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Catalytic Mechanism and Role of Hydroxyl Residues in the Active Site of Theta Class Glutathione *S*-Transferases

INVESTIGATION OF SER-9 AND TYR-113 IN A GLUTATHIONE *S*-TRANSFERASE FROM THE AUSTRALIAN SHEEP BLOWFLY, *LUCILIA CUPRINA**

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Spectroscopic and kinetic studies have been performed on the Australian sheep blowfly *Lucilia cuprina* glutathione *S*-transferase (*Lucilia* GST; EC 2.5.1.18) to clarify its catalytic mechanism. Steady state kinetics of *Lucilia* GST are non-Michaelian, but the quite hyperbolic isothermic binding of GSH suggests that a steady state random sequential Bi Bi mechanism is consistent with the anomalous kinetics observed. The rate-limiting step of the reaction is a viscosity-dependent physical event, and stopped-flow experiments indicate that product release is rate-limiting. Spectroscopic and kinetic data demonstrate that *Lucilia* GST is able to lower the pK_a of the bound GSH from 9.0 to about 6.5. Based on crystallographic suggestions, the role of two hydroxyl residues, Ser-9 and Tyr-113, has been investigated. Removal of the hydroxyl group of Ser-9 by site-directed mutagenesis raises the pK_a of bound GSH to about 7.6, and a very low turnover number (about 0.5% of that of wild type) is observed. This inactivation may be explained by a strong contribution of the Ser-9 hydroxyl group to the productive binding of GSH and by an involvement in the stabilization of the ionized GSH. This serine residue is highly conserved in the Theta class GSTs, so the present findings may be applicable to all of the family members.

Tyr-113 appears not to be essential for the GSH activation. Stopped-flow data indicate that removal of the hydroxyl group of Tyr-113 does not change the rate-limiting step of reaction but causes an increase of the rate constants of both the formation and release of the GSH conjugate. Tyr-113 resides on α-helix 4, and its hydroxyl group hydrogen bonds directly to the hydroxyl of Tyr-105. This would reduce the flexibility of a protein region that contributes to the electrophilic substrate binding site; segmental motion of α-helix 4 possibly modulates different aspects of the catalytic mechanism of the *Lucilia* GST.

Glutathione *S*-transferases (GSTs;[1] EC 2.5.1.18) are a wide group of isoenzymes able to catalyze the conjugation of GSH with a variety of electrophilic molecules (1–6). The cytosolic GSTs are dimeric enzymes subdivided into at least five main classes, Alpha, Mu, Pi (7), Theta (8), and Sigma (9, 10), and are characterized by a low sequence homology (less than 30%). Despite this heterogeneity, the overall polypeptide fold is very similar among the crystal structures so far obtained (6), and all GSTs are highly selective for the GSH molecule. An important conserved residue between classes is a tyrosine near the N-terminal region (Tyr-6 in rat GST M1–1 (11), Tyr-7 in human GST P1–1 (12), and Tyr-8 in human GST A1–1 (13)) that has been proposed to activate the bound GSH by stabilizing its thiolate form. The blowfly *Lucilia cuprina* infests Australian sheep flocks, and the GST isoenzyme purified from this species (14) is possibly involved in the mechanism of insecticide resistance. The *Lucilia* GST has been classified as a Theta class GST on the basis of its primary structure, but the crystal structure shows the equivalent tyrosine residue within the N-terminal region (Tyr-5) to be 13.9 Å away from the thiol group of GSH (15). Therefore, other residues could replace Tyr-5 and be involved in the activation of GSH. From the crystal structure, Ser-9 is found to be within hydrogen bond distance of the sulfur atom of GSH (3.9 Å), and the hydroxyl group of Tyr-113 may also form a hydrogen bond with the GSH sulfur atom through a water molecule. This tyrosine residue is not conserved in all of the Theta class isoenzymes, and it is absent in the human Alpha class GST. Data so far obtained on *L. cuprina* GST show that Tyr-113 is not significantly involved in catalysis (16). Nevertheless, an equivalent tyrosine residue plays an important catalytic role in the Mu class GST. X-ray diffraction of GST M1–1 in complex with a transition state analogue shows σ-complex stabilization by hydrogen-bonding interactions with the hydroxyl groups of Tyr-6 and Tyr-115 (17); moreover, the hydroxyl group of Tyr-115 is involved both in chemical and physical steps of catalysis, and its removal has different effects, depending on which of these steps is rate-limiting (18). In the GST P1–1, the equivalent Tyr-108 shows a multifunctional role in catalysis (19); the hydroxyl function of Tyr-108 stabilizes the transition state for the Michael addition of GSH to ethacrynic acid, whereas it has a negative influence when 7-chloro-4-nitrobenzene-2-oxa-1,3-diazole is used as cosubstrate. Muta-

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[1] The abbreviations used are: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; FDNB, 1-fluoro-2,4-dinitrobenzene.

