Role of the Glutamyl α-Carboxylate of the Substrate Glutathione in the Catalytic Mechanism of Human Glutathione Transferase A1-1[†]

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ABSTRACT: The Glu α-carboxylate of glutathione contributes to the catalytic function of the glutathione transferases. The catalytic efficiency of human glutathione transferase A1-1 (GST A1-1) in the conjugation reaction with 1-chloro-2,4-dinitrobenzene is reduced 15 000-fold if the decarboxylated analogue of glutathione, dGSH (GABA-Cys-Gly), is used as an alternative thiol substrate. The decrease is partially due to an inability of the enzyme to promote ionization of dGSH. The pK_a value of the thiol group of the natural substrate glutathione decreases from 9.2 to 6.7 upon binding to GST A1-1. However, the lack of the Glu α-carboxylate in dGSH raised the pK_a value of the thiol in the enzymatic reaction to that of the nonenzymatic reaction. Furthermore, K_M^{dGSH} was 100-fold higher than K_M^{GSH}. The active-site residue Thr68 forms a hydrogen bond to the Glu α-carboxylate of glutathione. Introduction of a carboxylate into GST A1-1 by a T68E mutation increased the catalytic efficiency with dGSH 10-fold and reduced the pK_a value of the active site bound dGSH by approximately 1 pH unit. The altered pK_a value is consistent with a catalytic mechanism where the carboxylate contributes to ionization of the glutathione thiol group. With Δ^5-androstene-3,17-dione as substrate the efficiency of the enzyme is decreased 24 000-fold while with 4-nitrocinnamaldehyde (NCA) the decrease is less than 150-fold. In the latter reaction NCA accepts a proton and, unlike the other reactions studied, may not be dependent on the Glu α-carboxylate for deprotonation of the thiol group. An additional function of the Glu α-carboxylate may be productive orientation of glutathione within the active site.

The glutathione transferases (GSTs)[1] are a family of detoxication enzymes, which play an important role in the cellular defense against a variety of electrophiles. There are both soluble and membrane-bound GSTs, which share the same enzymatic activity where the tripeptide (γ-Glu-Cys-Gly) glutathione (GSH) is used as a substrate to disarm the toxic electrophiles. Generally, the enzymes link the nucleophilic thiol group of glutathione to the electrophilic center of the second substrate.

The soluble GSTs are divided into several classes (*1*) essentially on the basis of their primary structure where the overall sequence identity within a class exceeds 50% (*2*). The mammalian classes are named alpha, kappa, mu, omega, pi, sigma, theta, and zeta (*1, 3*−*8*). Even though there is a great divergence in primary structure among the classes, the overall 3-dimensional fold has been conserved during evolution (*5, 9*−*11*). The soluble GSTs have a dimeric structure with one active site in each subunit. The active site has two substrate-binding pockets: the hydrophobic substrate-binding site (H-site), which binds the electrophile, and the glutathione-binding site (G-site).

The GSTs have a conserved tyrosine or a serine residue in the N-terminal part of the enzyme, which have been shown to be of fundamental importance in the catalytic mechanism (*12*−*17*). The hydroxyl group is considered to promote the reactivity of the thiol group of enzyme-bound glutathione. Apart from this tyrosine or serine residue, there are no conserved active site residues that have the same impact on catalytic activity of the different GST classes. However, it has been shown that the Glu α-carboxylate of the substrate glutathione is essential for catalysis to occur (*18*−*20*). An extensive investigation of the tolerance of alpha, mu, and pi class GSTs from rat to different modifications in the glutathione molecule demonstrated that the enzymes were generally more sensitive to alteration in the glutamyl part of the tripeptide than in the glycyl part. The decarboxyglutathione analogue, GABA-Cys-Gly (dGSH), did not give any detectable activity with rat GST A1-1, GST A3-3, GST M1-1, or GST M2-2 using 1-chloro-2,4-dinitrobenzene (CDNB) as the electrophilic substrate (*18*). Thus, apart from the thiol group, the Glu α-carboxylate is also a crucial functional group of the peptide substrate.

Whereas the catalytically important tyrosine or serine residues of GSTs have been extensively studied (*12*−*17, 21*−

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[1] Abbreviations: AD, Δ^5-androstene-3,17-dione; Boc, *t*-butoxycarbonyl; Bzl, benzyl; CDNB, 1-chloro-2,4-dinitrobenzene; GABA, 4-aminobutyric acid; dGSH, decarboxyglutathione (GABA-Cys-Gly); GSH, glutathione; GST, glutathione transferase; G-site, glutathione-binding site; H-site, hydrophobic substrate-binding site; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; NCA, 4-nitrocinnamaldehyde; TEA, triethylamine; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TNB, 1,3,5-trinitrobenzene.

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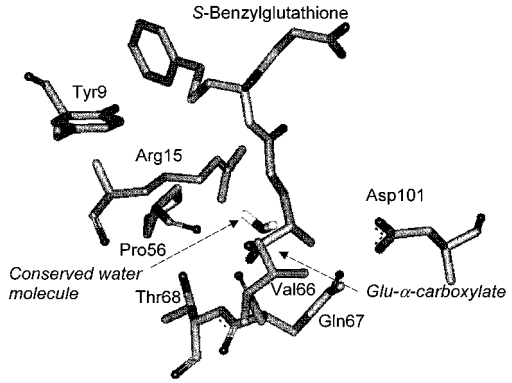


FIGURE 1: Part of the G-site in the structure of GST A1-1 in complex with *S*-benzylglutathione (*9*). The residues close to the Glu α-carboxylate of glutathione are shown.

38), the research conducted on the Glu α-carboxylate of the thiol substrate is limited. It has been suggested that the α-carboxylate functions as a proton acceptor during the ionization of glutathione (*39*) on the basis of an altered catalytic pH profile of a mutant of human GST A1-1 where the threonine residue, Thr68, which interacts with the α-carboxylate (*9*), was changed into a valine. The acidic limb of the pH profile was shifted more than 1 pH unit toward higher pH values.

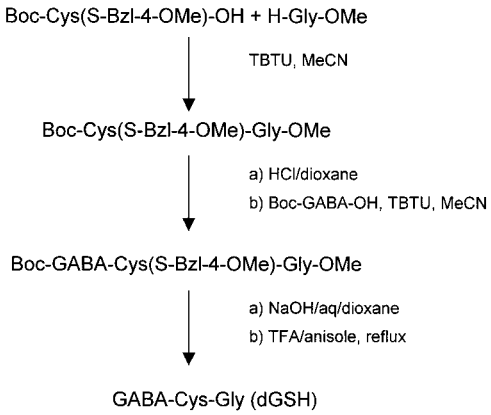
The aim of the present study was to further investigate the role of the Glu α-carboxylate of glutathione in the catalytic mechanism of the GSTs to clarify why the catalytic efficiency of the enzyme is so greatly affected by the deletion of the Glu α-carboxylate.

In crystal structures of human GSTs the Glu α-carboxylate of active-site-bound glutathione interacts with a hydroxyl group, either belonging to a threonine or a serine residue (*10*), and is hydrogen bonding to a water molecule that is conserved among different GST structures (Figure 1). In human GST A1-1, the residue interacting with the Glu α-carboxylate of glutathione is Thr68, which in the present study was mutated into an aspartate and a glutamate. Hence, a carboxylate was inserted into the position where the Glu α-carboxylate of glutathione is located upon binding to the G-site. Assuming that the carboxylate in position 68 could mimic the role of the Glu α-carboxylate of glutathione, the T68E mutant[2] would have higher activity toward dGSH than the wild-type enzyme. Indeed, the T68E mutation increased the activity toward dGSH 10-fold using CDNB as the electrophilic substrate.

