Mechanism of Selenium-Glutathione Peroxidase and Its Inhibition by Mercaptocarboxylic Acids and Other Mercaptans*

(Received for publication, May 23, 1983)

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In a systematic search for effectors of glutathione peroxidase, a number of mercaptocarboxylic acids and tertiary mercaptans were found to be strong and specific inhibitors of the enzyme glutathione peroxidase. Assessment of various models was made by linear and nonlinear least squares fitting techniques. The results support the formation of reversible enzyme-inhibitor complexes. The active site selenium is trapped by the rapid binding of the inhibitor in competition with GSH. Data are consistent with the formation of thioselenenate adducts of the active site. The kinetic model which best describes the observed inhibition by the very strong inhibitor mercaptosuccinate implies that a selenenic acid with a kinetically significant lifetime is not formed when hydroperoxide is reduced. A noncovalent binding site for GSH or the presence of a cysteine residue at the active site of the enzyme provides a mechanistic rationale for the observed kinetics. Three of the most potent inhibitors found in this study, mercaptosuccinate, penicillamine, and \( \alpha \)-mercaptopyrrolidglycine, are currently used as slow-acting drugs in the treatment of rheumatoid arthritis. Overall, the evidence suggests that glutathione peroxidase may be involved in the etiology of this disease.

Using GSH as the reducing equivalent, glutathione peroxidase (GSH:H\(_2\)O\(_2\) oxidoreductase, EC 1.11.1.9) reduces hydrogen peroxide to water (1) and organic hydroperoxides to the corresponding alcohols (2). The enzyme contains stoichiometric amounts of selenium (3, 4) and has been thought to account for the essentiality of selenium as a nutrient (5). Selenium in glutathione peroxidase has been shown to be in the form of selenocysteine (6, 7). This amino acid residue is within the polypeptide backbone (8, 9), and it is generally agreed that it is a major component of the active site (5, 10, 11). In recent years, various selenoproteins have been identified in animal tissues (12–14), suggesting the existence of selenoenzymes other than glutathione peroxidase. Understanding the chemistry of selenium at the active site of glutathione peroxidase may help to elucidate some general features of selenocysteine-mediated catalysis.

The kinetic data observed for hamster liver glutathione peroxidase, which was used in this study, are compatible with the ternary ping-pong mechanism reported for the enzyme from other various sources (15). Photoelectron spectroscopy (16) and the carboxymethylation of the active site (6, 15) support the existence of a selenocysteine selenolate as the resting form of the enzyme at physiological concentrations of GSH. However, the oxidized forms of the active site selenium during catalysis are controversial (17). Steady state kinetics do not separate the bimolecular rate constants for the two consecutive reactions of the oxidized forms of glutathione peroxidase with GSH in the absence of a specific effector of the enzyme. Inhibitors that interact with a given form of the enzyme may prove to be a useful tool to characterize the steps of the enzyme cycle.

Reversible enzyme inhibitors can be studied by conventional kinetics in more detail than irreversible effectors. Coenzyme A is the only compound reported to reversibly inhibit glutathione peroxidase at low concentration (18). The inhibition by coenzyme A is not well understood, but the sulfhydryl group and the nucleotidic moiety of the molecule seemed to be required for inhibition of glutathione peroxidase. Other reversible effectors of glutathione peroxidase include pyrimidine nucleotides, which were reported to behave as uncompetitive inhibitors of the enzyme from pig red blood cells (19). A few GSH analogs act as weak reducing substrates in the absence of GSH (20), and many mercaptans, such as mercaptopetothanol, will compete for GSH in the second reduction step of the enzyme cycle (21). A necessary reductive preactivation of the enzyme can also be performed by many mercaptans (22).

In practice, the above mentioned compounds have not afforded great insights into the catalytic cycle of glutathione peroxidase. The studies of these compounds strongly suggest, however, that a search for specific and reversible effectors of the active site of glutathione peroxidase should focus on bifunctional mercaptans. In making such a search, we discovered that mercaptocarboxylic acids and tertiary mercaptans are potent inhibitors of glutathione peroxidase. Herein is reported a detailed study of the inhibition by these mercaptans that provides new insight into the mechanism of glutathione peroxidase. A possible pharmacological significance of this inhibition is also briefly discussed.

**MATERIALS AND METHODS**

**Chemicals**—All mercaptans were >98% pure and were obtained from commercial sources. Mercaptosuccinate and \( \beta \)-mercaptovaline used for kinetic analyses were purchased from Sigma. \( \beta \)-Methylmercaptosuccinate was prepared by reaction of 10 mM sodium thiomalate with a 3-fold excess of methyl iodide in 0.1 M borate buffer, pH 9.0, until the sulfhydryl group was no longer detected with Ellman’s reagent (23). Excess methyl iodide was then hydrolyzed by adjusting the pH to 12 with sodium hydroxide.