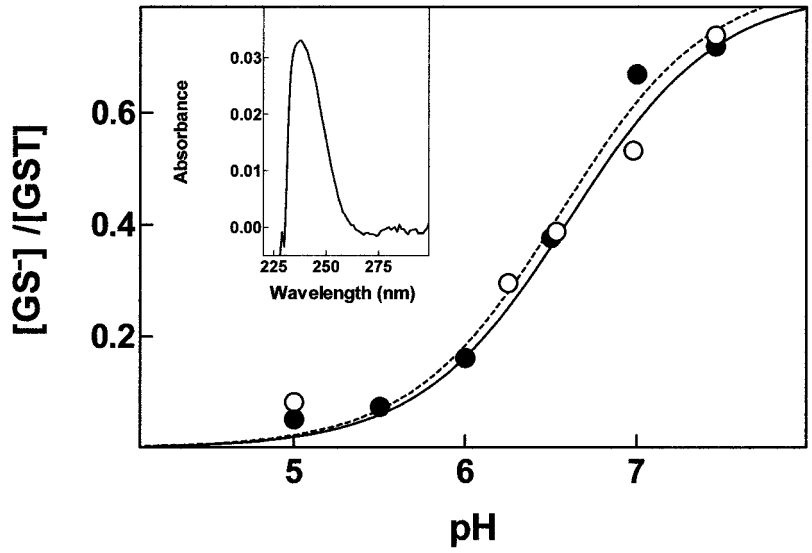

FIG. 1. **Spectroscopic evidence for GSH ionization.** Spectra of GS^- in the GST·GSH binary complex were obtained at different pH values as described under "Experimental Procedures." The amount of GS^- formed was calculated by assuming an $\epsilon_{240\ nm}$ of 5,000 M^{-1} cm^{-1}. Each experimental point is the mean of three determinations, and S.E. for each point does not exceed 5%. *Lines* are obtained by fitting the data to Equation 2. ○ (*solid line*), *Lucilia* GST wild type; ● (*dashed line*), Y113F mutant; *inset*, differential spectrum of GSH (0.5 mM) in the presence of *Lucilia* GST wild type (12 μM) in 0.1 M potassium-phosphate buffer, pH 7.0.

tion of Tyr-108 in Phe yields a 7-fold increase of the turnover number, and the additional link between Tyr-108 and the co-substrate may increase the internal friction of the protein lowering the k_{cat} (20). With regard to Ser-9, this residue is conserved in all Theta class isoenzymes, and its mutation to Ala strongly lowers the activity of *Lucilia* GST (16). The role in catalysis of the equivalent Ser-11 in human Theta GSTT2–2 has been recently studied (21); in that case, the contribution of serine varies with the nature of the second substrate. The aim of this paper is to investigate the catalytic mechanism of the *Lucilia* GST and the possible role of Ser-9 and Tyr-113 as suggested by the *Lucilia* GST crystal structure.

EXPERIMENTAL PROCEDURES

Materials—L. cuprina GST wild type, Y113F, and S9A mutants were expressed in *Escherichia coli* and purified as reported by Board *et al.* (14, 16). With regard to the S9A mutant, the enzyme was partially purified on a DE52 ion exchange column, since this mutant loses its activity almost completely after the GSH affinity column.

GSH, 1-chloro-2,4-dinitrobenzene (CDNB), and 1-fluoro-2,4-dinitrobenzene (FDNB) were purchased from Sigma. Glycerol was a BDH product.

Spectrophotometric measurements were performed with a double beam Uvikon 940 spectrophotometer Kontron equipped with a cuvette holder thermostatted at 25 °C.

Kinetic Experiments—Steady state kinetics of *Lucilia* GST with CDNB as cosubstrate were measured at 340 nm as previously reported (22). Kinetic experiments were carried out in 1 ml (final volume) of 0.1 M potassium phosphate buffer, pH 6.5, containing variable amounts of substrates and 0.2–0.8 μg of enzyme, except for the S9A mutant, where about 40 μg of enzyme was utilized. The reaction rates were measured at 0.1-s intervals for a total period of 12 s. Initial rates were determined by linear regression and corrected for the spontaneous reaction. $S_{0.5}^{CDNB}$ was obtained at fixed GSH (10 mM) and variable CDNB concentrations (from 20 μM to 2 mM). $S_{0.5}^{GSH}$ was obtained at fixed CDNB (1 mM) and variable GSH concentrations (from 10 μM to 20 mM). Data of v *versus* [S] were fitted to Equation 1,

$$v = V_{max}/(1 + K/[S]^{n_H}) \quad \text{(Eq. 1)}$$

and the best fit gave V_{max}, $S_{0.5}$ ($S_{0.5} = K^{1/n_H}$), and Hill coefficient (n_H) for each substrate.

