Glutathione S-Transferase A

A NOVEL KINETIC MECHANISM IN WHICH THE MAJOR REACTION PATHWAY DEPENDS ON SUBSTRATE CONCENTRATION

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SUMMARY

Glutathione transferase A has been purified from rat liver. The enzyme catalyzes the conjugation of glutathione with compounds bearing an electrophilic site, especially those in which the electrophilic site is on, or α to, an aromatic ring. The enzyme has a molecular weight of 45,000 and is composed of two similar subunits.

Initial velocity, product inhibition, and binding studies indicate a biphasic kinetic mechanism in which the reaction pathway depends on the concentration of the substrates. At high concentrations of GSH, an ordered sequential pathway predominates in which GSH binds first. At low concentrations of GSH, a ping-pong pathway predominates in which the electrophilic substrate adds first. In accordance with a prediction of the general rate equation for the over-all mechanism, the breakpoint in the biphasic double reciprocal plot for GSH saturation was found to shift to lower GSH concentrations as the concentration of the electrophilic substrate was lowered. A numerical rate equation was developed which describes initial velocities over the entire range of substrate concentrations. An appendix presents a method for distinguishing among several formal kinetic mechanisms which yield nonlinear double reciprocal initial velocity plots as a result of multiple reaction pathways.

Glutathione S-transferase A is one of four similar enzymes which we have purified to homogeneity in the course of an investigation into the glutathione-conjugating activity of rat liver (1-3). Each of the glutathione transferases catalyzes the reaction of GSH with compounds bearing an electrophilic site (3). The preparation and certain of the properties of transferase A are described here.

An initial kinetic evaluation of the enzyme revealed an unexpected complexity: the saturation with GSH in a double reciprocal plot was biphasic, yielding one apparent $K_m$ value for GSH in the high range of GSH concentrations and a different apparent $K_m$ value for GSH at low concentrations. As a first approximation we conducted separate experiments at both the high and low regions of GSH concentration. At high levels of GSH, approximately 0.15 to 5 mM, kinetic analysis indicated an ordered sequential mechanism, with GSH adding first; a similar ordered sequential mechanism was recently demonstrated for an enzyme conjugating menaphthyl sulfate with GSH (4). However, in the GSH concentration range of 0.1 mM or lower, the data with glutathione transferase A suggested a ping-pong mechanism in which the electrophilic substrate added first.

A rate equation has been developed for the system which is applicable to the entire range of GSH concentrations. The general form of the rate equation allowed a prediction which we were able to verify experimentally (see "Appendix" (5)). Thus, we were able to unify our observations with glutathione transferase A into a concept of a dual pathway mechanism which states that the flux is predominantly through the ordered pathway at high GSH concentrations and predominantly through the substituted-enzyme pathway at low GSH concentrations.

MATERIALS AND METHODS

Chemicals—[7-14C]Benzyl chloride (250 µCi, 13.92 mCi per mmole; New England Nuclear Corp.), a solution in 0.25 ml of benzene was diluted 1:10 into ethanol and stored at -15°. 1-Chloro-2,4-dinitro[U-14C]benzene (50 µCi per mmole, Amersham-Searle Corp.) was dissolved in 0.2 ml of ethanol prior to use.

S-(2-Chloro-4-nitrophenyl)glutathione was prepared by a modification of the published procedures (6, 7). Glutathione (4.0 g) was dissolved in 100 ml of 0.5 N H2SO4 and heated to 50°. Cuprous oxide (0.98 g) was triturated with 3 ml of acetic acid, in which it partially dissolved. The cuprous oxide suspension was slowly added dropwise to the heated glutathione solution, resulting in a silky white precipitate of the copper salt of glutathione. 2-Chloro-4-nitroaniline (Aldrich Chemical Co.) (2.24 g) was dissolved in 1 N H2SO4 at 50° and filtered to remove insoluble impurities. The solution was cooled to 0°, whereupon the 2-chloro-4-nitroaniline precipitated, yielding a suspension of flocculent crystals in a yellow solution. Sodium nitrite, 0.92 g, dissolved in 2 ml of water, was added to the suspension. The resultant colorless solution was slowly added dropwise to the heated glutathione solution, resulting in a light white precipitate of the copper salt of glutathione. After filtration, the filtrate was washed with a total of 750 ml of ether. Activated charcoal was added to the aqueous phase, removed by filtration, and washed with 1 liter of water. The product was eluted from charcoal with 500 ml of methanol-aqueous ammonia (20:1) and concentrated to a yellow oil. After acidification with acetic acid, the oil was dissolved in 50 ml of water and warmed to 50°. At 50°, ethanol was added until a slight cloudiness appeared.
After cooling to −15°, the compound crystallized and was re-crystallized from water-ethanol.

