfhydryl groups in the catalytic subunit and the extent of inactivation was examined at various concentrations of GSSG (Fig. 3). As the GSSG concentration increased, the sulfhydryl content of the enzyme was decreased in rough proportion to the loss of phosphatase activity. When one free sulfhydryl group was modified by GSSG at high concentrations above 20 mM, the phosphatase was completely (over 90%) inactivated. The above finding that only one sulfhydryl group reacted with DTNB in the absence of sodium dodecyl sulfate also supports the hypothesis that a single sulfhydryl group is important for enzyme activity. These results indicate that one of the two sulfhydryl groups in the catalytic subunit is involved in disulfide exchange with GSSG, resulting in inactivation of the phosphatase.

**Binding of [glycine-2-$^{3}H$]GSSG to Phosphatase Catalytic Subunit during Inactivation**—To characterize further the relationship between disulfide exchange and inactivation of the phosphatase, a binding study was performed using [glycine-2-$^{3}H$]GSSG. After 30-min incubation of phosphorylase phosphatase catalytic subunit with [$^{3}H$]GSSG, the incubation mixture was subjected to gel filtration on a Sephadex G-100 column (1.6 × 30 cm). Both the phosphatase activity and radioactivity were measured in the eluate (Fig. 4). One small peak of the phosphatase activity was detected when the eluate was assayed in the absence of 2-mercaptoethanol. However, when measured in the presence of 50 mM 2-mercaptoethanol, an approximately 4-fold increase in enzyme activity was observed in all fractions. The peak position of the enzyme activity was identical with that of native catalytic subunit, indicating that the apparent molecular weight of the enzyme did not change during inactivation with [$^{3}H$]GSSG.

Two peaks of radioactivity were obtained, the first coinciding with the peak of enzyme activity, while the second major peak corresponded to unreacted GSSG as demonstrated by a ninhydrin test and by position of the elution. From the total radioactivity bound to the enzyme, it is calculated that 9.73 eq of sulfhydryl group/mol of catalytic subunit (M, = 33,000) has been modified. This value corresponds to approximately 75% inactivation of the enzyme (see Fig. 3). Moreover, when the fractions containing inactivated enzyme were pooled, concentrated to 1 ml, treated with GSH (for 2-mercaptoethanol), and then dialyzed against 50 mM imidazole, 5 mM EDTA, 0.5 mM dithiothreitol buffer (pH 7.45), the radioactivity incorporated into the enzyme was lost during dialysis and the enzyme activity was restored.

**DISCUSSION**

In a previous study, we have demonstrated that GSSG is a natural inactivating factor of liver phosphorylase phosphatase, and that GSSG is involved in regulation of phosphorylase activity by causing inactivation of phosphorylase phosphatase in the liver (6). The mechanism of this inactivation has now been studied with the homogeneous catalytic subunit of phosphorylase phosphatase. Phosphorylase phosphatase was shown to be reversibly inactivated by GSSG and other disulfide compounds by interacting with one of the two sulfhydryl groups contained/mol of catalytic subunit. The inactivated phosphatase could be reactivated by addition of 2-mercaptoethanol, dithiothreitol, or GSH. The Mn$^{2+}$ requirement for maximal reactivation observed previously (6) was not confirmed in the present study. This is probably due to a differ-
ence in the enzyme preparation used. In the previous study, a partially purified enzyme that has a larger molecular weight (about 48,000) than the catalytic subunit \((M_r = 33,000)\) was used. Possibly, Mn\(^{2+}\) is necessary for the modulation of the regulatory subunit which might be contaminated in that enzyme preparation.

The present findings indicate that regulation of liver phosphorylase phosphatase activity involves disulfide exchange between one sulfhydryl group in the catalytic subunit and GSSG. Similar disulfide exchange as a possible regulatory mechanism for sulfhydryl enzymes has been studied with rabbit liver fructose diphosphatase (9), rat liver glycogen synthetase (7, 8), guinea pig liver tyrosine aminotransferase (14), and rat brain adenylate cyclase (15). The effect of disulfide formation on fructose diphosphatase is activation, whereas on the latter three enzymes it is inactivation.

The present results also suggest that the inactivation of liver phosphorylase phosphatase by GSSG is associated with the formation of a mixed disulfide. This assumption is based on the facts that (a) [glycine-\(^2\)H]GSSG is incorporated into the catalytic subunit of the enzyme, with roughly stoichiometric loss of enzyme activity, (b) the incorporated radioactivity is readily released by thiols, with concomitant restoration of enzyme activity, and (c) no apparent molecular weight change in enzyme occurs. Fig. 5 shows a presumed model for inactivation and activation of phosphorylase phosphatase. As mentioned above, liver phosphorylase phosphatase has two sulfhydryl groups in the catalytic subunit. One sulfhydryl group seems unrelated to the formation of mixed disulfide with GSSG. The other can interact with GSSG, thereby participating in regulation of the phosphorylase phosphatase activity.

REFERENCES

Regulation of liver phosphorylase phosphatase by glutathione disulfide.
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