Mechanism for the Several Activities of the Glutathione S-Transferases

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The catalyzed reactions of GSH with organic nitrate and thiocyanate esters and with a series of chloronitrobenzene substrates have been investigated and the results used to formulate a mechanism for glutathione S-transferase catalysis. All the homogeneous preparations of the glutathione transferases that have been tested catalyze the reaction of GSH with organic nitrates and thiocyanates. The nature of the reaction with nitrate esters, resulting in the formation of GSSG rather than a thioether, has been investigated further. The presence of an additional nonsubstrate thiol decreased the formation of GSSG to an extent that cannot be explained by disulfide interchange. These results are interpreted to reflect the enzymatic formation of an unstable glutathione sulfonyl nitrite that undergoes subsequent nonenzymatic decomposition.

Hammett plots of the catalytic constants of rat liver transferases B and C obtained with a series of 4-substituted 1-chloro-2-nitrobenzene substrates demonstrate a linear relationship with ơ- substituent constants, reflecting the nucleophilic nature of the enzymatic reactions and their strong dependence on the electrophilicity of the nonthiol substrate. These data suggest that the many diverse reactions catalyzed by the glutathione transferases may be formulated as a nucleophilic attack of enzyme-bound GSH on the electrophilic center of the second substrate. The final products observed reflect this primary event and the existence of subsequent nonenzymatic reactions.

The glutathione S-transferases (EC 2.5.1.18), a group of enzymes active in the initial step of mercapturic acid synthesis (1, 2), catalyze the formation of thiocyanide by the addition of GSH to a large number of compounds bearing an electrophilic carbon (1, 3). The five transferases isolated in homogeneous form from rat liver display a range of activity without rigorous specificity for either the leaving group or the carbon skeleton of the substrates; many compounds bearing nitro groups, halogens, epoxides, or sulfate esters are reactive (3–7). An equal number of glutathione S-transferases prepared in homogeneous form from human liver exhibit a similar broad spectrum of activity (9). Regardless of source, specificity was directed to GSH and not to the second substrate.

This pattern suggested that the transferases might be effective in reactions wherein GSH participates at electrophilic centers other than carbon. In a preliminary report (9) we noted that the reaction of nitroglycerin with GSH (Equation 1), ascribed to nitrate ester reductase (10, 11), and of GSH with organic thiocyanates (Equation 2) (12, 13), were both catalyzed by the glutathione S-transferases; the electrophilic atom is nitrogen and sulfur, respectively, for the two classes of compounds. Evidence extending these observations is presented here together with the results of an investigation of the effect of systematic substitution in a chloronitrobenzene substrate. The results lead to a hypothesis for a general mechanism of glutathione S-transferase action.

MATERIALS AND METHODS

Except where noted, all work with the glutathione S-transferases was carried out with homogeneous preparations isolated as previously described for transferases AA (14), A (6), B (5), and C (5) from rat liver and transferases ß and ơ (8) from human liver. [14C]Glutathione ([1-14C]glycine), 12 mCi/mmol, was purchased from Amersham/Searle. Isotopically labeled GSSG (1.9 mCi/mmol) was prepared from [14C]GSH by aerobic oxidation and was purified by descending paper chromatography on Whatman No. 1 paper. The solvent system, butanol/acetic acid/water (12/3/5), was used for the development of all paper chromatograms reported here. 1,4-Dichloro-2-nitrobenzene (m.p. 55°) was purified by distillation under reduced pressure. 4-Chloro-3-nitrobenzaldehyde (m.p. 63.5°) and 4-chloro-3-nitrocetophene (m.p. 99°) were recrystallized from boiling water and ethanol, respectively; 4-chloro-3-nitrobenzamide (m.p. 156°) was recrystallized from boiling ethanol with water added to the point of incipient turbidity. Nitroglycerin (glyceryl trinitrate), sorbitol dinitrate (1, 4, 5, 6-dianhydrosofritol 2,5 -dinitrate), and ethylene glycol dinitrate were gifts from T. Z. Bell and F. Cox of the Atlas Powder Co.; erythritol tetranitrate was a gift from W. B. Brownell of Burroughs Wellcome Co. The
nitrates, obtained in concentrations of between 10 and 25% in lactose, were extracted with 95% ethanol to produce a 0.1 M solution. The general method of Eriksson (15) was used for the preparation of ethylglutathione disulfide. The thionocarbonic derivative of glutathione disulfide was treated with ethanol and the product was purified by ascending paper chromatography on Whatman No. 3MM paper. Structure was confirmed by proton nuclear magnetic resonance spectroscopy. S-Cyanoglutathione was synthesized (16) by slow addition of 1 M potassium hydroxide to an equal volume of 20 mM sodium nitrite. Both were precipitated with 20 M potassium phosphate at pH 7.5. After incubation for 4 h, the solution was brought to pH 3 with acetic acid. The product was isolated by ascending chromatography on Whatman No. 3MM and quantitated by amino group (17) and sulfhydryl group (18) analysis.