**Preparation and Assays of Glutathione Peroxidase**—Glutathione peroxidase used in this study was highly purified (approximately 160-fold) from hamster liver as described elsewhere (15). The preparation was devoid of selenium-independent glutathione peroxidase activity.
and had a specific activity of 500 μmol of t-butylhydroperoxide reduced/min and per μmol of selenium at pH 7.6, 37 °C, and 0.25 mM GSH. A highly concentrated solution of the enzyme was stored at 4 °C in 50 mM Tris-HCl, 10% ethanol, pH 7.7. Initial rates of glutathione peroxidase activity were monitored at 37 °C with a thermostated spectrophotometer using a glutathione reductase-coupled assay (24). The decrease in NADPH absorbance was monitored for at least 2 min. Unless otherwise mentioned, initial concentrations of reactants in the cuvette were as follows: 0.25 mM GSH, 0.20 mM t-butylhydroperoxide (initiator), 0.12 mM NADPH, 1 unit/ml of glutathione reductase, 3.0 mM glutathione peroxidase, 50 mM Tris-HCl, 0.1 mM EDTA, pH 7.7. The mixed components, with the exception of hydroperoxide, were preincubated at 37 °C for at least 5 min.

For studying the structural requirements for inhibition, the above assay components were used in the presence of 0.2 mM effector. As a control of the validity of the assay, initial rates of glutathione reductase activity were measured in the presence of each effector. The decrease in NADPH absorbance was monitored for at least 2 min at 340 nm and 37 °C, with the following concentrations of reactants in the cuvette: 0.25 mM GSH, 0.20 mM GSSG (initiator), 0.20 mM NADPH, 0.02 unit/ml of glutathione reductase, and 50 mM Tris-HCl, 0.1 mM EDTA, pH 7.7.

**Steady State Kinetics of Inhibition**—The pH profiles of glutathione peroxidase activity with and without inhibitor were obtained using a three-component polybuffer. The buffer, containing 50 mM each of Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)-propane-1,3-diol, and hydrochloric acids, was titrated to pH with either sodium hydroxide or Tris, pH 7.4. The corresponding pH profiles of glutathione peroxidase activity, without inhibitor, were obtained using 1 unit/ml of glutathione reductase. Absorbance monitoring was continued for at least 1 min after completion of hydroperoxide reduction. Each curve of absorbance versus time was digitized with a model 4662 interactive digital plotter of a Tektronix 4051 computer. Time intervals between digitized points were between 3 and 6 s. Linear least squares fitting to the digitized data for the integrated rate equation were obtained by the use of a Tektronix 4051. The corresponding BASIC routine included a correction for nonenzymatic reduction of NADPH and variable inhibitor or vice versa. In each reaction system, glutathione peroxidase activity was initiated with approximately 45 μM t-butylhydroperoxide in the presence of 80 μM NADPH and 1 unit/ml of glutathione reductase. Absorbance monitoring was continued for at least 1 min after completion of hydroperoxide reduction. Each curve of absorbance versus time was digitized with a model 4662 interactive digital plotter of a Tektronix 4051 computer. Time intervals between digitized points were between 3 and 6 s. Linear least squares fitting to the digitized data for the integrated rate equation were obtained by the use of a Tektronix 4051. The corresponding BASIC routine included a correction for nonenzymatic reduction of NADPH and variable inhibitor or vice versa. In each reaction system, glutathione peroxidase activity was initiated with approximately 45 μM t-butylhydroperoxide in the presence of 80 μM NADPH and 1 unit/ml of glutathione reductase. Absorbance monitoring was continued for at least 1 min after completion of hydroperoxide reduction. Each curve of absorbance versus time was digitized with a model 4662 interactive digital plotter of a Tektronix 4051 computer. Time intervals between digitized points were between 3 and 6 s. Linear least squares fitting to the digitized data for the integrated rate equation were obtained by the use of a Tektronix 4051. The corresponding BASIC routine included a correction for nonenzymatic reduction of NADPH and variable inhibitor or vice versa. In each reaction system, glutathione peroxidase activity was initiated with approximately 45 μM t-butylhydroperoxide in the presence of 80 μM NADPH and 1 unit/ml of glutathione reductase. Absorbance monitoring was continued for at least 1 min after completion of hydroperoxide reduction. Each curve of absorbance versus time was digitized with a model 4662 interactive digital plotter of a Tektronix 4051 computer. Time intervals between digitized points were between 3 and 6 s. Linear least squares fitting to the digitized data for the integrated rate equation were obtained by the use of a Tektronix 4051. The corresponding BASIC routine included a correction for nonenzymatic reduction of NADPH and variable inhibitor or vice versa. In each reaction system, glutathione peroxidase activity was initiated with approximately 45 μM t-butylhydroperoxide in the presence of 80 μM NADPH and 1 unit/ml of glutathione reductase. Absorbance monitoring was continued for at least 1 min after completion of hydroperoxide reduction. Each curve of absorbance versus time was digitized with a model 4662 interactive digital plotter of a Tektronix 4051 computer. Time intervals between digitized points were between 3 and 6 s. Linear least squares fitting to the digitized data for the integrated rate equation were obtained by the use of a Tektronix 4051. The corresponding BASIC routine included a correction for nonenzymatic reduction of NADPH and variable inhibitor or vice versa. In each reaction system, glutathione peroxidase activity was initiated with approximately 45 μM t-butylhydroperoxide in the presence of 80 μM NADPH and 1 unit/ml of glutathione reductase.
Mechanism and Inhibition of Glutathione Peroxidase

