THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 43, Issue of October 28, pp. 26890–26897, 1994
Printed in U.S.A.

Active-site Tyrosyl Residues Are Targets in the Irreversible Inhibition of a Class Mu Glutathione Transferase by 2-(S-Glutathionyl)-3,5,6-trichloro-1,4-benzoquinone*

(Received for publication, April 13, 1994, and in revised form, July 5, 1994)

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The mode of inactivation of glutathione S-transferase isoenzyme 3-3 from rat by the active site-directed inhibitor 2-(S-glutathionyl)-3,5,6-trichloro-1,4-benzoquinone (GSTCBQ) has been investigated by a combination of site-specific mutagenesis and mass spectrometric analysis of the sites of reaction of the reagent with the enzyme. This very reactive reagent is shown to target 3 residues in or near the active site, including the hydroxyl groups of Tyr-6 and Tyr-115 and the sulfhydryl group of Cys-114. Although the covalent attachment of one 2-(S-glutathionyl)dichloro-1,4-benzoquinonyl group/active site is sufficient to inactivate the enzyme (<5% residual activity), the 1 mol of reagent appears to be distributed among all three target sites. Mutant enzymes in which the reactive functional groups of these 3 residues have been individually removed remain susceptible to GSTCBQ. Evidence from amino acid sequencing and peptide maps visualized by matrix-assisted laser desorption/ionization mass spectrometry suggests that both Tyr-6 and Tyr-115 are primary targets of the reagent in the native enzyme. Docking of a model of GSTCBQ in a model of the active site derived from the crystal structure of the enzyme indicates that the trichlorobenzoquinonyl group can be positioned so that both tyrosine hydroxyl groups can act as nucleophiles to add to the reagent or alternatively act as electrophiles to assist in the nucleophilic addition of the other. The reaction of GSTCBQ with Cys-114 appears to require a conformation different from that in the crystal structure.

The cytosolic glutathione S-transferases (EC 2.5.1.18) catalyze the conjugation reaction between the tripeptide GSH and numerous substrates that have electrophilic functional groups (Mannervik and Danielson 1988; Armstrong, 1991, 1994; Rushmore and Pickett, 1993). This conjugation reaction has been recognized to be crucial for the detoxification of several agents of both endogenous and exogenous origin. The GSH transferases appear to be organized into at least four distinct gene families designated alpha, mu, pi (Mannervik, 1985), and theta (Meyer *et al.*, 1991). Crystallographic studies of class alpha, mu, and pi enzymes indicate that the protein is organized into two domains, a GSH-binding domain (domain I) at the N terminus and a xenobiotic-binding domain (domain II) composed of the C-terminal two-thirds of the protein (Reinemer *et al.*, 1991; Ji *et al.*, 1992, 1994; Sinning *et al.*, 1993).

Although the GSH transferases serve a detoxification function, inhibitors of the enzymes are of considerable interest as a potential mechanism to overcome the resistance of certain tumor cells to some antineoplastic drugs (Tew *et al.*, 1988; Waxman, 1990; Black and Wolf, 1991; van Bladeren and van Ommen, 1991). A large number of reversible inhibitors of the enzymes are known (Mannervik and Danielson, 1988). The GSH transferases are also known for their sensitivity to irreversible inactivation by sulfhydryl group-directed reagents presumably through modification of cysteine residues (Askelof *et al.*, 1975; van Ommen *et al.*, 1989; Tamai *et al.*, 1990; Caccuri *et al.*, 1992). However, site-specific mutagenesis has shown that cysteine residues are not essential for catalysis (Widersten *et al.*, 1991; Chen *et al.*, 1992; Ji *et al.*, 1992), and they do not appear to be located in the active sites of class mu and pi isoenzymes.

The catalytic efficiency of the enzyme is dependent on a tyrosyl residue in domain I near the N terminus (Tyr-6 in the class mu enzymes) that facilitates the reaction by lowering the pK_a of the thiol of bound GSH (Liu *et al.*, 1992; Kolm *et al.*, 1992; Kong *et al.*, 1992). Mutation of Tyr-6 to phenylalanine does not completely abolish the catalytic activity of the enzyme, but reduces k_{cat} by a factor of ~100 at pH 6.5. This tyrosine appears to be conserved in virtually all sequences of the cytosolic isoenzymes. Residues in the xenobiotic substrate-binding domain are also directly involved in catalysis. For example, the hydroxyl group of Tyr-115, which resides at the end of the α4-helix in domain II of isoenzyme 3-3, participates in both chemical and physical steps in catalysis (Johnson *et al.*, 1993). Evidence from crystallographic studies and site-specific mutagenesis suggests that this residue is located in a position to directly assist in the chemical steps of various reactions including epoxide ring openings, Michael additions, and nucleophilic aromatic substitution reactions (Ji *et al.*, 1993, 1994). Although Tyr-115 or its equivalent is not strictly conserved in all GSH transferases, it does appear to be a common feature of the class mu and pi isoenzymes. The 2 tyrosyl residues are obvious targets for active site-directed inactivation of many GSH transferases. Each has been shown to be sensitive to chemical modification (Barycki and Colman, 1993; Meyer *et al.*, 1993).

Active site-directed affinity labeling of several rat and human isoenzymes with GSH conjugates of quinones, especially 2-(S-glutathionyl)-3,5,6-trichlorobenzoquinone (GSTCBQ),[1]

* This work was supported by Grant GM 30910 from the National Institutes of Health and NATO Collaborative Research Grant CRG 920113. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GSTCBQ, 2-(S-glutathionyl)-3,5,6-trichloro-1,4-benzoquinone; GSTCHQ, 2-(S-glutathionyl)-3,5,6-trichloro-

has been characterized (van Ommen *et al.*, 1988, 1991; Ploemen *et al.*, 1991). The parent quinones, which are known for their high chemical reactivity with sulfhydryl groups, reacted with a total of 3 amino acids of class mu isoenzyme 4-4. Indications were obtained that the 3 cysteine residues of this isoenzyme were involved in the modification, although the incorporation of just 1 mol of reagent was sufficient to inactivate the enzyme. The incorporation of 1 mol of the active site-directed conjugate GSTCBQ/mol of enzyme was also found to fully inactivate the enzyme. However, the conjugate was more efficient, suggesting that the glutathionyl portion of the molecule imparts a selectivity to the modification not evident with the parent quinones. The identity of the target residue(s) in the inactivation with GSTCBQ has not been established. Katusz and Colman (1991) and Katusz *et al.* (1992) have shown quite convincingly that a structurally related affinity label, *S*-(4-bromo-2,3-dioxobutyl)glutathione, reacts with Tyr-115 of class mu isoenzymes 3-3 and 4-4.