The product was purified by chromatography on Whatman No. 3MM paper with 1-butanol-acetic acid-water (12:3:5) to remove traces of both oxidized and reduced glutathione. Re-chromatography of a sample on an Eastman Chromagram-cellulose sheet with the same solvent revealed a single spot (Rf = 0.70) which was ultraviolet-absorbing and reacted with ninhydrin. The final concentration of the product in aqueous solution was determined by its absorbance (εmax = 8.5 × 103).

The other compounds used were obtained commercially or were re-crystallized separately, as described (3).

Methods—For sucrose gradient centrifugation, the enzyme was layered on top of a 5 to 20% sucrose gradient in 0.1 M potassium phosphate, pH 7.5, and centrifuged at 36,500 rpm for 20 hours in an SW 39 rotor (8). Standard enzyme assays, sedimentation equilibrium centrifugation, and sodium dodecyl sulfate-gel electrophoresis were performed as described in the preceding paper (3).

Preparation of Enzyme—The initial steps in preparation of glutathione transferase A from rat liver have been described (3) and are a part of a general method for separating the several related enzymes. These steps include homogenizing the tissue and applying the extract to DEAE and CM-cellulose columns. Fractions 180 through 195 from the CM-cellulose column (see Fig. 2 of Ref. 3) were pooled and concentrated to 20 ml by ultrafiltration with a Diaflo PM-10 membrane.

The concentrated enzyme was dialyzed for 18 hours against 500 ml of 0.01 M potassium phosphate, pH 6.7, containing 5 mM GSH, 1 mM EDTA, and 30% glycerol (Buffer B), with two changes of buffer. This material was applied to a column of hydroxylapatite (4 × 20 cm) equilibrated with Buffer B. The column was rinsed with 250 ml of Buffer B, followed by a linear phosphate gradient consisting of 750 ml of Buffer B and 750 ml of the same buffer, except that the concentration of potassium phosphate was 350 mM. Fractions of 12 ml were collected. A single major peak of activity, eluting in Fractions 70 through 79, was concentrated by ultrafiltration and dialyzed against Buffer B.

The resultant protein solution was applied to another hydroxylapatite column of the same size and under the same conditions as in the previous step and was eluted with the same gradient. The product of the second hydroxylapatite column was concentrated, dialyzed against Buffer B, and stored at −80°.

Purified transferase A was crystallized from ammonium sulfate by the general method previously described (9), using successive salt concentrations of 80, 75, and 67% of saturation; a yield of 80% was obtained at 67% ammonium sulfate. Since crystallization did not increase the specific activity of the enzyme, it was not used as a step in purification.

RESULTS

Purification—A summary of the results of purification is presented in Table I. A yield of 14% from the crude extract was attained with the use of 1, 2-dichloro-4-nitrobenzene as the assay substrate; it should be recalled that transferase A represents only one of the enzyme species active with this substrate which are present in the extract (2, 3).

Homogeneity and Molecular Weight—The enzyme is homogeneous by the criteria of sedimentation equilibrium centrifugation, sucrose gradient centrifugation, and sodium dodecyl sulfate-gel electrophoresis.

Sedimentation equilibrium analysis in Buffer B at two concentrations of protein (50 and 100 µg per ml) resulted in linear plots of r² versus the log of protein concentration. A molecular weight of 45,500 was calculated on the basis of a partial specific volume of 0.737 cm³ g⁻¹; the latter value was estimated (10) from the amino acid composition (3).

Upon sucrose gradient centrifugation, a single symmetrical peak of enzyme activity was observed at 3.5 S which corresponded exactly with that of horseradish peroxidase (40,000 daltons; partial specific volume (η) = 0.699 cm³ g⁻¹). On the assumption that the peroxidase and the GSH transferase have the same shape, a molecular weight of 45,000 was calculated for transferase A.

Gel electrophoresis in sodium dodecyl sulfate produced a single band corresponding to a molecular weight of 29,000 under conditions in which six standard proteins allowed a linear plot of mobility against log molecular weight.

When subjected to gel filtration with Sephadex G-75, the elution position of transferase A was symmetrical and indistinguishable from that of an ovalbumin standard (45,000; f = 0.75 cm³ g⁻¹).

Stability—Under refrigeration, transferase A is stable for at least 6 months in 0.1 M potassium phosphate, pH 7.0, containing 30% glycerol and 1 mM EDTA. The enzyme loses activity in the presence of 2-mercaptoethanol, although other sulfhydryl reagents, such as glutathione, thioglycolic acid, and dithiothreitol, act as stabilizing agents.