To find the basis for the kinetic effects the conjugation reaction between glutathione and CDNB has been dissected into its microscopic rate constants. The rate of glutathione binding has been measured in various ways, but the rate of dGSH binding could not be determined since the various probes used to monitor binding did not give any detectable signals with dGSH. Furthermore, the pre-steady-state and steady-state kinetics of the conjugation reaction with CDNB were measured to determine the rate-limiting step with the two different thiols. It was revealed that the deletion of the Glu α-carboxylate from the thiol substrate changed the rate-limiting step for the wild-type enzyme, from product release to the chemical step. For mutant T68E the rate-limiting step was the chemical step with both nucleophiles. The pH dependences of the different kinetic parameters show that the pK_a value of the active-site bound thiol group is greatly affected by the modification of the thiol substrate.

[2] Names of GST A1-1 mutants: T68E, threonine 68 mutated to glutamic acid; T68D, threonine 68 mutated to aspartic acid; Y9F, tyrosine mutated to phenylalanine.

Scheme 1



MATERIALS AND METHODS

Synthesis of the Thiol Substrate Peptide GABA-Cys-Gly (dGSH). The tripeptide GABA-Cys-Gly (dGSH) was obtained by chemical synthesis in solution from readily accessible protected precursors (Scheme 1). Supporting Information is available in the World Wide Web edition.

Thus, Boc-Cys(*S*-Bzl-4-OMe)-OH and H-Gly-OMe were condensed to the dipeptide Boc-Cys(*S*-Bzl-4-OMe)-Gly-OMe in excellent yield in dry acetonitrile using TBTU as coupling reagent. HCl smoothly accomplished the removal of the amine protection in dioxane in essentially quantitative yield, and the resulting H-Cys(*S*-Bzl-4-OMe)-Gly-OMe was coupled in high yield to Boc-GABA-OH. The protected tripeptide Boc-GABA-Cys(*S*-Bzl-4-OMe)-Gly-OMe was then converted quantitatively to Boc-GABA-Cys(*S*-Bzl-4-OMe)-Gly-OH by alkaline hydrolysis of the C-terminal ester function in aqueous dioxane using an equimolar amount of NaOH at ambient temperature. Finally, the remaining acid-labile protective groups were cleaved off in refluxing TFA under dry argon in the presence of excess anisole as scavenger. The crude deprotected tripeptide contained minor impurities as indicated by ^{1}H and ^{13}C NMR, HPLC, and amino acid analysis. For a more detailed description see the Supporting Information.

Construction, Expression, and Purification of T68 Mutants. Thr68 of human GST A1-1 was altered into Asp and Glu by site-directed mutagenesis using inverted PCR with *Pfu* DNA polymerase and the template pGNdeA1. pGNdeA1 is a pGEM-3Zf(+) vector (Promega Co., Madison, WI) with the cDNA encoding GST A1-1 inserted between the *Eco*RI and *Sal*I sites in the cloning cassette. Downstream of the *Eco*RI site of pGNdeA1, and as a part of the start codon of the cDNA, there is an *Nde*I site.

The mutants were identified by sequence analysis and the coding region was subcloned into pET21-a (Novagen, Inc., Madison, WI) using the restriction sites of *Nde*I and *Sal*I.

Expression and purification on HiTrap SP cation-exchange column (Amersham Pharmacia Biotech, Uppsala, Sweden) was conducted as previously described (*40*). Enzyme purity was >95% as judged from an SDS−PAGE gel (*41*). The

Y9F mutant was prepared according to a previously published protocol (*28*).

Steady-State Kinetics. Steady-state kinetics were studied for T68E, T68D, and wild-type GST A1-1 at varied concentrations of CDNB, dGSH, and glutathione in 0.1 M sodium phosphate buffer at pH 6.5. The Y9F mutant of GST A1-1 was also tested for activity toward dGSH at pH 7.0 and 8.25 using substrate concentrations of 1.6 mM CDNB and 8.6 mM dGSH.

The pH profiles of the steady-state parameters for T68E and wild-type GST A1-1 were measured under pseudo-first-order conditions, where the concentration of CDNB was held constant at both 5 and 30 °C using either glutathione or dGSH as the varied substrate. At pH values above 8.0, 0.1 M ethanolamine was used; otherwise 0.1 M sodium phosphate was used as buffer.

Steady-state kinetics were also measured using Δ^5-androstene-3,17-dione (AD) and 4-nitrocinnamaldehyde (NCA) as the electrophilic substrate. Both electrophilic substrates and glutathione or dGSH were varied. Measurements with AD were conducted as described by Pettersson and Mannervik (*42*) at pH 8.0 in 50 mM Tris-HCl buffer. Measurements with NCA were done as described by Widersten et al. (*39*) at pH 6.5 in 0.1 M sodium phosphate.

The k_{cat} and K_M values were obtained by fitting the Michaelis−Menten equation to the data using GraphPad Prism whereas k_{cat}/K_M was determined using a first-degree rational function. The steady-state kinetic parameters for AD were determined by fitting eq 1 to the data using the SIMFIT program package (*43*):

$$v = V_{max}[GSH][AD]/(K_M^{AD}K_S^{GSH} + K_M^{AD}[GSH] + K_M^{GSH}[AD] + [GSH][AD]) \quad (1)$$

where V_{max} is $k_{cat}[E]_{tot}$.

Determination of pK_a of the Hydroxyl Group of Tyr9. The pK_a value of Tyr9 in the active site of T68E and T68D was obtained by difference spectroscopy as earlier described (*44*). The ionization of Tyr9 was monitored by the increase in absorbance at 298−301 and 250−255 nm caused by the ionization of Tyr9 (*23*, *28*, *29*).

Stopped-Flow Experiments. Glutathione binding at pH 7.5 and 8.0 was monitored by the increase in absorbance at 239 nm caused by the deprotonation of glutathione in the active site of the enzyme (*44*, *45*). The measurements were conducted as previously described (*44*). Aliquots of 75−100 μL from each syringe were rapidly mixed, and the enzyme subunit concentration ranged between 30 and 40 μM.

The individual rate constants for σ-complex formation between 1,3,5-trinitrobenzene (TNB) and glutathione (*46*) were also determined using the stopped-flow technique. The experiments were conducted at 5 °C in 0.1 mM sodium phosphate buffer at different pH values. A solution of 10−20 μM GST A1-1 or T68E (final subunit concentration) was incubated with a constant concentration of 0.8 mM TNB (final concentration) and rapidly mixed with glutathione (0.1−10 mM) or dGSH (up to 20 mM) of different concentrations. The preequilibrium formation of the σ-complex was monitored by the change in absorbance at 455 nm. The observed rate constants (k_{obs}) and the amplitudes were obtained by fitting a single-exponential function to the experimental traces using the software supplied with the stopped-flow apparatus (from Applied Photophysics Limited, Leatherhead, U.K.).

