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Binding and Kinetic Mechanisms of the Zeta Class Glutathione Transferase*

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The Zeta class of glutathione transferases (GSTs) has only recently been discovered and hence has been poorly characterized. Here we investigate the substrate binding and kinetic mechanisms of the human Zeta class GSTZ1c-1c by means of pre-steady state and steady-state experiments and site-directed mutagenesis. Binding of GSH occurs at a very low rate compared with that observed for the more recently evolved GSTs (Alpha, Mu, and Pi classes). Moreover, the single step binding mechanism observed in this enzyme is reminiscent of that found for the Theta class enzyme, whereas the Alpha, Mu, and Pi classes have adopted a multistep binding mechanism. Replacement of Cys[16] with Ala increases the rate of GSH release from the active site causing a 10-fold decrease of affinity toward GSH. Cys[16] also plays a crucial role in co-substrate binding; the mutant enzyme is unable to bind the carcinogenic substrate dichloroacetic acid in the absence of GSH. However, both substrate binding and GSH activation are not rate-limiting in catalysis. A peculiarity of the hGSTZ1c-1c is the half-site activation of bound GSH. This suggests a primitive monomer-monomer interaction that, in the recently diverged GSTP1-1, gives rise to a sophisticated cooperative mechanism that preserves the catalytic efficiency of this GST under stress conditions.

Cytosolic glutathione transferases (GSTs,[1] EC 2.5.1.18) are a family of detoxifying enzymes characterized by similar tertiary structures and active site topologies (1, 2). GSTs are probably evolved from thioredoxin and glutaredoxin enzymes; in fact, the $\beta\alpha\beta\alpha\beta\beta\alpha$ motif of the N-terminal domain of all cytosolic GSTs superimposes well with the structures of the redox enzymes that are characterized by one or two cysteines in the active site essential for catalysis (3–5). A few GSTs still show a cysteine residue in or in close proximity of the active site, and their role has been the object of several studies. In particular, the bacterial GSTB1-1 and the human GSTO1-1 show a GSH molecule in mixed disulfide with the active site cysteine (6, 7). These enzymes, beside the classical GST activity, catalyze thiol-disulfide oxidoreductase reactions, so they could represent a transition structure between an ancestral redox enzyme and a GST-conjugating enzyme (8, 9). In the more recently diverged Alpha, Mu, and Pi class GSTs, the cysteine residues involved in the redox catalysis have been lost. Nevertheless, in GSTP1-1, Cys^{47} is an important residue lining the G-site. Cys^{47} is not essential for catalysis of GSTP1-1, but it is highly reactive and forms a peculiar ion pair with Lys^{54} that is responsible for the high affinity of the enzyme for the substrate GSH. Substitution of Cys^{47} with Ala decreases the affinity for GSH. In addition this substitution triggers a positive cooperativity for the binding of GSH, and this provided the first indication for a structural intersubunit communication in the GST superfamily (10). One of the most recently discovered GSTs is the Zeta class enzyme (GSTZ1-1), which shows very low or no activity toward the classical GST substrates (11). GSTZ1-1 is characterized by two unusual GSH-dependent reactions as follows: (i) the isomerization of maleylacetoacetate to fumarylacetoacetate, a crucial step in the tyrosine metabolism; (ii) the biotransformation of α-haloacids such as dichloroacetic acid (DCA), a potential carcinogenic contaminant of chlorinated drinking water, which is dehalogenated to glyoxylic acid (12, 13). The Zeta class GST is widely distributed in nature and contains a characteristic motif (SSC*X*(W/H)RVIAL) in the N-terminal region (11). Among these residues, the first three (Ser^{14}–Ser^{15}–Cys^{16}) line the active site pocket and have been investigated recently for their possible role in catalysis. In particular, the crystal structure of the human variant GSTZ1a-1a shows the sulfur atom of Cys^{16} close to the sulfur atom of GSH (14). It has been shown that C16A mutant is active with different substrates but displays a higher K_m value for GSH. Thus, Cys^{16} seems to play a role in a proper binding of GSH rather than a direct role in catalysis (15). Four polymorphic variants of the human GSTZ1-1 have been detected in the Caucasian population resulting from mutations at positions 32, 42, and 82 (16, 17). In the present study, binding and kinetic mechanisms of the most abundant variant, the human GSTZ1c-1c, have been carefully investigated using DCA and GSH as substrates. Moreover, binding and kinetic properties of its C16A mutant have been determined by disclosing multiple roles for the Cys^{16} residue.

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[1] The abbreviations used are: GST, glutathione *S*-transferase; TCHQ, tetrachlorohydroquinone; DCA, dichloroacetic acid.

EXPERIMENTAL PROCEDURES

Materials—GSH, DCA, NADH; and lactic dehydrogenase were obtained from Sigma; nickel-agarose matrix (nickel-nitrilotriacetic acid) was purchased from Qiagen (Valencia, CA).

Expression and Purification of GSTZ1c-1c and C16A Mutant Enzymes—Human GSTZ1c-1c and C16A mutant enzymes were expressed in *Escherichia coli* as His_6-tagged proteins and were purified by nickel-agarose column chromatography as described previously (16). Protein concentration was calculated from the absorbance at 280 nm assuming an $\epsilon_{1\ mg/ml}$ of 0.82. The extinction coefficient was calculated on the basis of the amino acid sequence as reported by Gill and von Hippel (18) and confirmed by the bicinchoninic acid method (Pierce). A molecular mass of 25.3 kDa per GST subunit was used in the calculations (19).