At pH 7.0, in the absence of glycerol and either EDTA or a sulfhydryl reducing agent, the enzyme gives rise to multiple forms which can be separated on hydroxylapatite or on isoelectric focusing gels (11) in the range of pH 7 to 9 (Fig. 1). The difference in the isoelectric point (pI) between these forms is on the order of 0.1 pH unit. The various forms are enzymatically active, sediment identically on a sucrose gradient, and are interconvertible by dialysis in the presence or absence of the stabilizing factors. The preparation used for further study was purified in the presence of the stabilizing factors and appears as a single species upon chromatography with hydroxylapatite. Gel isoelectric focusing in a pH range of 7 to 9 (11) resulted in one major band which was accompanied by a faint secondary band at a slightly lower isoelectric position. When the gel was sliced and the isolated major band again was electrofocused, the second faint band reappeared. Both bands were enzymatically active.

Inactivation by Iodosobenzoate and by Iodoacetate—Since some of the substrates of transferase A are classical sulfhydryl alkylating agents, it was not surprising to find that iodoacetate alone was completely ineffective in inactivating the enzyme. However, o-iodosobenzoate proved to be a good inactivator at a concentration as low as 30 µM, although the time course of inactivation was unusual. The logarithm of the percentage of the remaining activity, when plotted against time, was not linear, indicating that the process was not a simple first order reaction, but rather consisted of an initial rapid inactivation down to 20 to 30% of the initial activity, followed by a slower loss of the remaining activity (Fig. 2). Interestingly, when iodoacetate, ineffective

Table I

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume protein (ml)</th>
<th>Total activity (µg min⁻¹)</th>
<th>Specific activity (µg min⁻¹ µg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>1400</td>
<td>61,000</td>
<td>0.018</td>
</tr>
<tr>
<td>DEAE-Cellulose</td>
<td>150</td>
<td>37,000</td>
<td>0.014</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>190</td>
<td>7,100</td>
<td>0.12</td>
</tr>
<tr>
<td>CM-Cellulose</td>
<td>10</td>
<td>330</td>
<td>0.35</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>12</td>
<td>42</td>
<td>3.5</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>23</td>
<td>37</td>
<td>3.5</td>
</tr>
</tbody>
</table>

As assay by the standard system, with 1,2-dichloro-4-nitrobenzene as substrate.
FIG. 1. Chromatography on hydroxylapatite of transferase A maintained in the absence of stabilizing factors. Enzyme which had previously been dialyzed against 0.1 M potassium phosphate, pH 7.5, and stored for several months at -30° was chromatographed on hydroxylapatite with Buffer B. The enzyme was eluted with a linear gradient of 200 ml of Buffer B plus 200 ml of Buffer B containing 0.35 M potassium phosphate. Fractions (5 ml) were collected, starting with the emergence of the gradient. The standard 1,2-dichloro-4-nitrobenzene assay was used (3). The inset presents the results of gel isoelectric focusing in the pH range 7 to 9. a, Enzyme before addition of Buffer B, which contains the stabilizing factors; b, enzyme after addition of Buffer B, prior to being applied to the hydroxylapatite; c, enzyme from Fraction 27; d, enzyme from Fraction 30.

alone, was included with o-iodosobenzoate, inactivation went rapidly to completion.

Saturating levels of glutathione protected the enzyme to a substantial degree, probably by reacting with the o-iodobenzoate. However, 1,2-dichloro-4-nitrobenzene also protected the enzyme; whether it did so by reacting with the o-iodobenzoate or by binding to the enzyme was not determined (Fig. 2).

**Sulfhydryl Group Titration**—The enzyme was titrated with 5,5’-dithiobis(2-nitrobenzoate) (12), yielding 3.5 sulfhydryl groups per enzyme. Titration of sulfhydryl groups was closely paralleled by loss of enzymatic activity. Unfortunately, 1,2-dichloro-4-nitrobenzene and the other aromatic substrates interfere with the reaction, so that it was not possible to obtain meaningful data with the sulfhydryl reagent in the presence of substrates.

**Effect of pH**—Fig. 3 shows the pH dependence of the non-enzymatic and the corrected enzymatic rates for three substrates. Whereas the pH at which the nonenzymatic rate becomes significant is quite different for each of the compounds, the enzyme allows reaction of all three with GSH in the physiological range of pH.

**Stoichiometry and Reversibility**—The conversion of 1,2-dichloro-4-nitrobenzene to S-(2-chloro-4-nitrophenyl)glutathione is stoichiometric. 1,2-Dichloro-4-nitrobenzene (25 and 50 nmols), in the standard assay system, was completely converted to S-(2-chloro-4-nitrophenyl)glutathione, as judged by the change in absorbance at 345 nm. The extinction coefficient of the product at 345 nm is 8.5 mM⁻¹, which is the same as the Δε for the reaction, since the substrate does not absorb significantly at 345 nm.

