Identification of Tyrosine 79 in the Tocopherol Binding Site of Glutathione *S*-Transferase Pi†

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ABSTRACT: Alpha-tocopherol, the most abundant form of vitamin E present in humans, is a noncompetitive inhibitor of glutathione *S*-transferase pi (GST pi), but its binding site had not been located. Tocopherol iodoacetate (TIA), a reactive analogue, produces a time-dependent inactivation of GST pi to a limit of 25% residual activity. The rate constant for inactivation, k_{obs}, exhibits a nonlinear dependence on reagent concentration, with $K_I = 19$ μM and $k_{max} = 0.158$ min^{-1}. Complete protection against inactivation is provided by tocopherol and tocopherol acetate, whereas glutathione derivatives, electrophilic substrate analogues, buffers, or nonsubstrate hydrophobic ligands have little effect on k_{obs}. These results indicate that TIA reacts as an affinity label of a distinguishable tocopherol binding site. Loss of activity occurs concomitant with incorporation of about 1 mol of reagent/mol of enzyme subunit when the enzyme is maximally inactivated. Isolation of the labeled peptide from the tryptic digest shows that Tyr^{79} is the only enzymic amino acid modified. The Y79F, Y79S, and Y79A mutant enzymes were generated, expressed, and purified. Changing Tyr^{79} to Ser or Ala, but not Phe, renders the enzyme insensitive to inhibition by either tocopherol or tocopherol acetate as demonstrated by increases of at least 49-fold in K_I values as compared to the wild-type enzyme. These results and examination of the crystal structure of GST pi suggest that tocopherols bind at a novel site, where an aromatic residue at position 79 is essential for binding.

Glutathione *S*-transferases (GSTs,[1] EC 2.5.1.18) constitute a group of detoxification enzymes involved in the inactivation of a very broad range of electrophilic chemical compounds by catalyzing the addition of glutathione to noxious chemical species (*1*, *2*). Many of the GST substrates are genotoxic, causing mutations and cancer (*3*). GSTs consequently have an anticarcinogenic function, and the lack of adequate GST activity in an organism may predispose the individual to the development of cancer (*3*).

On the basis of amino acid sequence, substrate specificity, and immunological reactivity, the cytosolic GSTs have been classified into at least eight distinct classes (named alpha, kappa, mu, omega, pi, sigma, theta, and zeta) (*4*). All cytosolic isozymes exist as dimers in the crystalline state, with a subunit molecular mass of about 25 kDa (*1*−*3*, *5*). Each subunit contains an active site composed of a glutathione binding site and one or more electrophilic binding sites (*1*, *2*).

The pi isozyme of GST is of particular interest for several reasons. It is overexpressed in a variety of malignant tumors, and this isozyme has been implicated in the development of resistance toward various anticancer drugs (*6*−*9*). Furthermore, this enzyme is the most widely distributed GST isozyme in human tissue, with a notable absence in the liver, suggesting that it may be implicated in other physiological roles in addition to detoxification (*10*, *11*). For example, a new function has been reported in which GST pi forms complexes with other proteins such as Jun N-terminal kinase (*12*−*16*), 1-Cys peroxiredoxin (*17*, *18*), and tumor necrosis factor alpha (*19*). Several studies have shown that, in addition to the substrates, GST pi can bind various hydrophobic compounds that do not undergo metabolism (*20*−*25*). It has been proposed that GSTs may serve a transport function and direct these hydrophobic nonsubstrate ligands within the cell (*22*, *23*). Moreover, the binding of these nonsubstrate ligands has been shown to influence the activity of GST pi. Examples of some of the hydrophobic ligands that have been identified include bilirubin, bile acids (*20*−*23*), and tocopherol (*26*−*29*).

Alpha-tocopherol (Figure 1A), found commonly in nuts and vegetable oils, is the most abundant form of vitamin E present in humans and is a powerful biological antioxidant (*30*). This compound has been shown to inhibit the enzymes noncompetitively with respect to 1-chloro-2,4-dinitrobenzene and glutathione (*26*−*30*), and GSTs were proposed to have a role in the binding and transport of tocopherol derivatives. It has recently been reported that there is an increase in mortality from all causes among individuals receiving high-dose supplements of vitamin E (alpha-tocopherol) (*31*). The inhibition by vitamin E of glutathione *S*-transferase, with its ability to detoxify xenobiotics, was suggested as one explanation for the mortality data (*31*). The pi isozyme has

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[1] Abbreviations: GST, glutathione *S*-transferase; GST pi, Pi class glutathione *S*-transferase; GSH, glutathione; TIA, tocopherol iodoacetate; CDNB, 1-chloro-2,4-dinitrobenzene; ANS, 8-anilino-1-naphthalenesulfonate; HPLC, high-performance liquid chromatography; BSP, bromosulfophthalein; PIPES, 1,4-piperazinebis(2-ethanesulfonic acid); MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 4-morpholineethanesulfonic acid; CHES, *N*-cyclohexyl-2-aminoethanesulfonic acid; PDB, Protein Data Bank.

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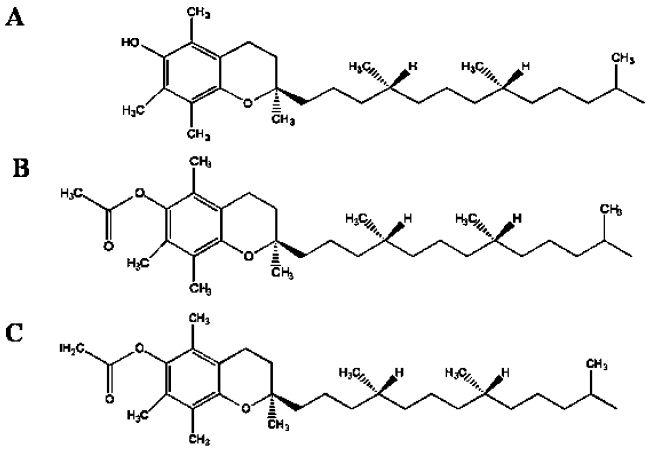

FIGURE 1: Molecular structure of tocopherols: (A) tocopherol; (B) tocopherol acetate; (C) tocopherol iodoacetate.

the highest affinity for tocopherol and is inhibited with an IC_{50} of less than 1 μM (*26*−*29*, *31*); however, the location of the binding site of alpha-tocopherol in GST pi was unknown. Since alpha-tocopherol is such a potent inhibitor of the pi isoform of GST, characterization of the binding site of tocopherol and its derivatives will not only provide insight into this function of GST but may present a basis for designing lead compounds for the development of a new class of inhibitors relatively specific for GST pi that can be used as adjuvants in cancer therapy, preventing decomposition of chemotherapeutic compounds.

