Identification of N-Acetylcysteine as a New Substrate for Rat Liver Microsomal Glutathione Transferase

A STUDY OF THIOL LIGANDS*

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N-Acetyl-l-cysteine serves as an efficient substrate for the rat liver microsomal glutathione transferase with 1-chloro-2,4-dinitrobenzene as second substrate (8.8 ± 0.37 μmol/min mg). The activity is actually higher than that obtained with glutathione (2.4 μmol/min mg). In examining the activity of liver subcellular fractions, no activity with N-acetyl-l-Cys could be detected in dialyzed or N-ethylmaleimide-treated (in order to remove endogenous glutathione) cytosol. The activity in rat liver microsomes was 0.11 ± 0.007 μmol/min mg, which is accounted for by the content of microsomal glutathione transferase. Thus, N-acetyl-l-Cys can be used as a specific substrate for determining the conjugating activity of microsomal glutathione transferase. N-Acetyl-l-Cys was also shown to function as a substrate for the enzyme when other second substrates than 1-chloro-2,4-dinitrobenzene (with varying electrophilicity) are used.

The pH dependence of microsomal glutathione transferase was studied. The \( k_{cat}/K_m(1\text{-chloro-2,4-dinitrobenzene}) \) dependence on pH with an apparent \( pK_a \) of 6, ≥9, and ≥8 with saturating glutathione, γ-L-Glu-L-Cys, and N-acetyl-l-cysteine, respectively. Apparently the enzyme has the ability to lower the \( pK_a \) of glutathione by 3 orders of magnitude. The \( k_{cat}/K_m(\text{thiol}) \) did not vary appreciably with pH (except for N-acetyl-l-cysteine), indicating that no rate-determining deprotonation occurs on the enzyme itself between pH 5.5 and 9.

The abilities of histidine-, lysine-, and arginine-selective reagents to inactivate the enzyme when N-acetyl-l-cysteine and γ-L-Glu-L-Cys were used as substrates were investigated. The activity toward N-acetyl-l-cysteine was decreased considerably less after treatment with the arginine-selective reagent phenylglyoxal, as compared to the activity toward GSH and γ-L-Glu-L-Cys. This indicates that an arginine makes contact with the γ-L-Glu residue in GSH. With the other reagent/substrate combinations tested the enzyme was inactivated almost completely.

The ability of microsomal glutathione transferase to stabilize the Meisenheimer complex formation between 1,3,5-trinitrobenzene and various glutathione analogues, including non-substrate thiols, has been examined. It is shown that, in general, substrates exhibited higher formation constants (approaching 50 ms⁻¹) than non-substrates (4.5 ± 1.7 ms⁻¹, \( n = 7 \)), whereas simpler thiols did not yield enzyme-bound complexes. The fact that the enzyme can stabilize Meisenheimer complexes from non-substrate thiol analogues of glutathione offers new possibilities for examining the substrate interactions of glutathione transferases.

Membrane-bound microsomal glutathione transferase (reviewed in Refs. 1 and 2) is characterized by its ability to be activated by sulfhydryl reagents (3) and trypsin (4). The enzyme has a unique amino acid sequence and immunological properties, in comparison to its cytosolic counterparts (5, 6). On the functional level, as is the case for other members of the glutathione transferase group of enzymes (see Refs. 7 and 8 for reviews), it is involved in the detoxication of numerous carcinogenic, mutagenic, toxic, and pharmacologically active compounds (9). Polyhalogenated hydrocarbons and (phospho-)-lipid hydroperoxides appear to be particularly interesting groups of substrates for the microsomal glutathione transferase (10, 11).

In a previous examination of the structural features of glutathione required for activity and activation utilizing various glutathione analogues (12), we had hoped to see some striking differences to cytosolic glutathione transferases (13). However, no specific substrate was found. In the present study we report that N-acetyl-l-cysteine is a unique and efficient substrate for the microsomal glutathione transferase.

Meisenheimer complex stabilization by glutathione transferase has been shown previously to occur with glutathione and 1,3,5-trinitrobenzene (14). In the present study we have investigated the Meisenheimer complex stabilization by microsomal glutathione transferase with 1,3,5-trinitrobenzene and glutathione analogues. Quite remarkably, even analogues that are inactive as substrates for the enzymatic reaction give rise to Meisenheimer complex stabilization by the enzyme. This finding offers new possibilities for studying the substrate-enzyme interactions of glutathione transferases.