The reaction is experimentally irreversible. Transferase A (3 μg) was incubated with 10 μM S-(2-chloro-4-nitrophenyl)glutathione in the absence of GSH in 1.0 ml of 0.1 M Tris-chloride, pH 7.5. No change in A₃₄₅ was detectable with a Cary 15 spectrophotometer adjusted for a full scale deflection of 0.1 absorbance, indicating that reversibility is less than 1%. Similarly, in the absence of GSH, [7-¹⁴C]benzyl chloride (0.1 mm, 13.92 μCi per mmole) was not converted to S-benzylglutathione in the presence of S-(2-chloro-4-nitrophenyl)glutathione (0.1 mm) and 3 μg of enzyme in 1.0 ml of 0.1 M potassium phosphate, pH 7.5; addition of 0.1 mM GSH to this system produced the expected S-benzylglutathione. Incubation mixtures were separated by chromatography on Whatman No. 1 paper with 1-butanol-acetic acid-H₂O (12:3:5) and assayed for radioactivity with a Vanguard strip counter.

**Substrate Specificity**—In the presence of saturating GSH (5 mM), 1,2-dichloro-4-nitrobenzene, 4-nitropyridine-N-oxide, and p-nitrobenzyl chloride showed apparently normal hyperbolic saturation of transferase A, with Kₘ values in the region of 1 mM (Table II). Because of their low solubility, it was not...
possible to achieve concentrations of these compounds greater than 1 mM. At a lower concentration of GSH (0.25 mM), chosen to minimize the nonenzymatic reaction, trans-4-phenyl-3-buten-2-one showed normal saturation at concentrations as high as 0.6 mM. 1-Chloro-2,4-dinitrobenzene also displayed normal saturation, with a $K_m$ of 0.06 mM with 1 mM GSH. The enzyme is also somewhat active with naphthalene oxide, p-nitrophenethyl bromide, and 1,2-epoxy-3-(p-nitrophenoxy)propene but does not catalyze conjugation with methyl iodide or menaphthyl sulfate (Table II). Other glutathione transferases have higher specific activities with these last five substrates (2, 3). Comparison of the relative rates of reaction from this limited sampling of substrates indicates that this enzyme favors substrates whose leaving group is on, or to, the aromatic ring.

Because of its clinical interest as an indicator of liver function, bromosulfophthalein was tested as a substrate with a spectrophotometric assay (3). The compound displays strong substrate inhibition (Fig. 4), with a $K_m$ of 2 $\mu$m and maximal activity in the region of 5 $\mu$m.

**GSH Saturation**—A double reciprocal plot of GSH saturation was biphasic, with a discontinuity near 0.1 mM GSH (Fig. 5). At concentrations higher than 0.15 mM, GSH shows normal hyperbolic saturation, yielding an apparent $K_m$ of 0.2 mM. In the region below 0.1 mM, saturation is also linear in the double reciprocal plot, yielding an apparent $K_m$ of 0.01 mM. The same biphasic curve was obtained when this saturation experiment was repeated in the presence of 1 mM dithiothreitol.

**Rate Equations for High GSH Concentrations**—Fig. 6, A and B, presents double reciprocal plots of the effect of substrate concentrations on the initial velocity. The range of GSH concentrations used was limited to that greater than 0.15 mM, i.e., those concentrations above the break in the saturation curve. The range of 1,2-dichloro-4-nitrobenzene was limited by low solubility to concentrations below its $K_m$. Despite these restrictions, the data produce an intersecting pattern of lines from which the rate equation (Equation 1) was developed (13).

![Diagram](https://example.com/diagram.png)

**Table II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity $a$</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2-Dichloro-4-nitrobenzene</td>
<td>4.1</td>
<td>1.10</td>
<td>390</td>
</tr>
<tr>
<td>p-Nitrobenzyl chloride</td>
<td>11.4</td>
<td>1.35</td>
<td>1200</td>
</tr>
<tr>
<td>4-Nitropyridine-N-oxide</td>
<td>1.7</td>
<td>1.05</td>
<td>160</td>
</tr>
<tr>
<td>1,2-Epoxy-3-(p-nitrophenoxy)-propene</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Nitrophenethyl bromide</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl iodide</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Menaphthyl sulfate</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-4-Nitropyridine-N-oxide</td>
<td>0.02</td>
<td>0.5$^b$</td>
<td></td>
</tr>
<tr>
<td>p-Nitrophenoethyl bromide</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bromosulfophthalein</td>
<td>0.53</td>
<td>0.002</td>
<td>72</td>
</tr>
<tr>
<td>1-Chloro-2,4-dinitrobenzene</td>
<td>60.0</td>
<td>0.04</td>
<td>3000</td>
</tr>
</tbody>
</table>

*Under standard assay conditions (3).*

*Apparent $K_m$ since GSH is not saturating under standard assay conditions.*

Equation 1 indicates $K_m$ values of 0.27 mM for GSH and 2.1 mM for 1,2-dichloro-4-nitrobenzene (DCNB).