GSH and DCA Binding to GSTZ1c-1c—Binding of both GSH and DCA to 2 μM GSTZ1c-1c in phosphate/acetate/borate buffer (50:50:50), pH 7.0, was measured in a single photon counting spectrofluorometer (Fluoromax, S.A. Instrument, Paris, France) with a sampler holder thermostated at different temperatures between 10 and 37 °C. Excitation was at 280 nm, and emission was collected at 333 nm. The affinities of GSTZ1c-1c for both GSH and DCA were determined by measuring the perturbation of the intrinsic fluorescence of the protein following the addition of substrate. Fluorescence data were corrected both for dilution and for inner filter effects and fitted to Equation 1, which provides the K_D value for the substrate,

$$\Delta F = \Delta F_{max}/(1 + K_D/[S]) \quad \text{(Eq. 1)}$$

where ΔF is the protein fluorescence change observed in the presence of a given amount of substrate, and ΔF_{max} is the maximum fluorescence change observed at saturating substrate concentrations.

GSH Activation in GSTZ1c-1c and in the C16A Mutant Enzymes—Difference spectra of GSH bound to both GSTZ1c-1c and C16A mutant enzymes were obtained with a Kontron double beam Uvikon 940 spectrophotometer thermostated at 25 or 37 °C. Spectra were corrected for the contribution of the free enzyme and free GSH, and the amount of GSH thiolate formed at the active site was calculated by assuming an $\epsilon_{240\ nm} = 5.0\ mM^{-1}\ cm^{-1}$. The dependence of thiolate formation on pH was obtained by adding saturating amounts of GSH to both GSTZ1c-1c (15 μM) and C16A (20 μM) mutant enzymes in phosphate/acetate/borate buffer (50:50:50) between pH 5.0 and 7.0. For the native enzyme 1 mM GSH was used in the 5.5–7.0 pH range, whereas 3 mM GSH was used at pH 5.0. Due to the low affinity of the mutant enzyme for GSH, 20 mM GSH was used in the 5.0–5.5 pH region and 10 mM between pH 6.0 and 6.5. Under these conditions, the spontaneous ionization of GSH was always negligible. The thiolate formed at pH 7.0 with the mutant enzyme was obtained under subsaturating GSH concentrations (1 mM) and extrapolated to saturating GSH concentrations by assuming an average K_D value for GSH of 800 μM.

Kinetics of the Binding of GSH and DCA to GSTZ1c-1c and C16A Mutant Enzymes—Rapid kinetic experiments were performed on an Applied Photophysics kinetic spectrometer stopped-flow instrument equipped with a thermostated 1-cm light path observation chamber. Binding of GSH was measured at different temperatures between 10 and 37 °C by rapid mixing of the enzyme (100 μM in phosphate/acetate/borate buffer (50:50:50), pH 7.0) with the same volume of GSH (from 0.006 to 20 mM) in phosphate/acetate/borate buffer (50:50:50), pH 7.0. The DCA binding was studied at 25 °C by rapid mixing of the enzyme (100 μM in phosphate/acetate/borate buffer (50:50:50), pH 7.0) with the same volume of DCA (from 0.006 to 2 mM) in the same buffer.

Binding of GSH to GSTZ1c-1c wild type and C16A mutant enzymes was followed by recording the increase of the intrinsic fluorescence at 340 nm and the increase of the absorbance at 240 nm due to the GSH thiolate formation. Binding of DCA to GSTZ1c-1c was followed by recording the quenching of the intrinsic fluorescence at 340 nm. No spectral changes were observed after the addition of DCA to the C16A mutant enzyme. Experimental traces were fitted to a single exponential equation, and pseudo first-order kinetic constants (k_{obs}) were calculated at different substrates concentrations. The dependence of k_{obs} on substrate (S) concentration was fitted to Equation 2,

$$k_{obs} = k_{on}[S] + k_{off} \quad \text{(Eq. 2)}$$

which gives the second-order rate constant for the formation of the enzyme-substrate complex (k_{on}), and the first-order rate constant for the release of the substrate from the active site (k_{off}). The dissociation constant (K_D) for each substrate was estimated from $K_D = k_{off}/k_{on}$. Moreover, in the mutant enzyme, the dependence of fluorescence perturbation on GSH concentration, was fitted to a simple isotherm binding equation to calculate the GSH dissociation constant (K_{D2}).

Determination of the pK_a Value for the Cys^{16} Sulfhydryl Group—The pK_a value for the Cys^{16} sulfhydryl group was determined from the spectral perturbation of the protein due to DCA binding. Difference spectra of both GSTZ1c-1c and C16A mutant enzymes bound to DCA were obtained with a Kontron double beam Uvikon 940 spectrophotometer thermostated at 25 °C. In a typical experiment 1 mM DCA was added to 20 μM enzyme in phosphate/acetate/borate buffer (50:50:50) between pH 4.5 and pH 8.0. Spectra were corrected for the contribution of the free enzyme and free DCA, and the amount of thiolate present at a given pH value was calculated from the decrease of the absorbance at 230 nm by assuming an $\epsilon_{230\ nm}$ of 5.0 $mM^{-1}\ cm^{-1}$. Experimental data were fitted to Equation 3, which provides the pK_a value for the Cys^{16} sulfhydryl group,

$$(Cys{-}S^-/GST) = ((Cys{-}S^-/GST)_{max})/(1 + 10^{pK-pH}) \quad \text{(Eq. 3)}$$

Dehalogenase Activity with DCA as Co-substrate—Enzyme activity was measured spectrophotometrically continuously at 340 nm and 37 °C using a lactate dehydrogenase/NADH-coupled assay (20). The standard assay mixture (1-ml final volume) contained phosphate/acetate/borate buffer (50:50:50), pH 7.0, 1 mM EDTA, 1 mM GSH (10 mM with the C16A mutant), 1 mM DCA (10 mM with the C16A mutant), 0.2 mM NADH, 2 units of lactate dehydrogenase, and catalytic amounts of GSTZ1c-1c or C16A mutant enzymes. The spontaneous reaction was determined under the same experimental conditions in the absence of enzymes. The amount of the lactate dehydrogenase, used as the coupling enzyme, was always enough to avoid underestimation of the dehalogenase reaction. One unit of dehalogenase activity is defined as the amount of enzyme that dehalogenates 1 μmol of DCA per min at 37 °C.