In this paper, we report the results of the affinity labeling of human GST pi using a reactive tocopherol analogue, tocopherol iodoacetate (TIA) (Figure 1C). TIA specifically targets Tyr^{79} of GST pi, and we present evidence that replacement of this residue reduces the affinity of this enzyme for tocopherol. Furthermore, molecular modeling studies and the above observations suggest a possible location for the tocopherol binding site within the pi isozyme.

EXPERIMENTAL PROCEDURES

Materials. Tocopherol, tocopherol acetate, iodoacetic acid, *N*,*N*′-dicyclohexylcarbodiimide, reduced glutathione, 1-chloro-2,4-dinitrobenzene (CDNB), *S*-hexylglutathione−Sepharose, *S*-hexylglutathione, *S*-methylglutathione, *S*-(*p*-nitrobenzyl)-glutathione, PIPES, MOPS, HEPES, MES, CHES, bromosulfophthalein, 8-anilino-1-naphthalenesulfonate (ANS), *N*-ethylmaleimide, ammonium bicarbonate, trypsin, and Sephadex G-50 were all obtained from Sigma Chemical Co. Trifluoroacetic acid was purchased from Aldrich. Oligonucleotides for mutagenesis and primers for DNA sequencing were purchased from Biosynthesis, Inc. Dye reagent concentrate was purchased from Bio-Rad. All other chemicals were of reagent grade.

Expression and Purification of Wild-Type and Mutant GST Pi. The full-length cDNA for human glutathione *S*-transferase P1-1 was encoded in a pUC120 plasmid, as described by Manoharan et al. (*32*), and was a gift from W. E. Fahl (University of Wisconsin, Madison). Site-directed mutagenesis was performed using the Stratagene QuikChange kit. The following oligonucleotides and their complements were used to incorporate the mutations (position of the mutation underlined): Y79S, 5′-ACC CTT GGG CTC TCT GGG AAG GAC CAG CAG; Y79A, 5′-ACC CTT GGG CTC GCT GGG AAG GAC CAG CAG; Y79F, 5′-ACC CTT GGG CTC TTT GGG AAG GAC CAG CAG. Mutations were confirmed by DNA sequencing (forward sequencing primer, 5′-CCG CCC TAC ACC GTG GTC TAT TTC CCA GTT, and reverse sequencing primer, 5′-CTG TTT CCC GTT GCC ATT GAT GGG GAG GTT), which was carried out at the University of Delaware Center for Agricultural Biotechnology using an ABI Prism model 377 DNA sequencer (PE Biosystems).

For the expression of GST, the WT and mutant plasmids were transformed into *Escherichia coli* JM105 and the cells grown and induced for expression of GST (*25*). Purification of all proteins was performed using an *S*-hexylglutathione−agarose affinity column, as described previously (*25*). In all cases, GST pi was eluted using buffer containing 2.5 mM *S*-hexylglutathione. All of the enzymes were purified to homogeneity, yielding a single peptide by N-terminal sequencing on an Applied Biosystems gas-phase sequencer (Model Procise) equipped with an on-line microgradient delivery system (Model 140C) and a computer (Model 610 Macintosh). All purified GSTs were stored at −80 °C in aliquots of 0.1 M potassium phosphate, pH 6.5, containing 1 mM EDTA.

Synthesis of Tocopherol Iodoacetate. Tocopherol iodoacetate (TIA) was synthesized from tocopherol and iodoacetic acid by procedures based on the method of Pons et al. (*33*). One molar equivalent of tocopherol, 1.1 molar equiv of iodoacetic acid, and 2 molar equiv of dicyclohexylcarbodiimide were combined in 15 mL of cellosolve. The reaction was initiated by the addition of a catalytic amount of pyridine (250 μL), and the reaction mixture was allowed to stir at room temperature overnight. The reaction was stopped by the addition of 3 mL of distilled water, and the mixture was centrifuged to remove the insoluble dicyclohexylurea. The organic layer, containing tocopoherol iodoacetate, was lyophilized and redissolved in 88% acetonitrile/8% 2-propanol/4% tetrahydrofuran. Tocopherol iodoacetate was purified by HPLC using a Varian 5000 LC equipped with a Phenomenex C18 column (0.46 × 25 cm) and a UV-100 detector, using an isocratic solvent of 88% acetonitrile/8% 2-propanol/4% tetrahydrofuran at 1 mL/min. The effluent was monitored at 297 nm, and tocopherol iodoacetate eluted at 12 min. For comparison, tocopherol elutes at 7 min and iodoacetate elutes at 4 min in the same isocratic system. Product purity was confirmed by the presence of a single mass peak of 638.2 amu, using a ThermoFinnigan model LCQ mass spectrometer equipped with an electrospray attachment. TIA has a UV absorption spectrum with a maximum at 297 nm. The extinction coefficient at 297 nm was measured to be 65 400 M^{-1} cm^{-1}.

Enzyme Assays. Enzymatic activity of GST toward 1-chloro-2,4-dinitrobenzene (CDNB) was measured in a total volume of 1.0 mL using a Hewlett-Packard 8453 spectrophotometer by monitoring the formation of the conjugate of CDNB (3 mM) and glutathione (2.5 mM) at 340 nm ($\Delta\epsilon = 9600$ M^{-1} cm^{-1}) in 0.1 M potassium phosphate buffer containing 1 mM EDTA (pH 6.5) at 25 °C, according to the method of Habig et al. (*20*). All measurements were corrected for the spontaneous nonenzymatic rate of formation of the conjugate of glutathione and CDNB.

To determine the apparent K_m value of glutathione, a range of glutathione concentrations (0.01−2 mM) was used at a

constant CDNB concentration (3 mM). Similarly, the apparent K_m value for CDNB was determined from a range of concentrations of CDNB (0.01−3.5 mM) at a constant glutathione concentration (2.5 mM) in 0.1 M potassium phosphate buffer containing 1 mM EDTA (pH 6.5).

To determine the kinetic parameters of GSH and CDNB in the presence of either tocopherol or tocopherol acetate, a final concentration of 1−4 μM tocopherol and 40−80 μM tocopherol acetate, both in 0.2% acetonitrile, was included in the enzymatic assays mentioned above. For all kinetic parameter determinations, the temperature was maintained at 25 °C. Data were analyzed by fitting directly to the Michaelis−Menten equation using a nonlinear curve fitting program (SigmaPlot from SPSS).