**Inhibition by Products at High GSH**—Under normal assay conditions, chloride ion was not inhibitory when tested at concentrations as high as 100 mM with GSH at saturation or with GSH near its $K_m$ level (0.05 mM). The other product, S-(2-chloro-4-nitrophenoxy)glutathione, is a potent inhibitor. Fig. 7 indicates that this compound is competitive with GSH, with a $K_i$ of 5 $\mu$m. The inhibition is noncompetitive with 1,2-dichloro-4-nitrobenzene (Fig. 7).
Inhibition by Benzyl Chloride at High GSH—Benzyl chloride is a substrate for the enzyme; the reaction may be followed titrimetrically at pH 6.5. The production of S-benzylglutathione was confirmed chromatographically. With 1 mM benzyl chloride, the rate of production of S-benzylglutathione was 1.2 μmoles min⁻¹ mg⁻¹ of protein, a rate in the range found with many other substrates in spectrophotometric assays (Table II). Because benzyl chloride did not interfere with the spectrophotometric assays and was available in radioactively labeled form for binding experiments, its behavior as an inhibitor of the standard 1,2-dichloro-4-nitrobenzene reaction was studied. Surprisingly, benzyl chloride was found to be noncompetitive with both 1,2-dichloro-4-nitrobenzene and GSH (Fig. 8).

Inhibition by trans-4-Phenyl-3-buten-2-one at High GSH—Because of the unexpected results of inhibition with benzyl chloride, another substrate which did not interfere with the 1,2-dichloro-4-nitrobenzene assay was tested as an inhibitor. trans-4-Phenyl-3-buten-2-one was competitive with 1,2-dichloro-4-nitrobenzene and uncompetitive with GSH (Fig. 9).

Rate Equation for Low GSH Concentrations—Reliable initial velocity data are difficult to obtain in the region of low GSH concentrations (between 0.015 mM and 0.1 mM) because of the low velocities involved. Nevertheless, a rate equation (Equation 2) could be calculated from the data shown in Fig. 6, C and D.

\[
\frac{v}{v_{\text{max}}} = \frac{1 + 0.119 \text{[GSH]} + 1.61 \text{[GSH]}}{\text{[GSH]} + 0.00923 \text{[GSH]}}
\]

(2)

The \(K_m\) values are similar to those obtained in the high GSH region, i.e. a \(K_m\) of 0.12 mM for GSH and of 1.4 mM for 3,4-dichloronitrobenzene. However, the numerator of the last term in the equation for the low GSH region is 1 order of magnitude smaller than that for the high GSH region. Because of this small numerator, the lines described by the equation in Fig. 6, C and D, appear to be parallel, or nearly so.

Inhibition by Product at Low GSH—Because of the low solubility of 1,2-dichloro-4-nitrobenzene, it was not possible to achieve concentrations of this compound which were greater than its \(K_m\). In product inhibition studies, therefore, it was difficult to distinguish competitive from mixed noncompetitive inhibition when 1,2-dichloro-4-nitrobenzene was the variable substrate. However, after repeated inhibition studies, we found that at low GSH concentrations (0.03 mM), S-(2-chloro-4-nitrophenyl)glutathione is competitive with 1,2-dichloro-4-nitrobenzene; the data are presented in Fig. 10 for evaluation. The \(K_i\) for the product was 4 μM, which is the same as that determined at high GSH concentrations. With GSH at low concentrations (i.e. less than 0.1 mM) as the variable substrate, product inhibition was of the mixed noncompetitive type (Fig. 11).

Binding of [7,2-C]Benzyl Chloride—Enzyme was incubated in the presence of 0.05 mM radioactive benzyl chloride for 15 min at room temperature, and the mixture was passed through a column of Sephadex G-75 to remove free benzyl chloride (Fig...
The amount of radioactivity which remained bound to the enzyme corresponded to 0.9 mole of benzyl chloride bound per mole of enzyme. The labeled enzyme was divided into two portions, one of which was reapplied to the Sephadex column. Approximately one-half of the benzyl chloride remained bound, i.e. 0.45 mole of benzyl chloride per mole of enzyme (Fig. 12B). The second portion of labeled enzyme was treated with 5 mM GSH before being reapplied to the Sephadex column. The radioactive benzyl chloride was completely separated from the enzyme by GSH (Fig. 12C).

