Properties and Regulation of Glutathione Peroxidase*

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SUMMARY

GSH peroxidase has been purified approximately 2500-fold from pig's blood. With cumene hydroperoxide as substrate, kinetic analysis on the purified enzyme gave nonlinear Lineweaver-Burk plots for the hydroperoxide substrate. Linear Lineweaver-Burk plots were obtained for GSH and a limiting $K_m$ value of approximately 3 mM was obtained for GSH. A limited analysis of the rate data has been carried out and a tentative mechanism for GSH peroxidase is given.

A wide range of nucleotides inhibited the enzyme, with pyrimidine nucleotides being the most effective. Also, the inhibitory effectiveness increased with the number of phosphate groups in the nucleotide. Nucleotide inhibition was competitive with respect to GSH whereas increased levels of hydroperoxide enhanced the inhibition.

The sensitivity of the enzyme to nucleotide inhibition could be substantially decreased by x-ray, ethanol, or trypsin treatment, or aging with a lesser decrease of catalytic activity. Conversely, heat, $p$-chloromercuribenzoate, or sodium lauryl sulfate preferentially abolished the catalytic function with a lesser effect on the nucleotide response. It was concluded that nucleotides interact with the enzyme at a site other than the active center and hence that GSH peroxidase is an allosteric enzyme.

A glutathione peroxidase (hydrogen peroxide: GSH oxidoreductase, EC 1.11.1.9) was first described by Mills and Randall in erythrocytes (1) and later in other tissues (2). Cohen and Hochstein (3) have provided evidence that peroxidase in erythrocytes may be able to break the autocatalytic chain reaction of lipid peroxidation and thus act as an antioxidant.

The properties of a preparation of glutathione peroxidase from a hemolysate were first investigated by Mills (11). Little and O'Brien (8, 12), using a preparation from liver, investigated in more detail the specificity for both substrates, the stoichiometry of the reaction, pH optima, and the effects of various inhibitors. The kinetics of the enzyme, however, were investigated under substrate concentrations where the reaction rate was first order and a tentative mechanism for GSH peroxidase is given.

The following experiments were made repeatedly to ensure that the peroxidase concentration rather than GSSG reductase was rate-limiting. Occasionally the assay method was further checked by assaying the inhibitory effectiveness increased with the number of phosphate groups in the nucleotide. Nucleotide inhibition was competitive with respect to GSH whereas increased levels of hydroperoxide enhanced the inhibition.

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EXPERIMENTAL PROCEDURE

Materials

All nucleotides, coenzymes, glutathione reductase, trypsin, $p$-chloromercuribenzoate, iodosaccharate, iodine, GSH, and GSSG were purchased from Sigma. ROOH was supplied by Matheson, Coleman, and Bell. All other reagents used were of analytical reagent grade of purity. Deionized water was used for making all solutions.

Assay of Enzymic Activity

GSH peroxidase activity was assayed as described previously (8, 13). The enzyme is coupled to NADPH via GSSG reductase and the rate of NADPH oxidation is measured spectrophotometrically at 340 nm. Unless otherwise stated, the reaction mixture (1 ml) contained 0.120 mM NADPH, 0.1 M Tris-HCl buffer (pH 7), 0.25 mM GSH, 0.2 mM ROOH, 3 mM EDTA, and a large excess of GSSG reductase. Throughout the experiments checks were made repeatedly to ensure that the peroxidase concentration rather than GSSG reductase was rate-limiting. Occasionally the assay method was further checked by assaying

1 The symbol ROOH is used to denote cumene hydroperoxide:

$$\text{C}_9\text{H}_8-\text{C}(\text{CH}_3)_2-\text{O}+\text{OH}^-$$
residual GSH concentration at different times during the reaction by the method of Boyer (14).

In the absence of peroxidase, an uncatalyzed reaction typically <5% and never >10% of the total rate was observed. This background rate varied with GSH and ROOH concentrations. Enzymic rates were calculated from

\[ \text{Enzymic rate} = \text{total rate} - \text{rate without peroxidase} \]

**Irradiation Conditions**

The enzyme was irradiated in flat bottomed glass tubes in the presence of air at 0°. The irradiation source was a Philips MG 100 x-ray machine. The irradiation parameters were 100 kv, 10 ma with 0.5-mm Be filtration. The dose rate was 2 kR per min.