EXPERIMENTAL PROCEDURES

Materials

Glutathione, glutathione sulfonate, l-cysteine, and N-ethylmaleimide were purchased from Sigma. N-Acetyl-l-cysteine (Boehringer, Mannheim, Germany), γ-L-Glu-L-Cys (Kohjin Co. Ltd., Tokyo, Japan), and L-cysteinyl-glycine (Bachem Feinchemikalien AG, Bubendorf, Switzerland) were from the sources indicated. N-Acetyl-l-cysteine was a kind gift from Dr. Ian Cotgreave (Department of Toxicology, Karolinska Institute). 1-Chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene were obtained from Merck Co. (Darmstadt, Germany). 4-Chloro-3-nitrobenzenecarboxylic acid was from Aldrich-Chemie (Steinheim, Germany). 4-Chloro-3-nitrobenzamide was from the Alfred Bader Library of Rare Chemicals (a division of Aldrich). Glutathione analogues were synthesized as described (15) and gener-

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Thiol Substrates of Microsomal Glutathione Transferase

Table 1

| Substrate | Enzyme          | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | Specific activity |
|-----------|-----------------|----------|-------|---------------|------------------|
| γ-γ-Glu-L-Cys | Unactivated     | 0.2 ± 0.02 | 0.33 ± 0.01 | 0.54 | 0.58 ± 0.02 |
|            | Activated       | 1.0 ± 0.04 | 0.7 ± 0.06 | 1.5  | 3.4 ± 0.09 |
| N-acetyl-L-Cys | Unactivated     | 8.6 ± 0.22 | 25 ± 0.8  | 0.35 | 8.2 ± 0.37 |
|            | Activated       | 5.0       | 54      | 0.1  | 2.6 ± 0.1 |
| N-Acetyl-t-Cys | Unactivated     |           |         |      | <0.02   |
|            | Activated       |           |         |      | <0.001  |
| L-Cys-Gly   | Unactivated     | 3.7       | 1.7     | 1.8  | 3.4 ± 0.1 |
|            | Activated       | 30        | 2.2     | 9.5  | 8.6 ± 0.8 |

* Calculated from the $k_{cat}$ and $K_m$ values.

* Enzyme activated by N-ethylmaleimide as described under "Methods."

* Not detectable with unactivated and activated enzyme representing the limit of detection.

* From Ref. 12.

Methods

Male Sprague-Dawley rat liver subcellular fractions were prepared according to (16). In order to remove endogenous glutathione, cytosol was dialyzed overnight against 0.25 M sucrose or treated with 1 mM N-ethylmaleimide at 4 °C. In order to activate the glutathione transferase activity of microsomes, these were treated with 1 mM N-ethylmaleimide at 4 °C prior to assay.

Rat liver microsomal glutathione transferase was purified as described (3).

Activation of the purified enzyme with 2–5 mM N-ethylmaleimide was performed at 4 °C in 10 mM potassium phosphate, pH 8, 1% Triton X-100, 0.1 mM EDTA, 1 mM GSH, 20% glycerol, and 0.1 M KCl. When maximal activity was reached (within 5 min.), the reaction was terminated by addition of GSH to give a final concentration of the free thiol of approximately 1 mM.

Glutathione was removed from the purified enzyme by dialysis against 10 mM potassium phosphate, pH 7, 0.1 mM EDTA, 20% glycerol, and 50 mM KCl (two daily changes). Removal was checked by testing Meisenheimer complex stabilization without the addition of GSH and took 48–72 h. The glutathione-free enzyme was activated by addition of 0.5 mM NEM.1

1 The abbreviations used are: NEM, N-ethylmaleimide; CDNB, 1-chloro-2,4-dinitrobenzene.

Enzyme inactivation with group-selective reagents was performed with the glutathione-free enzyme (that had been activated with NEM) at room temperature by the inclusion of diethyl pyrocarbonate (10 mM), trinitrobenzenesulfonate (2 mM), pyridoxal 5'-phosphate (2 mM), and phenylglyoxal (5 mM). These reagents are selective toward histidine, lysine, lysine, and arginine, respectively. NEM treatment of the enzyme prevented the known sulphydryl reaction of trinitrobenzenesulfonate. Aliquots were withdrawn for assay at different time points as indicated in Fig. 3 (a–d).