The preequilibrium kinetics of the S_NAr reaction between CDNB and glutathione were measured essentially as described above for TNB. The formation of the product was monitored at 340 nm, and a single-exponential function combined with a linear function was used to determine both k_{obs} for the pre-steady-state formation of GS•CDNB conjugate and the initial rate of the steady-state phase. No burst was seen for T68E and a linear function was used to determine the steady-state rate.

RESULTS

Steady-State Kinetics. The deletion of the Glu α-carboxylate from glutathione strongly reduced the rate of the reactions catalyzed by GST A1-1. In contrast, the rates of the nonenzymatic reactions of the alternative thiols with the electrophiles used were not significantly different (data not shown). The apparent k_{cat}/K_M^{thiol} value decreased 82 000-fold for CDNB, 24 000-fold for AD, and 150-fold for NCA (Table 1). With CDNB and NCA these values overestimate the decreases since the K_M values of CDNB and NCA were increased. This could not be compensated for in the measurements due to the limited solubilities of the substrates. k_{cat}/K_M^{dGSH} measured with CDNB could be estimated to approximately 0.03 s^{-1} mM^{-1}, and hence, the decrease in k_{cat}/K_M^{thiol} with CDNB was about 15 000-fold. The k_{cat} value decreased approximately 130 times when the 10-fold increased K_M^{CDNB} value was taken into account. However, as shown below, the rate-limiting step of the reaction was altered from product release to the chemical step. The rate constant of the chemical step was therefore reduced at least 500-fold at pH 6.5 (assuming that k_{cat}^{dGSH} is a measure of the chemical step). K_M^{thiol} was increased 100-fold using CDNB as electrophilic substrate.

The K_M^{NCA} value could not be determined in the measurements with dGSH, and therefore, the apparent k_{cat}/K_M^{thiol} and k_{cat} values could not be compensated for with respect to the change in saturation of the enzyme with NCA.

The attempt to increase the activity of human GST A1-1 with dGSH by introduction of a carboxylate in the form of a Glu or Asp residue at position Thr68 was successful. T68E displays a catalytic efficiency that is 1 order of magnitude higher, and T68D 3-fold higher, than that of wild-type GST A1-1 with CDNB (Table 1). Since T68E displayed higher activity than T68D with dGSH, the former mutant was used for a more thorough characterization.

T68E also displayed a higher k_{cat}/K_M^{dGSH} in the isomerization reaction with AD (Table 1). However, the mutation made no difference in the k_{cat} and K_M^{dGSH} values for the Michael addition of dGSH to NCA.

The Y9F mutant of GST A1-1 did not catalyze the conjugation reaction between dGSH and CDNB at any measurable rate.

To understand the dramatic differences in steady-state kinetics with dGSH as compared to glutathione, a thorough characterization of wild-type GST A1-1 and the T68E mutant was undertaken. Preequilibrium kinetics of thiol binding was investigated, and the reaction mechanism with CDNB was elucidated using pre-steady-state kinetics.

Table 1: Kinetic Constants of the Wild-Type GST A1-1 and the Mutants T68E and T68D at 30 °C Measured with the Three Different Electrophiles CDNB, AD, and NCA, Using the Two Thiol Substrates Glutathione and dGSH[a]

	thiol substrate			electrophile substrate		
enzyme	k_{cat} (s^{-1})	K_M (mM^{-1})	k_{cat}/K_M ($s^{-1}\,mM^{-1}$)	k_{cat} (s^{-1})	K_M (mM^{-1})	k_{cat}/K_M ($s^{-1}\,mM^{-1}$)
	Varied [GSH] (0.1−10 mM) + 1.6 mM CDNB			Varied [CDNB] (0.25−2.0 mM) + 5 mM GSH		
wild type	53 ± 2	0.12 ± 0.02	470 ± 40	88 ± 3[b]	0.56 ± 0.04[b]	160 ± 8.0[b]
T68E	62 ± 2	2.2 ± 0.2	28 ± 1	120 ± 30	3.4 ± 1.0	36 ± 4
T68D			0.35 ± 0.05			1.1 ± 0.1
	Varied [dGSH] (0.5−8.0 mM) + 1.6 mM CDNB			Varied [CDNB] (0.25−2.0 mM) + 5 mM dGSH		
wild type	0.076 ± 0.018	13 ± 4	0.0057 ± 0.0006	0.18 ± 0.06	7 ± 3	0.026 ± 0.002
T68E	0.83 ± 0.07	12 ± 2	0.066 ± 0.003	1.0 ± 0.2	5 ±1	0.21 ± 0.02
T68D	0.22 ± 0.04	12 ± 3	0.019 ± 0.002	nd	nd	nd
	Varied [GSH] (0.025−6.0 mM) + Varied [AD] (0.010−0.40 mM)[c]					
wild type[d]	29 ± 1	0.16 ± 0.01	180 ± 10	29 ± 1	0.058 ± 0.004	500 ± 40
T68E	22 ± 2	2.3 ± 0.5	10 ± 0.2	22 ± 2	0.18 ± 0.03	120 ± 20
	Varied [dGSH] (0.50−10 mM) + 0.20 or 0.40 mM AD			Varied [AD] (0.050−0.40 mM) + 6.2 mM dGSH		
wild type			0.0076 ± 0.0007	0.21 ± 0.03	0.060 ± 0.013	3.5 ± 1.5
T68E	0.81 ± 0.15	9.4 ± 2.7	0.087 ± 0.009	0.22 ± 0.02	0.21 ± 0.04	1.1 ± 0.1
	Varied [GSH] (0.10−20 mM) + 0.40 mM NCA			Varied [NCA] (0.05−0.40 mM) + 5 or 20 mM GSH		
wild type	0.39 ± 0.01	0.267 ± 0.017	1.45 ± 0.08	0.70 ± 0.06	0.29 ± 0.05	2.4 ± 0.2
T68E	0.59 ± 0.03	7.0 ± 1.0	0.084 ± 0.007	0.27 ± 0.08	1.5 ± 0.5	0.17 ± 0.01
	Varied [dGSH] (0.4−18 mM) + 0.40 mM NCA			Varied [NCA] (0.025−0.40 mM) + 9.3 mM dGSH		
wild type	0.038 ± 0.003	3.7 ± 0.7	0.010 ± 0.001			0.22 ± 0.04
T68E	0.031 ± 0.002	3.1 ± 0.5	0.010 ± 0.001			0.083.± 0.016

[a] Parameter values and their standard deviation were obtained by nonlinear regression analysis. [b] From Widersten et al. (*51*). [c] Parameters were obtained by fitting eq 1 to the data. [d] From Pettersson & Mannervik (*42*). nd = not determined. Data could not be obtained.

Scheme 2

$$E + GSH \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} E{\cdot}GSH \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} E{\cdot}GS^- + H^+$$

Determination of pK_a of Tyr9 and Active-Site Bound Glutathione. The pK_a value of Tyr9 was measured for T68E since Tyr9 has previously been shown to be important for lowering the pK_a value of the glutathione thiol group (*28*). The value was determined to 8.3 ± 0.1 and, hence, was essentially unchanged by the T68E mutation. The wild-type value is 8.1−8.2 (*28*, *44*). An attempt was made to measure the pK_a of glutathione bound to the active site of T68E, but data could not be collected at saturating concentrations of glutathione since there was a very large contribution from free glutathione thiolate to the spectrum. Likewise, the pK_a of active-site bound dGSH could not be measured.