In this paper, we describe a set of experiments designed to determine the targets for the reaction of GSTCBQ in the active site of isoenzyme 3-3 of GSH transferase. Evidence is presented that this highly reactive affinity reagent reacts with 3 residues, Tyr-6, Tyr-115, and Cys-114, in the vicinity of the active site. The native enzyme is inactivated more rapidly than either the Y115F or Y6F mutant, suggesting that the hydroxyl groups of these residues are not only targets for GSTCBQ, but may provide anchimeric assistance in the inactivation reactions. The results are consistent with molecular modeling studies that indicate that both tyrosyl residues are appropriately positioned to participate in the chemical modification of the enzyme.

EXPERIMENTAL PROCEDURES

General Materials—Tetrachloro-1,4-benzoquinone (TCBQ) was purchased from Merck (Darmstadt, Germany). GSH was obtained from Boehringer (Mannheim, Germany). [^{35}S]Glutathione (166 μCi/mmol) was purchased from Sigma. Solvents, buffer salts, and chemical reagents were of the highest quality commercially available.

Enzymes—Recombinant isoenzyme 3-3 was prepared as described previously (Liu *et al.*, 1992). Preparation of the cysteine mutants C86S, C114S, and C173S has been described before (Ji *et al.*, 1992). The preparation of the two tyrosine mutants Y6F and Y115F has been described by Liu *et al.* (1992) and Johnson *et al.* (1993), respectively.

Synthesis of Inhibitor—GSTCBQ was prepared as described previously by van Ommen *et al.* (1988). ^{35}S-Labeled GSTCBQ was prepared by the dropwise addition of 1.1 μmol of [^{35}S]GSH in 40 μl of H_2O to a vigorously stirred solution of 11.1 μmol of TCBQ in 0.3 ml of acetone. The mixture was stirred for 1 min, and the resulting GSTCBQ was purified by preparative reversed-phase HPLC on a 250 × 21.2-mm Zorbax ODS column eluted at a flow rate of 3 ml/min for 15 min with 50% of a solution of 0.05% (v/v) formic acid in H_2O and 50% methanol, followed by a linear gradient to 100% methanol (k' = 2.1 and 3.3 for GSTCBQ and TCBQ, respectively). Fractions (6 ml) were collected on ice and concentrated under a stream of dry N_2. The ^{35}S-labeled conjugate had the same retention time as GSTCBQ on analytical HPLC (van Ommen *et al.*, 1988). An impurity (~20%) was present that had a retention time corresponding to that of 2-(*S*-glutathionyl)-3,5,6-trichloro-1,4-hydroquinone (GSTCHQ).

Kinetic Analysis of Inhibition—Typically, a 1 μM solution of enzyme was incubated with at least a 5-fold excess of GSTCBQ. Reactions were terminated at the appropriate times by the rapid addition of GSH to a final concentration of 2 mM. The quenched reactions were placed on ice until the catalytic activity was determined. The catalytic activities of native and mutant enzymes toward 1-chloro-2,4-dinitrobenzene were determined by the method of Habig *et al.* (1974) with a saturating concentration of GSH, usually 2–5 mM. All kinetic experiments were carried out in 0.1 M potassium phosphate (pH 6.5) at 25 °C.

Covalent Incorporation of [^{35}S]GSTCBQ—The covalent incorporation of GSTCBQ was examined at 25 °C and pH 7.4 by the following procedure. Enzyme, typically 1 μM, was rapidly mixed with [^{35}S]GSTCBQ and quenched after the appropriate time by the addition of ascorbic acid (final concentration of 1 mM). A 0.4-ml aliquot of a 20% (w/v) solution of trichloroacetic acid was added, followed by 20 μl of a 20 mg/ml solution of bovine serum albumin to increase the total protein concentration. The protein precipitate was collected by centrifugation. The supernatant was discarded, and the precipitate was washed three times with 0.4 ml of 10% (w/v) trichloroacetic acid. The pellet was dissolved overnight at 40 °C in 0.5 ml of Soluene 350 (Packard Instrument Co.). Scintillation fluid (9 ml, Hionic fluor, Packard Instrument Co.) was used to screen the samples for radioactivity. Blank samples containing no enzyme were treated in the same way.

Peptide Mapping—A 3.0-ml solution (pH 6.5) of 10 μM native or mutant enzyme was incubated with 100 μM GSTCBQ for 10 min at 25 °C. The reaction was stopped with ascorbic acid (final concentration of 10 mM) and dialyzed three times overnight against three changes of 3 liters of a 0.1% (v/v) solution of formic acid. The sample was lyophilized. The GSTCBQ-modified enzyme and unmodified controls were digested with protease V8 from *Staphylococcus aureus* (protease/enzyme ratio of 1:20 (w/w)) in 0.05 M ammonium acetate (pH 4) for 5 h at 37 °C (Smith, 1988) or with CNBr at a 1:50 molar ratio of methionine to CNBr in 70% (v/v) formic acid for 24 h (Smith, 1988). The incubation mixtures were lyophilized and analyzed by mass spectrometry.

Mass Spectrometry—The masses of intact enzymes and peptides in the digests were determined by matrix-assisted laser desorption/ionization mass spectrometry using a VISION 2000 reflector-type time-of-flight laser desorption instrument (Finnigan MAT, Bremen, Germany) equipped with a nitrogen laser at 337 nm. The matrix used 2,5-dihydroxybenzoic acid at a concentration of 10 g/liter in water for the intact enzymes and in 0.1% (v/v) trifluoroacetic acid for the peptide mixtures from digested enzymes. Sample solutions with a typical concentration of 1–10 pmol/μl were then mixed with the matrix solution (1:1 (v/v)). One μl of the matrix/sample solution was applied to a stainless steel target, air-dried, and introduced into the mass spectrometer. The assumption was made that 1 chlorine atom of GSTCBQ is displaced upon reaction with either cysteine or tyrosine and that the bound adduct was in the hydroquinone oxidation state due to reduction of the quinone with ascorbate. The expected mass gain of the enzyme or peptide upon adduct formation is 482 atomic mass units.

N-terminal Sequence Analysis—Sequencing of the N terminus of native, mutant, and modified enzymes, which had been dissolved in 5% (v/v) acetic acid, was done by automated Edman degradation on an Applied Biosystems Model 475 peptide sequencer interfaced with a Model 120A PTH analyzer.