**Binding of 1-Chloro-2,4-dinitro[U-14C]benzene—Enzyme (33 μM)** was incubated with 0.57 mM radioactive 1-chloro-2,4-dinitrobenzene for 15 min at 25°C in 1.0 ml of 0.1 M potassium phosphate (pH 7.5) containing 0.1 mM EDTA. The incubation mixture was then applied to a column of Sephadex G-75, with the results shown in Fig. 13A: 0.40 mole of 1-chloro-2,4-dinitrobenzene was bound per mole of enzyme. The fraction containing the peak enzyme activity was divided into three parts. One part was incubated for 5 min at 25°C with 1 mM dithiothreitol and then was reapplied to the Sephadex column. The second part was incubated with 1 mM GSH and the third was incubated without any addition. The results are shown in Fig. 13. The untreated enzyme retained 0.32 mole of 1-chloro-2,4-dinitrobenzene per mole of enzyme. Dithiothreitol was less effective (0.22 mole of substrate per mole of enzyme) than GSH in releasing bound substrate.

**Experimental Verification of Prediction of Over-all Rate Equation**—Because of the difficulties associated with kinetic analysis in the presence of low concentrations of GSH and because we wished to determine whether our separate observations at high and low ranges of GSH concentration were consistent with a single over-all mechanism, we consulted Dr. John Westley, who developed the general over-all rate equation described as Scheme 1 under "Appendix" (5). The general rate equation predicts that the position of the apparent "breakpoint" in the double reciprocal plot of GSH saturation is a function of the concentration of the second substrate. Specifically, the concentration of GSH at which the breakpoint occurs will be lower as the concentration of 1,2-dichloro-4-nitrobenzene is decreased.

This prediction may be rationalized in the following way: as 1,2-dichloro-4-nitrobenzene is decreased, the pathway which requires that this compound bind first becomes relatively less significant when compared with the pathway in which GSH binds first. The results of an experiment to test this prediction are shown in Fig. 14. At 1 mM 1,2-dichloro-4-nitrobenzene, the breakpoint (indicated by the intersection of the broken lines) appears to be in the region of 0.25 mM GSH, whereas at 0.6, 0.4, 0.2, and 0.1 mM 1,2-dichloro-4-nitrobenzene the breakpoints are near 0.20, 0.13, 0.08, and 0.06 mM GSH, respectively, in accord with the prediction.

**Correlation of Experimental Data with Numerical Rate Equation**—The curves drawn in Fig. 14 were generated from the initial velocity equation shown in Scheme 1. This equation is an expansion of the general rate equation given in Scheme 1 of "Appendix," and was derived by the method of King and Altman (14) from Scheme 2.

\[
\frac{1}{v} = k_4 \left[ [k_1 k_2 k_3 k_4 k_5 k_6] + k_2 [k_3 k_4 k_5 k_6] + k_1 k_2 k_3 k_4 k_5 k_6] \right]
\]

**Scheme 1**
FIG. 11 (left). Secondary plots of noncompetitive inhibition by S-(2-chloro-4-nitrophenyl)glutathione with GSH as the variable substrate (0.1 to 0.015 mM) and 1,2-dichloro-4-nitrobenzene concentration constant at 0.5 mM.

FIG. 12 (center). Binding of benzyl chloride. A, elution profile on Sephadex G-75 of 14 nmoles of enzyme, incubated for 15 min at 25° with 0.65 mM [ring]-benzyl chloride in 0.4 ml of 0.1 M potassium phosphate (pH 7.5) containing 30% glycerol. (Enzyme had been previously freed from GSH by gel filtration.) B, one-half of the enzyme from the peak in A was reapplied to the column.

In the diagram and rate equation, G represents GSH, A represents 1,2-dichloro-4-nitrobenzene, C represents S-(2-chloro-4-nitrophenyl)glutathione, and a represents Cl-. Rate constants were estimated from the experimental data of Fig. 14: \( k_1 = 3500 \text{ min}^{-1} \text{ M}^{-1} \), \( k_2 = 150 \text{ min}^{-1} \), \( k_3 = 7.5 \times 10^6 \text{ min}^{-1} \text{ M}^{-1} \), \( k_4 = 150 \text{ min}^{-1} \), \( k_{-1} = 15 \text{ min}^{-1} \), \( k_{-2} = 3 \times 10^4 \text{ min}^{-1} \text{ M}^{-1} \), \( k' = 1 \times 10^5 \text{ min}^{-1} \text{ M}^{-1} \), \( k'_{-1} = 1.6 \times 10^4 \text{ min}^{-1} \text{ M}^{-1} \), \( k'_4 = 30 \text{ min}^{-1} \), \( k'_{-2} = 2 \text{ min}^{-1} \), and \( k''_{-2} = 58 \text{ min}^{-1} \). These constants were used in the initial velocity equation to produce the curves shown in Fig. 14. Although these constants are compatible with the experimental data, they are only estimates and have not been uniquely determined. Nevertheless, the mathematically constructed curves in Fig. 14 correlate well with the experimental data.