Preequilibrium Binding of Glutathione and dGSH. The preequilibrium kinetics of thiol-substrate binding was studied to elucidate how binding and deprotonation of the thiol substrate were affected by the lack of the Glu α-carboxylate.

Previously, the microscopic rate constants for binding of the natural glutathione to wild-type GST A1-1 have been investigated (*44*). The data were supplemented in the present study to get a better view of the pH dependence of these rate constants. The rate constants are defined in Scheme 2 in agreement with the numbering of the kinetic constants in a previously published scheme that includes a rapid equilibrium between two enzyme conformations as a first step (*44*).

The observed rate constants, obtained from following thiolate formation at 239 nm at pH 7.0 or 7.5, as a function of glutathione concentration are presented in Figure 2. The maximum k_{obs} obtained from nonlinear regression analysis equals the rate constant, k_3, for glutathione deprotonation (*44*) within the active site of GST A1-1 (Scheme 2, Figure 2). Due to the detection limit of the stopped-flow apparatus

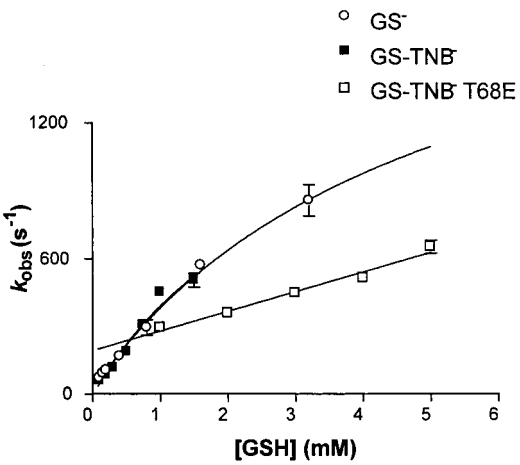


FIGURE 2: Observed rate constants for glutathione binding and deprotonation as function of [GSH]. The data were obtained by following the absorbance at 239 nm, monitoring glutathione deprotonation (○), as well as the absorbance at 455 nm, monitoring the formation of the σ-complex between glutathione and TNB in the active site of wild-type GST A1-1 at pH 7.5 (■) and in the active site of T68E at pH 7.0 (□). For wild-type GST A1-1, the initial slope of the curve is equal to the on-rate constant for glutathione binding, k_2 (Scheme 2), and the maximum observed rate constant obtained at infinite concentration of glutathione is the deprotonation rate constant, k_3 (Scheme 2). T68E displays a much higher off-rate constant (the intercept with the *y* axis) and a higher value of $K_{0.5}$, which equals the concentration of glutathione giving the half-maximum observed rate constant, than the wild-type enzyme. Therefore the maximum k_{obs} value for T68E could not be determined, and consequently, the rate constant for glutathione deprotonation could only be estimated. The graph shows that the rate constant is ≥1000 s^{-1}.

(dead-time 1 ms) it was difficult to obtain accurate values of the observed rate constants at high glutathione concentrations. However, the data in the present study support the earlier determined value for k_3 of at least 1000 s^{-1} at 5 °C (*44*).

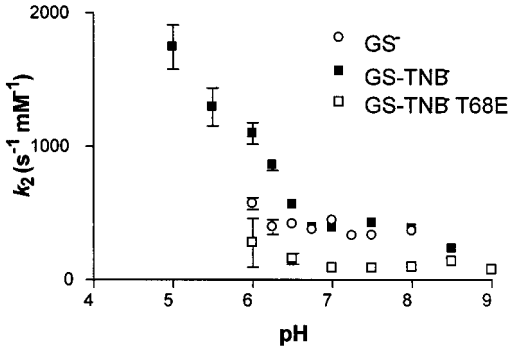


FIGURE 3: Rate constant for glutathione binding, k_2 (Schemes 2 and 3), as a function of pH. Data were obtained by monitoring glutathione ionization (○) as well as formation of the GS−TNB$^-$ complex in the active site of GST A1-1 (■) and of mutant T68E (□). For T68E only a minimum value of k_2 could be obtained since the glutathione binding rate could not be measured in any other way than by following the σ-complex formation between TNB and glutathione where the initial slope is a function of several rate constants: $k_2k_3/(k_3 + k_{-2})$ (Scheme 3).

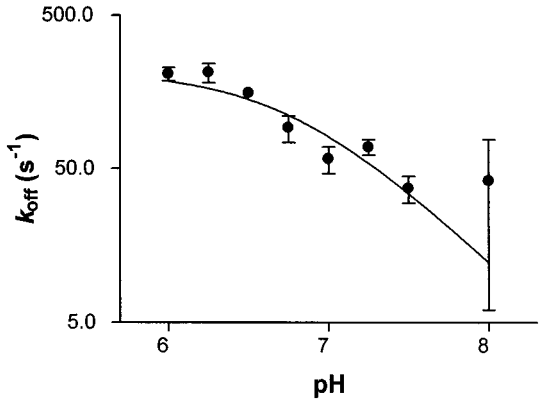


FIGURE 4: Off-rate constant for the binding of glutathione to GST A1-1 as a function of pH. Glutathione thiolate formation was monitored at 239 nm at different pH values, and eq 2 was fitted to k_{off} versus [H$^+$]. The best-fit curve corresponds to a pK_a value of 6.8, which reflects the ionization of the glutathione thiol group.

The association-rate constant for glutathione binding, k_2, is obtained from the initial slope of the hyperbolic curve obtained of k_{obs} on [GSH] (Figure 2). The value was determined to 350−450 s^{-1} mM^{-1} and does not seem to vary within the pH interval of 6.3 and 8.0 (Figure 3). A slightly higher value of 570 ± 40 s^{-1} mM^{-1} was obtained at pH 6.0 indicating that the rate might increase at low pH values.

The off-rate constant, obtained from the analysis of the k_{obs} of glutathione deprotonation, was earlier described as being a combination between the release of GS$^-$ from the active site and the protonation of the thiolate anion within the active site (*44*). However, since the concentration of glutathione is much higher than that of GS$^-$ at the pH values studied, it is reasonable to assume that the contribution of GS$^-$ release to the off-rate is negligible as compared to protonation of GS$^-$ in the enzyme active site. The off-rate is then described by a function of the proton concentration (Figure 4), which can be simplified to eq 2.

$$k_{off}^{GSH} = k_{-2}[H^+]/((k_3 + k_{-2})/k_{-3} + [H^+]) \qquad (2)$$

The initial slope of the pH dependence of k_{off}^{GSH}, $k_{-3}(k_{-2}/k_{-2} + k_3)$, was determined as approximately 10^9 s^{-1} M^{-1}. The maximum, or true glutathione off-rate constant, k_{-2} may itself vary slightly with pH but is on the order of 100 s^{-1} as previously determined by measuring the fluorescence quenching caused by glutathione binding to GST A1-1 (*44*). The deprotonation rate constant, k_3, is approximately 1000 s^{-1}, and hence, the protonation rate constant, k_{-3}, is of the order of 10^{10} s^{-1} M^{-1}. The concentration where half the maximum k_{obs} is obtained, $(k_3 + k_{-2})/k_{-3}$, can be simplified to k_3/k_{-3} since k_3 is larger than k_{-2} (Table 2). k_3/k_{-3} is also the equilibrium constant for ionization of active-site bound glutathione. The pK_a value thus was 6.8, which is similar to the ionization constant of the glutathione thiol, 6.7 ± 0.1, determined previously by difference spectroscopy (*44*).