UV Difference Spectroscopy and Fluorescence Titrations—UV difference spectra were determined in 0.1 M PIPES (pH 6.5) using a double-beam Perkin-Elmer Lambda 4B UV-visible spectrophotometer with high performance optics. Native enzyme was added to the sample cuvette to a final concentration of 12.9 μM and a volume of 1.0 ml, and the appropriate mutant enzyme was added to the reference cuvette to the same final concentration and volume. A difference spectrum was recorded between 250 and 500 nm as a control to ensure that the protein concentrations were equivalent. For example, the C114S mutant resulted in a flat difference spectrum, whereas the Y115F and Y6F mutants gave distinctive spectra of a single protonated tyrosyl residue. Forty μl of a solution of GSTCBQ was added to each cuvette to give a final concentration of 70 μM. The samples were allowed to react for 10 min, after which no further changes in the absorption spectra occurred. The difference spectra of the GSTCBQ-modified proteins were then recorded.

The equilibrium constants for the noncovalent preassociation of GSTCBQ with the native and mutant enzymes were estimated by fluorescence titration of the proteins with the nonreactive reduced hydroquinone analogue (GSTCHQ) by a procedure previously described (Zhang and Armstrong, 1990). A 0.2 μM solution of enzyme in 10 mM potassium phosphate (pH 6.5) was titrated at 25 °C in the presence of 21 μM ascorbate (to ensure that the titrant remained reduced) by the incremental addition of GSTCHQ in small (3–10-μl) aliquots. The fluorescence data were fit to a hyperbola using the program HYPER (Cleland, 1979).

Molecular Modeling—The molecular structure of GSTCBQ was generated in Quanta/CHARMm (Molecular Simulations Inc., Waltham, MA). The glutathionyl portion of the molecule was copied from the crystal structure of isoenzyme 3-3 (Ji *et al.*, 1992) and attached to the 3,5,6-trichloro-1,4-benzoquinonyl moiety. A restrained geometry of the GSTCBQ model was optimized by fixing the conformation of the peptide

1,4-hydroquinone; GSDCHQ, 2-(*S*-glutathionyl)dichloro-1,4-hydroquinone; GSMCHQ, 2-(*S*-glutathionyl)monochloro-1,4-hydroquinone; TCBQ, tetrachloro-1,4-benzoquinone; HPLC, high pressure liquid chromatography; PTH, phenylthiohydantoin; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

TABLE I
Stoichiometry of labeling and residual activity of native and mutant enzymes by GSTCBQ

Experimental conditions for the modifications are described under "Experimental Procedures."

Enzyme or mutant	[^{35}S]GSTCBQ incorporated	Residual activity toward CDNB[a]
	mol/mol active sites	%
3-3	1.25 ± 0.08	3.4 ± 2.8
C86S	1.00 ± 0.02	2.0 ± 0.6
C114S	1.24 ± 0.03	6.5 ± 5.7
C173S	1.32 ± 0.14	2.1 ± 0.1
Y6F	0.93 ± 0.20	9.2 ± 5.1
Y115F	0.99 ± 0.02	16.0 ± 9.1

[a] CDNB, 1-chloro-2,4-dinitrobenzene.

and minimizing the energy of the 3,5,6-trichloro-1,4-benzoquinonyl moiety using steepest decent and conjugate gradient methods. This model of GSTCBQ was docked in the active site of the type 3 subunit by superposition of the glutathionyl moiety with that in the crystal structure. To find a set of reasonable conformations of GSTCBQ in the active site, a conformational search of two torsion angles was carried out. The torsion angle (Cα–Cβ–S–C2) about the Cβ–S bond was varied from 0° to −130° in increments of 10°, and at each position, the torsion angle (Cβ–S–C2–C1) about the S–C2 bond between the peptide and quinone was rotated 360° in 10° increments, generating 504 conformations. The interatomic distances between the phenolic oxygen atom of 1 tyrosyl residue and a chlorine-bearing carbon atom of the quinone ring and the distance of the other tyrosyl oxygen and the carbonyl oxygen *meta* to the chlorine-bearing carbon above were plotted along with the relative potential energies for the 36 conformers at each Cα–Cβ–S–C2 torsion angle. These plots were then evaluated for conformations with reasonable distances and energies constituting possible productive interactions for chemical modification of one or the other tyrosyl hydroxyl group.

RESULTS

Covalent Incorporation of GSTCBQ in Native and Mutant Enzymes—The notion that covalent modification and inhibition of GSH transferases by GSTCBQ are mediated by 1 or more of the cysteine residues was tested by examination of the inhibition of three site-specific mutants, namely C86S, C114S, and C173S, by the reagent. The results, given in Table I, suggest that none of the cysteinyl residues are crucial to the inactivation process. With respect to Cys-86 and Cys-173, this result is not too surprising since these 2 residues are 25 and 22 Å from the active site, respectively, as measured to the sulfur of bound GSH. Since it was anticipated that GSTCBQ would be an active site-directed reagent by virtue of the peptidyl portion of the molecule, Cys-114 appears to be the only reasonable cysteine target, with the distance between the sulfur of the side chain and the sulfur of bound GSH being 13.5 Å.

That the C114S mutant was inactivated by GSTCBQ prompted the examination of two other potentially reactive groups in the active site, namely the hydroxyl groups of Tyr-6 and Tyr-115, which lie 3.2 and 7.5 Å from the sulfur of GSH. However, both the Y6F and Y115F mutants are inactivated by GSTCBQ, with the incorporation of ~1 mol of GSTCBQ/active site as indicated in Table I. Although it could be concluded from these data that neither of these residues is involved in the inactivation process, it is no less logical to conclude that *both* and perhaps even Cys-114 participate in the inactivation of the enzyme. Removal of 1 residue could simply redirect the inhibitor to another target site.