The protein concentration was obtained from the protein absorbance at 280 nm assuming an $\epsilon_{1\ mg/ml}$ of 1.41 for Theta wild type and 1.36 for the Y113F mutant; extinction coefficients were calculated on the basis of the amino acid sequence as reported by Gill *et al.* (23). For the S9A mutant, the absorbance at 280 nm of the enzyme partially purified on a DE52 ion exchange column was corrected for the relative amount of the S9A mutant derived from SDS-polyacrylamide gel electrophoresis analysis.

A molecular mass of 23.9 kDa per GST subunit was used in the calculations.

TABLE I
pH dependence of kinetic and spectroscopic parameters

$k_{cat}/S_{0.5}^{lim}$ represents the limiting value of the specificity constant at high pH values; $[GS^-]/[GST]^{lim}$ represents the limiting value of the relative amount of GSH thiolate at high pH values.

	Kinetic parameters		Spectroscopic parameters	
	pK_a	$k_{cat}/S_{0.5}^{lim}$	pK_a	$[GS^-]/[GST]^{lim}$
		$s^{-1}M^{-1}$		
Wild type	6.48 ± 0.14	(5.3 ± 0.5) × 10^5	6.60 ± 0.11	0.82 ± 0.07
Y113F	6.33 ± 0.15	(15.3 ± 1.1) × 10^5	6.55 ± 0.08	0.84 ± 0.05
S9A	7.57 ± 0.03	(17.2 ± 0.3) × 10^3		

pH Dependence of Kinetic and Spectroscopic Parameters—The pH dependence of $k_{cat}/S_{0.5}^{CDNB}$ was obtained as reported above by recording the enzymatic reaction in the following buffers (0.1 M): sodium acetate buffer between pH 4.5 and pH 5.5 and potassium phosphate buffer between pH 6.0 and pH 8.7. Control studies showed that the affinity of the enzyme toward GSH does not change in the pH range utilized.

The pH dependence of the GS^- bound to *Lucilia* GST was obtained by difference spectroscopy. In a typical experiment, 0.5 mM GSH was added to 12 μM GST active sites in 1 ml of 0.1 M suitable buffer. The amount of thiolate formed at each pH was monitored with the peak-to-trough amplitude between 240 and 300 nm ($\epsilon_{240\ nm}$ = 5,000 M^{-1} cm^{-1}) after subtraction of the spectral contributions of free enzyme and of free GSH (in a serum bovine albumin solution showing an absorbance at 240 nm similar to that of GST sample).

pK_a values were obtained from kinetic and spectroscopic experiments by fitting the data to Equation 2.

$$y = y_{lim}/(1 + 10^{pK_a - pH}) \quad \text{(Eq. 2)}$$

Binding Experiments—Binding of GSH to *Lucilia* GST was studied by difference spectroscopy by following the amount of GS^- formed in the active site. In a typical experiment, GSH (ranging from 5 μM up to 4 mM) was added to 1 ml containing 25 μM GST active sites in 0.1 M potassium-phosphate buffer, pH 6.5. The amount of GS^- bound was monitored with the peak-to-trough amplitude between 245 and 300 nm ($\epsilon_{245\ nm}$ = 3,800 M^{-1} cm^{-1}) after subtraction of the spectral contributions of free enzyme and of free GSH (in a serum bovine albumin solution showing an absorbance at 245 nm similar to that of GST sample). The 245-nm wavelength was selected to minimize the absorbance of the high amount of free GSH utilized. Data were fitted to Equation 1, where v and V_{max} were substituted by GS^- bound and total GST active sites, respectively.

Fluoride/Chloride Leaving Group Substitution—The second order kinetic constants at pH 6.5 for the spontaneous reaction of GSH with CDNB and FDNB and the turnover numbers at pH 6.5 for *Lucilia* GST with CDNB and FDNB as cosubstrates were obtained as previously reported (24).

Viscosity Effect on Kinetic Parameters—Kinetic parameters were obtained at 25 °C by using 0.1 M potassium phosphate buffer, pH 6.5, containing variable glycerol concentrations as already described (25).