**Enzyme Assays**—Details of assays with the standard substrates, 1,2-dichloro-4-nitrobenzene and 1-chloro-2,4-dinitrobenzene, have been presented (5). Enzyme units for all substrates reported here are in terms of micromoles of product formed/min at 25°C. Maximal velocities of a series of 4-substituted 1-chloro-2-nitrobenzenes were calculated from data obtained by varying the concentration of GSH and the second substrate in a constant ratio (19, 20) in 0.1 M potassium phosphate at pH 7.0. Nonlinear regression analyses were performed by means of an interactive curve-fitting program, MLAB, developed for a PDP-10 digital computer (21).

Enzyme assays with organic thiocyanates were conducted in glass tubes (10 × 65 mm) in a total volume of 0.1 M potassium phosphate at pH 7.5, 1 mM GSH, 0.75 mM thiochyanate, 2.5% ethanol, and an appropriate amount of enzyme. After 10 min, the reaction was terminated by addition of 0.5 ml of 0.3 M N-ethylmaleimide in 0.1 M potassium phosphate at pH 7.5 and the solution was allowed to incubate for 1 min. Loss of volatile cyanide under these conditions was less than 5% and no correction was applied. Following addition of 10 μl of 0.1% chloramine-T and additional incubation for 3 min, 0.4 ml of a pyrazolone reagent was added (22). The pyrazolone reagent was prepared by mixing 200 volumes of a saturated, filtered solution of 1-phenyl-3-methyl-5-pyrazolone in distilled water with 1 volume of a fresh solution of 0.1% 3,3'-dimethyl-1,1'-diphenyl-4,4'-bi-2-pyrazolone-5,5'-dione in pyridine. After 20 min of incubation the absorbance of the assay solution was determined at 618 nm. The concentration of a sodium cyanide standard solution was calibrated by titration with silver nitrate using a potassium iodide indicator (23). The absorbance of the assay solution was determined at 618 nm. The concentration of a sodium cyanide standard solution was calibrated by titration with silver nitrate using a potassium iodide indicator (23).

Absorbance was a linear function of cyanide concentration in the range of 0.4 to 10 nmol; neither ethyl, octyl, nor benzyl thiochyanate at 0.8 μmol interfered with the assay. Inorganic thiochyanate reacted in this assay with a color value approximately 80% that of cyanide.

Assay conditions with nitrate esters have been presented (9). The product, nitrite, was determined (24) as previously described (5); 1.0 nmol of nitrite was equivalent to approximately 4 Klett units. Neither nitrate nor GSSG decreased color development; GSH at a final concentration of less than 0.1 mM also did not interfere.

**Extinction Differences of Substrates**—The magnitude and wavelength of maximal extinction differences between the 1-chloro-2-nitrobenzene derivatives and their respective glutathione conjugates were determined by comparing product spectra, corrected for glutathione absorbance, with substrate spectra (Table I). The GSH conjugates were prepared by incubation of a 0.1 mM concentration of each substrate with 10 mM GSH, 0.2 mM EDTA, 1.9% ethanol, and 0.1 M sodium carbonate at pH 9.0. Before determining the extinction coefficients, aliquots of 1.0 ml were neutralized with 20 μl of 85% phosphoric acid and treated with a stream of argon.

**Determination of Disulfide Interchange**—At 1 mM each, 1°C glutathione disulfide (1.9 mM/mmol) and 2-mercaptoethanolamine were incubated in 0.1 M potassium phosphate at pH 6.5. Aliquots were removed at 1-min intervals for 5 min and the reaction in each sample was stopped by addition of N-ethylmaleimide to provide a 10-fold greater reaction mixture. The reaction mixture was fractionated by descending paper chromatography on Whatman No. 1 paper; isopropically labeled exchange products were located with a Searle Actigraph III chromatogram scanner. The S(N-ethylsucinimido)glutathione formed was quantitated with an integrating chromatogram scanner (Searle) or by elution and counting with a Beckman LS-200 liquid scintillation spectrometer.