Enzyme Assays—Activities were assayed in 0.1 M potassium phosphate, pH 6.5, containing 0.1% Triton X-100 at 30 °C and varying glutathione analogue concentrations with 0.5 mM 1-chloro-2,4-dinitrobenzene. All experiments involved duplicate or triplicate determinations and were repeated at least once. $k_{cat}$ and $K_m$ values were derived by non-linear regression with the program Pennzyme (17). In the case of N-acetyl-L-cysteine and activated enzyme, these values are from Lineweaver-Burk plots. Activites with 4-substituted 1-chloro-2-nitrobenzene and 1,2-dichloro-4-nitrobenzene (0.5–1 mM) were assayed in 0.1 M potassium phosphate, pH 6.5, containing 0.1% Triton X-100 at 30 °C with 10 mM N-acetyl-L-cysteine at the wavelengths described in Ref. 18. The non-enzymatic rates were measured and subtracted from the observed enzymatic activities. In no case were they more than 10% of the enzyme-catalyzed rates (except for t-Cys: 550%).

The pH dependence of $k_{cat}/K_m$ was determined in the following buff-
The decrease in catalytic efficiency for N-acetyl-L-Cys upon NEM-treatment of the enzyme agrees well with previous findings that the free amino function in the γ-L-Glu group of GSH is important for activation (12). In keeping with this prediction, activation did occur with γ-L-Glu-L-Cys.

The pH rate behavior of microsomal glutathione transferase with GSH, γ-L-Glu-L-Cys, and N-acetyl-L-Cys was investigated in terms of kcat/Km for both CDNB and the thiois. The kcat/Km for the thiol at saturating CDNB concentration should reflect the pH behavior of the enzyme-CDNB complex and the free thiol substrate. It is evident (Fig. 1) that there were very small effects of pH on kcat/Km (thiol). The conclusions that can be drawn from this experiment are: 1) no rate-determining ionizations of the free enzyme appear to occur between pH 5.5 and 8; 2) the deprotonation of the sulfhydryl has little influence on kcat/Km, implying that the enzyme has no preference for the substrate in its thiolate anion form (except perhaps in the case of N-acetyl-L-cysteine). The kcat/Km for CDNB at saturating thiol should reflect the pH dependence of the enzyme-thiol complex and in that way reveal any effect of the enzyme in lowering the pKs of these thios (e.g. if no rate-determining titrating group is present on the enzyme itself, which appears to be the case). As can be seen in Fig. 2, the pKs of GSH was lowered to approximately 8.5-9 and ≥7.5, respectively. Thus, the pKs for GSH in complex with the enzyme appears to be lowered from that of the parent compound (pK = 8.9-9.1; Ref. 21) by 3 pH units. The kcat/Km for CDNB with saturating γ-L-Glu-L-Cys and N-acetyl-L-Cys, on the other hand, displayed an increase with pH, where γ-L-Glu-L-Cys approached the value for GSH at high pH. This indicates that the thiolate anion of this compound can substitute efficiently for glutathione and that the binding energy available in the glycine part of glutathione is utilized by the enzyme to lower the pKs of the thiol. Although it is technically difficult to measure rates at higher pH levels, the enzyme does not appear to be able to lower the pKs of γ-L-Glu-L-Cys and N-acetyl-L-Cys to a great extent (if at all) and the pKs values might thus resemble those of the parent compounds.

The activated and unactivated enzyme are affected similarly by pH, except in the case of kcat/Km for CDNB with glutathione as the saturating substrate. In this case there is a sharper decline of the activated enzyme at low pH, actually becoming lower than the value for the unactivated enzyme. This effect can only be partly attributed to a lower stability of the activated enzyme at low pH. It is interesting to note that the differences between glutathione and γ-L-Glu-L-Cys resemble those that were obtained with cystolic glutathione transferase 3-3 wild type and the in vitro mutant tyrosine to phenylalanine where the enzyme loses the ability to lower the pKs of glutathione (22). In our case the substrate change brings about the same effect.