TABLE I
Effect of mercaptocarboxylic acids and related compounds on glutathione peroxidase activity

<table>
<thead>
<tr>
<th>Effector (0.2 mM)</th>
<th>Enzyme activity*</th>
<th>pKₐ (SH) at 25 °C</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Mercaptoethanol</td>
<td>73.0 ± 4.8</td>
<td>9.44, 9.6</td>
<td>34, 35</td>
</tr>
<tr>
<td>Cysteine</td>
<td>56.0 ± 3.3</td>
<td>8.31*</td>
<td>36</td>
</tr>
<tr>
<td>Coenzyme A</td>
<td>15.2 ± 0.8</td>
<td>9.6</td>
<td>35</td>
</tr>
<tr>
<td>Mercaptosuccinate</td>
<td>0.0 ± 0.0</td>
<td>Not found</td>
<td>4</td>
</tr>
<tr>
<td>2,3-Dimercapto succinate</td>
<td>0.0 ± 0.0</td>
<td>Not found</td>
<td></td>
</tr>
<tr>
<td>Mercaptoacetate</td>
<td>36 ± 2.5</td>
<td>10.11</td>
<td>37</td>
</tr>
<tr>
<td>2-Mercaptopropanoate</td>
<td>17.3 ± 0.4</td>
<td>10.11</td>
<td>37</td>
</tr>
<tr>
<td>2-Mercaptobenzonate</td>
<td>0.8 ± 0.6</td>
<td>9.96</td>
<td>34</td>
</tr>
<tr>
<td>3-Mercaptopropionate</td>
<td>25.1 ± 1.0</td>
<td>10.54, 10.80</td>
<td>34, 37</td>
</tr>
<tr>
<td>β-Mercaptocysteine</td>
<td>0.5 ± 0.3</td>
<td>10.46, 10.58*</td>
<td>34, 37</td>
</tr>
<tr>
<td>t-Butylmercaptan</td>
<td>0.0 ± 0.0</td>
<td>11.22</td>
<td>36</td>
</tr>
<tr>
<td>β-Mercaptosuccinate</td>
<td>73.4 ± 3.2</td>
<td>9.25*</td>
<td>This work</td>
</tr>
<tr>
<td>β-Mercaptopyruvate</td>
<td>16.9 ± 1.7</td>
<td>Not found</td>
<td></td>
</tr>
<tr>
<td>Methyl(3-mercaptopropionate)</td>
<td>59.9 ± 3.4</td>
<td>10.3*</td>
<td>This work</td>
</tr>
<tr>
<td>Methylmercaptoacetate</td>
<td>99.7 ± 2.4</td>
<td>10.3*</td>
<td>This work</td>
</tr>
<tr>
<td>α-Mercaptopropanoylglycine</td>
<td>61.0 ± 6.2</td>
<td>10.25*</td>
<td>This work</td>
</tr>
<tr>
<td>2-Mercaptobenzenesulfonate</td>
<td>90.3 ± 6.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-Methylmercaptosuccinate</td>
<td>86.0 ± 5.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Thiazolidine-4-carboxylate</td>
<td>102.3 ± 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malate</td>
<td>105.7 ± 8.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>100.0 ± 3.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Glutathione peroxidase activity is expressed as percentages of the control (mean + range of two independent measurements) at 0.25 mM GSH and 37 °C.

† Preference was given to compilations of pKₐ (SH) values. Most of the numbers are average values computed from original data obtained at 0.1 ionic strength.

‡ Assignment of macroscopic constants of aminothiols is tentative.

§ pKₐ (SH) neither measurable by titration nor found in literature.

* Titration.

Other β-mercaptocarboxylic acids. The much smaller inhibition by cysteine may be explained by a preferential interaction of the sulfhydryl group with the amino group, as occurs with mercaptoethylamine. It is difficult to account for the lack of strong inhibition with 3-mercaptopropionate.