All enzyme assays were carried out at 23° and all other reactions at 0° unless otherwise stated.

Protein was determined by the method of Lowry et al. (15).

**Purification of Enzyme**

**Preparation of Hemolysate**—Pig blood (3 to 4 liters) containing EDTA as anticoagulant was allowed to stand overnight. The blood was then centrifuged at 1,500 \( \times \) g for 30 min and the cells were washed twice with an equal volume of 0.9% NaCl solution and centrifuged as before. The buffy coat layer was sucked off. The erythrocytes were then hemolyzed by 30 min exposure to saponin (0.05%, w/v). The mixture was centrifuged at 20,000 \( \times \) g for 15 min and the precipitate was discarded.

**Solvent Precipitation**—The hemolysate was adjusted pH 6.5 with acetic acid and the hemoglobin was removed by exposure to ethanol-chloroform at -10° according to the method of Keilin (16). The supernatant fluid was then treated with 35% acetone at -20° for 30 min and the precipitate was removed by centrifugation (20,000 \( \times \) g for 15 min). The precipitate was dissolved in 50 mM potassium phosphate buffer, pH 6.8.

**Sephadex CM-50**—The solution was applied to a column (5 \( \times \) 40 cm) of Sephadex CM-50 ion exchange resin and eluted with the above solvent buffer. The colorless fractions containing GSH-peroxidase activity were pooled and concentrated by pressure filtration through an Amicon PM30 ultrafiltration membrane.

**Sephadex G-100**—Concentrated active fraction from the previous step was applied to a column (10 \( \times \) 90 cm) of Sephadex G-100 and eluted with 20 mM Tris-HCl buffer, pH 7. Then 150 ml of eluant containing the peak of enzyme activity were collected.

**DEAE-Sephadex**—The eluant was loaded onto a column (8 \( \times \) 40 cm) of DEAE-Sephadex and a continuous gradient of potassium phosphate buffer, pH 6.8, from 0.005 M to 0.18 M was applied. The enzyme eluted from the column at approximately 0.07 M buffer. Fractions containing activity were pooled and concentrated by pressure filtration as above.

The enzyme was stored as a 1 mg per ml solution in 20% glycerol at -16°. After 1 month, the enzyme retained 70 to 60% of its original activity. Marked losses in the sensitivity of the purified enzyme to nucleotide inhibition (see below) were noted during storage. Less purified samples of enzyme were, however, much more stable to storage.

**RESULTS**

**Purification of Enzyme**—The results of a typical purification procedure are summarized in Table I. Certain anomalies were observed during the purification. First, significant variations were noted in the specific activity of the hemolysates from different samples of pig's blood. These variations may arise from the effects of hormonal and sex factors on the levels of GSH peroxidase (17). Second, anomalously high recoveries of activity were obtained after certain purification steps, especially gel filtration. It is possible that an inhibitor of GSH peroxidase is removed at this stage. Evidence for this theory was that, when the active Sephadex G-100 eluant fractions were combined with the inactive fractions, a decrease in the total units of activity was noted. Thus, purification of the enzyme, as measured by increases in specific activity, may be due in part to inhibitor removal.

It was also noted that the specific activity of the final preparation could be increased by 2.5- to 3-fold by incubation in 25 mM GSH, at pH 7, for 30 min at 30°.

**Enzyme Kinetics**—The dependence of the reaction rate on the concentration of hydroperoxide substrate was first examined. It

![Fig. 1. Lineweaver-Burk plots for GSH peroxidase. A, effect of GSH on saturation kinetics for ROOH. B, effect of ROOH on saturation kinetics for GSH. The conditions of assay were as described under "Assay of Enzymic Activity," except that the level of GSH and ROOH were varied.](image)