Reaction of TIA with GST Pi. GST pi (0.3 mg/mL) was incubated in 90 mM phosphate buffer (pH 7.5) at 37 °C with various concentrations of TIA by the addition of appropriate stock solutions of TIA in acetonitrile. The concentration of TIA was determined from the absorbance at 297 nm using $\epsilon_{297nm} = 65400\ M^{-1}\ cm^{-1}$. The volume of acetonitrile was maintained at 10% of the total volume of the reaction mixture. In control experiments, the enzyme was incubated under the same conditions including 10% acetonitrile but without TIA. In every case, aliquots of the reaction mixture were removed at specified times and assayed for enzymatic activities using CDNB as substrate. For the assay, aliquots of the reaction mixture were diluted 10-fold with 0.1 M potassium phosphate buffer (pH 6.5) at 25 °C and were assayed by the addition of 20 μL to the cuvette, containing 1.0 mL of assay solution. In the preparation of modified and control enzyme, excess reagent was separated from the enzyme by the gel centrifugation method of Penefsky (*34*) in which aliquots (0.5 mL) of the reaction mixture at a given time were applied to a 5 mL column of Sephadex G-50 equilibrated with 90 mM potassium phosphate buffer (pH 7.5) and centrifuged. The protein concentration in the filtrate was determined using the Bio-Rad protein reagent, which is based on the dye-binding method of Bradford (*35*). Purified GST pi was used as the protein standard, and absorbance at 600 nm was measured using a Bio-Rad model 2550 RIA reader.

Measurement of Incorporation of TIA into GST Pi. GST pi (1.0 mg/mL) was incubated for the indicated time with 100 μM TIA, with or without the addition of protectants under standard reaction conditions. Excess reagents were removed by gel filtration columns, and the protein concentration was determined by the Bio-Rad method, as described above. The amount of reagent incorporated was determined from the absorbance at 297 nm using $\epsilon_{297nm} = 65400\ M^{-1}\ cm^{-1}$, which is the characteristic absorbance for the tocopherol moiety. The contribution of GST pi to the absorbance at 297 nm was subtracted by using $\epsilon_{297nm} = 6710\ M^{-1}\ cm^{-1}$, the calculated extinction coefficient for GST pi alone.

Trypsin Digestion of Modified GST Pi. GST pi (1.0 mg/mL) was incubated for 60 min with 20 μM TIA under standard reaction conditions. Excess reagent was removed by gel filtration as described above. Solid urea (to give 6 M as the final concentration) and *N*-ethylmaleimide (final concentration, 10 mM) were added to the enzyme and incubated for 30 min at 25 °C. The enzyme solution was then dialyzed overnight against 4 L of 50 mM ammonium bicarbonate (pH 7.8). The enzyme solution was lyophilized and then resolubilized in 250 μL of 8 M urea in 50 mM ammonium bicarbonate (pH 7.8) and incubated at 37 °C for 2 h. Ammonium bicarbonate (750 μL, 50 mM, pH 7.8) was then added to the solution to dilute the urea to 2 M. Trypsin [2.5% (w/w)] and the enzyme sample were incubated for 2 h at 37 °C. A second aliquot of the trypsin solution was added, and incubation was continued for another 2 h at 37 °C.

HPLC Separation of Modified Peptides. The tryptic digest was injected onto a Varian 5000 LC HPLC (Varian, Walnut Creek, CA) equipped with a Phenomenex C18 reverse-phase column (0.46 × 25 cm) equilibrated with solvent A (0.1% trifluoroacetic acid in water). After elution with solvent A (0% solvent B) for 10 min, a linear gradient was run to 40% solvent B (0.075% trifluoroacetic acid in acetonitrile) at 235 min followed by a linear gradient to 100% solvent B at 265 min, and finally the column was eluted for 10 min with 100% solvent B. The flow rate was 1 mL/min. The eluate was monitored at 220 and 297 nm with 1 mL fractions collected.

Analysis of Isolated Peptides. The purified labeled peptides from the HPLC separation (as described previously) were lyophilized and redissolved in 0.1% trifluoroacetic acid/H_2O. The amino acid sequences were determined on an Applied Biosystems gas-phase sequencer (Model Procise) equipped with an on-line microgradient delivery system (Model 140C) and a computer (Model 610 Macintosh). Peptide molecular weights were determined using a ThermoFinnigan model LCQ mass spectrometer equipped with an electrospray attachment.

Molecular Modeling. Modeling was conducted using the program Insight II from Biosym Technologies on a Silicon Graphics Indigo 2 workstation. The molecular model of tocopherol acetate was built and energy-minimized using the Builder module of the Insight II program. The approach of the tocopherol acetate molecule to Tyr^{79} was modeled by positioning the chromanol moiety of tocopherol at a site close to the aromatic side chain of Tyr^{79} of human GST pi (PDB code 18GS) (*36*) by sequentially rotating and translating it along the *x*, *y*, and *z* axes. The intermolecular energy in terms of both van der Waals' and electrostatic interactions as well as the interatomic distance between tocopherol acetate and the enzyme was continuously monitored for conformations, with reasonable distances and potential energies constituting possible productive interactions for chemical modification of the tyrosyl hydroxyl group. All of the models constructed were submitted to the Discover program from Biosym for extensive energy minimization using steepest descent and conjugate gradient methods to relieve residual van der Waals' overlaps and optimize the structures.

RESULTS

Inactivation of Human Glutathione S-Transferase Pi by TIA. Incubation of human GST pi (0.3 mg/mL) with 20 μM tocopherol iodoacetate (TIA) at pH 7.5 and 37 °C results in a time-dependent loss of enzyme activity that reaches a limit of 25% of the original activity, as is illustrated in Figure 2A. After 60 min, excess reagent was removed, and a second addition of 20 μM TIA was added; no further decrease in activity occurred. Because the activity levels off at 25% at long incubation times and over a range of TIA concentrations, the data for all TIA concentrations were calculated using 25% as the end point (Figure 2B). Control enzyme