Table 2: Rate Constants of the CDNB Reaction (Scheme 4) at pH 6.5 and 5 °C

rate const	wild type	T68E
k_2	4.2×10^5 s^{-1} M^{-1}	$\geq 1.6 \times 10^5$ s^{-1} M^{-1}
k_{-2}	70 s^{-1}	a
k_3	1000−2000 s^{-1}	>1000 s^{-1}
k_{-3}	10^{10} s^{-1} M^{-1}	a
k_4	40 s^{-1}	10 s^{-1}
k_5	8 s^{-1}	>10 s^{-1}

[a] Data could not be determined.

Scheme 3

$$\text{E·TNB} + \text{GSH} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \text{E·GSH·TNB} \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} \text{E·GS}^-\text{·TNB} \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} \text{E·GS-TNB}^-$$

The kinetics of glutathione binding could not be determined for the T68E mutant. There were no changes in the fluorescence of the enzyme, and the background absorbance of free GS$^-$ was too high, at glutathione concentrations that saturate T68E, to detect the small contribution of GS$^-$ formed in the enzyme active site.

Furthermore, no change in absorbance was observed at 239 nm upon rapidly mixing dGSH with either the wild-type enzyme or with T68E. Hence, this method could not be used for measuring binding and deprotonation of dGSH in the active site of the two variant enzymes.

Preequilibrium Formation of the σ-Complex in the Active Site. Instead of using the increase in absorbance at 239 nm as a direct measure of thiol deprotonation, the formation of the σ-complex between TNB and the thiol was used as a probe for thiolate formation. TNB is a more reactive electrophile than CDNB due to the additional nitro group in the *ortho* position in the aromatic ring. However, since TNB does not possess a good leaving group, the formation of the σ-complex, GS−TNB$^-$, is reversible and an equilibrium is reached (Scheme 3).

When glutathione is rapidly mixed with the binary TNB·GST A1-1 complex, the experimental trace is, within the experimental error, indistinguishable from the trace obtained when directly monitoring the formation of the thiolate within the active site of GST A1-1 (Figure 2). This implies that the σ-complex formation is much faster than the deprotonation of the thiol ($k_4 \gg k_3$). The expression for the apparent rate constant for binding therefore equals the equation describing the ionization of glutathione.

$$k_{obs}^{GS-TNB^-} = k_{off}^{GS-TNB^-} + k_3[GSH]/(K_{0.5} + [GSH]) \qquad (3)$$

The deprotonation rate constant, k_3, was 1000−2000 s^{-1} at

Scheme 4

$$\text{E-CDNB} + \text{GSH} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \text{E-CDNB-GSH} \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} \text{E-CDNB-GS}^- \xrightarrow{k_4} \text{E-GS-DNB} \underset{k_{-5}}{\overset{k_5}{\rightleftarrows}} \text{E} + \text{GS-DNB}$$

5 °C throughout the pH interval investigated. The errors are large, but the data agree well with the deprotonation rates given by the direct measurements of glutathione binding.

The initial slope of the hyperbolic dependence (eq 3) is described by $k_3/K_{0.5}^{GSH}$ where $K_{0.5}^{GSH}$ equals $(k_3 + k_{-2})/k_2$. But k_3 is larger than k_{-2} and therefore the pH profile of the initial slope (Figure 3) mirrors the glutathione-binding on-rate. For wild-type GST A1-1, the data correlate well with the binding-rate constants determined by measuring fluorescence quenching (*44*) and the ionization of the thiolate at pH values between 6.5 and 8.0 (Figure 3). However, at pH values below 6.5 the rate constant k_2 increases. A cause of this could be a more open active site at low pH values. As previously discussed (*44*) the relatively low rate constant for glutathione binding is probably due to different conformations adopted by the C-terminus of GST A1-1 of which certain conformations block the access to the active site. At low pH values the conformation of the C-terminus might be shifted toward structures that give a more open active site increasing its accessibility for glutathione. A similar effect on the on-rate constant at low pH values has been observed for rat GST T2-2 (*47*).

The deprotonation rate constant of glutathione, k_3 (Scheme 3, eq 3), in the active site of T68E could not be obtained accurately from the experiments involving TNB since the rates were too large at saturating concentrations of glutathione. However, the highest observed rate constant determined was 650 s^{-1}, and since no curvature was seen in the plot of k_{obs} versus [GSH] (Figure 2), the maximum observed rate constant is at least twice as high. Hence, the value for k_3 for T68E was estimated as equal to or higher than the corresponding value for the wild-type enzyme.

For T68E the expression of the initial slope, $k_2k_3/(k_3 + k_{-2})$, cannot be simplified to just k_2, since k_{-2} is probably greater in the mutant due to the repulsion between the carboxylate of Glu68 and the Glu α-carboxylate of glutathione. Thus, the slope of the plot of k_{obs} against [GSH] (Figures 2 and 3) is only a minimum value of the glutathione binding rate constant, k_2.

No increase in absorbance at 455 nm was obtained when mixing the TNB−enzyme complex with dGSH. Hence, the formation of the σ-complex between TNB and dGSH could neither be used to determine the rate of dGSH binding to the two variant enzymes nor to estimate the rate of deprotonation of dGSH. These results imply that dGS^- does not accumulate within the active site. The highest concentration of dGSH used in the study was 20 mM, which is about twice the K_m value of both wild-type GST A1-1 and T68E. Therefore, the lack of signal is probably not due to a low degree of saturation, with regard to the rate-limiting step.

Pre-Steady-State Kinetics of the Conjugation Reaction between CDNB and Glutathione. The pre-steady-state kinetics of the conjugation reaction between CDNB and glutathione were studied to determine the rate-limiting step of the catalyzed reaction. The different rate constants for the conjugation reaction between glutathione and CDNB are defined in Scheme 4.

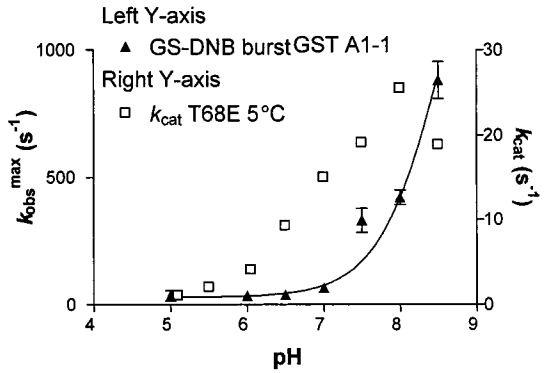


FIGURE 5: pH dependence of the rate constant for the chemical step, k_4 (Scheme 4). For wild-type GST A1-1 (▲, left *y* axis) the rate constant is determined from the maximum k_{obs} for the burst of product formation from CDNB and glutathione bound to the enzyme (eq 4). T68E (□, right *y* axis) does not display a pre-steady-state burst. The chemical step is instead reflected by k_{cat} obtained from the steady-state kinetics of the CDNB reaction.

The scheme is drawn for the stopped-flow experiment where the enzyme was preincubated with the electrophile before being mixed with glutathione.