Kinetics of Inhibition of Native and Mutant Enzymes by GSTCBQ—The kinetics of inhibition of the native enzyme and three mutants (Y6F, Y115F, and C114S) by GSTCBQ is illustrated in Fig. 1 for a typical set of reaction conditions. It should be noted that the catalytic efficiency of the unmodified Y6F mutant is considerably lower than that of the native enzyme or the other two mutants. The time course of inactivation for all

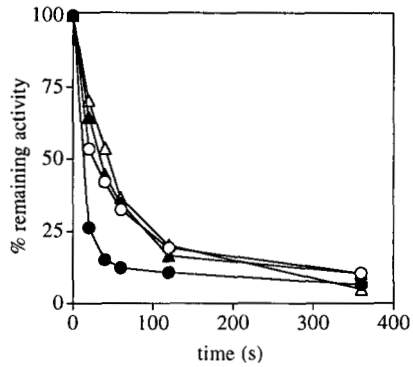


FIG. 1. **Time course for the inactivation of isoenzyme 3-3 (●) and the Y6F (○), Y115F (△), and C114S (▲) mutants at pH 6.5 and 25 °C.** The concentrations of the enzymes were 1 μM with [GSTCBQ] = 5 μM. The turnover numbers for unmodified isoenzyme 3-3, Y6F, Y115F, and C114S with 1-chloro-2,4-dinitrobenzene are 20, 0.28, 72, and 24 s^{-1}, respectively.

four proteins was complex and did not appear to vary appreciably with the concentration of the inhibitor under conditions where [GSTCBQ] was >2 μM. All four enzymes had <10% of their original activity after 15 min. Semilog plots (not shown) of the data were curved in all cases, suggesting multiple phases in the inactivation reactions. Attempts to describe the inactivation under a variety of conditions as a biphasic (two-exponential) process did not yield satisfactory results. The inactivation data were not of sufficient precision to justify fitting the kinetics to a three-exponential or higher order process.

The covalent modification reaction is almost certainly preceded by an equilibrium binding of GSTCBQ to the active site of the enzyme. Unfortunately, it was not possible to study the inactivation kinetics at sufficiently low concentrations of enzyme and GSTCBQ to determine the equilibrium constant for the preassociation. However, the dissociation constants for all four proteins could be estimated by fluorescence titration of the enzymes with the reduced nonreactive hydroquinone analogue (GSTCHQ). All four dissociation constants were quite similar and in the range of 0.5–1 μM. Thus, under the conditions of inactivation, the enzyme and the three mutants are essentially saturated with GSTCBQ.

Although the inactivation reaction eluded a precise quantitative description, it is apparent from Fig. 1 that the native enzyme is more rapidly inactivated than are the three mutants. This general observation pertains to a wide variety of reaction conditions including pH, temperature (0 or 25 °C), and concentration of GSTCBQ (2–250 μM) and suggests that all 3 residues are involved, either directly or indirectly, in the inactivation process and that they may cooperate with one another in the reaction. It is also clear that none of the 3 residues is a unique target for GSTCBQ.

UV-visible Difference Spectroscopy of Modified Mutants—In principle, the spectroscopic characteristics of individual reaction sites on the protein can be determined by difference spectroscopy, in which the spectrum of the modified mutant enzyme is subtracted from the spectrum of the modified native enzyme. This analysis assumes that an individual active site can only be modified once and that the mutation does not alter the partitioning of the reagent between multiple reaction sites in the active site. While the former assumption is likely correct, the latter is probably not. Even so, if a mutated side chain does not participate in the chemical modification either by direct reaction or by assisting in the reaction with another residue, it would be expected to be spectroscopically silent. It is clear from the UV-visible difference spectra in Fig. 2 that the side chains of Tyr-6, Cys-114, and Tyr-115 all contribute to the spectrum of

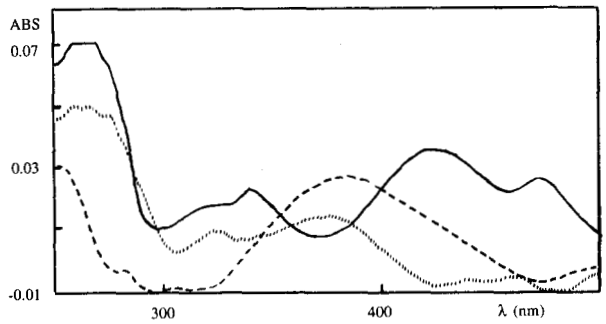


FIG. 2. **UV-visible difference spectra of modified native and modified mutant enzymes.** The difference spectra of GSTCBQ-modified isoenzyme 3-3 minus GSTCBQ-modified Y6F (——), C114S (– – –), and Y115F (· · · ·) are superimposed on the same plot. The enzyme concentrations were 12.9 μM. *ABS*, absorbance.

the modified native enzyme. Thus, it can be concluded that all three side chains are either targets for modification by the reagent or participate in the modification of one of the other side chains.

Evidence for Reaction at Tyrosine 6—Edman degradation of the unmodified and modified enzymes provided the first evidence that the side chain of Tyr-6 is modified by GSTCBQ. The average yield of PTH-tyrosine (cycles 5 and 6) in the degradation of unmodified isoenzyme 3-3, C114S, and Y115F was 79 ± 19%, which compares favorably with the average yield of 92 ± 12% for cycles 2–5 for the same proteins. In contrast, the yields of PTH-tyrosine from GSTCBQ-modified isoenzyme 3-3 and the C114S and Y115F mutants were substantially less and averaged 21 ± 7%. The yields in degradation cycles 2–5 for the modified enzymes were normal (96 ± 14%). Although it was not possible to identify a modified PTH-tyrosine in cycle 6 of the Edman degradation of the modified enzymes, the dramatically reduced yield of PTH-tyrosine suggests that 60–85% of Tyr-6 is modified by GSTCBQ in the native enzyme and the C114S and Y115F mutants. The extent of modification of Tyr-6 estimated from Edman degradation of the modified and unmodified enzymes is summarized in Table II. It appears that Tyr-6 is the principal target of GSTCBQ, with the remainder of the reagent partitioning between the other possible reaction sites, presumably Cys-114 and/or Tyr-115.

Location of Covalent Modifications by Mass Spectrometry—Matrix-assisted laser desorption/ionization mass spectrometry of the intact enzymes and those that had been labeled with GSTCBQ suggested that approximately one 2-(*S*-glutathionyl)-dichloro-1,4-hydroquinonyl group was attached to the native enzyme as well as to the three mutants (Table III). The average measured increase in molecular mass was 482 ± 63 atomic mass units as compared with an expected increase of 482 atomic mass units for the incorporation of one label. These results are in good agreement with the extent of incorporation of radiolabeled reagent in the proteins (Table I).

Peptide maps of unmodified isoenzyme 3-3 and the three mutants by matrix-assisted laser desorption/ionization mass spectrometry were fully consistent with the known sequences of the proteins. As expected, peptides from the mutant enzymes that harbored the Y6F, C114S, or Y115F mutation were found to be 16 atomic mass units smaller than the corresponding peptides of the native enzyme. Peptide maps visualized by matrix-assisted laser desorption/ionization mass spectrometry clearly revealed multiple sites of covalent modification of the enzyme by GSTCBQ, as illustrated in Fig. 3 for the protease V8 map of the C114S mutant. The observation of both unmodified and modified peptides containing Tyr-6 (*e.g.* V1, residues 1–24) is consistent with the results of Edman degradation of the N-terminal sequence, which indicated partial (75–95%) reaction at this residue. Similarly, peptide V10-11 (residues 101–125), which contains the target site Tyr-115, but not Cys-114, is also partially labeled, a fact that corroborates the conclusion from the UV-visible difference spectra that Tyr-115 is modified.