The dependence of dehalogenase activity on pH was determined in phosphate/acetate/borate buffers (50:50:50) between pH 5.0 and 8.0 in the presence of saturating substrate concentrations (1 mM for the native enzyme and 10 mM for the C16A mutant with the exception of pH 5.0, where the GSH concentrations were 3 and 20 mM for the native and the C16A mutant enzymes, respectively).

Steady-state kinetic analysis was performed with both GSTZ1c-1c and C16A mutant enzymes at pH 7.0 and 37 °C by varying both GSH and DCA from 0.02 (0.2 mM with the C16A mutant) to 3 mM (10 mM with the C16A mutant) over a matrix of 25 substrate concentrations. The dependence of velocity on concentration of the variable substrate was fitted to a simple isotherm binding equation which provides $V_{max(app)}$ and $K_{D(app)}$ values for the variable substrate at fixed co-substrate concentrations. Data were reported as double-reciprocal plots to better evaluate the steady-state kinetic mechanism.

In the native enzyme, replots of $K_{D(app)}/V_{max(app)}$ ratios and of $1/V_{max(app)}$ intercept values *versus* the reciprocal of the co-substrate concentration provide the affinity of both DCA and GSH toward the free enzyme (K_D) and toward the enzyme saturated with the co-substrate (αK_D), respectively (21). In the C16A mutant, replot of $K_{D(app)}$ for DCA *versus* the reciprocal of the fixed GSH concentrations provides the affinity of GSH toward the free enzyme and the affinity of DCA toward the enzyme in complex with GSH. Moreover, a plot of $K_{D(app)}/V_{max(app)}$ ratios, when GSH is the variable substrate, *versus* 1/DCA indicates the order of addition of substrates to the enzyme (21).

RESULTS

Binding of GSH and DCA to GSTZ1c-1c—Isothermic binding of both GSH and DCA to GSTZ1c-1c was studied at different temperatures between 10 and 37 °C and at pH 7.0 by following the perturbation of the intrinsic fluorescence of the protein at different concentrations of substrates. Fluorescence data were fitted to Equation 1, and K_D^{GSH} values of 11, 59, and 132 μM were found at 10, 25, and 37 °C, respectively, revealing a strong dependence of the dissociation constant for GSH on the temperature. Binding experiments with the second substrate DCA give a K_D^{DCA} of 26 μM at 25 °C, which is comparable with the value obtained for GSH at the same temperature.

Unfortunately, in the C16A mutant, only a small blue shift of the intrinsic fluorescence was observed after addition of GSH, whereas DCA did not cause any spectral change. Thus, in the mutant enzyme, binding of substrates was not detectable by using this technique.

GSH Activation in GSTZ1c-1c and in C16A Mutant—The

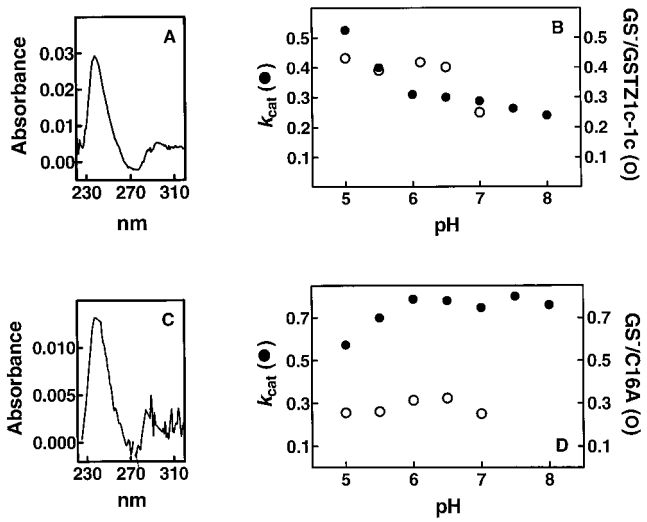


FIG. 1. **Spectroscopic and kinetic evidence for GSH ionization.** Spectra of GS^- in the binary complex GST-GSH were obtained at 25 °C and at different pH values as described under "Experimental Procedures." *Panel A*, spectrum of GS^- (1 mM GSH) bound to 15 μM GSTZ1c-1c at pH 6.0. *Panel C*, spectrum of GS^- (1 mM GSH) bound to 20 μM C16A mutant enzyme at pH 7.0. The amount of GS^- formed at the active site was calculated by assuming an $\epsilon_{240\ nm} = 5.0\ mM^{-1}\ cm^{-1}$ and was reported as function of pH. The turnover number (k_{cat}) was obtained at 37 °C and at different pH values under saturating GSH and DCA concentrations as described under "Experimental Procedures." *panel B*, pH dependence of GS^-/GSTZ1c-1c (○) and pH dependence of k_{cat} (●) in the native enzyme. *Panel D*, pH dependence of GS^-/C16A (○) and pH dependence of k_{cat} (●) in the C16A mutant enzyme. Because of the strong contribution of the spontaneous ionization of GSH at neutral alkaline conditions, the thiolate formed at pH 7.0 in the mutant enzyme was obtained with 1 mM GSH, which is a subsaturating concentration. The thiolate value was then extrapolated at saturating GSH by assuming an average K_D value for GSH of 800 μM (see Table I).