A pre-steady-state burst of product formation was obtained for wild-type GST A1-1 when the conjugation reaction between glutathione and CDNB was monitored at 340 nm. At pH values equal to or below 8.0, the maximum k_{obs} of the burst is substantially lower than the deprotonation rate constant for glutathione, k_3 (determined from the preequilibrium binding of glutathione to GST A1-1). The fast deprotonation step can therefore be excluded in the derivation of the rate equation for the k_{obs} of the burst. The observed rate constant will then be described by

$$k_{obs}^{burst} = k_4[\text{GSH}]/(K_{0.5} + [\text{GSH}]) \qquad (4)$$

The maximum value of the observed burst rate constant, at infinite glutathione concentration, is thus the rate constant for the chemical step, k_4 (Scheme 4). This rate constant is 40−65 s^{-1} at pH values between 6.0 and 7.0, but at pH 7.0 the rate for the chemical step starts to increase significantly (Figure 5). The apparent pK_a value of the pH dependence of k_4 is 8.6 ± 0.5.

No pre-steady-state burst of product formation was obtained with T68E. Hence, the mutation has caused a shift in the rate-limiting step of the reaction, which needs to be taken into account when the steady-state kinetics are interpreted.

Steady-State Kinetics of the Conjugation Reaction between CDNB and Glutathione. The pre-steady-state burst of product formation proves that the rate-determining step follows the chemical step of the CDNB reaction, suggesting it to be product release, k_5 (Scheme 4). The steady-state rate constant k_{cat} is approximately 10 s^{-1} at pH 6.5 and 5 °C, which is a reasonable value for the release of the GSH•CDNB conjugate from the active site, considering the additional interactions formed between the enzyme and the conjugate as compared to unconjugated glutathione. Glutathione displays a somewhat larger off-rate constant of 70 s^{-1}, at pH 6.5.

The pH profiles of the steady-state kinetic parameters at 30 °C follow the pH profiles at 5 °C for both the wild-type

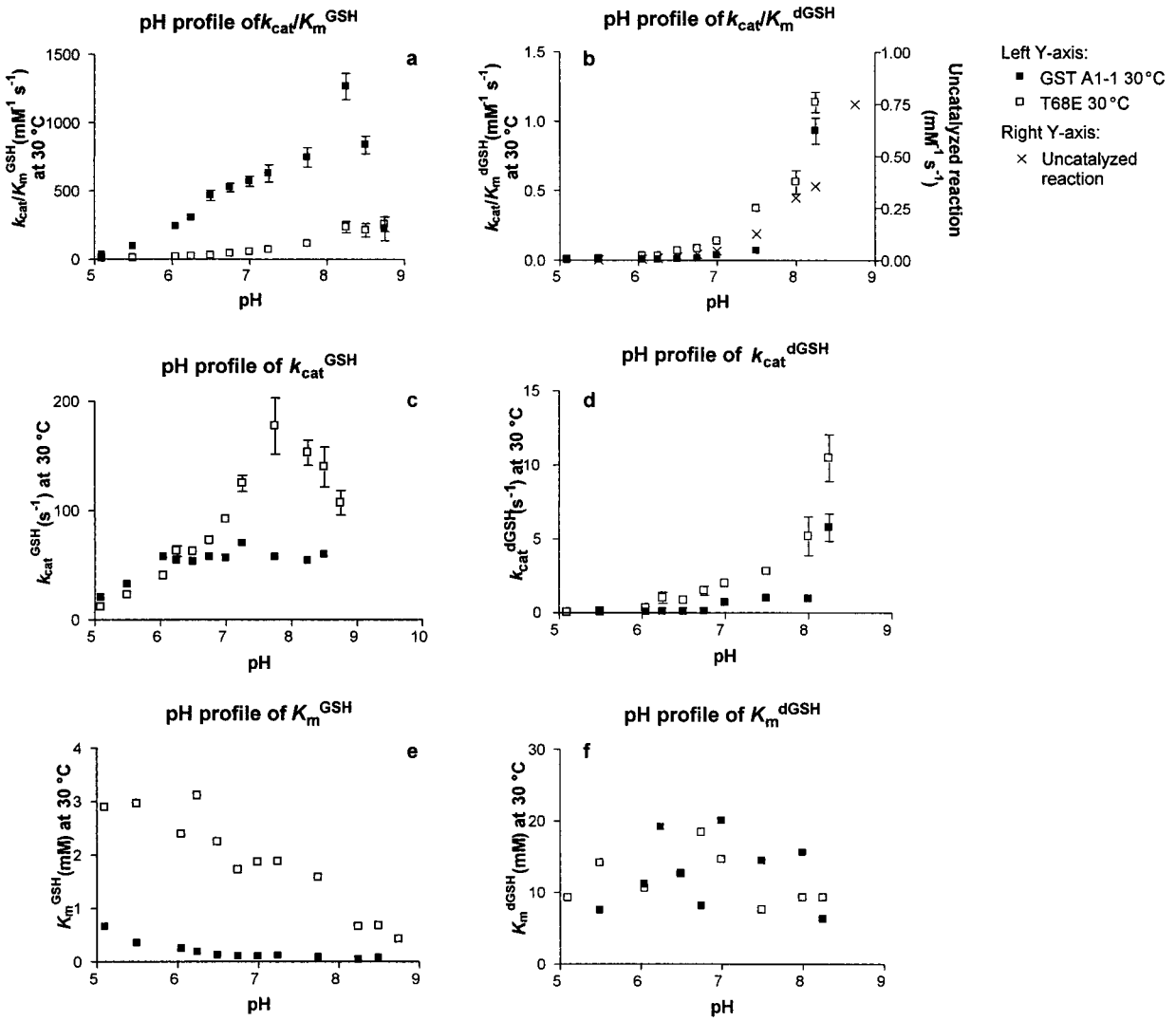


FIGURE 6: pH profiles of the steady-state parameters obtained for wild-type GST A1-1 (■) and mutant T68E (□) with glutathione and dGSH at 30 °C. Data were obtained at constant concentrations of CDNB (1.6 mM) and at varying concentrations of glutathione (left panels) or of dGSH (right panels). The first-order rate constant for the nonenzymatic reaction between dGSH and CDNB (×) is plotted against pH in the right *y* axis in panel b together with the pH profiles of k_{cat}/K_M^{dGSH}. The pH profile of the nonenzymatic reactions has only a slightly higher pK_a value than those of k_{cat}/K_M^{dGSH} for the wild-type enzyme and the T68E mutant.

enzyme and T68E. Therefore, it is assumed that the rate-limiting step is the same at the two temperatures.

At pH values above 6.5, the k_{cat} values obtained for T68E exceed those of the wild-type enzyme. No pre-steady-state burst of product formation was obtained with T68E showing that the mutation has caused a shift in the rate-determining step, from product release to the chemical step. Therefore, for T68E, the pH profile of k_{cat} reflects titrations in the enzyme−substrate complex (*48*). k_{cat} of T68E displays two apparent pK_a values (Figures 5 and 6). The first one, at pH 7.4 ± 0.2, probably mirrors the pK_a value of the active site bound glutathione (Figure 6). The second pK_a value is approximately 8.2 ± 2 and may reflect the deprotonation of Tyr9.

Pre-Steady-State Kinetics of the Conjugation Reaction between CDNB and dGSH. No pre-steady-state burst of product formation between dGSH and CDNB was obtained for either wild-type GST A1-1 or T68E. Hence, the lack of the Glu α-carboxylate in the thiol substrate shifts the rate-limiting step from product release to either the deprotonation of the dGSH thiol group or to the chemical step for the wild-type enzyme. For T68E the rate-limiting step either remains the chemical step or is shifted to deprotonation of dGSH.