TABLE II
Estimates of the extent of modification of Tyr-6 by Edman degradation

Enzyme	Tyr-6 modified	Cys-114 and/or Tyr-115 modified[a]
	%	%
Isoenzyme 3-3	65–85	15–35
Y6F	0	100
C114S	75–95	5–25
Y115F	60–80	20–40

[a] Deduced from the extent of modification of Tyr-6 and the assumption that 1 mol of reagent is incorporated in each active site.

The peptide mapping results from two different digests of the modified native enzyme and three mutants are summarized in Table IV. Taken together, they clearly suggest that there are three target sites located near the active site including Tyr-6, Cys-114, and Tyr-115. The persistence of the label in peptides C7 and V10-11, containing targets sites Cys-114/Tyr-115 in the C114S and Y115F mutants, strongly suggests that both residues react with the reagent. The reaction of GSTCBQ with another residue in this region cannot strictly be ruled out. However, examination of the sequence of peptides C7 and V10-11 does not suggest any obvious alternative reaction sites located near the active site. In this regard, it is interesting to note that peptide C7 also appears to be labeled with a species that has lost an additional chlorine atom (C7+GSMCHQ). This species could arise from a cross-linking of Cys-114/Tyr-115 by the reagent. Finally, the CNBr digest provides evidence for modification of Cys-86 by the reagent. This particular residue, located on the surface of the protein some 25 Å from the active site, has been previously shown to be easily modified by sulfhydryl-specific reagents (Hsieh *et al.*, 1991).

Although the attachment of the inhibitor to a particular region of the protein can be positively determined by observation of the appropriate labeled peptide in the mass spectrum of the digested protein, the extent of labeling cannot be quantified from the mass spectral data, and the absence of a signal for a labeled peptide does not prove that the peptide is not labeled. The exact site of attachment can only be inferred from the inability to observe labeled peptide with a mutant target site. For example, peptide V1 was found to be labeled to some extent in the native protein and the C114S and Y115F mutants, but not in Y6F, where the presumed point of attachment has been removed. Again, it must be emphasized that the extent of labeling of any of the three sites cannot be inferred from the relative intensities of the peaks in the mass spectra.

Molecular Modeling—A model of the GSTCBQ molecule was fit into a model of the active site of isoenzyme 3-3 derived from the crystal structure of the protein (Ji *et al.*, 1992). A conformational search, in which the peptidyl portion of the inhibitor was fixed in the GSH-binding site and the torsion angles about the Cα–Cβ–S–C2 and Cβ–S–C2–C1 bonds were varied, revealed that it was possible to dock the trichlorobenzoquinonyl group into the hydrophobic pocket without grievous violation of the van der Waals surface of the active site cavity. One such docked structure is shown in Fig. 4, in which the proximity of the side chains of active-site residues Tyr-6, Tyr-115, and Ser-209 are illustrated. Both the hydroxyl groups of Tyr-6 and Tyr-115 approach within hydrogen bonding distances (2.3–3.4 Å) of carbonyl oxygens of the trichlorobenzoquinonyl group and are positioned close to (3.4–3.6 Å) ring carbons bearing chlorine atoms. The side chain of Cys-114 is more distant (~8 Å from the ring) and points away from the cavity. Ser-209 is also located

TABLE III

Stoichiometry of labeling estimated by matrix-assisted laser desorption/ionization mass spectrometry of intact modified and unmodified enzymes

The measured masses are the average of two independent measurements externally calibrated with chymotrypsinogen.

Enzyme	Calculated mass, unmodified	Measured mass		Mass difference (modified – unmodified)	No. of GS-DCHQ
		Unmodified	Modified		
3-3	25,783	26,012	26,483	471	~1
Y6F	25,767	25,966	26,379	413	~1
C114S	25,767	25,951	26,427	476	~1
Y115F	25,767	25,906	26,472	566	~1

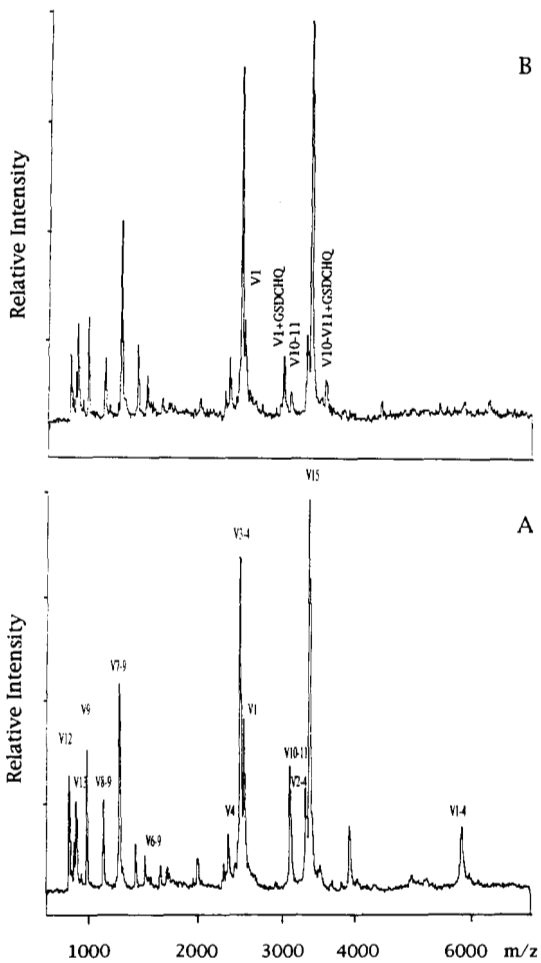


FIG. 3. **Matrix-assisted laser desorption/ionization mass spectra of the unmodified C114S mutant (*A*) and the GSTCBQ-modified C114S mutant (*B*) digested with protease V8.** Peptides V1 and V10-11 in the spectrum of the modified enzyme (*B*) illustrate the partial labeling of Tyr-6 and Tyr-115, respectively.

close to the trichlorobenzoquinonyl ring and in some conformations approaches within hydrogen bonding distance of the carbonyl oxygens.