**RESULTS AND DISCUSSION**

The activities and kinetic parameters of rat liver microsomal glutathione transferase with various truncated glutathione derivatives and CDNB and CDNC are shown in Table I. L-Cysteine was a very poor substrate displaying a low specific activity, which is important for activation (12).

| Fraction          | Specific activity | GSH (μmol/min mg) | N-Acetylcysteine (μmol/min mg) |
|-------------------|-------------------|-------------------|-------------------------------|
| Microsomes        | 0.11 ± 0.007      | 0.14 ± 0.008      |                               |
| Microsomes (NEM-treated) | 0.003 ± 0.001 | 0.08 ± 0.07       |                               |
| Cytosol           |                   | 0.23 ± 0.22       |                               |
| Cytosol (NEM-treated) | ND                | 2.59 ± 0.22       |                               |
| Cytosol (dialyzed) | ND                | 1.38 ± 0.12       |                               |

*Not detectable.*

**Table II**

| CDNB conjugating activity of rat liver cytosol and microsomes with GSH or N-acetyl-L-cysteine as the thiol substrate

**Table III**

| Substrate          | Enzyme form | N-Acetyl-L-Cys* | Activation/deactivation | GSH* |
|--------------------|-------------|----------------|-------------------------|------|
| CDNB               | Unactivated | 5.6 ± 0.5      | -2.8-fold               | 2.6  |
| 4-Chloro-3-nitrobenzaldehyde | Activated | 3.0 ± 0.2      | 3.9                      |
| 4-Chloro-3-nitrobenzacetophenone | Unactivated | 1.1 ± 0.08 | -10-fold               | 46   |
| 4-Chloro-3-nitrobenzamide | Activated | 0.11 ± 0.003   | 2.0                      |
| 2,5-Dichloronitrobenzene | Unactivated | 0.062 ± 0.0003 | -19-fold               | 15   |
| 1,2-Dichloro-4-nitrobenzene | Activated | 0.020 ± 0.001 | 1.9                      |
|                   | Activated | 0.063 ± 0.004 | +3-fold                 | 1.9  |
|                   | Unactivated | 0.11 ± 0.02   | +14-fold                | 1.0  |
|                   | Activated | 0.16 ± 0.02   | +8-fold                 | 0.06 |

*The results are expressed as the mean ± S.D. of four determinations.

From Ref. 26.
Cytosolic glutathione transferases purified from rat liver are active with \(\gamma\)-Glu-l-Cys, but not with N-acetyl-l-Cys (23). Rat liver cytosol and microsomes were used in order to test whether N-acetyl-l-Cys is a selective substrate for microsomal glutathione transferase. As can be seen in Table II, no N-acetyl-l-Cys conjugating activity could be detected in cytosol that had been freed from endogenous GSH by dialysis or NEM treatment. Rat liver microsomes, on the other hand, displayed a high activity (Table II), which was fully accounted for by their content of microsomal glutathione transferase. Upon NEM treatment of microsomes, this activity was still significant, but decreased to a much lower level than expected from the activity decrease observed with the purified NEM-treated enzyme (approximately 10%). This could reflect the presence of an endogenous inhibitor (that has been suggested to be present in microsomes; Ref. 24) that inhibits N-acetyl-l-Cys conjugation by the NEM-treated enzyme by as much as 90%. Al-NEM treatment of microsomes, this activity was still significant, but decreased to a much lower level than expected from the content of microsomal glutathione transferase. Upon treatment with NEM, the activity decrease observed with the purified NEM-treated enzyme is, in the exception of glutaryl-L-Cys-Gly (3 m\(^{-1}\)), which displays a formation constant similar to those of poor substrates and non-substrates. The absorbances of the Meisenheimer complexes between the magnitude of the formation constant and the catalytic efficiency seems to exist (14). Meisenheimer complexes obtained in the absence of enzyme (0.028 \(m\)1 cm\(^{-1}\)) is large compared to that reported in the absence of enzyme (0.028 \(m\)1 cm\(^{-1}\)) and is within the range of those reported for the cytosolic isoenzymes 3-3 and 4-4 (51 and 0.7 \(m\)1 cm\(^{-1}\), respectively) (14). In general, a correlation between the magnitude of the formation constant and the catalytic efficiency seems to exist (14). Meisenheimer complexes between glutathione analogues and 1,3,5-trinitrobenzene are also stabilized by the microsomal glutathione transferase (Table IV) and, quite remarkably, even with analogues that are not substrates for catalysis. Formation constants for good substrates are in general above a threshold value of 8 \(m\)1 cm\(^{-1}\), with the exception of glutaryl-L-Cys-Gly (3 \(m\)1 cm\(^{-1}\)), which displays a formation constant similar to those of poor substrates and non-substrates. The absorbances of the Meisenheimer complexes obtained in the absence of enzyme are very low and have been subtracted. Differences between the activated and unactivated enzyme are, in general, small, indicating that the conformational change that accompanies activation (4) does not increase the complementarity of the enzyme to the dead-end complex and, to the degree that this resembles the transition state, does not appear to increase catalytic efficiency by increasing transition state complementarity. The extinction coefficients (Table IV) are consistently lower than those expected from the Meisenheimer complex of GSH in solution (30) and to those ob-