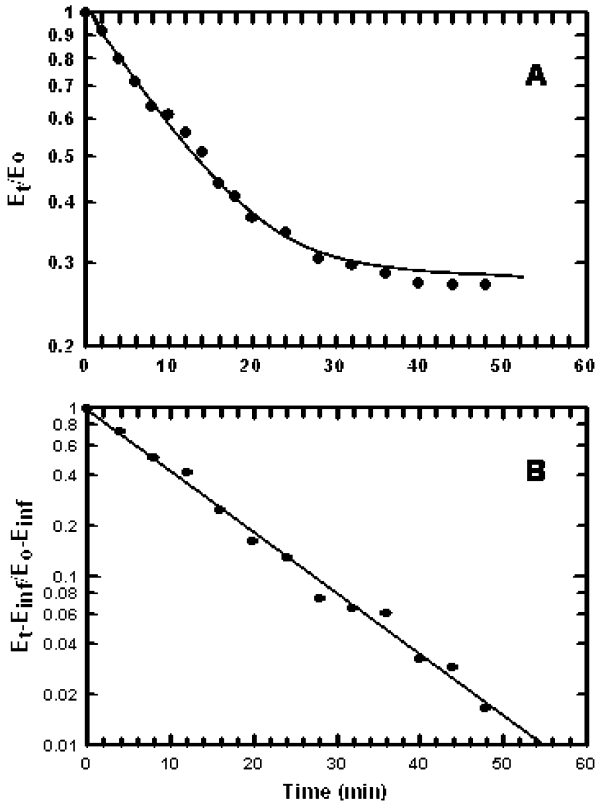

FIGURE 2: Inactivation of GST pi by TIA. A solution of 0.3 mg/mL GST 1-1 was incubated with 20 μM TIA in 90 mM potassium phosphate buffer, pH 7.5, at 37 °C. Activity was measured using the substrates 1-chloro-2,4-dinitrobenzene and glutathione, as described under Experimental Procedures. (A) Semilog plot of enzyme activity at time t (E_t/E_0) versus time. (B) Semilogarithmic plot of $[E_t - E_\infty]/[E_0 - E_\infty]$ versus time, where E_0 is the original enzyme activity and E_∞ is the enzyme activity at long times, which $= 0.25(E_0)$. The apparent rate constant (k_{obs}) determined from this graph was 0.0836 min^{-1}.

incubated under the same conditions but with no reagent present shows no loss of activity. The k_{obs} for inactivation was calculated from the slope of $\ln([E_t - E_\infty]/[E_0 - E_\infty])$ versus time, where E_t is the enzyme activity at time t, E_0 is the original enzyme activity, and E_∞ is the enzyme activity at long times, which is equal to $0.25(E_0)$. The reaction obeys pseudo-first-order kinetics with a rate constant of 0.0836 min^{-1} (Figure 2B). The observation of a limit to the inactivation suggests that reaction does not occur directly in the active site.

Concentration Dependence of the Rate of Inactivation. GST pi (0.3 mg/mL) was incubated with 15−100 μM TIA as described above to determine the rate of inactivation at various reagent concentrations (Figure 3). The apparent rate constant k_{obs} exhibits a nonlinear dependence on reagent concentration. This type of curve is typical of an affinity label, suggesting that a reversible enzyme−reagent complex is formed prior to the irreversible modification of the enzyme. The curve can be described by the equation $k_{obs} = k_{max}/(1 + K_I/[TIA])$, where K_I is the apparent dissociation constant of the enzyme−reagent complex and k_{max} is the maximum rate of inactivation at saturating concentrations of the reagent. A least-squares fit of the observed data yields $K_I = 19 \pm 3$ μM and $k_{max} = 0.158 \pm 0.008$ min^{-1}.

Effect of Ligand Analogues on the Inactivation Rate of GST Pi by TIA. The effect of various ligand analogues on the inactivation rate of GST pi (0.3 mg/mL) by 20 μM TIA was investigated. The results, given in Table 1, are expressed as the ratio of the inactivation rate constant measured in the presence of a particular ligand (k_{+L}) to the inactivation rate constant measured in the absence of ligands (k_{-L}). Addition of glutathione analogues (Table 1, lines 2−4) does not offer complete protection against inactivation. Since the concentrations of the analogues used are high relative to their K_I values, this partial protection must reflect an indirect effect, and the

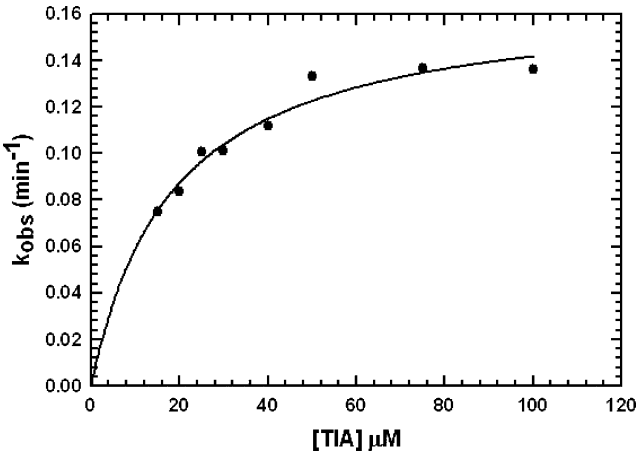

FIGURE 3: Concentration dependence of k_{obs} for the inactivation of GST pi by TIA. GST pi (0.3 mg/mL) was incubated with a range of concentrations of TIA under the same conditions as Figure 2. At each concentration k_{obs} was calculated as illustrated in Figure 2B, with $E_\infty = 0.25(E_0)$. The points are experimental, and the line is the theoretical fit to $k_{obs} = k_{max}/(1 + (K_I/[TIA]))$. A least-squares fit of the data yields $K_I = 19 \pm 3$ μM and $k_{max} = 0.158 \pm 0.008$ min^{-1}.

Table 1: Effects of Enzyme Ligands on the Rate Constant for Inactivation by 20 μM TIA[a]

protecting ligand	concn	k_{+L}/k_{-L}
1. none		1.00
glutathione derivatives		
2. *S*-methylglutathione	5 mM	0.61
3. *S*-hexylglutathione	5 mM	0.54
4. *S*-(*p*-nitrobenzyl)glutathione	5 mM	0.22
xenobiotic substrate analogues		
5. dinitrophenol	10 mM	0.98
6. *S*-(hydroxyethyl)bimane	330 μM	1.01
hydrophobic nonsubstrate ligands		
7. 8-anilino-1-naphthalenesulfonate	60 μM	2.03
8. bromosulfophthalein	100 μM	1.60
tocopherol derivatives		
9. tocopherol	50 μM	0.39
10. tocopherol	150 μM	0.09
11. tocopherol acetate	100 μM	0.12
12. tocopherol acetate	150 μM	0.02
buffers		
13. PIPES	100 mM	4.2
14. MOPS	50 mM	0.58
15. MOPS	100 mM	0.25
16. MOPS	200 mM	0.12
17. HEPES	100 mM	0.42
18. HEPES	200 mM	0.44
19. MES	50 mM	0.46
20. MES	100 mM	0.26
21. MES	200 mM	0.27
22. CHES	50 mM	0.39
23. CHES	100 mM	0.29
24. CHES	200 mM	0.06

[a] GST pi (0.3 mg/mL) was incubated with 20 μM TIA in 90 mM KPO_4, pH 7.5 at 37 °C, in the presence of the various ligands shown. Activity was assayed using CDNB as substrate.

target site of TIA must be distinct from the glutathione site.