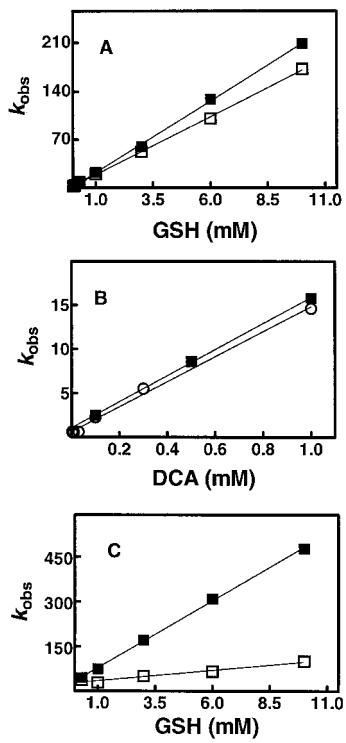


FIG. 2. **Kinetics of substrates binding to GSTZ1c-1c and C16A mutant enzymes.** The observed rate constants (k_{obs}) for the binding of both GSH and DCA at 25 °C were obtained by following the fluorescence perturbation of the protein at 340 nm (■) and the UV perturbation at 240 nm (□) as described under "Experimental Procedures." Binding of GSH to both GSTZ1c-1c and C16A mutant is reported in *panels A* and *C*, respectively. Binding of DCA to GSTZ1c-1c is shown in *panel B*. In the same panel are also reported the observed rate constants (k_{obs}) obtained at 230 nm following the protonation of Cys^{16} upon DCA binding (○). Linear regression analysis of the dependence of k_{obs} on substrate concentration gives the second-order rate constant for the formation of the enzyme-substrate complex (k_{on}) and the first-order rate constant for the release of the substrate from the active site (k_{off}).

crystal structure of hGSTZ1 indicates that the thiol group of the enzyme-bound GSH is located almost directly over the N-terminal end of helix α1 (14). Therefore, it has been suggested that the proximity of a Cys side chain to the positive end of a helix dipole may decrease the pK_a of the thiol as suggested for the GSH-stabilizing residue in other GSTs (15). Direct evidence that thiol ionization really occurs in the Zeta class isoenzyme comes from UV difference spectroscopy. Difference spectra of GS^- bound to either GSTZ1c-1c or C16A mutant enzymes were recorded between 220 and 320 nm and show an absorption band at 240 nm which is typical of the mercaptide ion (Fig. 1, *panels A* and *C*).

Difference spectra, recorded at different pH values under saturating GSH concentrations, reveal that the ionization of the bound GSH in the C16A mutant is comparable with that of the native enzyme, and both trends are nearly pH-independent (Fig. 1, *panels B* and *D*). Moreover, at both 25 and 37 °C, the amount of ionized GSH does not exceed 0.5 GS^- per active site both in the native and mutated enzymes. We must also stress that saturation of C16A mutant below pH 7.0 is obtained only at 15–20 mM GSH. In fact, at pH 5.0 and 1–5 mM GSH, the amount of GSH thiolate is almost undetectable.

Kinetics of GSH Binding—Rapid kinetic experiments were performed to dissect the binding mechanism of GSH to both GSTZ1c-1c and C16A mutant enzymes. Kinetics of the binding of GSH was studied by following the perturbation of the intrinsic fluorescence of the protein at 340 nm due to GSH binding and the absorbance change at 230–240 nm due to the GSH thiolate formation. Moreover, the stopped-flow approach allowed us to follow the binding process to the mutant enzyme, which causes only a small fluorescence perturbation. Experiments were performed at 10, 25, and 37 °C and pH 7.0, and in Fig. 2 are shown the results obtained at 25 °C. The apparent first-order rate constants (k_{obs}) for both the fluorescence perturbation and the thiolate formation follow a linear dependence on GSH concentration up to 10 mM. This suggests that GSH forms a Michaelis complex by a simple bimolecular interaction both in the native and in C16A mutant enzymes. In GSTZ1c-1c, the fluorescence perturbation is synchronous with the thiolate formation at 25 and 37 °C, whereas at 10 °C the GSH binding and the thiolate formation appear as two distinct kinetic events. In the C16A mutant, this nonsynchronous behavior is observed at both 10 and 25 °C, with the thiolate formation significantly out of step with the GSH binding (Fig. 2, *panel C*).

The microscopic kinetic constants k_{on} and k_{off} for the binding of GSH were estimated from fluorescence data by linear regression analysis of the k_{obs} dependence on substrate concentration (see "Experimental Procedures"); K_D values were calculated from the k_{off}/k_{on} ratio (see Table I). These K_D values agree with those obtained from steady-state experiments, despite that former values are affected by large standard deviations, and confirm a remarkable decrease of affinity for GSH induced by Cys^{16} mutation both at 10 and 25 °C

Most interesting, substitution of Cys^{16} with Ala causes an increase of both k_{on} and k_{off} values for GSH binding. However, k_{on} value increases 2-fold, whereas about a 30-fold increase has been observed for the k_{off} value. Thus, a faster release of GSH from the G-site seems to be the determinant of the decreased affinity toward GSH in the C16A mutant and suggests a role for Cys^{16} in the maintenance of an high affinity active site

TABLE I
Kinetic parameters for GSH and DCA binding

k_{off} and k_{on} were calculated from stopped-flow experiments made as reported under "Experimental Procedures"; K_D was calculated from the k_{off}/k_{on} ratio. K_{D1} was calculated from the fluorescence perturbation observed in the binding experiments at equilibrium; K_{D2} was calculated from the fluorescence perturbation observed in the stopped-flow experiments. ND indicates not done.