At least three conformations could be identified in which the hydroxyl group of 1 tyrosyl residue was poised ~3.5 Å from one of the chlorine-bearing carbons, while the hydroxyl group of the other residue was within hydrogen bonding distance of the carbonyl oxygen *meta* to the chlorine-bearing carbon. The three conformations had a Cα–Cβ–S–C2 torsion angle of –100° and Cβ–S–C2–C1 torsion angles of –30°, –60°, and –140°. Two of these conformations are illustrated in Fig. 5. The most intriguing aspect of the modeled structures is that they appear to suggest that while one tyrosyl hydroxyl group acts as a nucleophile, attacking the trichlorobenzoquinonyl ring, the other may act as an electrophile and assist in the reaction by protonation or stabilization of the enolate *meta* to the position being attacked. The models are consistent with the fact that the native enzyme bearing both tyrosines is more rapidly inactivated than either the Y6F or Y115F mutant.

TABLE IV

Identification of peptides from GSH transferases modified with GSTCBQ by matrix-assisted laser desorption/ionization mass spectrometry of cyanogen bromide and protease V8 digests

The symbols +, 0, and – indicate a clear signal, weak signal, or absence of signal, respectively, for the indicated peptides. Quantification of the extent of labeling of each peptide is not possible by this technique.

Digest[a] and peptide (residues)[b]	Target site	Enzyme			
		3-3	Y6F	C114S	Y115F
CNBr					
C2 (3 –34)	Tyr-6	+	+	+	+
C2 (3 –34)+GSDCHQ	Tyr-Y6	+	–	+	–
C4 (77 –104)	Cys-86	+	+	+	+
C4 (77 –104)+GSDCHQ	Cys-86	+	+	+	+
C7 (113 –134)	Cys-114/ Tyr-115	0	0	+	+
C7 (113 –134)+GSDCHQ	Cys-114/ Tyr- 115	+	+	+	+
C7 (113 –134)+GSMCHQ	Cys-114/ Tyr-115	+	+	–	–
V8					
V1 (1 –21)	Tyr-6	+	+	+	+
V1 (1 –21)+GSDCHQ	Tyr-6	+	–	+	+
V1-2-3-4 (1 –48)	Tyr-6	+	+	+	+
V1-2-3-4 (1 –48)+GSDCHQ	Tyr-6	+	–	–	+
V10-11 (101 –125)	Cys1-14/ Tyr-115	+	–	+	+
V10-11 (101 –125)+GSDCHQ	Cys-114/ Tyr-115	–	–	+	+

[a] Proteins were digested with either CNBr or protease V8 as described under "Experimental Methods."

[b] The first and last residues for each peptide are given in parentheses. The +GSDCHQ and +GSMCHQ designations indicate the added masses of 482 and 447 atomic mass units to the peptides by appended 2-(*S*-glutathionyl)dichloro-1,4-hydroquinonyl and 2-(*S*-glutathionyl)-monochloro-1,4-hydroquinonyl moieties, respectively.

Finally, the rather long distance between any of the optimized model structures and the sulfur of Cys-114 suggests that this residue probably reacts in a protein conformation other than the one represented by the crystal structure. The sulfur is not exposed on the surface of the protein, so it is unlikely that it reacts with GSTCBQ from outside the active site as does Cys-86.

DISCUSSION

Role of Cysteinyl Residues in Inactivation—Previous proposals regarding the target sites for reaction of halobenzoquinone-type inhibitors such as TCBQ and GSTCBQ and GSH transferases have focused on cysteinyl residues (van Ommen *et al.*, 1988, 1989). Although it has been obvious that cysteine modifications occur with these types of inactivators, the exact relationship to the inactivation of the enzyme has remained elusive. The addition of the glutathionyl group (*e.g.* GSTCBQ) has been shown to impart a higher degree of specificity and efficiency to this class of inhibitors, presumably by guiding the halobenzoquinonyl group to the active site (van Ommen *et al.*,