**Table I**

<table>
<thead>
<tr>
<th>No. and fraction</th>
<th>Volume</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>units</td>
<td>mg</td>
<td>units/mg</td>
<td>%</td>
</tr>
<tr>
<td>1. Hemolysate</td>
<td>1,200</td>
<td>285,000</td>
<td>204,000</td>
<td>1.1</td>
<td>100</td>
</tr>
<tr>
<td>2. Solvent precipitation</td>
<td>160</td>
<td>72,000</td>
<td>1,312</td>
<td>54</td>
<td>25</td>
</tr>
<tr>
<td>3. Carboxymethyl Sephadex</td>
<td>135</td>
<td>82,000</td>
<td>432</td>
<td>190</td>
<td>29</td>
</tr>
<tr>
<td>4. Sephadex G-100</td>
<td>170</td>
<td>154,500</td>
<td>306</td>
<td>505</td>
<td>54</td>
</tr>
<tr>
<td>5. DEAE-cellulose</td>
<td>75</td>
<td>118,000</td>
<td>34</td>
<td>3470</td>
<td>41</td>
</tr>
</tbody>
</table>
Properties of GSH Peroxidase

The enzyme was assayed in the presence of different amounts of the various nucleotides and the nucleotide concentration causing 30% inhibition was obtained. Assays were carried out as described under "Assay of Enzymic Activity."

In Table II the inhibitory power of various nucleotides for the enzyme was compared. All nucleotides studied caused some degree of enzyme inhibition. In general, the pyrimidine nucleotides were the most effective. Of the nonpyridine nucleotides adenosine nucleotides seemed more effective than those of other bases. It is also apparent from Table II that the number of phosphate groups in a nucleotide seems to influence its inhibitory effect. Thus NADP+ was a better inhibitor than NAD+. In addition, the inhibitory power of the adenosine nucleotides increased in the order adenosine, AMP, ADP, ATP, adenosine 5'-tetraphosphate.

The inhibition of GSH peroxidase by ATP was studied in more detail. Fig. 3 shows the effect of the two enzyme substrates on ATP inhibition. The data are plotted after the fashion of Webb (21). It is apparent from Fig. 3 that the interaction between GSH and ATP is of a conventional, purely competitive type with a calculated $K_i$ value of 2.9 mM ATP. However, the interaction between cumene hydroperoxide and ATP is of a mixed type with increased levels of the hydroperoxide enhancing rather than decreasing the inhibitory effectiveness of ATP.

Physical and Chemical Modification—Nucleotides might inhibit GSH peroxidase by interacting at or very near to the active center or at a separate site. To gain more information on the site of nucleotide interaction, the enzyme was subjected to various chemical and physical treatments and the effects on the catalytic activity and the ATP sensitivity were measured and compared. Table III shows that whereas certain treatments of the enzyme preferentially destroyed the catalytic activity others preferentially destroyed the ATP response. Thus, heat, $p$-chloromercuibenzoate, or sodium lauryl sulfate decreased the

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**TABLE II**

**Inhibition of GSH peroxidase by nucleotides**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Concentration required for 30% inhibition (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH</td>
<td>0.26</td>
</tr>
<tr>
<td>5'-Adenosine tetraphosphate</td>
<td>0.5</td>
</tr>
<tr>
<td>NADP+, 5'ATP</td>
<td>0.7</td>
</tr>
<tr>
<td>3'-AMP</td>
<td>1.0</td>
</tr>
<tr>
<td>5'-ADP, NAD+</td>
<td>1.5</td>
</tr>
<tr>
<td>3',5'-AMP, GTP, ITP</td>
<td>2.4</td>
</tr>
<tr>
<td>2'-AMP</td>
<td>3.0</td>
</tr>
<tr>
<td>CTP, 5'-AMP</td>
<td>7.5</td>
</tr>
<tr>
<td>Adenosine</td>
<td>12.0</td>
</tr>
</tbody>
</table>

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Fig. 2. Hill plots of the data in Fig. 1. A, Hill plot of rate data for ROOH. The bar lines indicate maximum scatter of points. The curve was drawn through the average values. B, Hill plot of rate data for GSH. Above 42 mM ROOH all points fell on the same straight line. The bar lines indicate maximum observed scatter of points.

In Fig. 2 the data from Fig. 1 are plotted after the fashion of Hill (20). At the point of inflection of the Hill plot for cumene hydroperoxide the slope of the curve gives a value of $n = 0.5$. At either side of the point of inflection $n = 1$. The Hill plots for the data relating to GSH are straight lines whose slopes give values of $n = 1$. At levels of ROOH sufficient to give the limiting $K_m$ values for GSH, the Hill plots are coincident.