That a sulfhydryl group is essential for inhibition is supported by two lines of evidence. First, analogs of mercaptocarboxylic acids that do not bear a sulfur-containing group, such as malate and succinate, are not inhibitors. Second, substitution of an S-alkyl group for a sulfhydryl group also relieves the inhibition. This is illustrated by the effects of S-methylmercaptoacetamide, carboxymethylmercaptosuccinate, and the cyclic L-thiazolidine-4-carboxylate (thioproline). Similarly, the importance of the carboxylate group is supported by the moderate inhibition observed with methyl-3-mercaptopropionate and by the lack of inhibition observed with methylmercaptoacetate. That a sulfonate group cannot fulfill the function of the carboxylate group is supported by the weak inhibition observed with 2-mercaptoethanesulfonate.

Inspection of pKₐ(SH) values reported in Table I shows that all inhibitors have rather weakly acidic sulfhydryl groups. A high pKₐ(SH) is probably not a determinant of the effect because of the lack of a quantitative relationship. The high pKₐ(SH) values of most mercaptocarboxylic acids may rather reflect a slight tendency to form cyclic structures involving hydrogen bonding between the carboxylate and sulfhydryl groups. If accessibility to such a structure was an important feature of these inhibitors, one would expect β-mercaptocarboxylic acids to be somewhat stronger inhibitors than their α-isomers because of the greater thermodynamic stability of six-membered rings over five-membered rings. On the other hand, given its highly hindered position, the sulfhydryl group of β-mercaptopovaline should not easily adopt this type of conformation. This suggested that tert-mercapanes might be a second family of inhibitors. This hypothesis was tested with β-mercaptopoisoleucine or t-butylmercaptan. As shown in Table I, t-butylmercaptan was a very potent inhibitor of glutathione peroxidase. However, more hindrance near the sulfhydryl group seems to relieve the inhibition, as shown with β-mercaptopoisoleucine. Kinetic studies were not done to establish the mechanism of inhibition by the tertiary mercaptoacids in this study.

As discussed below, kinetic data support the assumption that mercaptocarboxylic acids react with an oxidized form of the active site selenocysteine in glutathione peroxidase. The specificity of this type of inhibition is confirmed in Table II. This table shows that two enzymes that also decompose hydroperoxides (catalase and selenium-independent glutathione peroxidase), two essential sulphydryl enzymes (alcohol dehydrogenase and isocitrate dehydrogenase), and glutathione reductase, which bears an essential dithiole disulfide bond at its active site, were either not inhibited or only moderately inhibited by a high concentration of either mercaptosuccinate or α-mercaptopropionylglycine. Mercaptosuccinate was selected to investigate the kinetics of inhibition of glutathione peroxidase because it could be used at concentrations much lower than the hydroperoxide and because the inhibition apparently reached steady state in a few seconds.

As shown in Fig. 2, the pH profile of glutathione peroxidase...
activity with and without mercaptosuccinate indicates that inhibition is not pH-dependent around neutral pH. The range of pH that could be used with the reductase-coupled assay is below the pK_a(SH) of mercaptosuccinate. Therefore, the thiolate form of this inhibitor is not required in the mechanism of inhibition.

**Steady State Kinetics**—Studies of steady state kinetics are often the only way to obtain a description of enzyme inhibition without detailed understanding of the chemistry involved. With non-Michaelian kinetics or with complex inhibition, theoretical models require expensive sets of initial rate data for analysis. The information obtained from progress curves is equivalent to information obtained from initial rate assays when product inhibition is negligible, but much information is obtained at low substrate concentrations where initial rate measurements are difficult. The information is obtained in fewer assays by following progress curves. Following progress curves is of particular advantage when the amount of enzyme is limiting.

In this study, interpretation of velocities along entire progress curves was not hampered by product inhibition. Glutathione peroxidase initial rates were not affected by up to 15% ethanol, and glutathione reductase prevented the accumulation of significant amounts of GSSG. The enzyme mechanism was first assessed in the absence of inhibitor using Models I and II on Fig. 3. Model I has generally been considered to be the correct model for glutathione peroxidase (22, 39, 15), but it had not been assessed previously by a nonlinear least squares fitting technique. As shown in Table III, the reverse step is not substantiated by the kinetic data. The reversible pathway in Model II was considered because it might have explained the apparent complexity of the inhibition by mercaptosuccinate.