The electrophilic (xenobiotic) substrate analogues dinitrophenol and *S*-(hydroxyethyl)bimane (Table 1, lines 5 and 6), do not protect against inactivation, demonstrating that TIA does not react within the electrophilic substrate binding site. In addition to its catalytic function, GST pi has a high affinity for a variety of nonsubstrate compounds (*21*, *24*, *25*). 8-Anilino-1-naphthalenesulfonate and bromosulfophthalein are two compounds known to bind to nonsubstrate-binding sites in GST pi (*37*, *38*); the addition of these ligands to the reaction mixture increases the rate constant about 2-fold (Table 1, lines 7 and 8). These results indicate that ANS and BSP bind at an enzyme site distinct from that targeted by TIA but, when bound, increase the exposure of the reaction site of TIA on GST pi.

Inclusion in the reaction mixture of tocopherol or tocopherol acetate (Figure 1B) causes a marked decrease in the rate of inactivation by TIA (Table 1, lines 9−12). These results indicate that the site of reaction is within a tocopherol binding site, distinguishable from the active site and hydrophobic nonsubstrate site of the enzyme.

In crystals of human GST pi, the buffer ions *N*-morpholinoethanesulfonic acid (MES) and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) bind in a site in each subunit distinct from the substrate sites (*39*, *40*). It is possible that tocopherol binds at a site occupied by certain buffers. When tested, CHES, MES, HEPES, and MOPS (Table 1, lines 14−24) yield significant protection against inactivation by TIA, but only when high concentrations of these buffers (i.e., >100 mM) are present in the reaction mixture. These results suggest that the buffers examined do not bind in the same site as TIA; rather, the protective effect observed against inactivation is likely an indirect effect, resulting from weak binding at a distinct site. In contrast, the addition of PIPES to the reaction mixture increases the rate constant about 4-fold (Table 1, line 13), similar to the results observed with the addition of ANS and BSP to the reaction mixture. These results suggest that PIPES may bind at or near the ANS and BSP site in the enzyme and similarly, when bound, increases the exposure of the reaction site of TIA on GST pi.

Incorporation of TIA by GST Pi. GST pi was incubated with 100 μM TIA in the absence or presence of added protectants for various time periods. Subsequently, the modified enzymes were isolated, the residual activity was determined, and the incorporation of tocopherol acetate was measured from its characteristic absorbance at 297 nm. The incorporation of TIA into GST pi was measured over the time period 0−80 min in the absence of added ligands, and the reagent incorporation was plotted as a function of loss of activity at the same time (Figure 4). Extrapolation to totally inactive enzyme yields about 1 mol of reagent/mol of enzyme subunit at 100% of maximum inactivation. These results suggest that inactivation results from modification of at most one amino acid residue per subunit of enzyme.

The effect of the addition of ligands on the incorporation of TIA into GST pi was investigated. Incubation of GST pi with 100 μM TIA for 80 min affords a modified enzyme that is ~24% active and contains 1 mol of reagent/mol of enzyme subunit. The presence of either tocopherol or tocopherol acetate during the incubation of the enzyme with TIA yields an enzyme that retains appreciable activity (94% and 92%, respectively) and has a dramatic decrease in incorporation to no more than 0.08 mol of reagent/mol of subunit. These results support the proposal that the TIA reaction occurs at the tocopherol binding site of GST pi.

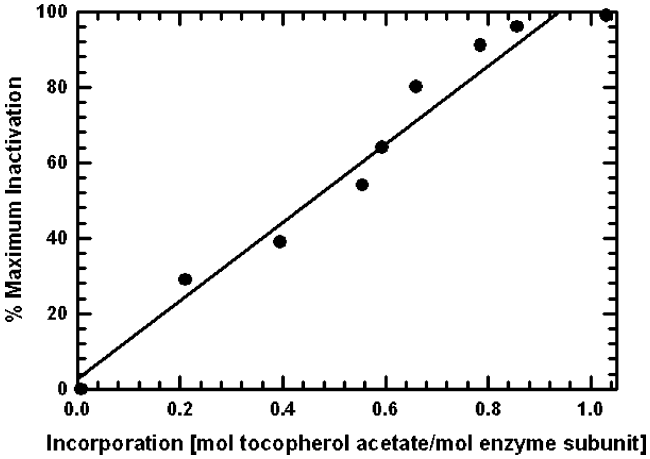

FIGURE 4: Incorporation of TIA into GST pi as a function of the percentage of maximum inactivation. GST pi (1.0 mg/mL) was incubated with 100 μM TIA in this experiment. Extrapolation to the maximum inactivation of the enzyme reveals an incorporation of about 1.0 mol of reagent/mol of enzyme subunit.

Isolation and Characterization of Tryptic Peptides from TIA-Modified GST Pi. Maximally inactivated GST pi was prepared and digested with trypsin. The digest was fractionated by HPLC using a reverse-phase column (C18) equilibrated with 0.1% trifluoroacetic acid and an acetonitrile gradient (Figure 5A). One peptide peak, which elutes at 26% acetonitrile, exhibits the characteristic tocopherol absorbance of 297 nm (Figure 5B). The fractions corresponding to this peak were pooled, lyophilized, and subjected to gas phase amino acid sequencing. The results are shown in Table 2. This peak contained two peptides. The first peptide is the expected product of a tryptic digestion if cleavage occurs after Arg^{74}, yielding Thr-Leu-Gly-Leu-modified Tyr-Gly-Lys corresponding to residues 75−81. However, the second peptide was an unexpected tryptic fragment yielding Leu-Gly-Leu-modified Tyr-Gly-Lys corresponding to residues 76−81 in the known amino acid sequence. None of the standard phenylthiohydantoin derivatives were detected in cycles 4 and 5 where, according to the amino acid sequence, a phenylthiohydantoin-Tyr should have eluted. Thus, Tyr^{79} of GST pi is the amino acid target of TIA.