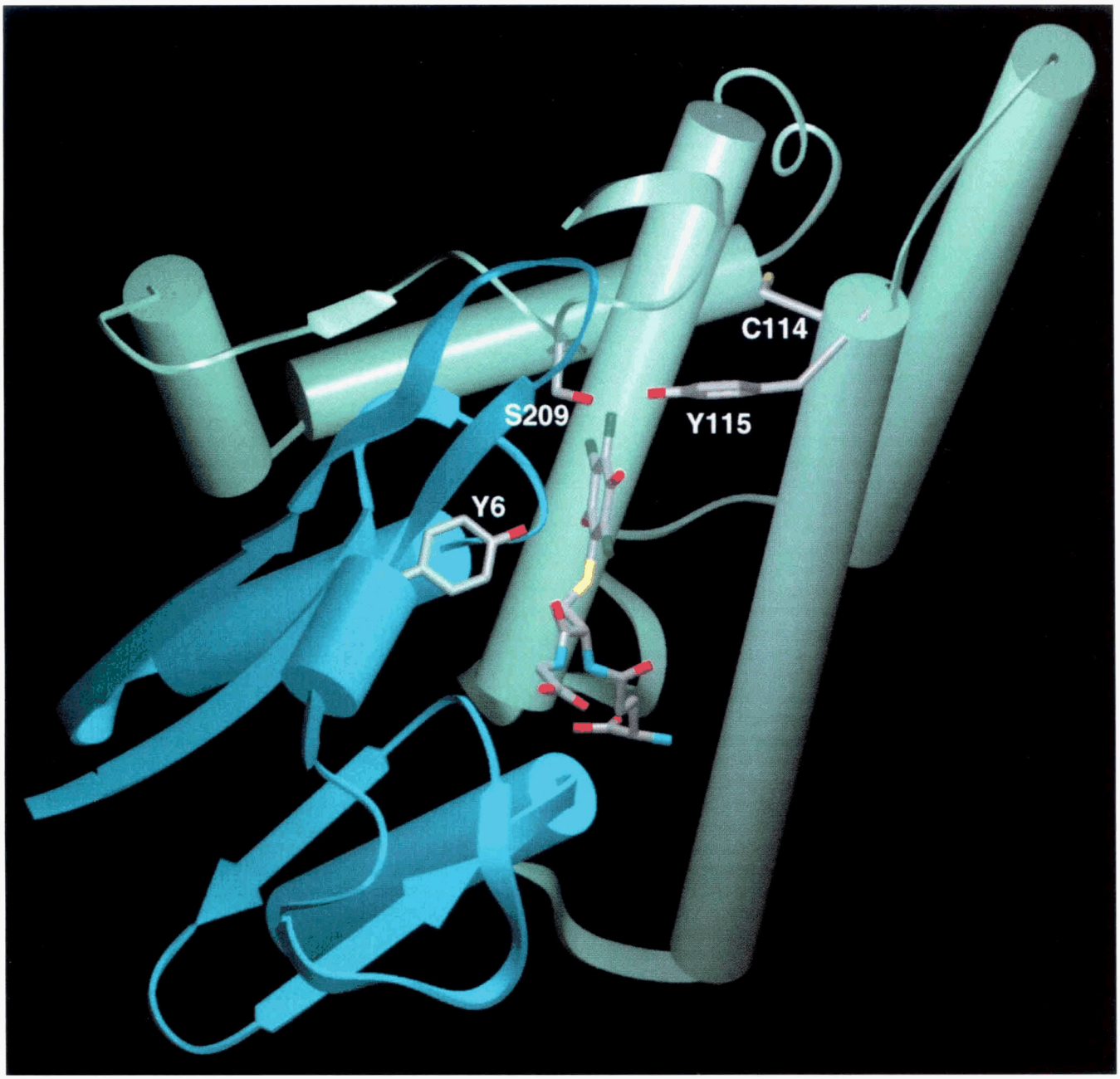


FIG. 4. **SETOR representation (Evans, 1993) of the relative locations of the side chains of Tyr-6, Cys-114, Tyr-115, and Ser-209 in one of the docked structures of isoenzyme 3-3 and GSTCBQ.** The N-terminal GSH-binding domain is illustrated in *blue*, and the C-terminal xenobiotic substrate-binding domain is shown in *light green*. The *arrows* represent β-strands, while the *cylinders* represent α-helices. Atoms of the side chains and GSTCBQ are color-coded *gray* for carbon, *red* for oxygen, *blue* for nitrogen, *yellow* for sulfur, and *green* for chlorine. Note that the side chain torsion angle of Cys-114 points the sulfur away from the active-site cavity.

1988, 1989). The three-dimensional structure of isoenzyme 3-3 revealed that only 1 of the 3 cysteinyl residues (Cys-114) is anywhere near the active site. The remote surface locations of Cys-86 and Cys-173 suggested that, although these residues might be modified under some circumstances, they were unlikely to be involved in the inactivation of the enzyme.

Fig. 4 suggests that even though Cys-114 is located near the active site of the enzyme, the sulfur is pointed away from the substrate-binding cavity. Thus, the side chain is partly occluded from the active site. It is apparent that there needs to be some alteration in the conformation of the protein to allow the trichlorobenzoquinonyl group of an active site-bound inhibitor to react with Cys-114. This is not an unreasonable possibility particularly considering the much higher than average crystallographic temperature factors for the residues near the C-terminal end of the α4-helix (Ji *et al.*, 1992). If the temperature factors are any indication, this region of the protein containing Cys-114 and Tyr-115 is fairly flexible. Alternative conformations in which the cysteinyl side chain is more exposed to the active site-bound reagent are likely. Given the much higher nucleophilicity of sulfur as compared with oxygen, these alternative conformations would not need to be populated extensively for the reaction at sulfur to be competitive with the reaction with the tyrosyl hydroxyl groups. Although the reaction of GSTCBQ with Cys-114 from the outside cannot be ruled out, it seems unlikely since the sulfhydryl group is buried ~9 Å below the surface of the protein.

The reason that the C114S mutant is inactivated more slowly than the native enzyme is not clear. There are several possibilities. The mutation could alter the conformation of the active

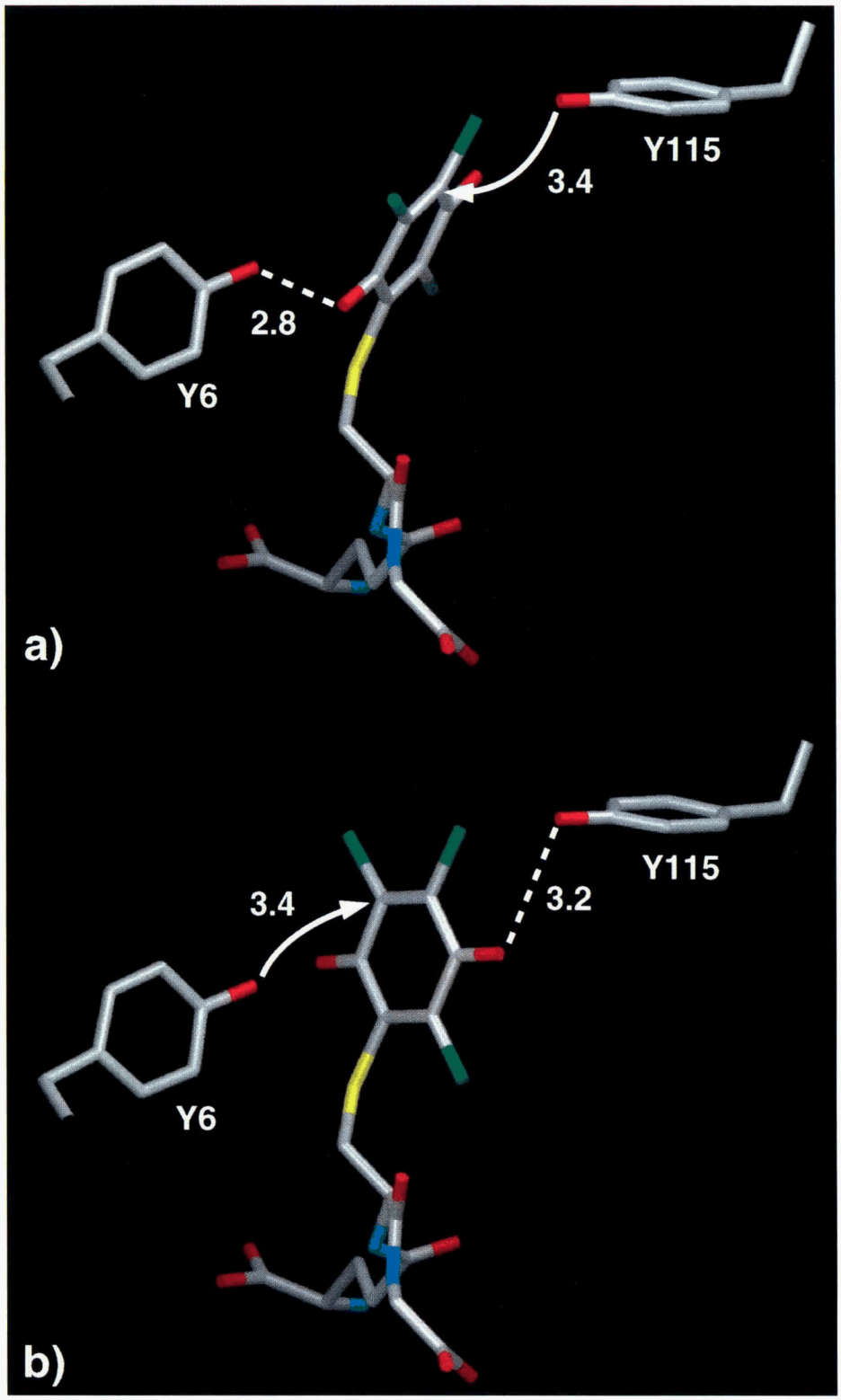
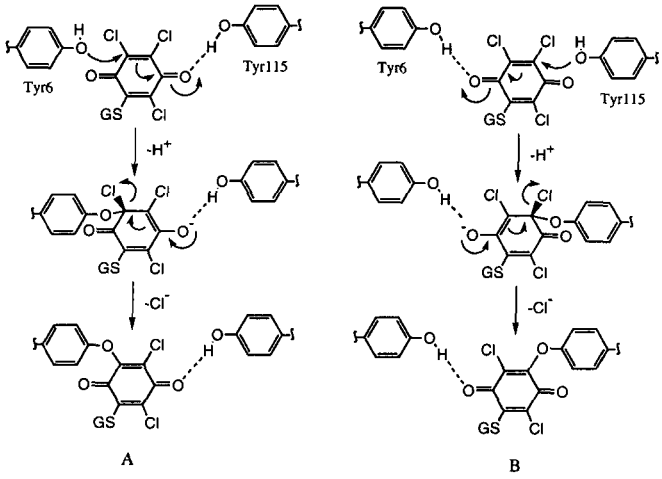