### Table IV

| Thiol compound            | Enzyme form | \(K_f\) \(m^{-1}\) (n=3) | \(E_{abs}\) \(m^{-1}\) cm\(^{-1}\) (n=2) | Inhibition (5 mM GSO\(_2\)) |
|---------------------------|-------------|--------------------------|----------------------------------------|----------------------------|
| Glu-l-Cys-Gly             | Unactivated | 3.0 ± 1.7 (n=8)          | 2.2 ± 1.3 (n=8)                        | 56 ± 13 (n=3)              |
| NEM-treated 4             | NEM-treated | 3.5 ± 1.9 (n=6)          | 1.3 ± 0.8 (n=6)                        | 71 ± 12 (n=4)              |
| NEM-treated 5             | NEM-treated | 2.2 ± 0.5 (n=3)          | 4.1 ± 0.5 (n=3)                        | ND                        |
| NEM-treated 6             | NEM-treated | 5.0 ± 1.4 (n=3)          | 1.3 ± 0.3 (n=3)                        | 83                        |
| NEM-treated 7             | NEM-treated | 5.0 ± 1.5 (n=5)          | 2.0 ± 0.1 (n=5)                        | 84                        |
| NEM-treated 8             | NEM-treated | 10.1 ± 0.7 (n=6)         | 3.3 ± 0.7 (n=6)                        | 24                        |
| NEM-treated 9             | NEM-treated | 9.3 ± 3.9 (n=4)          | 4.2 ± 3 (n=4)                          | 87                        |
| NEM-treated 10            | NEM-treated | 5.1 ± 2.4 (n=3)          | 1.5 ± 0.4 (n=3)                        | 68                        |
| NEM-treated 11            | NEM-treated | 2.4 ± 1.6 (n=2)          | 1.0 ± 0.1 (n=2)                        | 86                        |
| NEM-treated 12            | NEM-treated | 5.7 ± 2.2 (n=2)          | 1.3 ± 0.1 (n=2)                        | 25 ± 19 (n=2)             |
| NEM-treated 13            | NEM-treated | 15 ± 1 (n=2)             | 0.3 ± 0.1 (n=2)                        | 44                        |
| NEM-treated 14            | NEM-treated | 6.5 ± 6.8 (n=2)          | 1.2 ± 0.0 (n=2)                        | 45                        |
| NEM-treated 15            | NEM-treated | 4.6 ± 2.5 (n=3)          | 1.7 ± 1.0 (n=3)                        | 30                        |
| NEM-treated 16            | NEM-treated | 2.9 ± 1.9 (n=2)          | 0.7 ± 0.3 (n=2)                        | 78                        |
| NEM-treated 17            | NEM-treated | 41 ± 3.9 (n=3)           | 14 ± 1 (n=3)                           | 14 ± 2 (n=3)              |
| NEM-treated 18            | NEM-treated | 26 ± 3 (n=3)             | 13 ± 1 (n=3)                           | 58 ± 8 (n=3)              |
| NEM-treated 19            | NEM-treated | 10 ± 3 (n=3)             | 5.1 ± 1.4 (n=3)                        | 89 ± 5 (n=3)              |
| NEM-treated 20            | NEM-treated | 6.8 ± 4.1 (n=3)          | 1.8 ± 1.2 (n=3)                        | 85 ± 5 (n=3)              |
| NEM-treated 21            | NEM-treated | 2.3 ± 0.4 (n=2)          | 2.1 ± 0.5 (n=2)                        | 24 ± 1 (n=2)              |
| NEM-treated 22            | NEM-treated | 1.5 ± 1.6 (n=2)          | 0.6 ± 0.3 (n=2)                        | ND                        |
| NEM-treated 23            | NEM-treated | 13 ± 2 (n=3)             | 10 ± 2 (n=3)                           | 42 ± 13 (n=2)             |