T	k_{on}	k_{off}	K_D	K_{D1}
°C	$mM^{-1}\ s^{-1}$	s^{-1}	μM	μM
Binding of GSH to GSTZ1c-1c				
10	8.15 ± 0.07	0.3 ± 0.1	37 ± 12	11 ± 2
25	20.87 ± 0.40	1.0 ± 0.3	48 ± 13	59 ± 5
37	28.55 ± 0.70	4.7 ± 1.1	165 ± 37	132 ± 18
Binding of DCA to GSTZ1c-1c				
25	14.18 ± 1.90	0.8 ± 0.3	56 ± 12	26 ± 10
Binding of GSH to C16A mutant				K_{D2}
10	13.90 ± 0.25	3.0 ± 0.6	216 ± 37	103 ± 0.6
25	45.30 ± 3.22	33.5 ± 7.8	740 ± 119	917 ± 80
37	ND	ND	ND	ND

structure by limiting the number of its potential conformations.

Kinetics of DCA Binding—Rapid kinetic experiments were performed to dissect the binding mechanism of DCA to both GSTZ1c-1c and C16A mutant enzymes. Kinetics of the binding of DCA was studied by following the quenching of the intrinsic fluorescence of the protein at 340 nm due to DCA binding. Kinetic and equilibrium binding experiments with DCA can be affected by the occurrence of a covalent interaction with the thiol group of Cys^{16} as observed previously (19). Despite this possibility, in the temporal range of the present experiments we did not detect any covalent interaction with the enzyme as suggested by the preservation of the enzyme activity at the end of the experiments (data not shown).

The apparent first-order rate constants for the fluorescence perturbation follow a linear dependence on DCA concentration up to 1 mM, and linear regression analysis gives k_{on} = 14.2 $mM^{-1}s^{-1}$, k_{off} = 0.8 s^{-1}, and a K_D value of 56 μM at 25 °C and pH 7.0 (Fig. 2 and Table I). The kinetic and thermodynamic constants found for DCA are almost coincident to the values found for the binding of GSH, indicating that GSTZ1c-1c binds and releases both substrates at comparable rates.

Kinetics of the co-substrate binding (DCA) was studied only in the wild type enzyme because no spectral changes were observed after the addition of DCA to the C16A mutant enzyme.

Acidic Properties of Cys^{16} Residue—Cys^{16} is selectively modified by DCA under appropriate conditions. The covalent bond involves one Cys^{16} per dimer and induces a complete enzyme inactivation (19). This reaction only occurs in the presence of GSH as shown by Tzeng and co-workers (20). In an attempt to study the noncovalent interaction of the enzyme with DCA, we analyzed the UV spectral perturbation due to the co-substrate binding. Notably, DCA induces an instantaneous disappearance of a sharp absorption band centered at 230 nm (Fig. 3, *panel A*). A very similar fast spectral perturbation was observed after incubation of the enzyme with the alkylating agent bromopyruvic acid (data not shown). However, in the case of DCA, a fast covalent modification of Cys^{16} does not occur due to the absence of GSH in the incubation mixture and proved by the full recovery of the enzyme activity at the end of the experiments. No relevant spectral change was observed when DCA was added to the C16A mutant enzyme; thus, binding of DCA probably induces the protonation of the Cys^{16} thiolate suppressing its spectral contribution in the UV region. The UV-absorption band is proportional to the amount of the thiolate present at each pH value, and its pH dependence provides a pK_a value of 5.5 ± 0.2 (Fig. 3, *panel B*), a value about 4 units lower than that of the free cysteine in solution. Assuming an extinction coefficient at 230 nm close to that of the thiolate anion of GSH (ϵ = 5.0 mM^{-1} cm^{-1}), the limiting value of the Cys^{16} thiolate, measured at alkaline pH, accounts for about 0.4 eq per active site. The present data suggest that only one Cys^{16} per dimer shows acidic properties, and this may explain the half-site reactivity of this residue observed previously (19). Most interesting, kinetics of Cys^{16} protonation upon DCA binding (as observed by stopped-flow analysis) overlaps kinetics of fluorescence perturbation due to the co-substrate binding (see Fig. 2, *panel B*).

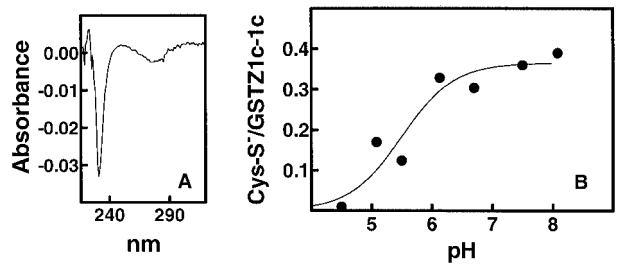


FIG. 3. **pK_a determination of Cys^{16} residue.** The spectrum of GSTZ1c-1c in complex with DCA was measured at different pH values between pH 4.5 and pH 8.0 as described under "Experimental Procedures." *Panel A* shows the differential spectrum of 20 μM GSTZ1c-1c in the presence of 1 mM DCA at pH 6.0; the negative band is centered at 230 nm. The amount of the Cys^{16} thiolate present at a given pH value was obtained from the decrease of the absorbance at 230 nm by assuming an $\epsilon_{230\,nm}$ of 5.0 mM^{-1} cm^{-1}. Experimental data were fitted to the equation $(Cys\text{-}S^-/GST) = ((Cys\text{-}S^-/GST)_{max})/(1 + 10^{pK-pH})$, which fulfills a p$K_a$ value for the Cys^{16} sulfhydryl group of 5.5 ± 0.2 (*panel B*).