Additional evidence for the chemical modification of this peptide was provided by electrospray mass spectrometry. Peptides having masses of 1222.9 (the major peak), 750.8, 1120.8, and 648.1 amu were detected. The mass of 1222.9 amu is identical to the predicted mass of the modified peptide corresponding to residues Thr^{75} to Lys^{81} in which the iodide group of TIA had been displaced by the hydroxyl group of Tyr^{79}. The mass of 750.8 amu is that of the unmodified form of the peptide, while the mass of 1120.8 amu corresponds to the predicted mass of the modified peptide corresponding to residues Leu^{76} to Lys^{81}, and 648.1 amu is the mass of this same peptide without the modification. A mass of 473.4 amu was also detected in this sample equivalent to the mass of tocopherol acetate which was probably cleaved from the peptides during fragmentation of the sample. This information, in conjunction with the primary sequence of the enzyme,

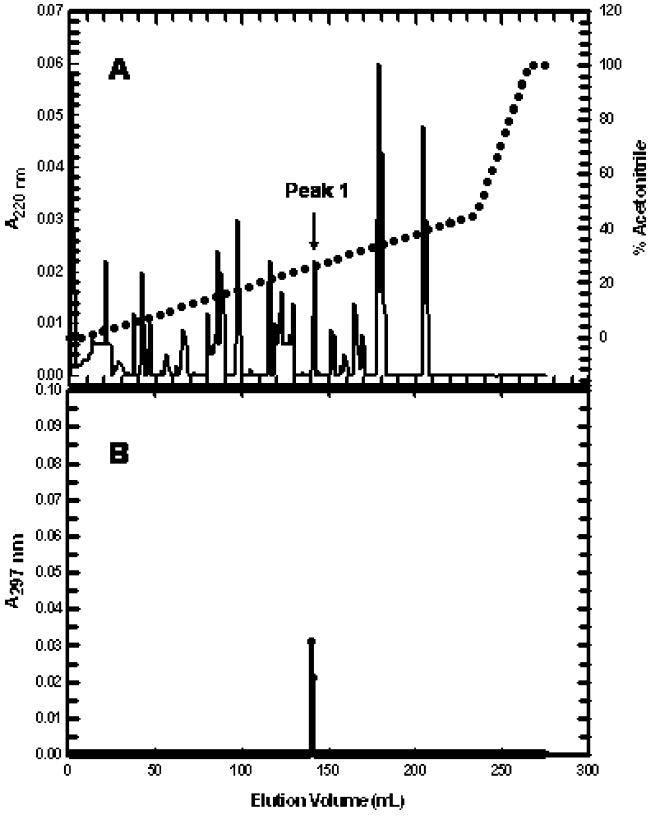

FIGURE 5: HPLC separation of tryptic digests of protein resulting from the 60 min modification of GST pi by TIA. The enzyme was modified with 20 μM TIA at pH 7.5 and 37 °C for 60 min and subsequently digested with trypsin. The digest was fractionated on a C18 column, as described under Experimental Procedures, and the effluent was monitored at either (A) A_{220nm} or (B) A_{297nm}.

Table 2: Representative Amino Acid Sequence of Modified Peptide Present in the Inactivated Enzyme[a]

cycle no.	amino acid (pmol),[b] peak 1	
1	Thr^{75} (1669)	Leu^{76} (363)
2	Leu^{76} (3785)	Gly^{77} (511)
3	Gly^{77} (2349)	Leu^{78} (966)
4	Leu^{78} (3561)	modified Tyr
5	modified Tyr	Gly^{80} (568)
6	Gly^{80} (2093)	Lys^{81} (579)[c]
7	Lys^{81} (2379)[c]	

[a] This is the sequence of the only peak, shown in Figure 5B, with absorbance at 297 nm. [b] The yield of each PTH-amino acid in picomoles is shown in parentheses. [c] Peptide ends.

identifies Tyr^{79}as the target of TIA; its modification is responsible for the loss of enzymatic activity.

Tocopherol and Tocopherol Acetate as Noncompetitive Inhibitors of GST Pi. To determine whether tocopherol and tocopherol acetate bind at sites distinguishable from the CDNB and GSH substrate sites, each molecule was tested for its ability to compete kinetically with the substrates GSH and CDNB. Measurement of the recombinant human GST pi's kinetic parameters in the presence of tocopherol reveals it is a noncompetitive inhibitor with respect to GSH and CDNB as substrates. As shown in Table 3, over the 1−4 μM concentration range of tocopherol, it has no effect on the K_m but decreases the V_{max} of the wild-type enzyme, yielding an average K_I value of 0.8 μM which agrees with the IC_{50} reported for tocopherol as an inhibitor of GST pi (*27*). As shown in Table 4, when the kinetic parameters were

Table 3: Tocopherol Is a Noncompetitive Inhibitor of Wild-Type GST Pi with Respect to CDNB and GSH as Substrates[a]

	CDNB		GSH[b]	
tocopherol (μM)	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})
0	837 ± 31	85 ± 7	55 ± 5	81 ± 4
1	1180 ± 54	50 ± 4	54 ± 3	44 ± 2
2	981 ± 70	37 ± 5	48 ± 2	31 ± 3
3	1010 ± 97	21 ± 2	51 ± 4	17 ± 1
4	886 ± 69	16 ± 3	43 ± 2	11 ± 2

[a] The K_m values were determined under saturating conditions of the second substrate, and the V_{max} values were determined by extrapolation of the K_m kinetic data to infinite concentrations of CDNB or GSH using SigmaPlot for data analysis. [b] GSH kinetics was measured using CDNB as the electrophilic substrate.

Table 4: Tocopherol Acetate Is a Noncompetitive Inhibitor of Wild-Type GST Pi with Respect to CDNB and GSH as Substrates[a]

	CDNB		GSH[b]	
tocopherol acetate (μM)	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})
0	837 ± 31	85 ± 7	55 ± 5	81 ± 4
40	1080 ± 64	43 ± 3	49 ± 4	48 ± 5
50	1160 ± 81	37 ± 5	51 ± 3	42 ± 4
60	990 ± 109	31 ± 3	50 ± 5	29 ± 3
80	846 ± 69	26 ± 3	53 ± 2	28 ± 2

[a] The K_m values were determined under saturating conditions of the second substrate, and the V_{max} values were determined by extrapolation of the K_m kinetic data to infinite concentrations of CDNB or GSH using SigmaPlot for data analysis. [b] GSH kinetics was measured using CDNB as the electrophilic substrate.

tested in the presence of tocopherol acetate, the same effect was observed in that the K_m of the substrates did not change, but the V_{max} decreased. These results suggest that tocopherol acetate is also a noncompetitive inhibitor with respect to GSH and CDNB as substrates (Table 4). However, the average K_I of 36 μM for tocopherol acetate is higher than the reported IC_{50} for this molecule (*27*). Thus, GST pi has a higher affinity for tocopherol than for tocopherol acetate.