**Notes:**
- As described under "Methods."
- ND, not determined.
Thiol Substrates of Microsomal Glutathione Transferase

Fig. 3. Inactivation of microsomal glutathione transferase by diethyl pyrocarbonate (a), trinitrobenzenesulfonate (b), pyridoxal 5'-phosphate (c), and phenylglyoxal (d). Activity was determined with glutathione (●), γ-L-Glu-L-Cys (□), and N-acetyl-L-Cys (○), respectively, with CDNB as the second substrate. Experimental conditions are described under “Methods.”

Maintained with cytosolic glutathione transferases (14), and the reason for this is not known. The peaks in the spectrum were centered around 450 and 560 nm and were not significantly different for the analogues and activated-unactivated enzyme (not shown). Glutathione sulfonate (an inhibitor of the enzyme) was added last in some of the titrations to demonstrate the reversibility of the complex stabilization (Table IV). L-Cysteinylglycine, N-acetyl-D-Cys, cysteamine, and mercaptoethanol did not result in any detectable enzymatic contribution to complex stabilization, showing that the enzyme will not bind any Meisenheimer complex formed in solution to its hydrophobic binding site. The fact that non-substrate glutathione analogues can be utilized to probe the active site of glutathione transferases provides an additional tool for in-depth analysis of future in vitro mutagenesis experiments.

The structural changes in glutathione and its analogues result in changes in $k_{cat}/K_m$ and $K_f$, which can be used to calculate the corresponding changes in binding energies to the enzyme (8, 19). It is of particular interest to compare the $k_{cat}/K_m$ and $K_f$ for the same pair of substrates. The α-amino group in the γ-L-Glu residue in GSH is, for instance, responsible for a binding energy of 3.2 kJ/mol (based upon comparing the $k_{cat}/K_m$ values of glutaryl-L-Cys-Gly and GSH (12) with the unactivated enzyme), whereas comparison of the formation constants yields a value of 3.6 kJ/mol. The agreement of these values implies that the binding energy of the amino group is utilized equally effectively in complex stabilization and catalysis and contributes to catalysis in this manner. On the other hand, comparison of $k_{cat}/K_m$ values for γ-L-Glu-L-Cys and GSH indicates that the glycine moiety contributes 3 kJ/mol in binding energy, whereas removal of the glycine actually yields a tighter Meisenheimer complex (2.8 kJ/mol increase in binding energy). Clearly, changes in binding energies determined by kinetic and dead-end complex binding studies are not comparable for gross structural changes, but appear to be quite accurate for quantification of more subtle substrate/substrate analogue enzyme interactions. The value of being able to examine non-substrate ligands can be exemplified by comparing the $K_f$ values for 4-Abu-L-Cys-Gly (which is not a substrate) and GSH, from which it can be estimated that the free carboxyl group of the γ-L-Glu residue stabilizes the complex to the unactivated enzyme by 4.4 kJ/mol.

Another valuable approach utilizing analogues of glutathione lies in the possibility of determining specific enzyme-substrate interactions, demonstrated here by the experiments involving group selective reagents. In Fig. 3d it can be seen that phenylglyoxal almost totally inactivated the microsomal glutathione transferase when glutathione or γ-L-Glu-L-Cys are used as substrates. On the other hand, the activity with N-acetyl-L-Cys decreased by only 50% after 30 min of phenylglyoxal treatment. These observations indicate a possible function for an arginine residue in substrate binding through interaction with the γ-L-Glu residue of glutathione. Furthermore, since it has been observed (31) that phenylglyoxal can also react with lysine, it is interesting to note that lysine reagents did not yield the same result, indicating that both arginine and lysine residues are involved in substrate binding. Histidine and lysine reagents showed no difference in their inhibition characteristics with any of the substrates tested. The introduction of bulky amino acid-modifying reagents into the active site of an enzyme can, of course, be a much more drastic change than substitution of active site amino acids by in vitro mutagenesis. Therefore,
the techniques described here involving alternate substrates and analysis of Meisenheimer complex stabilization with non-substrate glutathione analogues can be particularly revealing in combination with in vitro mutagenesis experiments.

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