Steady-state Kinetics of the Reaction between DCA and GSH—Although a few co-substrates can be used to characterize the kinetic mechanism of the dehalogenase reaction of this enzyme (*e.g.* 2-bromo-3-(4-nitrophenyl) propionic acid (15)), dissection of the catalytic mechanism of GSTZ1c-1c with DCA is of considerable interest, because this carcinogenic molecule is a contaminant of chlorinated drinking water. Thus, we utilized a continuous spectrophotometric method to measure the rate of glyoxylic acid formation after reduction of this molecule by the lactate dehydrogenase/NADH-coupled system (21).

The turnover number (k_{cat}) of the reaction measured at 37 °C and at pH 7.0 is 0.3 s^{-1} for the native enzyme and 0.8 s^{-1} for the C16A mutant. Both in the native and in the C16A mutant enzymes, the k_{cat} is quite pH-independent and parallels the pH-independent behavior of the GSH thiolate (Fig. 1, *panels B* and *D*). This finding suggests that the rate-limiting step may be the chemical event occurring after the GSH activation and that mutation of Cys^{16} does not change the rate-limiting step of reaction.

The reciprocal plots of the initial rate data obtained at 37 °C

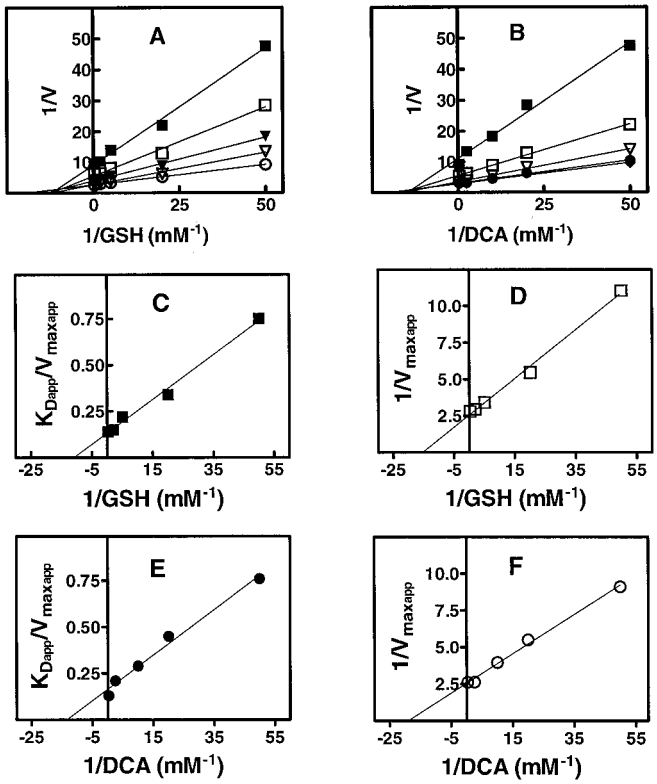
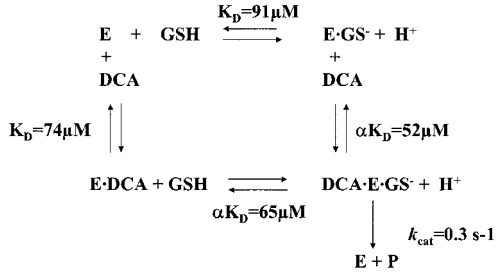


FIG. 4. **Steady-state kinetic analysis of the dehalogenase reaction catalyzed by GSTZ1c-1c.** The reciprocal plots of the initial rate data were obtained at 37 °C and pH 7.0. *Panel A*, GSH was the varied substrate, and DCA concentrations (millimolar) were fixed at 0.02, 0.05, 0.1, 0.4, and 3.0. *Panel B*, DCA was the varied substrate, and GSH concentrations (millimolar) were fixed at 0.02, 0.05, 0.2, 0.5, and 3.0. Replots of $K_{D(app)}/V_{max(app)}$ ratios *versus* the reciprocal of the co-substrate concentration fulfill the affinity of GSH (*panel C*) and of DCA (*panel E*) toward the free enzyme. Replots of $1/V_{max(app)}$ intercept values *versus* the reciprocal of the co-substrate concentration yield the affinity of GSH (*panel D*) and of DCA (*panel F*) toward the enzyme saturated with the co-substrate. Velocities were expressed as ΔA/min at 340 nm. Each data point represents the mean of three different experiments, and standard error does not exceed 5%.

and pH 7.0, by varying both DCA and GSH, give intersecting lines with both the native and the C16A mutant. The family of plots for the varied GSH concentration is symmetrical to that obtained for varied DCA concentration in the native enzyme (Fig. 4, *panels A* and *B*), and this is compatible with a rapid equilibrium random sequential mechanism in which DCA and GSH bind randomly to the enzyme. In this case, replots of $K_{D(app)}/V_{max(app)}$ ratios and of $1/V_{max(app)}$ intercept values *versus* the reciprocal of the co-substrate concentration yield the affinity of both DCA and GSH toward the free enzyme (K_D) and toward the enzyme saturated with the co-substrate (αK_D), respectively (Fig. 4) (22). The dissociation constants (K_D) of GSH and DCA from the binary complex GSTZ1-GSH and GSTZ1-DCA are K_D^{GSH} = 91 μM and K_D^{DCA} = 74 μM. The dissociation constants (αK_D) of GSH and DCA from the ternary complex DCA-GSTZ1-GSH are αK_D^{GSH} = 65 μM and αK_D^{DCA} = 52 μM. From these data the factor α is calculated to be 0.7, thus indicating that the affinity toward one substrate is increased by the binding of the co-substrate to the enzyme. A proposed catalytic mechanism for GSTZ1c-1c is reported in Scheme 1.

