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 rather than catalase protects hemoglobin from oxidation to methemoglobin by hydrogen peroxide. Hydrogen peroxide may be the toxic agent in the drug-induced anemia associated with a genetic deficiency of erythrocyte glucose 6-phosphate dehydrogenase (4). Recently, Hochstein and Utley (5) have shown that peroxidase in the liver supernatant is also able to compete effectively with catalase for hydrogen peroxide. The oxidized glutathione formed by the peroxidase may be responsible for the toxic agent in the drug-induced anemia associated with a genetic deficiency of erythrocyte glucose 6-phosphate dehydrogenase.

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Little and O’Brien (8) have found that glutathione peroxidase is probably responsible for most of the decomposition of lipid peroxide in the liver cell and may thus protect the cell from the deleterious effects of peroxides. Catalase had no effect on the lipid peroxide (9). Christophersen (10) has identified the lipid products formed by the decomposition of lipid peroxides by glutathione peroxidase and has further suggested that glutathione peroxidase 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 with respect to GSH and zero with respect to peroxide. In the following the kinetics have been investigated over a wider range of substrate concentrations. Evidence is also presented for an allosteric inhibition by nucleotides.

EXPERIMENTAL PROCEDURE

Materials

All nucleotides, coenzymes, glutathione reductase, trypsin, p-chloromercuribenzoate, iodosacate, 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 mM 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

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The symbol ROOH is used to denote cumene hydroperoxide:

$$C_6H_5—C(CH_3)_3—O—OH$$
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

Enzymic rate = total rate - 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 MG100 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 x g for 30 min and the cells were washed twice with an equal volume of isotonic 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 saponine (0.05%, w/v). The mixture was centrifuged at 20,000 x 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 x g for 15 min). The precipitate was dissolved in 50 mM potassium phosphate buffer, pH 6.8.

Sephadex G-100—The solution was applied to a column (5 x 40 cm) of Sephadex G-100 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 x 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 (5 x 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 -15°. After 1 month, the enzyme retained 50 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 in vivo 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.](http://www.jbc.org/issue-29/1/issue-29-1.html)
Fig. 2. Hill plots of the data in Fig. 1. A, Hill plot of rate data for ROOH. The bar lines indicate the maximum scatter of points. The curve was drawn through the average values. B, Hill plot of rate data for GSH. Above 42 μM ROOH all points fell on the same straight line. The bar lines indicate maximum observed scatter of points.

**TABLE II**

**Inhibition of GSH peroxidase by nucleotides**

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."

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Concentration required for 30% inhibition</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>

was noted that with H₂O₂ (18) or linoleic acid hydroperoxide (12) very low substrate concentrations (<10⁻⁵ M) were required before the latter became rate-limiting. Consequently, with these compounds, kinetic studies would be difficult. With ROOH, higher substrate concentrations were rate-limiting and therefore subsequent studies on the enzyme were carried out with this substrate. Fig. 1A shows the dependence of reaction rate on hydroperoxide concentration measured at various concentrations of GSH, the second substrate. The results are plotted on the double reciprocal after the fashion of Lineweaver and Burk (19). It is apparent that significant deviations from the classical linear plot result. The reaction rate seems to be enhanced at high hydroperoxide concentrations. Without curve analysis it is not possible to derive from Fig. 1A meaningful quantitative data concerning K_m values. However, it seems that increased GSH concentrations will increase the K_m of the enzyme for hydroperoxide.

Analogous data for GSH are plotted in Fig. 1B. Increased hydroperoxide concentrations increase the K_m of the enzyme for GSH, with the latter value having a limiting value of approximately 3 mM GSH.

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 II). 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-chloromercuribenzoate, or sodium lauryl sulfate decreased the...
for GSH peroxidase. A ping-pong mechanism is postulated with
and \( V_{\text{max}} \). However, the enzyme has not yet been purified
enhance the enzyme activity. This behavior could arise from
abolished the ATP response. Also, as mentioned under 'LPurifi-
view of the high specificity of the enzyme for GSH (8)) binding is rather than the body of the substrate.

The relative concentrations of each form would depend on the
sufficiently to establish this possibility. Alternatively, the en-
zyme may exist in two or more forms with different activities.
Conversely, trypsin, ethanol, and \( \alpha \)-irradiation preferentially
inhibition of the treated enzyme samples by \( 3 \text{ mM} \) ATP was meas-
ured and expressed as percentage of that of the control.

catalytic activity significantly more than the ATP sensitivity. Conversely, trypsin, ethanol, and \( \alpha \)-irradiation preferentially abolished the ATP response. Also, as mentioned under "Purifi-
cation of Enzyme," on storage the ATP response of the highly
purified enzyme was more labile than the enzymic activity. It
seems, therefore, that the catalytic function and the nucleotide
response of GSH peroxidase are well differentiated.

discussion

GSH peroxidase shows nonlinear Lineweaver-Burk plots for
ROOH substrate. High levels of hydroperoxide apparently
enhance the enzyme activity. This behavior could arise from
the presence of two enzymes with quite different values of \( K_m \)
and \( V_{\text{max}} \). However, the enzyme has not yet been purified
sufficiently to establish this possibility. Alternatively, the en-
zyme may exist in two or more forms with different activities.
The relative concentrations of each form would depend on the
relative levels of GSH and ROOH.