**Inhibition by Nucleotides**—Since there is an increasing number of enzymes whose activity is modified by nucleotides, the effect of a wide range of nucleotides on GSH peroxidase activity was investigated (Table III). All nucleotides studied caused some degree of enzyme inhibition. In general, the pyrimidine nucleotides were the most effective. Of the nonpyridine nucleotides adenosine nucleotides seemed more effective than those of other bases. It is also apparent from Table II that the number of phosphate groups in a nucleotide seems to influence its inhibitory powers. Phosphate ions alone were found to have only a small inhibitory effect. Thus NADP+ was a better inhibitor than NAD+. In addition, the inhibitory powers of the adenosine nucleotides increased in the order adenosine, AMP, ADP, ATP, adenosine 5'-tetraphosphate.

The inhibition of GSH peroxidase by ATP was studied in more detail. Fig. 3 shows the effect of the two enzyme substrates on ATP inhibition. The data are plotted after the fashion of Webb (21). It is apparent from Fig. 3 that the interaction between GSH and ATP is of a conventional, purely competitive type with a calculated $K_i$ value of 2.9 mM ATP. However, the interaction between cumene hydroperoxide and ATP is of a mixed type with increased levels of the hydroperoxide enhancing rather than decreasing the inhibitory effectiveness of ATP.

**Physical and Chemical Modification**—Nucleotides might inhibit GSH peroxidase by interacting at or very near to the active center or at a separate site. To gain more information on the site of nucleotide interaction, the enzyme was subjected to various chemical and physical treatments and the effects on the catalytic activity and the ATP sensitivity were measured and compared. Table III shows that whereas certain treatments of the enzyme preferentially destroyed the catalytic activity others preferentially destroyed the ATP response. Thus, heat, $p$-chloromercuibenzoate, or sodium lauryl sulfate decreased the...
for GSH peroxidase. A ping-pong mechanism is postulated with the two stable forms of the enzyme being $\cdot$SH and $\cdot$SSG. In view of the high specificity of the enzyme for GSH (8), binding is indicated to involve the body of the molecule rather than the $\cdot$SH. The very low specificity shown for hydroperoxide substrates (8,24) suggests that binding involves the $\cdot$OOH function rather than the body of the substrate.
Properties of GSH Peroxidase

Vol. 245, No. 14

nucleotides. Thus, two sites in the enzyme, one responsible for catalytic activity and another involved in binding nucleotides, may be clearly differentiated.

As is the case with most allosteric enzymes, the modifier affected substrate affinity at the active site. Double reciprocal plots showed that ATP inhibition was competitive with respect to GSH.

A replot of the data according to the method of Webb (21) suggests total exclusion of GSH from the active site rather than a simple reduction in the affinity of the enzyme for GSH. A similar analysis of ATP and ROOH interactions reveals that the inhibition is enhanced by increasing levels of ROOH, thus suggesting an uncompetitive or coupling inhibition type.

It is interesting to note that GSH peroxidase does not appear to exhibit the sigmoidal relationship between rate and substrate concentration or degree of inhibition and inhibitor concentration shown by most allosteric enzymes. Thus, GSH peroxidase may not fit the classical concept of an allosteric enzyme which shows both homologous and heterologous interactions.

The enzyme shows $K_m$ values for $\text{H}_2\text{O}_2$ and lipid peroxides of the order of 1 to 10 $\mu\text{M}$ with the $K_m$ value for GSH being approximately 3 $\text{mM}$. It is therefore possible that intracellular levels of GSH and hydroperoxides may be sufficient to saturate the enzyme substantially. Thus, the intracellular levels of certain nucleotides may be the major mechanism for regulation of GSH peroxidase activity. The normal concentration of ATP is 1.2 $\text{mM}$ in erythrocytes (25) and in the liver cell may be as high as 5 $\text{mM}$ (26). Thus changes in the intracellular levels of ATP could modify GSH peroxidase and thus influence the intracellular levels of lipid hydroperoxides (19).

REFERENCES

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