FIG. 5. **SETOR (Evans, 1993) depiction of two possible orientations of GSTCBQ in the active site of isoenzyme 3-3 of glutathione transferase.** The Cβ–S–C2–C1 torsion angles are −30° in *A* and −140° in *B*. Only the 2 tyrosines and GSTCBQ are shown. The atoms and bonds are color-coded *gray*, *red*, *blue*, *yellow*, and *dark green* for carbon, oxygen, nitrogen, sulfur, and chlorine, respectively. The *broken lines* depict hydrogen bonds, and the *curved arrows* indicate attack of the nucleophile. *A* shows Tyr-115 as the nucleophile and Tyr-6 as the electrophilic catalyst. In *B*, the tyrosyl residues have the opposite roles.

site. This seems unlikely since the C114S mutant appears to be indistinguishable from the native enzyme with respect to its catalytic activity (Ji *et al.*, 1992). The sulfhydryl group may participate as a catalyst in the modification of 1 of the tyrosyl residues in a manner similar to that discussed below. The geometry of the modeled complex of inhibitor and protein does not support this idea since it would necessarily involve a conformation other than that represented by the crystal structure. Perhaps the most reasonable explanation is a statistical one. Removing one of the three target sites simply lowers the probability for inactivation.

Electrophilic Catalysis of Nucleophilic Attack of Tyrosyl Residues—Active-site tyrosyl residues have been shown by others (Katusz and Colman, 1991; Katusz *et al.*, 1992; Barycki and Colman, 1993; Meyer *et al.*, 1993) to be targets of chemical modification by affinity labels, so it is not surprising to find tyrosine to be a target for GSTCBQ. The reagent is a bit unusual in that it obviously reacts with multiple target sites. This is a function of the fact that the trichlorobenzoquinonyl group has multiple electrophilic sites of very high reactivity. The fact that the native enzyme is inactivated more efficiently than either the Y6F or Y115F mutant could be statistical, as sug-



SCHEME 1.

gested above, or could be due to an anchimeric participation of 1 tyrosyl residue catalyzing the nucleophilic addition of the other as shown in Scheme 1. In one instance (Scheme 1*A*), the conjugate addition of Tyr-6 at the position *meta* to the sulfur could be assisted by protonation of, or hydrogen bonding to, the carbonyl oxygen *meta* to the sulfur by Tyr-115. The alternative mode of attack shown in Scheme 1*B* reverses the roles of the 2 tyrosyl residues so that protonation of the carbonyl *ortho* to the sulfur by Tyr-6 facilitates nucleophilic attack by Tyr-115 at the carbon *para* to the sulfur.

The general idea of a tyrosyl-assisted nucleophilic addition to an enone is not unusual. In fact, the altered catalytic properties of the Y115F mutant have been taken to suggest that the hydroxyl group of Tyr-115 facilitates the addition of GSH to enones such as 4-phenyl-3-buten-2-one by acting as an electrophilic catalyst (Ji *et al.*, 1994). The obvious difference in the inhibition reaction shown in Scheme 1*A* is that the nucleophile is an active-site residue rather than the substrate, GSH. It might be possible to exploit this proposed catalytic effect to design efficient specific inhibitors of the GSH transferases.

In principle, an inhibitor such as GSTCBQ with multiple electrophilic sites can form cross-links between target sites on a protein. In spite of an explicit search, no cross-link was observed between Tyr-6 and Tyr-115 in the peptide maps of GSTCBQ-modified enzymes. However, a cross-link between Tyr-115 and Cys-114 along the α4-helix is implicated by the observation of a CNBr cleavage peptide with an attached species with a mass that corresponds to that of GSTCBQ and the loss of 2 chlorine atoms. It seems unlikely that this putative cross-link would form on cleavage and work-up of the protein since the inactivation reaction is quenched by reduction of the quinone to the nonreactive hydroquinonyl species. The model of the docked inhibitor (Fig. 4) suggests that, if this cross-link does occur, it must form from a protein conformation other than that represented by the crystal structure.

Conclusions—The inactivation of the enzyme by the highly reactive affinity reagent GSTCBQ appears to involve 3 active-site residues, Tyr-6, Cys-114, and Tyr-115. Altered enzymes that harbor a single mutation at 1 of these 3 residues are also inactivated by GSTCBQ, but at a slightly slower rate. Molecular models suggest that each tyrosyl residue helps to catalyze the nucleophilic addition of the other to the trichlorobenzoquinonyl group in much the same way that Tyr-115 has been proposed to assist in the addition of GSH to enones. In principle, it should be possible to design molecules that take advantage of the anchimeric participation of the adjacent tyrosyl residues and that act as isoenzyme-specific covalent inhibitors of the GSH transferases. Work is underway to exploit this potential.

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