Lineweaver-Burk plots for GSH substrate yield straight lines.
However, at low ROOH levels a parallel pattern seems to emerge.
As ROOH is increased, the slopes change and the lines converge and intersect the abscissa, giving a \( K_m \) value for GSH of approxi-
mately \( 3 \text{ mM} \). It may be of interest to note that Schneider and Flöhè (22) could obtain no \( K_m \) value for GSH with bovine
eerythrocyte enzyme and \( \text{H}_2\text{O}_2 \) as second substrate. The change
from parallel to converging lines may suggest that at low ROOH
levels there is a strict binding sequence implying strong interac-
tions between the ROOH and the GSH sites or binding to differ-
ent forms of the enzyme separated by one or more irreversible
steps. At high ROOH a change may occur to apparent random
binding. Further experiments on this point are currently in
progress.

The stoichiometric equation for the enzyme reaction (8) is

\[
\text{ROOH} + 2\text{GSH} \rightarrow \text{ROH} + \text{GSSG} + \text{H}_2\text{O}
\]

The question arises whether there are two separate binding sites
for GSH so that an ordered or concerted reaction between 1
ROOH and 2 GSH molecules could occur or whether there is a
sequence wherein only one binding site for GSH exists. In the
latter case one GSH and one ROOH would react with the en-
yme, yielding an enzyme intermediate (possibly ESSG) as
opposed to a Michaelis complex. The intermediate would then
react with a 2nd molecule of GSH, thereby releasing GSSG and
the original form of the enzyme, i.e. a ping-pong mechanism.
The Hill plot of rate against GSH concentration yields straight
lines of unit slope over a wide range of ROOH concentrations.
Thus, the reaction would appear to be first order in GSH over a
wide range of ROOH concentrations.

The present data are insufficient to differentiate between
ordered and ping-pong mechanisms. However, evidence for the
latter mechanism is currently being prepared for publication.

The Hill plot of the rate data for ROOH shows a sigmoidal
curve with a slope through the inflection point of less than unity.
This behavior may suggest either negative cooperativity between
enzyme subunits (23) or that a second allosteric binding site for
ROOH exists. At either side of the inflection point in Fig. 2A
the slope approaches unity, suggesting either a single binding
site for ROOH with different affinities depending upon the en-
yme form (ping-pong), two completely independent catalytic
sites (two enzymes), or a separate allosteric site of the strictly
\( V_{\text{max}} \) type. Calculations of \( R_h \), which may be taken as a measure
of cooperativity of binding between different sites (23) gave
values between 96 and 122. This may be interpreted to mean
that, if separate ROOH binding sites exist, their cooperativity
is of a very low order and hence their interaction is not of the
\( K_m \) type. A pictorial representation of a suggested mechanism
for the enzyme is given in Fig. 4.

Although there is no good evidence that an allosteric site for
ROOH exists, there is excellent evidence for a separate allosteric
site for nucleotides. Thus, Table III shows that various physical
or chemical treatments of the enzyme may preferentially
abolish either the catalytic activity or the enzyme response to

\[
\begin{align*}
\text{E} - \text{SH} & \leftrightarrow \text{GSH} \\
\text{ROOH} & \leftrightarrow \text{E} - \text{SH} \\
\text{E} - \text{SH} & \leftrightarrow \text{ROH} \\
\text{GSH} & \leftrightarrow \text{E} - \text{SH} \\
\text{E} - \text{SH} & \leftrightarrow \text{GSH} \\
\end{align*}
\]

Fig. 4. Diagrammatic representation of suggested mechanism
for GSH peroxidase. A ping-pong mechanism is postulated with
the two stable forms of the enzyme being \( \text{E} - \text{SH} \) and \( \text{E} - \text{SSG} \). 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
\(-\text{SH} \). The very low specificity shown for hydroperoxide sub-
strates (8, 24) suggests that binding involves the \(-\text{OOH} \) function
rather than the body of the substrate.
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 $H_2O_2$ and lipid peroxides of the order of 1 to 10 $\mu$m with the $K_m$ value for GSH being approximately 3 $\mu$m. 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 $\mu$m in erythrocytes (25) and in the liver cell may be as high as 5 $\mu$m (26). Thus changes in the intracellular levels of ATP could modify GSH peroxidase and thus influence the intracellular levels of lipid hydroperoxides (12).

REFERENCES
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