**RESULTS**

All of the glutathione transferases tested catalyzed the formation of cyanide from organic thiocyanates and of nitrous acid from nitrate esters. The wide range of activities observed for the several proteins under standard assay conditions (Table II) reflected intrinsic differences in the catalytic parameters of the enzymes with the several substrates (Table III); for comparison, data are also presented in Table II for two substrates that form thioethers. The reaction product formed from organic thiocyanates is cyanide; neither chemically synthesized glutathione thiocyanate nor inorganic thiocyanate added to the assay system could account for the acid-volatile product observed. In both this reaction and that with nitrate esters, the specificity of the enzyme for GSH is as great as it is in those reactions in which a thioether is formed (5, 8, 25). GSH could not be replaced by L-cysteine, N-acetyl-L-cysteine, 2-mercaptoethanolamine, or dithiothreitol.

Based on activity-precipitant curves with antibody to transferase A (9), it had been inferred that the activity toward thiochyanate esters, nitrate esters, and the classic transferase substrate 1-chloro-2,4-dinitrobenzene, resided in a single protein. Identity has been further substantiated by activity elution profiles of rat liver extracts from a column of CM-cellulose. When thiochyanate and nitrate ester cleavage were assayed, both activities always coincided with the several transferases.

**Table I**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>λ</th>
<th>Δε</th>
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<tr>
<td>1,4-Dichloro-2-nitrobenzene</td>
<td>383</td>
<td>0.7</td>
</tr>
<tr>
<td>4-Chloro-2,4-dinitrobenzene</td>
<td>304</td>
<td>2.3</td>
</tr>
<tr>
<td>4-Chloro-3-nitrobenzenesulfone</td>
<td>370</td>
<td>0.3</td>
</tr>
<tr>
<td>4-Chloro-3-nitroacetophenone</td>
<td>297</td>
<td>11.9</td>
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**Table II**

<table>
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<tr>
<th>Substrate</th>
<th>Transferase</th>
<th>A</th>
<th>AA</th>
<th>B</th>
<th>C</th>
<th>β</th>
<th>δ</th>
</tr>
</thead>
<tbody>
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<td>Ethyl thiocyanate</td>
<td>0.076</td>
<td>0.20</td>
<td>0.019</td>
<td>0.057</td>
<td>0.28</td>
<td>0.13</td>
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<tr>
<td>Ocytl thiocyanate</td>
<td>0.12</td>
<td>0.12</td>
<td>0.647</td>
<td>0.16</td>
<td>0.067</td>
<td>0.094</td>
<td></td>
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<tr>
<td>Benzyl thiocyanate</td>
<td>0.59</td>
<td>0.96</td>
<td>0.084</td>
<td>1.1</td>
<td>0.25</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>Nitroglycerin</td>
<td>0.06</td>
<td>0.14</td>
<td>0.09</td>
<td>0.37</td>
<td>0.05</td>
<td>0.32</td>
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<tr>
<td>Erythritol</td>
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<td>0.36</td>
<td>0.15</td>
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<tr>
<td>Tetranitrate</td>
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<td>0.002</td>
<td>0.004</td>
<td>0.014</td>
<td>0.003</td>
<td>0.019</td>
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<tr>
<td>Isosorbide</td>
<td>0.013</td>
<td>0.031</td>
<td>0.020</td>
<td>0.060</td>
<td>0.012</td>
<td>0.102</td>
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</table>
expected peaks of activity (5, 14) with 1-chloro-2,4-dinitrobenzene (Fig. 1). These data, the cited immunological results (9), and the extensive characterization of the homogeneity of the glutathione transferase preparations (5, 6, 14), are in accord with the assignment of all of these functions to each of the transferases.

**Nitrate Ester Reaction**—We have confirmed the stoichiometry of the reaction previously attributed to nitrate ester reductase (Equation 1 and Refs. 10 and 11) with glutathione transferase B. Because GSSG formation by the transferases has been observed with other substrates, except as an artifact (13, cf. Ref. 9), we questioned its direct production in this reaction. As an alternative formulation, attack of GSH on the electrophilic nitrogen of the nitrate ester to form a glutathione sulfenyl nitrite intermediate (Equation 3) is suggested by known glutathione transferase reactions (3) and is consistent with nonenzymatic reactions of thiols. The intermediate thus formed could react nonenzymatically with additional GSH to yield the observed reaction products (Equation 4).