As mentioned above, the kinetic pattern of hamster liver glutathione peroxidase is similar to the one of the rat liver enzyme. It may be described by the following Dalziel equation:

$$ E_0/V = \phi_1/A + \phi_2/B $$

where $E_0$ is total enzyme concentration, $V$ is enzyme velocity, $A$ is hydroperoxide concentration, $B$ is GSH concentration, and $\phi_1 = 1/k_1$ and $\phi_2 = (k_3 + k_4)/k_2$ using the second order rate constants defined in Fig. 3. In the conditions of the reductase-coupled assay, $B$ is constant and the following integrated rate equation can be obtained (39):

$$ (E_0 \times t)/P_t = \phi_1 \times (\ln(A_0/A_0 - P_t)/P_t) + \phi_2/B $$

where $t$ is time, $P_t$ is product concentration at time $t$, and $A_0$ is initial hydroperoxide concentration. Since GSH concentration is always constant in the conditions of the coupled assay, the form of a similar integrated rate equation can be deduced from any theoretical mechanism of inhibition.

As shown in Fig. 4, at constant GSH concentration and for various mercaptosuccinate concentrations, the kinetic pattern

![Fig. 3. Two possible models for glutathione peroxidase cycle. E is the most reduced form of the enzyme. F is obtained after reduction of the hydroperoxide and release of alcohol. A and B are the concentrations of hydroperoxide and GSH, respectively; and $k_1$, $k_2$, and $k_3$ are bimolecular rate constants, whereas $k_4$ is monomolecular. These vector diagrams were used for King-Altman calculations. Note that enzyme-substrate complexes in addition to E, F, and G are not identifiable by steady state kinetics.](http://www.jbc.org/content/111/6/1046/F3)

![Fig. 4. Linearization of integrated rate Equation 1 for 1-butylhydroperoxide at 2 mM constant GSH and various concentrations of mercaptosuccinate. Each line was obtained by least squares linear regression of the points (each r $>$ 0.99). From top to bottom, mercaptosuccinate concentrations are 18, 15, 12, 9, 6, 3, and 0 mM, respectively.](http://www.jbc.org/content/111/6/1046/F4)

of glutathione peroxidase is still consistent with Equation 2, with the apparent $\phi_1$ unchanged (constant slope) and the apparent $\phi_2$ being a linear function of inhibitor concentration (intercept pattern). Since the ordinates on Figs. 4 and 5 are

![Table III. Statistical evaluation of rate equations used for nonlinear least squares fitting.](http://www.jbc.org/content/111/6/1046/TableIII)

<table>
<thead>
<tr>
<th>Model</th>
<th>Rate equation</th>
<th>Degree of freedom in reduced equation</th>
<th>Sum of squares $\times 10^6$</th>
<th>$\sigma \times 10^6$</th>
<th>Rejection criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>$E_0/V = 1/k_A + 1/k_B + 1/k_3$</td>
<td>2</td>
<td>1.2</td>
<td>4.9</td>
<td>Accepted</td>
</tr>
<tr>
<td>II</td>
<td>$E_0/V = 1/k_A + 1/k_B + 1/k_3$</td>
<td>2</td>
<td>1.1</td>
<td>4.9</td>
<td>C_2, C_6</td>
</tr>
<tr>
<td>III</td>
<td>$k_4(k_5 + k_4)B^2 + k_4(k_5 + k_4)B$</td>
<td>3</td>
<td>15</td>
<td>12</td>
<td>C_1, C_6</td>
</tr>
<tr>
<td>IV</td>
<td>$k_3(k_4 + k_5)B^2 + k_4(k_5 + k_4)B$</td>
<td>3</td>
<td>7.0</td>
<td>7.3</td>
<td>C_1, C_6</td>
</tr>
<tr>
<td>V</td>
<td>$k_3(k_4 + k_5)B^2 + k_4(k_5 + k_4)B$</td>
<td>4</td>
<td>4.2</td>
<td>5.7</td>
<td>C_1, C_6</td>
</tr>
<tr>
<td>VI</td>
<td>$k_3(k_4 + k_5)B^2 + k_4(k_5 + k_4)B$</td>
<td>4</td>
<td>6.4</td>
<td>7.0</td>
<td>C_1, C_6</td>
</tr>
<tr>
<td>VII</td>
<td>$E_0/V = 1/k_A + 1/k_B + 1/k_3$</td>
<td>3</td>
<td>6.5</td>
<td>7.2</td>
<td>C_1, C_6</td>
</tr>
<tr>
<td>VIII</td>
<td>$E_0/V = 1/k_A + 1/k_B + 1/k_3$</td>
<td>3</td>
<td>30</td>
<td>15</td>
<td>C_1, C_6</td>
</tr>
<tr>
<td>IX</td>
<td>$E_0/V = 1/k_A + 1/k_B + 1/k_3$</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>C_2</td>
</tr>
<tr>
<td>X</td>
<td>$E_0/V = 1/k_A + 1/k_B + 1/k_3$</td>
<td>5</td>
<td>4</td>
<td>3.8</td>
<td>4.0</td>
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<tr>
<td>XI</td>
<td>$E_0/V = 1/k_A + 1/k_B + 1/k_3$</td>
<td>6</td>
<td>6.6</td>
<td>7.1</td>
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<td>XII</td>
<td>$E_0/V = 1/k_A + 1/k_B + 1/k_3$</td>
<td>5</td>
<td>3.3</td>
<td>5.0</td>
<td>C_1, C_6</td>
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<tr>
<td>XIII</td>
<td>$E_0/V = 1/k_A + 1/k_B + 1/k_3$</td>
<td>6</td>
<td>3.6</td>
<td>5.3</td>
<td>Accepted</td>
</tr>
</tbody>
</table>