Steady-State Kinetics of the Conjugation Reaction between CDNB and dGSH. Since the pre-steady-state data showed that the rate-limiting step of the conjugation reaction had changed due to the lack of the Glu α-carboxylate from the thiol substrate, the pH dependences of the steady-state parameters were investigated to see how they were affected.

The steady-state kinetic parameters for the conjugation reaction between CDNB and dGSH were measured at 5 and 30 °C at various pH values. The pH profiles of the different kinetic parameters for both the wild-type enzyme and T68E were similar at both temperatures, and consequently, it was assumed that the rate-limiting step is the same at both temperatures.

Compared to the reaction with glutathione, the K_M^{thiol} value is about 100-fold increased for the wild-type and 5-fold increased for T68E at pH 6.5 using CDNB as electrophile (Table 1). K_M^{dGSH} does not vary with pH (within experimental error) for any of the two enzyme variants (Figure 6).

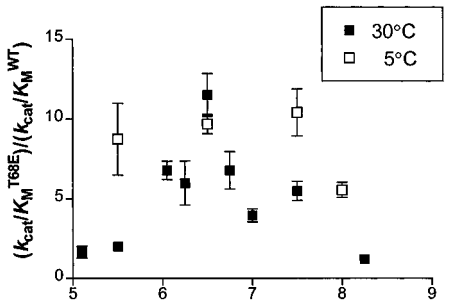


FIGURE 7: Ratio between k_{cat}/K_M^{dGSH} for T68E and wild-type GST A1-1 as a function of pH in the reaction of dGSH with CDNB. The values of the catalytic efficiency obtained for T68E at 5 °C (□) and at 30 °C (■) were divided by the respective wild-type values.

Scheme 5

$$\text{E•dGSH •CDNB} \xrightarrow{k_{cat}} \text{E•dGS-DNB}$$

In contrast, k_{cat}/K_M^{dGSH} displayed a great increase at pH values above 6.5 for T68E and above 7.5 for the wild-type enzyme. There were difficulties in determining accurate pK_a values of the k_{cat}/K_M^{dGSH} profiles using nonlinear regression analysis. However, simulation of the curves shows that T68E has a pK_a value between 8.0 and 8.2, whereas the wild-type value is similar to the pK_a for the nonenzymatic reaction, somewhere between 8.8 and 9.2.

Since K_M does not display a pH dependence, the pH profile of k_{cat}^{dGSH} is similar to that of k_{cat}/K_M^{dGSH} (Figure 6) and pK_a values obtained from simulations are the same.

The resemblance between the pH profiles of k_{cat} and k_{cat}/K_M^{dGSH} implies that binding of dGSH to the enzyme•CDNB complex does not alter the ionization behavior of the catalytically important groups. Hence, the ionization constants of free dGSH and the enzyme•CDNB complex affect the catalytic step in which the active-site bound substrates are converted to products (Scheme 5). This is further supported by the resemblance of the two profiles with the pH profile of the uncatalyzed reaction where the pK_a value of free dGSH is reflected.

If the ratios of the k_{cat}/K_M^{dGSH} value for T68E to the wild-type value at different pH values are compared, it is found that the greatest difference between the two is around pH 6.5−7.5 where T68E is a 10-fold better catalyst with dGSH than is the wild-type enzyme (Figure 7).

DISCUSSION

The Glu α-carboxylate of glutathione is fundamental to the catalytic effect of the GSTs (*18*−*20*). As shown in this study, the deletion of this carboxylate caused a great loss in the catalytic efficiency of wild-type GST A1-1 enzyme with all three substrates used in the present investigation, CDNB, AD, and NCA (Table 1).

The carboxylate introduced in position 68 of the enzyme could partially restore the activity lost by the deletion of the α-carboxylate. At pH 6.5, k_{cat}/K_M^{dGSH} of the T68E mutant is about 10-fold higher than the wild-type value in the reaction with CDNB (Figure 7).

This study has been aimed at investigating the cause and mechanism behind this loss of activity and the reason the T68E mutation partially restores the lost activity.

The pre-steady-state and the steady-state kinetic investigations of the GST A1-1 catalyzed conjugation of glutathione to CDNB suggested the enzyme to be rate-limited by product release. k_{cat} is 10 s^{-1} at pH 6.5 and 4 °C. This value agrees well with the dissociation-rate constant for the analogous GSH conjugate with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole that has been determined to 15 s^{-1} at 15 °C for the rat GST A1-1 (*38*).

The absence of a pre-steady-state burst of product formation in the catalyzed reaction between CDNB and dGSH shows that the lack of the Glu α-carboxylate shifts the rate-limiting step to the chemical or an earlier step. The rate of this was decreased more than 500-fold at pH 6.5 and 5 °C, as shown by a comparison between k_{cat}^{dGSH} and the rate constant of the chemical step with glutathione, k_4, obtained from the pre-steady-state kinetics.

Since the rate-limiting step is not the same for the catalyzed reaction with GSH and dGSH, the pH profiles of the two different k_{cat} values (Figure 6) reflect different underlying processes. With GSH, the pH dependence of product release is monitored whereas with dGSH the titration of catalytically important ionizable groups in the ternary E•dGSH•CDNB complex is measured (Scheme 5). The pH profiles of k_{cat}/K_M^{dGSH} and k_{cat}^{dGSH} both follow that of the nonenzymatic reaction (Figure 6). The similarity between the curves implies that lack of the Glu α-carboxylate abolishes the ability of the enzyme to lower the pK_a value of the thiol substrate. In agreement with a raised pK_a value of the steady-state parameters, no increase in absorbance at 239 nm was detected when mixing GST A1-1 with dGSH in the stopped-flow experiments. Additionally, no accumulation of *σ*-complex between dGSH and TNB was seen in the active sites.

The at least 500-fold decrease of the rate constant of the chemical step at pH 6.5 for the wild-type enzyme could be explained by the increased pK_a value of the active-site-bound thiol group. For the binary GST A1-1-glutathione complex the pK_a value is 6.7, as determined by difference spectroscopy (*44*). However, the apparent ionization constant of the ternary enzyme−glutathione−CDNB complex seems to be lower than 5.0, as reflected by the rate constant for the chemical step, k_4 (Figure 5). This parameter was measured between pH 5.0 and 8.5 and displays one pK_a value at 8.6 ± 0.5 which is too high to be associated with the titration of the thiol group, since the reaction is efficiently catalyzed at much lower pH values. It is unlikely that the protonated thiol form of glutathione reacts with the electrophile. Thus, there has to be at least one additional pK_a value below pH 5.0. There is no obvious explanation for the increase of k_4 at high pH values, but it could result from changes in the tertiary structure of GST A1-1 or reflect the titration of Tyr9, which has a pK_a value of 9.2 in the presence of glutathione (*28*). This issue was not further investigated since it was not in the scope of this study.

The pK_a value of the ternary enzyme−dGSH−CDNB complex is above pH 8.8 as judged from the pH profile of k_{cat}^{dGSH}. Thus, at pH 6.5 the concentration of dGS^- in the active site should be at least 100-fold lower than the corresponding concentration of GS^-, and this is probably the explanation for the decreased rate of the chemical step.