Mutation of Cys^{16} causes a change in the kinetic mechanism; in the C16A mutant, the family of plots for varied DCA intersects the 1/*v* axis, and this is indicative of a rapid equilibrium ordered mechanism in which GSH binds first (Fig. 5, *panel A*). In this case, replot of $K_{D(app)}$ for DCA *versus* the different fixed 1/GSH concentrations yields the affinity of GSH toward the



SCHEME 1. **Proposed kinetic mechanism for the DCA dehalogenase reaction catalyzed by GSTZ1c-1c.**

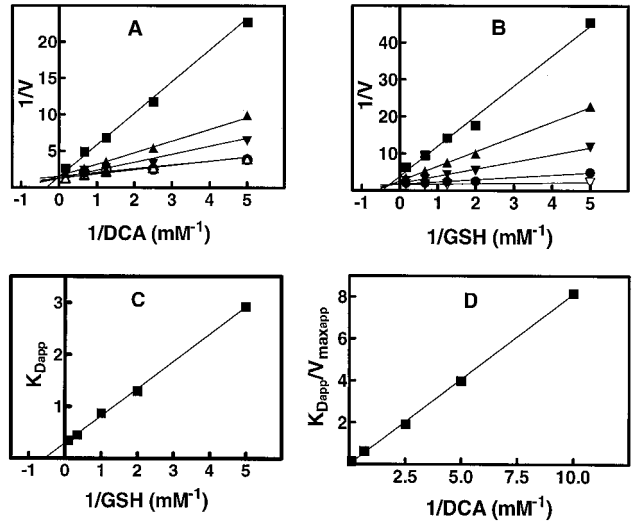
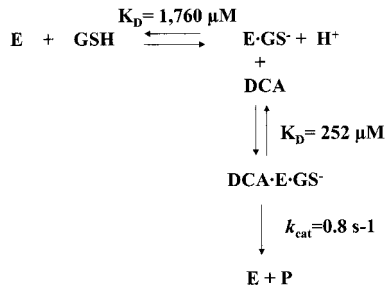


FIG. 5. **Steady-state kinetic analysis of the dehalogenase reaction catalyzed by C16A mutant.** The reciprocal plots of the initial rate data were obtained at 37 °C and pH 7.0. *Panel A*, DCA was the varied substrate, and GSH concentrations (millimolar) were fixed at 0.2, 0.5, 1.0, 3.0, and 10.0. *Panel B*, GSH was the varied substrate, and DCA concentrations (millimolar) were fixed at 0.1, 0.2, 0.4, 1.5, and 10.0. Plot of $K_{D(app)}$ for DCA *versus* the different fixed 1/GSH concentrations (*panel C*) yields the affinity of GSH toward the free enzyme and the affinity of DCA toward the enzyme in complex with GSH. *Panel D* is the plot of $K_{D(app)}/V_{max(app)}$ ratios, when GSH is the variable substrate, *versus* 1/DCA which confirms the order of addition of substrates to the enzyme. Velocities were expressed as ΔA/min at 340 nm. Each data point represents the mean of three different experiments, and standard error does not exceed 5%.

free enzyme (K_D = 1,760 μM) and the affinity of DCA toward the enzyme in complex with GSH (K_D = 252 μM) (Fig. 5, *panel C*). Moreover, a plot of $K_{D(app)}/V_{max(app)}$ ratios, when GSH is the variable substrate *versus* 1/DCA concentrations, is linear and intercepts at the origin (when DCA is infinitely high) (Fig. 5, *panel D*). This behavior is a further indication of an ordered mechanism with GSH adding to the enzyme before DCA (22). A proposed catalytic mechanism for C16A mutant is reported in Scheme 2.

DISCUSSION

Functional characterization of GSTZ1c-1c has been performed, and the role of the conserved Cys^{16} residue in the biotransformation of DCA has also been investigated. The Zeta class GSTs from a range of species contain a consensus motif in the N-terminal region that includes the following two residues found in the active site of the enzyme TCHQ dehalogenase: Cys^{13} and Ser^{11}. This enzyme shows a GSH-binding site similar to those found in GSTs, and it has been hypothesized to be related to the Zeta class GST (21). TCHQ dehalogenase converts TCHQ into dichlorohydroquinone in a two-step reaction in which the first one involves the nucleophilic attack of GSH on the electrophilic substrate, and the second step requires the



SCHEME 2. **Proposed kinetic mechanism for the DCA dehalogenase reaction catalyzed by C16A mutant.**

nucleophilic attack of the Cys^{13} residue. In the reaction catalyzed by GSTZ1c-1c with DCA, the first step involves the nucleophilic attack of GSH on the α-haloacid but Cys^{16} (equivalent to Cys^{13} in TCHQ dehalogenase) does not appear to be essential for the second catalytic step. Among GSTs, the presence of a cysteine residue in the active site has been observed in the Beta (Cys^{10}) and Omega (Cys^{32}) classes, and both cysteine residues are close to the position of Cys^{16} in GSTZ1-1 active site (14). The Beta and Omega class GSTs are characterized by a GSH molecule in mixed disulfide with the active site cysteine and catalyze glutaredoxin-like reactions (7, 9). In particular, the Cys^{10} residue in the Beta class GST is essential for this reaction, and replacement by alanine abolishes the enzymatic activity. The crystal structure of the human Zeta class GST shows that Cys^{16} is in close proximity to the sulfur atom of the bound GSH (14), but the enzyme is not able to catalyze redox reactions with disulfides. From all these results it is clear that the active site cysteine in this enzyme is no longer a catalytic residue. However, the Cys^{16} sulfhydryl group is characterized by the very low pK_a value of 5.5, about 4 units lower than that of free cysteine in solution. Similarly, in Pi class GST (GSTP1-1), Cys^{47} is characterized by a very low pK_a value (10). In this protein the sulfhydryl group of Cys^{47} is linked in an ion pair with the protonated group of Lys^{54}, and this interaction limits the structural fluctuations of the G-site resulting in an optimized affinity for GSH. Likewise in the GSTZ1c-1c, Cys^{16} is mainly in the ionized form at physiological pH values, and this may be caused by the presence of a basic residue in the G-site. Indeed, the active site, compared with other GSTs, is characterized by high electropositivity, and the positive charge of Arg^{19}, together with polar interactions from the side chain of Gln^{111}, may contribute by stabilizing the Cys^{16} thiolate in the absence of the co-substrate.