* Numbering refers to the vector diagrams that are represented in Figs. 3 and 6.

1. Rate equations:

   $$ E_0/V = \phi_1/A + \phi_2/B $$

   $$ (E_0 \times t)/P_t = \phi_1 \times (\ln(A_0/A_0 - P_t)/P_t) + \phi_2/B $$

2. Reduced equation:

   $$ E_0/V = 1/k_A + 1/k_B + 1/k_3 $$

   $$ (E_0 \times t)/P_t = \phi_1 \times (\ln(A_0/A_0 - P_t)/P_t) $$

3. Rejection criteria: C_1, large sum of squares and standard error; C_2, large relative uncertainty in parameters (>100%); C_3, negative parameter; C_4, term rejected by F test; C_5, unacceptable visual fit; C_6, no convergence; C_7, no model.
proportional to \( E_0 \), the observation of parallel lines when \( E_0 \) is fixed rules out noncompetitive types of inhibition (with respect to GSH). It also rules out interaction with \( E \). On Fig. 5, the slope, the apparent \( \phi_{11} \), is also independent of GSH concentration, whereas the apparent \( \phi_2 \) decreases nonlinearly with GSH concentration.

Four simple mechanisms of inhibition were then considered, and the corresponding King-Altman patterns were drawn for derivation of rate equations. These four patterns are shown in Fig. 6 as III through VI. They involve either the formation of a dead-end complex with an oxidized form of the enzyme or the appearance of an alternate pathway between two enzyme forms. \( E \) is the most reduced form of the enzyme, and it has the properties of a selenocysteine selenolate (5, 15, 16). Although complete knowledge of the redox states of \( F \) and \( G \) is not required, for discussion purposes they may be assigned the properties of a selenenic acid, PSeOH, and of a thioselenolate, PSeSR, respectively.

Inspection of the four rate equations derived from models III to VI (Fig. 6) indicates that they are all compatible with both Equation 2 and a slope independent of inhibitor concentration. After nonlinear least squares fitting, models III to VI were rejected as shown in Table III. The corresponding fits were poor at either constant GSH or constant inhibitor concentration, and in model V a negative constant was obtained. These results suggested that a more complex function of GSH concentration was necessary to describe the observed inhibition.

A comparison of these equations as descriptors of the data is summarized in Table III. Clearly, none of the equations derived from models VII to XI was acceptable. In model X, the term in \( (I)/G \) was systematically rejected (cf Table III). An arbitrary deletion of this term led to Equation Xx in Table III. As shown in Table III, this fit was statistically acceptable. All efforts to generate a viable model corresponding to this equation failed. The step responsible for the existence of an \( (I)/G \) term was, therefore, arbitrarily modified by replacing \( k_{-4} \) with \( k_{-4}(B) \) to yield model XIII. The corresponding equation gave a good fit to the entire set of data, as detailed in Table III. However, model XIII imposes the new constraint that \( F \) should contain one molecule of bound GSH or be obtained from the decomposition of an intermediate which contained GSH. This constraint is incompatible with \( F \) having selenium in the form of a selenenic acid. As discussed below,
other mechanistic interpretations will yield models whose corresponding rate equations are kinetically equivalent to Equation XIII. The actual fit is shown in Fig. 7, where 14 curves (total of 136 points) were simultaneously adjusted to this equation by nonlinear regression. The highest relative uncertainty in the parameters involved was 17%, which is generally considered as acceptable (40). Since each set of absorbance curves was obtained with different solutions of enzyme and chemicals, a significant level of experimental fluctuations would be expected. For all of the previous models tested, \( k_{-4} \) was replaced with \( k_{-4}(B) \). The reduced equations usually resulted in a form that had been tried previously and gave unsatisfactory results for all equations.