For mutant T68E the absence of a burst phase in the pre-steady-state kinetic measurements shows that the chemical

step presumably is rate determining with both GSH and with dGSH. Thus, the pH profiles of k_{cat} probably reflect the pK_a value of the active-site-bound thiol group, which was determined to 7.3 with glutathione and approximately 8.0−8.2 with dGSH. The ratio between k_{cat}^{GSH} and k_{cat}^{dGSH} for T68E, at 5 °C and pH 6.5, is approximately 80, and the raised pK_a value of the thiol group partially accounts for this loss of activity. If compared to the pH dependence of k_{cat}^{dGSH} for the wild-type enzyme, the pK_a value of the pH profile with dGSH is shifted about 1 pH unit to a lower value, which may explain the 10-fold higher k_{cat} value for T68E at pH 6.5. Hence, the carboxy residue in position 68 lowers the pK_a values of the kinetic parameters using dGSH as thiol substrate. This presumably reflects the pK_a value of the dGSH thiol group.

The pK_a values of the pH profile of k_{cat}^{dGSH} and k_{cat}/K_M^{dGSH} for T68E are similar to the pK_a value, 8.3, of the Tyr9 hydroxyl group. It could be speculated that Tyr9 assists as a base in the deprotonation process. However, the Tyr9 hydroxyl group may also serve in adjusting the orientation of the thiolate so that it becomes better positioned for nucleophilic attack on the electrophile. The Y9F mutant of GST A1-1 did not display any detectable activity with dGSH, emphasizing the importance of this residue in the conjugation reaction between dGSH and CDNB.

The K_M^{CDNB} value for wild-type GST A1-1 was 10-fold increased by the lack of the Glu α-carboxylate in the thiol substrate. The shift in rate-determining step partially accounts for this effect.

The K_M^{dGSH} value obtained from the steady-state measurements with CDNB was approximately 12 mM for both the variant enzymes. K_M^{dGSH} is likely to reflect the dissociation constant of the molecule, since k_{-2} (Scheme 4) should be greater than the rate constant of the chemical step. Glutathione interacts with 8 different amino acid residues within the active site of GST A1-1, forming about 10 different salt bridges and hydrogen bonds (*9*). Two of the hydrogen bonds are lost by the lack of the α-carboxylate (Figure 1), the two hydrogen bonds between the Glu α-carboxylate and Thr68. Nevertheless, mutating residue 68 to a valine increases the K_M^{GSH} value less than 2-fold (*39*). This mutation only eliminates one of the two hydrogen bonds; the interaction between the backbone amide and the Glu α-carboxylate is still present. Therefore, either the latter hydrogen bond is of fundamental importance for binding the thiol substrate or some other mechanism operates that greatly affects the interaction between the thiol substrate and the enzyme. In the previous studies of alpha class rat GSTs (*18*) it was demonstrated that the glutathione analogue lacking the amino group of the γ-Glu residue (glutaryl-Cys-Gly) but retaining the carboxylate gave substantial activity, supporting the hypothesis of a functional role of the Glu α-carboxylate in the catalyzed reaction.

Interestingly, the different reactions catalyzed by GST A1-1 are differentially affected by the thiol substrate. The ratio of k_{cat}/K_M^{GSH} over k_{cat}/K_M^{dGSH}, using CDNB or AD as the electrophilic substrate, is 15 000 and 24 000, respectively, whereas the same ratio for NCA is lower than 150 (Table 1). For all three substrates the K_M value for the thiol substrate is substantially increased, but with NCA the k_{cat} value is not as strongly affected by the deletion of the Glu α-carboxylate; it is reduced less than 10-fold. One explanation could be that GST A1-1 is less active toward NCA, and therefore, the effect is lower for this substrate since two poor enzyme activities are compared. Another interpretation could be that there is no proton released in the reaction with NCA. The Michael addition reaction is not dependent on an ionization of the thiol group prior to the chemical step if the nucleophilic attack of the thiolate occurs in concert with the addition of the thiol proton to the second carbon of the double bond. The glutathione thiolate has been suggested to function as a base in the isomerization reaction with AD (*42*), and in the CDNB reaction the mechanism also involves the release of a proton from the active site. Assuming that the Glu α-carboxylate assists in the ionization process, deletion of this group would have a larger effect on reactions in which a proton is released.

The T68E mutation positively influences the activity toward AD, whereas k_{cat}/K_M^{dGSH} with NCA is equal to the activity displayed by the wild-type enzyme (Table 1). As discussed above, the CDNB and AD reactions appear more constrained by the inability of the enzyme to deprotonate the thiol group than is the NCA reaction. Hence, since the effect of the T68E mutation is a lowered pK_a value of the active-site-bound thiol group of dGSH, the reaction rate with NCA would benefit less from this if the deprotonation of the thiol group occurs in concert with the addition step.

The questions of why the pK_a value of the thiol is raised on deletion of the Glu α-carboxylate and how the T68E mutation increases the reactivity of dGSH remain. As previously suggested, the Glu α-carboxylate may participate in the deprotonation of glutathione (*39*). In crystal structures solved for different GSTs the glutamyl part of the glutathione molecule is fixed and displays a similar conformation (*49*). However, when the mobility of the tripeptide within the active site of human GST P1-1 was investigated by NMR, the Glu residue was poorly defined and indications were found for a precomplex, in which the Glu residue is bound in a different fashion (*50*). Hence, the Glu residue might adopt conformations in addition to those shown by the X-ray structures. If the role of the α-carboxylate is to facilitate the ionization of the thiol substrate, then the T68E mutation could fulfill the same function (although with less flexibility) and partially rescue the activity lost by the deletion of the α-carboxylate from the glutathione molecule.

Another possibility is that the Glu α-carboxylate is influencing the binding mode of glutathione within the active site and that introduction of the carboxy residue in T68E helps to orient the dGSH such that the molecule aligns in a manner more suitable for catalysis. For example, the thiol group might come closer to the hydroxyl group of Tyr9, which could favor deprotonation of the thiol.

A third alternative is that the conserved water molecule, interacting with the Glu α-carboxylate in the structure of GST A1-1, is fixed in its position by the α-carboxylate and that this particular water molecule is crucial for catalysis. The water molecule forms hydrogen bonds to the backbone carbonyl oxygen atoms of Val66 and Pro56. The latter is in cis conformation and is one of the few GST residues that is strictly conserved throughout evolution. The residues around the Glu α-carboxylate are in general highly conserved which emphasizes the importance of this part of the G-site for enzyme function. Assuming that the location of this water molecule is shifted by the deletion of the Glu α-carboxylate,

the Glu68 carboxylate in T68E could help to restore the water molecule to a position favorable for catalysis.

In summary, the lack of the Glu α-carboxylate from the thiol substrate causes a drastic loss of the rate of reactions catalyzed by GST A1-1, which most probably is due to a combination of a lowered affinity for the thiol substrate and a raised pK_a value of the active-site-bound thiol group. Furthermore, the K_M value of small electrophiles is raised, which could partially be explained by the change in rate-limiting step and structural effects on the C-terminus of the enzyme. The introduction of a carboxylate in the G-site of the enzyme in a location normally occupied by the Glu α-carboxylate rescued some of the activity lost by the deletion of the α-carboxylate from glutathione, since the ability to ionize the thiol was improved by the T68E mutation.

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SUPPORTING INFORMATION AVAILABLE

A detailed description of the synthesis of dGSH. This material is available free of charge via the Internet at http://pubs.acs.org.

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