This numerical rate equation also indicates that no curvature will be apparent in a double reciprocal plot of 1,2-dichloro-4-nitrobenzene saturation, provided that the concentration of 1,2 dichloro-4-nitrobenzene does not exceed its \( K_m \) (2 mM). Since the limit of solubility of this compound is 1 mM, the apparently normal hyperbolic saturation observed with this compound is also compatible with the proposed mechanism.

DISCUSSION

Glutathione transferase A, which has been prepared in a homogeneous form, is characterized by a high turnover number with compounds the electrophilic site of which is on, or \( \alpha \) to, an
activity. The enzyme is a dimer of 45,000 molecular weight which requires approximately four sulfhydryl groups for activity.

This enzyme displays a complex kinetic mechanism. Under conditions extant in normal rat liver, i.e., in the presence of between 3 and 10 mM GSH (15), transferase A activity reflects the kinetics observed for the region of a high ordinary ping-pong mechanism (16). The order of addition is derived from the finding that trans-4-phenyl-3-buten-2-one, the product, S-(2-chloro-4-nitrophenyl)glutathione, is competitive with GSH and noncompetitive with the other substrate, 1,2-dichloro-4-nitrobenzene. Also in accord with the proposed order of binding is the finding that trans-4-phenyl-3-buten-2-one, a competitive inhibitor with respect to 1,2-dichloro-4-nitrobenzene, is unconpétitive with GSH.

Under conditions of low GSH concentrations, the initial velocity pattern is that of a series of nearly parallel lines (Fig. 6, A and B). In this range, an ordered sequential pathway predominates in which glutathione is the first substrate to react with the enzyme. This is shown by the intersecting initial velocity plots in the high GSH region, together with the stoichiometric nature of the reaction, which rules out side reactions (16). The order of addition is derived from the finding that the product, S-(2-chloro-4-nitrophenyl)glutathione, is competitive with GSH and noncompetitive with the other substrate, 1,2-dichloro-4-nitrobenzene. Also in accord with the proposed order of binding is the finding that trans-4-phenyl-3-buten-2-one, a competitive inhibitor with respect to 1,2-dichloro-4-nitrobenzene, is unconpétitive with GSH.

Under conditions of low GSH concentrations, the initial velocity pattern is that of a series of nearly parallel lines (Fig. 6, C and D), suggesting an emerging ping-pong mechanism. At low GSH concentrations, S-(2-chloro-4-nitrophenyl)glutathione is competitive with 1,2-dichloro-4-nitrobenzene and noncompetitive with GSH, a finding in accord with the idea that the electrophilic substrate is the first to bind at low GSH concentrations. Since reliable kinetic data are difficult to obtain in this region of low activity, i.e. at 0.1 mM GSH or lower, confirmation of the ping-pong hypothesis was sought in binding experiments in the absence of GSH. It had been observed that 1,2-dichloro-4-nitrobenzene protected the enzyme against inactivation by o-iodosobenzoate, offering the possibility that the aromatic substrate would bind to the enzyme in the absence of GSH. Subsequently it was shown that addition of 1 mM benzyl chloride, in the absence of GSH, produced a 5% decrease in the intrinsic fluorescence of the protein (excitation, 280 nm; emission, 355 nm). The binding and release by GSH of radioactive benzyl chloride and 1-chloro-2,4-dinitrobenzene support the idea of a substituted enzyme at low GSH. However, it must be admitted that the binding detected with radioactive electrophilic substrates may not necessarily be mechanistically significant, especially in view of the failure of GSH to release all of the bound 1-chloro-2,4-dinitrobenzene. Since 1-chloro-2,4-dinitrobenzene does inactivate the enzyme with time, it is suggested that this reagent can alkylate the enzyme irreversibly. Thus, the 1-chloro-2,4-dinitrobenzene which cannot be removed by GSH may be due to nonspecific alkylaition, whereas that which is removed may be bound as substrate.