Mechanistic Interpretation—Indirect information on the redox forms of the enzyme has been obtained in several ways. First, the most reduced form of the enzyme, \( E \), apparently contains selenium in the form of a selenocysteine selenolate at physiological concentration of GSH (5, 15, 16). Second, x-ray diffraction patterns of glutathione peroxidase from bovine red blood cells support the complete equivalence of the 4 atoms of selenium/tetramer and rule out the possibility of intramolecular diselenide formation (41). Accordingly, starting from a selenocysteine selenolate, a two-electron reduction of a single molecule of hydroperoxide (kinetic constraint) to the corresponding alcohol would yield a (+II) oxidation state of selenium. This oxidized form, \( F \), of glutathione peroxidase should be a selenenic acid (PSeOH). This would not preclude that the latter is kinetically unobserved due to very rapid transformation into another selenium (+II) derivative, e.g. a thioselenenate, PSeSR, or an ester. Indeed, if a sulfhydryl group, e.g. a cysteine residue or a noncovalently bound GSH, were present at the active site of \( E \), it would be unlikely that a selenenic acid could be formed with a significant lifetime since this type of electrophile should react very rapidly with sulfhydryl groups. Instead, a pseudo ter-reactant system would directly yield a thioselenenate, \( F \). This reasoning led to the two proposed mechanisms, \( a \) and \( b \), in Fig. 8. However, these are not the only mechanisms consistent with the observed kinetics.

As further support of either interpretation, no trapping of a selenenic acid was observed with 20 mM of either cyanide, thiocyanate, hydrazine at pH 7.7, or dimedone at pH 9.0, all of which are used to trap protein sulfenic acids (42). On the other hand, the active site selenium can be slowly trapped by cyanide at alkaline pH with subsequent loss of selenium when the enzyme has been air-oxidized in the presence of GSH (43). This may indicate the hydrolytic cleavage of a thioselenenate and the trapping of the resulting selenenic acid, which is not normally observed in the catalytic cycle. The need for a sulfhydryl group in proximity to the selenium can be further rationalized as necessary to prevent overoxidation of the selenium, which would inactivate the enzyme. Additionally, one of the oxidized forms of glutathione peroxidase apparently contains one GSH bound at the active site (17). Amino acid analysis indicates the presence of one cysteine residue in glutathione peroxidase (15), which is required for model \( b \) to be feasible. The high specificity observed for the first reduction step from \( F \) to \( G \) supports the existence of a specific recognition site for GSH although GSH binding could not be demonstrated with carrier-free (35S)GSH (17). Finally, one histidine residue has been identified at the active site of glutathione peroxidase (41); one common function of histidine is to modulate the protonation state of sulfhydryl groups.

For mechanism \( b \) to be valid, the inhibitor sulfhydryl must
preferentially attack the sulfur and GSH must preferentially attack the selenium. This interpretation can be rationalized as a result of the high specificity for GSH in the first reduction step. Furthermore, if the inhibitor reacted with the selenium, the mechanism would no longer fit the observed kinetics. Steps determined to be kinetically irreversible would be reversible as the same intermediate would be formed in two portions of the mechanism. These arguments cause some bias toward mechanism a, but this bias is insufficient to reject b.

Other interpretations of the kinetic data in this study would imply higher oxidation states for the selenium and covalently bound GSH. Although not disproved, these mechanisms are more complex because they would preclude the existence of a selenolate group within the catalytic cycle and imply the formation of selenium (+IV) intermediates within the enzyme cycle.

When the parameters obtained from the nonlinear least squares program are analyzed to obtain estimates of the rate constants as per model XIII, it is evident that there are more degrees of freedom than constraints. However, certain generalities can be made. $k_5$ is 730,000 M$^{-1}$ min$^{-1}$, with a relative uncertainty as estimated in the nonlinear least squares analysis of 1.4%. The reduction steps, $k_2$ and $k_4$, are both greater than 70,000 M$^{-1}$ min$^{-1}$ as $k_2 \times k_4/(k_0 + k_4)$ = 75,000 M$^{-1}$ min$^{-1}$ with a relative uncertainty of 4.0%. Additionally, $(k_2 \times k_3 - k_4)/(k_2 \times k_3 + k_2 \times k_4 + k_3 \times k_4 + k_2 \times k_3) = 0.72$, with a relative uncertainty of 9.4%. The following expressions are useful to examine the relative magnitudes of the rate constants: $k_3 = k_2 \times k_4/[0.0037 \times k_0 - k_2 - k_3]$ and $k_4 > 270 \times (k_2 + k_4)$. These constraints allow all rate constants in models a and b to have values that are positive and much less than the diffusion limit.