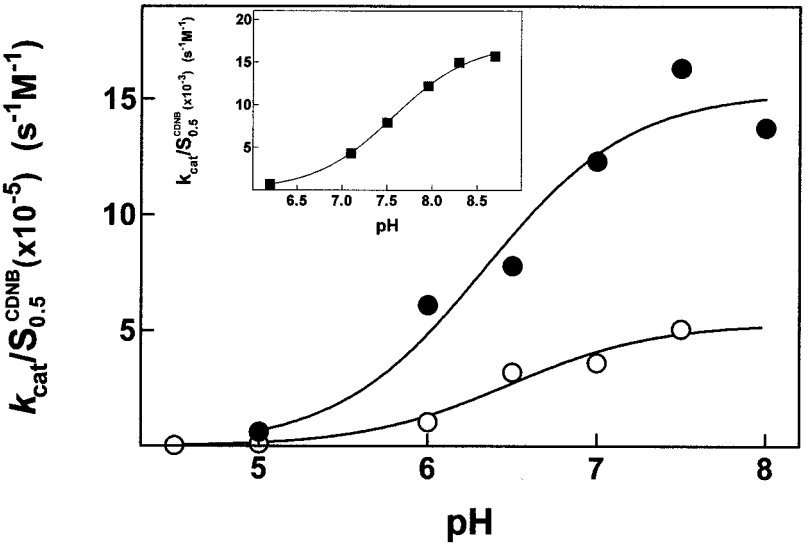

FIG. 2. **Kinetic evidence for GSH ionization.** pH dependence of $k_{cat}/S_{0.5}^{CDNB}$ for the *Lucilia* GST wild type (○) and Y113F mutant (●) is shown. *Inset*, pH dependence of $k_{cat}/S_{0.5}^{CDNB}$ for the S9A mutant. Kinetic parameters were obtained at 25 °C under saturating GSH (10 mM) and variable CDNB concentrations as reported under "Experimental Procedures." Each experimental point is the mean of three determinations, and S.E. for each point does not exceed 5%. *Lines* were obtained by fitting the data to Equation 2.

Stopped-flow Analysis—Rapid kinetic experiments were performed on an Applied Photophysics kinetic spectrometer stopped-flow instrument equipped with a temperature-regulated observation chamber with a 1-cm light path and 1-ms dead time. In a typical experiment, *Lucilia* GST (22 μM), in 0.1 M potassium phosphate buffer, pH 6.5, containing GSH (20 mM), was mixed with CDNB (2 mM) dissolved in the same buffer. The reaction was followed spectrophotometrically at 340 nm and at different temperatures (between 2.2 and 25 °C). The time course of the product (P) formation was fitted to Equation 3,

$$[P] = [E_0](k_1/(k_1 + k_2))^2 - [E_0](k_1/(k_1 + k_2))^2 \exp(-(k_1 + k_2)t) + [E_0](k_1/(k_1 + k_2))k_2t \quad \text{(Eq. 3)}$$

which gives the pseudo-first order rate constant for the formation of the enzyme-bound product (k_1) and the first order rate constant for the release of product from the active site (k_2) (26).