\[
\text{GSH} + \text{RONO} \rightarrow [\text{GS-NO}] + \text{ROH} \tag{3}
\]

\[
[\text{GS-NO}] + \text{GSH} \rightarrow \text{GSSG} + \text{HNO}_3 \tag{4}
\]

The existence of an intermediate was examined by assessing the effect of an additional thiol, itself not a substrate of the enzyme, on the stoichiometry. The second thiol should partition the intermediate between two disulfides so that the amount of GSSG produced would be significantly less than that of nitrate. In the absence of a second thiol, the reaction of GSH with erythritol tetranitrate as catalyzed by transferase B resulted in close equivalence of nitrate and GSSG formation (Fig. 2A). Upon inclusion of 2-mercaptoethylamine, in concentration equal to that of GSH, GSSG formation was markedly inhibited without significant effect on nitrite release (Fig. 2B). That this result did not reflect inhibition of the assay system for GSSG by asymmetric glutathionyl disulfides (26) is demonstrated by the quantitative response to GSSG added at the conclusion of the experiment (arrow in Fig. 2B).

These findings would also be obtained if facile disulfide interchange occurred between enzymatically produced GSSG and the second thiol to yield an asymmetric disulfide (Equation 5).

\[
\text{GSSG} + \text{RSH} \rightarrow \text{GSSR} + \text{GSH} \tag{5}
\]

However, the rate of disulfide interchange was minimized by use of low pH and low concentration of 2-mercaptoethylamine,
A Mechanism for Glutathione Transferases

A Mechanism for Glutathione Transferases

**FIG. 2.** Product formation in the nitrate ester reaction in the presence and absence of additional thiol. A, formation of glutathione disulfide (○) and nitrite (●) upon incubation of 0.87 μM transferase B with 1 mM glutathione and 1 mM erythritol tetranitrate in 0.1 M potassium phosphate at pH 6.5. The reaction was performed in the presence of 0.9 unit/ml of glutathione reductase, 0.18 mM TPNH, and 0.5 mM EDTA. The control consisted of a reaction mixture lacking erythritol tetranitrate. B, as in A but with addition of 1 mM 2-mercaptoethylamine. A- - - A, the calculated amount of GSSG expected based on 1:1 reaction stoichiometry with nitrite (as in A) and the existence of nonenzymatic disulfide interchange with 2-mercaptoethylamine. The arrow indicates the addition of 20 nmol of GSSG.

Additionally, TPNH and glutathione reductase were present to serve as a trap as well as an assay system for GSSG.

A quantitative estimate of the amount of GSSG that might nonetheless be lost through disulfide interchange was calculated as the product of three factors: the determined bimolecular rate constant for the forward reaction of Equation 5 (where RSH is 2-mercaptoethylamine); the known 2-mercaptoethylamine concentration; and the estimated concentration of GSSG over the course of the incubation. This calculation yielded a maximal value for loss of GSSG by disulfide interchange because the reverse reaction of Equation 5 was intentionally neglected. The rate constant for the forward reaction was found to be 22 M⁻¹ min⁻¹ by observing the initial rate of release of glutathione from GSSG on incubation with 2-mercaptoethylamine at pH 6.5. The steady state concentration of GSSG was estimated to be less than 10 μM at all times, a conservative estimate in view of the presence of a large excess of glutathione reductase and the close equivalence of GSSG and nitrite observed throughout the course of reaction (Fig. 2A). The amount of interchange, calculated for each time point, was subtracted from the theoretical amount of GSSG produced, i.e. an amount equivalent to that of inorganic nitrite in Fig. 2B, to yield the **dashed line** in Fig. 2B. It is apparent that disulfide interchange cannot account for the decrease in GSSG observed upon addition of 2-mercaptoethylamine. Rather, the results are consistent with the two-step reaction summarized by Equations 3 and 4.

**Thioether Formation**—We have chosen a group of 1-chloro-2-nitrobenzenes, substituted in position 4, for an evaluation of the effect of electrophilicity on reaction rate. The displacement of chloride and the formation of the corresponding thioether of GSH for this series of compounds was evaluated for the spontaneous reaction and that catalyzed by transferases B and C. As expected of activated nucleophilic aromatic substitution reactions (27), a Hammett plot of the second order rate constants for the nonenzymatic reaction of GSH with 4-substituted chloronitrobenzenes shows good correlation with resonance σ⁻ values. The **line** in Fig. 3, top, drawn by least squares regression analysis (r = 0.99), has a Hammett ρ value of 3.1. In contrast, a similar statistical analysis for relation to σ° values (not shown) provides poor correlation (r = 0.74).