The reason most $\beta$-mercaptocarboxylic acids are able to compete so efficiently with GSH in trapping the oxidized forms of glutathione peroxidase is not clear. It is noteworthy that these compounds do not reduce the reduced form of glutathione peroxidase by attack on sulfur and formation of a mixed disulfide of GSH but, instead, yield H. This indicates a strong preference for attack on selenium. Of great interest are the recent reports that mercaptocarboxylic acids are able to trap otherwise unstable $\text{Se}^{(+II)}$ and $\text{Te}^{(+II)}$ salts (44, 45). Hence, the possibility that they could also very rapidly trap organic $\text{Se}^{(+II)}$. This suggests that mercaptocysuccinate could interact with other selenoproteins. The particularly strong inhibition observed with mercaptocysuccinate may be due to the presence of two carboxylate groups. This could enhance competition with GSH for an electrostatic binding site or yield a transition state analog (46).

In conclusion, two significant results of this study are (i) the kinetic demonstration that a selenenic acid cannot be one active site. Of great interest is the finding of 1.4%. The reduction steps, $k_2$ and $k_4$, are both greater than 70,000 M$^{-1}$ min$^{-1}$ as $k_2 \times k_4/(k_0 + k_4)$ = 75,000 M$^{-1}$ min$^{-1}$ with a relative uncertainty of 4.0%. Additionally, $(k_2 \times k_3 - k_4)/(k_2 \times k_3 + k_2 \times k_4 + k_3 \times k_4 + k_2 \times k_3) = 0.72$, with a relative uncertainty of 9.4%. The following expressions are useful to examine the relative magnitudes of the rate constants: $k_3 = k_2 \times k_4/[0.0037 \times k_0 - k_2 - k_3]$ and $k_4 > 270 \times (k_2 + k_4)$. These constraints allow all rate constants in models a and b to have values that are positive and much less than the diffusion limit.

The reason most $\beta$-mercaptocarboxylic acids are able to compete so efficiently with GSH in trapping the oxidized forms of glutathione peroxidase is not clear. It is noteworthy that these compounds do not reduce the reduced form of glutathione peroxidase by attack on sulfur and formation of a mixed disulfide of GSH but, instead, yield H. This indicates a strong preference for attack on selenium. Of great interest are the recent reports that mercaptocarboxylic acids are able to trap otherwise unstable $\text{Se}^{(+II)}$ and $\text{Te}^{(+II)}$ salts (44, 45). Hence, the possibility that they could also very rapidly trap organic $\text{Se}^{(+II)}$. This suggests that mercaptocysuccinate could interact with other selenoproteins. The particularly strong inhibition observed with mercaptocysuccinate may be due to the presence of two carboxylate groups. This could enhance competition with GSH for an electrostatic binding site or yield a transition state analog (46).

In conclusion, two significant results of this study are (i) the kinetic demonstration that a selenenic acid cannot be one of the three redox forms of the enzyme and (ii) the finding that mercaptocarboxylic acids are able to form unproductive complexes with the oxidized forms of glutathione peroxidase active site.

The potential applications of mercaptocarboxylic acid inhibitors may eventually prove interesting. Mercaptosuccinate, dimercaptosuccinate, $\beta$-mercaptocysteine, and $\alpha$-mercaptopropionylglycine can be used in vitro specifically to block selenium-glutathione peroxidase activity. With high specificity, it may be possible to obtain new insights as to the function of selenium and/or glutathione peroxidase by using some of these inhibitors in vivo. Given its high specificity, mercaptocysuccinate may not affect other biological components to a significant extent. This is not to say that all $\beta$-mercaptocarboxylic acids are devoid of known effects; for example, thiosalicylate ($2$-mercaptobenzoate) serves as a potent inhibitor of respiratory chains through uncoupling of oxidative phosphorylation (47).

It is interesting to note that three of the most potent inhibitors found in this study, namely, thiomalate (mercaptosuccinate), penicillamine ($\beta$-mercaptocysteine), and $\alpha$-mercaptopropionylglycine, are currently used as slow-acting drugs in the treatment of rheumatoid arthritis. Whereas thiomalate has mostly been used as a complexing agent for gold (in the drug Myochrysine), recent studies indicate that thiomalate may be an active part of aurothiomalate (48, 49). The mechanism of action of such drugs is unknown. However, intracellular GSH concentration was shown to increase in the erythrocytes of a patient treated with penicillamine (48). It is generally accepted that the glutathione peroxidase pathway accounts for a major part of GSH consumption (50). Given the apparent importance of neutrophils and macrophages in this inflammatory disease, it seems relevant that (i) GSSG has recently been shown to be an essential activator of leukocyte collagenase (51) and (ii) GSH is a limiting substrate in the biosynthetic pathway to leukotrienes (52). These findings justify further attempts to assess the possibility that Se and/or glutathione peroxidase is involved in the etiology or evolution of rheumatoid arthritis.
Mechanism and Inhibition of Glutathione Peroxidase

Mechanism of selenium-glutathione peroxidase and its inhibition by mercaptocarboxylic acids and other mercaptans.

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