Two other kinetic observations deserve comment. The first is that benzyl chloride is noncompetitive with both GSH and 1,2-dichloro-4-nitrobenzene. This behavior may be due to benzyl chloride's forming a complex with free enzyme as well as with enzyme-GSH complex. The second observation is that BSP shows strong substrate inhibition. BSP has a comparatively low K_m, so that it is possible to approach saturation with this compound. Saturating concentrations of BSP would tend to bind to free enzyme as well as to enzyme-GSH complex, even in the presence of relatively high GSH concentrations. If the velocity through the ping-pong pathway were slower than that through the ordered sequential pathway, substrate inhibition would result. Substrate inhibition by BSP, viewed in this way, confirms the ordered rather than random nature of the sequential pathway.

Because of the complexity of the proposed mechanism, we tested whether a rate equation developed for the formal mechanism would be compatible with our initial velocity observations at both high and low GSH concentrations. The general rate equation also yielded a prediction on the behavior of the GSH breakpoint. As demonstrated in Fig. 14, both the initial velocities and movement of the breakpoint are consistent with the formal mechanism.

Enzymes generally have a single reaction pathway which is the most efficient pathway for a specific catalytic function. (An exception of which we are aware is human hypoxanthine phosphoribosyltransferase, which also displays a dual, ordered, sequential ping-pong mechanism (17). In this case, the primary ping-pong mechanism is supplanted by a ternary complex mechanism when Mg²⁺ is limiting.) The complex dual pathway mechanism of glutathione transferase A may be a consequence of the need to maintain maximal flexibility in substrate specificity for this enzyme, the presumed function of which is to intercept harmful compounds of unknown structure. A highly refined and efficient single pathway mechanism may not be compatible with the necessary substrate flexibility.

The kinetic mechanism of glutathione transferase A is an example of a system in which nonhyperbolic kinetics, displaying apparent "substrate activation" or "negative cooperativity," may be explained purely as a result of multiple reaction pathways, without the need to invoke allosterism, conformational changes, or association-dissociation phenomena. This and other examples of multiple pathway mechanisms are discussed more fully under "Appendix" (5).

1 The abbreviation used is: BSP, bromosulfophthalein.
REFERENCES

APPENDIX

DISTINCTION AMONG FORMAL MECHANISMS THAT YIELD DOUBLE RECIPROCAL PLOTS CONCAVE FROM BELOW*

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Steady state data for several enzymes with double displacement capability, i.e. the capacity to form a substituted enzyme as an intermediate in the catalytic cycle, have yielded double reciprocal plots for one or more substrates that curve downward as they approach the ordinate. Inspection of the initial velocity patterns in such cases can be used to distinguish among the several formal mechanisms that have been suggested to explain this behavior.

COMBINED SINGLE-DOUBLE DISPLACEMENT FORMS

A formal mechanism which combines single and double displacement cycles has been proposed by Pabst et al. (1) for glutathione transferase A. This mechanism is given in its simplest form in Scheme 1. The similar formal mechanisms in which the substrates enter the single and double displacement cycles in the same order rather than in the opposite order (Scheme 2) have been discussed briefly as theoretical possibilities in an earlier publication (2). All of the combined single-double forms are capable of generating either convex or concave curvature in double reciprocal plots for the donor substrate, i.e. the second substrate entering the double displacement cycle, depending on the values of the various rate constants and the accessible concentration ranges of the substrates. In experiments in which curvature concave from below is obtained, the “breakpoint,” which marks the change from flux mainly through the double displacement to flux mainly through the single displacement, is seen to vary with concentration of the fixed substrate, appearing to drift off to the left as that concentration is increased (Fig. 1).

The same order and opposite order forms are distinguishable from each other in several ways. The criterion that is simplest in principle is based on the fact that mechanisms in which the substrates enter the single and double displacement cycles in opposite order (Scheme 1) can generate nonlinear double reciprocal plots for the donor substrate as well as for the acceptor. In contrast, the mechanisms in which the substrates enter the single and double displacement cycles in the same order (Scheme 2) yield only linear, intersecting, initial velocity patterns for the donor. However, in practice, especially when accessible substrate concentration ranges are limited, it may be more practical to determine the order of substrate entry into the single displacement cycle directly by means of product inhibition and dead-end inhibition studies in the high range of acceptor concentration, as Pabst et al. have done (1). The order of substrate entry into a double displacement cycle is generally obvious.

BRIDGED FORMS

Folk and his collaborators (3, 4) have established as operative with transglutaminase a formal mechanism that resembles a double displacement with a “bridge” across the middle (Scheme
Glutathione S-Transferase A: A NOVEL KINETIC MECHANISM IN WHICH THE MAJOR REACTION PATHWAY DEPENDS ON SUBSTRATE CONCENTRATION
Michael J. Pabst, William H. Habig and William B. Jakoby


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