As evaluated kinetically by nonlinear regression analysis, higher maximal velocities for the enzyme-catalyzed reactions were found as the result of increasing the electron-withdrawing capacity of para substituents. Quantitatively, a Hammett plot of the catalytic constants for transferase B (Fig. 3, bottom) describes a straight line (ρ = 1.8, r = 0.96) although 1-chloro-2,4-dinitrobenzene (not included in regression analysis) is a more active substrate than expected. The results with transferase C (Fig. 3, bottom) also demonstrate a linear relationship (ρ = 1.6, r = 0.98) although here, the p-chloro derivative (not included in regression analysis) is less active as a substrate than expected.

**DISCUSSION**

Whereas earlier investigations have focused on transferase-catalyzed reactions with substrates bearing electrophilic car-
bon atoms, results with compounds having electrophilic sulfur or nitrogen atoms are reported here. The data provide support for a model of glutathione S-transferase catalysis that can account for all of the superficially dissimilar catalytic activities that have been observed. It is proposed that the transferases act primarily by enhancing the nucleophilicity of the thiol function of enzyme-bound GSH, thereby facilitating its attack on the electrophilic center of the second substrate. The latter is suggested to occupy a nonspecific, predominantly hydrophobic binding site, which may in itself provide an environment facilitating reaction (28).

Organic thiocyanate substrates undergo attack by enzyme-bound GSH at the electrophilic thiocyanate sulfur, resulting in the formation of an asymmetric glutathionyl disulfide, a reaction that also occurs in the absence of enzyme (29). With nitrate ester substrates, nucleophilic attack on the strongly electrophilic nitrogen atom has been inferred from data presented here and is analogous to earlier proposals for the mechanisms of related nonenzymatic processes: the reactions of hydroxylamine with nitrate esters (30) and of thiols with tetranitromethane (31, 32). Similarly, the appearance of nitrite on incubation of nitroglycerin with glyceraldehyde-3-phosphate dehydrogenase, in the absence of exogenous thiol, has been suggested to involve reaction with an active enzyme-bound sulphydryl group (33). Although a thioperoxide intermediate was suggested, we believe it equally likely that hydrolytic decomposition of a protein sulfenyl nitrite can account for the observed products.

The data for the chloronitrobenzene derivatives demonstrate that electron-withdrawing para-substituents facilitate both the uncatalyzed and enzymatic reactions, confirming the nucleophilic nature of the reactions. It is suggested that activated aromatic nucleophilic substitution, with substantial introduction of negative charge into the aromatic ring in the transition state, serves as a model for the enzyme catalyzed process. Furthermore, the operation of similar catalytic pathways for transferases D and C are quantitatively implied by the comparable $p$ values; that these are less than that of the nonenzymatic reaction is expected (34). The rate enhancement observed with transferase C, calculated as the ratio of the catalytic constant and the nonenzymatic bimolecular rate constant for reaction of 1-chloro-2,4-dinitrobenzene with GSH at pH 7.0, is 510 M, a relatively small value (35).

The transferases appear to act through a specific interaction with GSH, kinetically the first substrate bound in the Ordered Bi Bi reaction (6, 36), but with little specificity for the nonthiol substrate. In fact, the linearity of the Hammett plots demonstrates that, within a series of closely related compounds, the catalytic constant depends primarily on the electrophilicity of the non-thiol substrate. The rate determining step is, therefore, likely to be the bond-altering one in the ternary complex, for it is unlikely that a conformational change, or slow release of either the first or second products (chloride or $S(2$-nitro-4-X-phenyl)glutathione, respectively), would show strict dependence on reaction center electrophilicity and generate the observed results. Conversely, it is significant that random or horizontal (37) Hammett plots, which might reflect these situations, were not observed.

This large range of substrates, of diverse chemical composition and reaction center bond geometry, is mirrored by an equally large number of compounds that strongly interact with the transferases but are not chemically altered (38). That all of the known ligands possess a significant hydrophobic region, are bound to the transferases, and serve as inhibitors of the catalytic reactions with 1-chloro-2,4-dinitrobenzene (39) provides further evidence for a nonspecific binding site whose salient characteristic is hydrophobicity. This, and the modest rate enhancements noted above, are consistent with the hypothesis that the main catalytic device of the glutathione transferases is the enhancement of glutathione thiol nucleophilicity.

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