The present study reveals that substitution of Cys^{16} with alanine has multiple consequences on the binding and kinetic mechanisms of the Zeta class GST, highlighting the importance of this residue. Binding of GSH to the native enzyme occurs at a very low rate compared with that observed for the more recently diverged Alpha, Mu, and Pi class GSTs (23). In these enzymes the microscopic rate constants of the GSH binding process (k_{on}) at 5 °C are about 3 orders of magnitude higher than those found for the Zeta enzyme at 10 °C. The k_{on} and k_{off} values observed for the Zeta class GST are similar to those obtained for the Theta class GSTT2-2 (k_{on} = 27 mM^{-1} s^{-1} and k_{off} = 36 s^{-1} at 37 °C) (24). Notably, in the Theta enzyme the G-site is mostly obscured by the C-terminal portion of the protein, whereas in the Zeta class GST, GSH is buried in a very deep crevice between the two domains. Most interesting, the single step binding mechanism observed in the Zeta enzyme is also found in the Theta enzyme, whereas the more recently evolved GSTs have adopted a multistep binding mechanism (23).

Mutation of Cys^{16} increases the rate of GSH release from the active site explaining the almost 10-fold decrease of affinity toward GSH at pH 7.0. Moreover, the mutant enzyme shows very low affinity toward GSH in the acidic pH range. Thus at physiological GSH concentrations, binding and activation of GSH are almost undetectable in this pH region. At saturating GSH, the k_{cat} of the mutant enzyme is about 2.5-fold higher than that of the native enzyme in the 6–8 pH range, suggesting that mutation does not prevent the productive binding of GSH and probably increases the active site flexibility. A more drastic effect of mutation is observed on the binding of the co-substrate DCA. The C16A mutant enzyme has lost the ability to bind DCA, and binding occurs only in the enzyme bound to GSH. Consequently, the steady-state kinetics of the reaction between DCA and GSH is also affected by the mutation and shifts from a mechanism in which DCA and GSH bind randomly to the enzyme to an ordered mechanism in which GSH binds first. The crystal structure of hGSTZ1-1 is a complex with GSH and a sulfate ion derived from the crystallization medium. The thiol moiety of Cys^{16} forms direct interactions with the side chains of Arg^{19} and Gln^{111} and with the GSH thiol (distance of 2.8 Å), as well as numerous van der Waals interactions with the GSH backbone. In turn the GSH thiol moiety makes numerous contacts including the side chains of Ser^{15} and Gln^{111}. Most important, it also makes contacts with the sulfate ion that is thought to mimic the binding of DCA. In turn the sulfate ion makes direct interactions with Arg^{175}, thought to be a key residue in orientating DCA into the active site based on modeling studies. Thus the sulfhydryl group of GSH appears to play an important role, together with Arg^{175}, in the correct binding of DCA (14). Possibly, the sulfhydryl group of Cys^{16} may partially replace the sulfhydryl group of GSH to properly orientate binding of DCA in the wild type enzyme.

It is noteworthy that the amount of the GSH thiolate bound to hGSTZ1c-1c does not exceed 0.5 eq per active site under saturating GSH concentrations. The half-site GSH activation may be explained by a strong negative cooperativity among the two enzyme subunits, only one being competent to stabilize the GSH thiolate. This finding is reminiscent of the Theta class GSTT2-2 in which GSH is quantitatively deprotonated, but only half of the enzyme population is catalytically competent (24). The existence of a cooperative half-site behavior in the GST superfamily is a fascinating aspect of GST activity; it appears typical of the less phylogenetically evolved GSTs that utilize a serine residue to stabilize the GSH thiolate, whereas in the recently diverged GSTs, which use a tyrosine residue for GSH activation, it is transformed into a more sophisticated mechanism of regulation as observed in GSTP1-1 and most likely in the Alpha and Mu classes as well. GSTP1-1 is able to bind and activate GSH on both subunits but uses negative cooperativity to gain self-preservation when the first subunit is modified by chemical or physical factors (25). In addition, this enzyme shows a temperature-dependent homotropic regulation of substrate binding, which compensates for the deleterious effects of very high or low temperatures on GSH binding. In this enzyme, one subunit displays a temperature-dependent affinity constant for GSH with a positive ΔH_{K1} value of about 50 kJ/mol, whereas binding of GSH to the second subunit is quite temperature-independent with a ΔH_{K2} value approaching zero (26).

In GSTZ1c-1c, binding of GSH to the first subunit shows an apparent enthalpy of 40 kJ/mol, a value similar to that found for GSTP1-1, but a clear difference is observed in the modulation of the second subunit. In the Zeta class GST the second subunit becomes silent; it is not able to activate GSH, and this may reflect an inability to bind GSH. This primitive cooperativity could be the cost for a gain of structural stability of the enzyme, whereas in GSTP1-1, it seems to be evolved toward a

mechanism that minimizes the effect of strong inhibitors or temperature changes by preserving the catalytic efficiency of this enzyme.

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Binding and Kinetic Mechanisms of the Zeta Class Glutathione Transferase

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