Kinetic Properties of Mutant Enzymes. Since the affinity labeling study points to Tyr^{79} as the target residue of TIA, mutant enzymes were constructed with substitutions at position 79 to study the influence of tocopherol and tocopherol acetate on the activity of mutant enzymes with respect to GSH and CDNB as substrates. Tyrosine features both an aromatic and a phenolic group. Therefore, three mutants were constructed to study each major functional group present in the tyrosine side chain. Y79F was constructed to retain the aromatic moiety while eliminating the hydroxyl group. Y79S was engineered to retain a hydroxyl group while eliminating the aromatic component. Y79A was constructed to eliminate both the aromatic and the hydroxyl groups. These mutant enzymes were expressed at 25 °C in *E. coli* strain JM105 and then isolated and purified to homogeneity using *S*-hexylglutathione−agarose affinity chromatography. The substrates we focused on were glutathione and CDNB. The data for the kinetic parameters for the wild-type and mutant enzymes are shown in Table 5. For wild-type GST pi, the K_m for CDNB is about 15 times that of GSH (Table 5). For all mutants, the K_m values of CDNB do not change appreciably. For glutathione, Y79A is the only mutant that exhibits a modest increase in K_m (∼4 times). The most notable change observed in the mutants is in the V_{max}: Y79F and Y79S

Table 5: Kinetic Parameters for the Glutathione (GSH) Conjugation to 1-Chloro-2,4-dinitrobenzene by Wild-Type and Mutant GST Pi

	CDNB		GSH[a]	
enzymes	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})
wild type	837 ± 31	85 ± 7	55 ± 5	81 ± 4
Y79F	990 ± 84	39 ± 5	99 ± 7	42 ± 4
Y79S	960 ± 79	28 ± 4	71 ± 5	21 ± 3
Y79A	1200 ± 104	169 ± 12	243 ± 12	170 ± 7

[a] GSH kinetics was measured using CDNB as the electrophilic substrate.

Table 6: Inhibition Constants for Tocopherol and Tocopherol Acetate with Wild-Type and Mutant GST Pi[a]

	K_I for tocopherol		K_I for tocopherol acetate	
enzymes	from v_i vs [CDNB] (μM)	from v_i vs [GSH] (μM)	from v_i vs [CDNB] (μM)	from v_i vs [GSH] (μM)
wild type	0.8 ± 0.1	1.0 ± 0.1	36 ± 2	47 ± 3
Y79F	2.6 ± 0.5	3.3 ± 0.5	41 ± 3	49 ± 5
Y79S	39 ± 5	48 ± 4	>100[b]	>100[b]
Y79A	>100[b]	>100[b]	>100[b]	>100[b]

[a] The K_I values were determined by measuring the V_{max} at various concentrations (1−100 μM) of either tocopherol or tocopherol acetate. [b] The highest concentration of either inhibitor that could be measured is 100 μM.

enzymes exhibit V_{max} values that are respectively 49% and 30% that of the wild-type enzyme. In contrast, Y79A exhibits a 2-fold increase in V_{max}.

For the wild-type enzyme, tocopherol and tocopherol acetate were determined to be noncompetitive inhibitors (Table 3). We have now found that tocopherol or tocopherol acetate is also a noncompetitive inhibitor for the Y79F and Y79S mutants. The inhibition constants are shown in Table 6. Y79F exhibits a small increase in the K_I value for tocopherol, and no change in K_I for tocopherol acetate. In contrast, for Y79S the K_I value for tocopherol exhibits a substantial increase (at least 49-fold). Moreover, the K_I for tocopherol acetate could not be determined due to limitations in solubility, indicating that the inhibition constant for this compound is above 100 μM. Similarly, tocopherol and tocopherol acetate did not inhibit Y79A in the presence of up to 100 μM of either compound. Thus, changing Tyr[79] to Ala rendered the enzyme insensitive to inhibition by tocopherol or tocopherol acetate. These results indicate that the residue labeled by tocopherol iodoacetate is within the tocopherol binding site and such binding is governed by hydrophobic interactions with the amino acid at position 79.

DISCUSSION

Studying the inhibition of glutathione *S*-transferase pi is important because it can have both advantageous and disadvantageous effects. It can be advantageous to inhibit the detoxification activity of the GST pi isozyme because overexpression of GST pi in tumor cells has been shown to correlate with resistance to chemotherapeutic drugs through GSH conjugation of the active compounds. Therefore, the task of defining the ligand binding specificity of pi-class GST becomes a critical component in the rational design of highly potent GST pi-selective inhibitors that may increase the effectiveness of commonly used anticancer agents. Currently, the inhibitors that have been developed for GST pi have not yet had clinical success (*41*). As confirmed in this study, alpha-tocopherol is an effective inhibitor of GST pi. A potent inhibitor that can be used in chemotherapy has to enter into the cytosol of the cell; thus it has to be lipophilic or utilize a carrier system for uptake; tocopherol is lipophilic and can diffuse through the cellular membrane. Thus, the importance in identifying the binding site of tocopherol in GST pi is evident.

We have synthesized tocopherol iodoacetate (TIA), which is structurally similar to alpha-tocopherol, and have found that it acts as an affinity label of GST pi. Upon incubation of the enzyme with TIA, a time-dependent loss of activity is observed, yielding a maximum loss of 25% of the original activity. The rate of inactivation exhibits nonlinear dependence on reagent concentration, as is typical of an affinity label, for which an enzyme−reagent complex forms prior to irreversible modification. Upon maximum inactivation, about 1 mol of reagent is incorporated per mole of enzyme subunit, and Tyr[79] is the only amino acid that is modified. Iodide can be displaced from the iodoacetoxy group by nucleophilic attack of several amino acids, including cysteine, aspartate, lysine, tyrosine, and histidine (*42*, *43*). The specificity of reaction of TIA with a particular tyrosine must be attributed to the binding of TIA close to Tyr[79] within the tocopherol binding site. Protection against inactivation is afforded by tocopherol and tocopherol acetate but not by glutathione derivatives, electrophilic substrate analogues, buffers, or nonsubstrate hydrophobic ligands indicating that TIA is binding in a distinct site away from those that have been identified thus far in GST pi. A number of studies on the effects of site-directed mutagenesis of GST have been carried out; as a result, several tyrosine residues have been implicated in the function of GST. The first and most important residue targeted was the active site tyrosine, which stabilizes the thiolate form of GSH (*44*−*46*). In human GSTP1-1 this Y8F mutation reduces the specific activity to 0.3% of the wild-type value (*46*). Tyrosine[108] was also analyzed by site-directed mutagenesis, steady-state kinetic analysis, and crystallographic studies, and it was concluded that it contributes to the electrophilic substrate specificity of GST pi (*47*). Moreover, in a previous study, we identified tyrosine[103] as part of a distinguishable electrophilic substrate site distinct from that occupied by 1-choro-2,4-dinitrobenzene and monobromobimane (*25*). Additionally, Stenberg et al. suggested that tyrosine 50, located at the dimer interface, is important for the proper structure and function of GST pi (*48*). The present investigation suggests for the first time that tyrosine 79 may have a functional role in GST pi. Alignment of representative sequences of the major isozymes of human alpha, mu, and pi glutathione *S*-transferases shows that tyrosine 79 is conserved, supporting its possible importance in enzyme function. Therefore, mutants of this residue were constructed to examine its function in the enzyme. Examination of the kinetic characteristics of Y79F, Y79S, and Y79A enzymes showed that the K_m values for CDNB do not change, suggesting the Tyr[79] does not affect the binding of the electrophilic substrate. Moreover, the K_m values for GSH do not change much for Y79F and Y79S and only increase ∼4−5-fold for Y79A. These results indicate that Tyr[79] does not influence the enzyme's overall affinity for GSH and the change observed for the Ala mutant

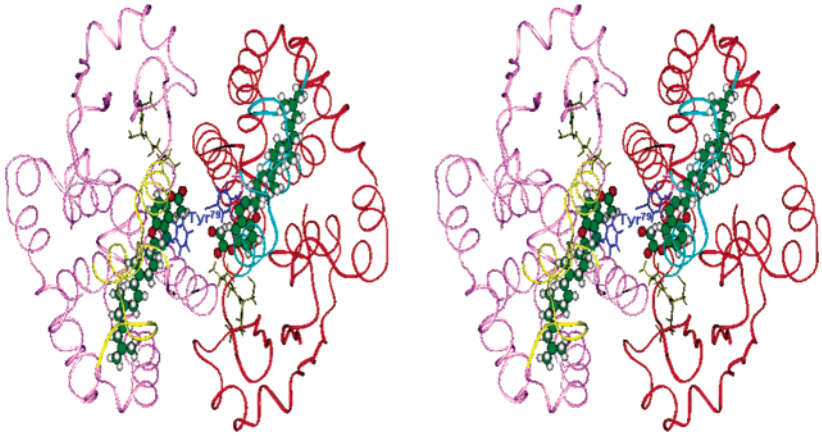

FIGURE 6: Stereoview showing human GST pi (PDB code 18GS) complexed with tocopherol acetate, constructed as described under Experimental Procedures. The structures are colored as follows: orange, GST pi subunit A; pink, GST pi subunit B; cyan, hydrophobic pocket in GST pi subunit A; yellow, hydrophobic pocket in GST pi subunit B; stick figure of dinitrobenzylglutathione in light green; for tocopherol acetate: green, carbon; red, oxygen; white, hydrogen. Atoms of protein side chains are shown in purple. The chromanol head of tocopherol acetate was positioned to interact with the aromatic moiety of Tyr^{79} while the phytyl tail was docked to interact with the highlighted hydrophobic pockets.

is probably an indirect effect. The most notable changes are observed in the V_{max} values. For the Y79A mutant, the decrease in affinity for GSH is accompanied by an increase in V_{max}, probably reflecting enhanced product release in this mutant due to the less than optimal affinity for the glutathione molecule. In contrast, Y79F and Y79S show a 2−3-fold decrease in V_{max}, which can be attributed to possible conformational changes in the enzyme as a result of these interfacial mutations. However, the most striking effect observed in these mutants is their decreased ability to interact with tocopherol and tocopherol acetate, as demonstrated by the dramatic increase in K_I for both compounds. Therefore, we propose that Tyr^{79} of GST pi is within a discrete binding site for tocopherol and its derivatives. Furthermore, the binding of vitamin E molecules within this site as well as replacements of Tyr^{79} has significant effects on the V_{max} of the enzyme. Thus, we suggest that Tyr^{79}, in addition to being part of the tocopherol binding site, may also function as a regulatory site of GST pi. Our results can be interpreted in terms of the crystal structure of GST pi where we have manually docked and energy-minimized the structure of the complex of tocopherol acetate and GST pi. The assumptions made were that that the chromanol head of tocopherol acetate interacts with the aromatic side chain of Tyr^{79} through pi−pi interactions and that 1 mol of tocopherol acetate binds per subunit of GST pi. The structure shown in Figure 6 reflects these requirements, yielding a distance of 3.4 Å between the phenol group of Tyr^{79} and the chromanol group of tocopherol acetate. Although Tyr^{79} is located near the subunit interface of the enzyme, evaluation of the GST pi crystal structure shows that binding of the entire tocopherol molecule is unlikely to occur in the contact region of the two subunits because of the presence of an electrostatic region at the interface. However, there is a hydrophobic pocket just below the G-site composed of amino acid sequences 15−22 and 68−79 highlighted in cyan and yellow in subunits A and B, respectively, where the hydrophobic tocopherol molecule could bind (Figure 6). Although the location of the tocopherol site is based on a model rather than being determined directly, it does provide a reasonable explanation of the experimental results obtained in this paper. Specifically, the model suggests that the phytyl tail of the tocopherol molecule has direct contact with Ala^{18}, Leu^{21}, Leu^{22}, Ala^{23}, Ile^{69}, Leu^{70}, and Leu^{73}. The binding of tocopherol and tocopherol derivatives in this region may affect the activity of the enzyme by introducing conformational changes. This could lead to the indirect distortion of the GSH and the xenobiotic sites, resulting in changes in V_{max} rather than $K_{m,}$ since the tocopherol acetate molecule is approximately 16.4 Å away from the active site.

In summary, tocopherol iodoacetate functions as an affinity label of the tocopherol binding site of human pi class glutathione *S*-transferase, mimicking (but irreversibly) the reversible inhibition of tocopherol and tocopherol acetate. Upon incubation with TIA, the enzyme loses ∼75% of its activity, incorporates about 1.0 mol of reagent/enzyme subunit, and is modified only at Tyr^{79}. Protection against inactivation by TIA is best provided by tocopherol and tocopherol acetate, indicating that Tyr^{79} is within the binding site of tocopherol molecules. On the basis of analysis of molecular models, this tocopherol site is located within the hydrophobic pocket between the two subunits of the enzyme.

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