RESULTS AND DISCUSSION

pH Dependence of Kinetic and Spectroscopic Parameters—A well documented property of GSTs is the ability to lower the pK_a of the sulfhydryl group of the bound GSH. The acidity constant of GSH in the active site ranges between 6.0 and 6.5 for the Alpha (27), Mu (11, 28), and Pi (12) class GSTs. Tyr-6 in rat GST M1–1 (11), Tyr-7 in human GST P1–1 (12), and Tyr-8 in human GST A1–1 (13) have a crucial role in stabilization of the thiolate form of GSH. Whether *Lucilia* GST activates the bound GSH in a manner that is similar to the other GST isoenzymes is still an open question. The crystal structure of Theta GST from *L. cuprina* blowfly (15) shows the equivalent tyrosine, near the N terminus (Tyr-5), is 13.9 Å away from the thiol group of bound GSH but suggests that two active site residues, either Ser-9 or Tyr-113, may replace Tyr-5 in the GSH activation. Direct evidence for the forced deprotonation of GSH in the active site of *Lucilia* GST comes from spectroscopic experiments. The differential spectrum of the binary complex GST·GSH, obtained under nonsaturating GSH concentration and at pH 7.0 (Fig. 1, *inset*), shows an absorption band centered at 240 nm that is typical of a thiolate anion. The absorbance value suggests that more than 50% of the bound GSH is deprotonated at neutral pH values. The pH dependence of the thiolate band shows a pK_a of 6.6 for the bound GSH (Fig. 1, Table I). The pH dependence of $k_{cat}/S_{0.5}^{CDNB}$, which should reflect a kinetically relevant ionization of the GST·GSH complex, gives an apparent pK_a value of 6.5 (Fig. 2 and Table I). These spectroscopic and kinetic experiments provide the first evidence that a Theta-like isoenzyme lowers the pK_a of the sulfhydryl group of the bound GSH as occurs in other GST classes despite replacement of the essential tyrosine residue in the latter with a serine residue in the former (11, 12, 27, 28). To check the influence of the proximal Tyr-113 residue, difference spectroscopy experiments were performed on the Y113F mutant. As shown in Fig. 1, the GSH thiolate band is still present, and the pH dependence of the mercaptide absorption band at 240 nm indicates a pK_a value of about 6.5, very close to that found with the wild type enzyme (Table I). Again, the pH dependence of $k_{cat}/S_{0.5}^{CDNB}$ parallels the spectroscopic data showing a pK_a value of 6.3 (Fig. 2 and Table I). Thus, the hydroxyl group of Tyr-113 does not seem to play a role in the forced deprotonation of the bound GSH. Ser-9 seems to be involved; the S9A mutant has been only partially purified, and direct spectroscopic evidence for the ionization of the bound GSH cannot be obtained. However, the pH dependence of $k_{cat}/S_{0.5}^{CDNB}$ identifies an apparent pK_a of 7.6, about 1 pH unit higher than that found for the wild type (Fig. 2, *inset*, and Table I).

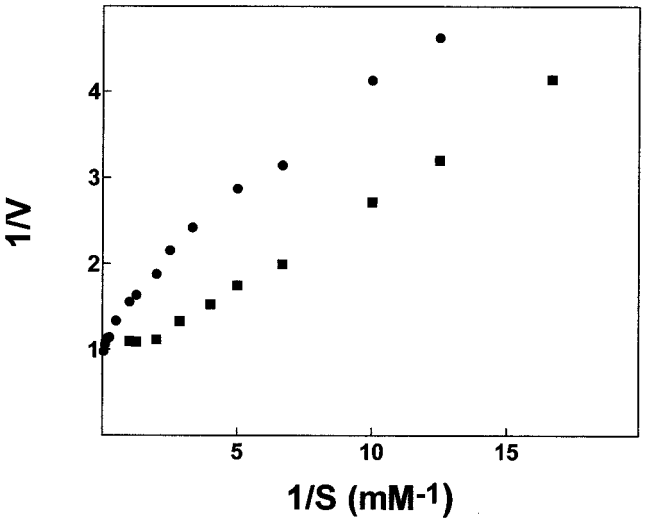

FIG. 3. **Initial velocity plot for the CDNB/GSH conjugation.** Reactions were performed at 25 °C in 1 ml of 0.1 M potassium phosphate buffer, pH 6.5, in the presence of about 0.8 μg of *Lucilia* GST. GSH varied between 80 μM and 2 mM at a constant CDNB concentration (1 mM) (●); CDNB varied between 60 μM and 1 mM at a constant GSH concentration (10 mM) (■). Velocities are expressed as $\Delta A_{340\ nm}$ min^{-1}.

Steady State Kinetics and Binding Experiments—Steady state kinetics of *Lucilia* GST do not strictly obey the Michaelis-Menten equation. When the concentration of GSH or CDNB is varied, at fixed cosubstrate concentrations, double reciprocal

TABLE II
Kinetic and thermodynamic parameters at pH 6.5

Kinetic and binding parameters were determined as described under "Experimental Procedures." $S_{0.5}$ represents the substrate concentration that yields half of the V_{max} value.

	k_{cat}	$k_{cat}/S_{0.5}$	$S_{0.5}^{CDNB}$	n_H[a]	$S_{0.5}^{GSH}$	n_H[b]	K_D^{GSH}	n_H[c]
	s^{-1}	$s^{-1}M^{-1}$	μM		μM		μM	
Wild type	53 ± 1	(3.2 ± 0.2) × 10^5	165 ± 8	1.24 ± 0.03	530 ± 41	0.72 ± 0.02	107 ± 11	0.90 ± 0.02
Y113F	144 ± 2	(7.8 ± 0.5) × 10^5	185 ± 10	1.17 ± 0.01	320 ± 20	0.83 ± 0.02		
S9A	0.28 ± 0.02	(1.3 ± 0.1) × 10^3	208 ± 30	1.10 ± 0.01	540 ± 71	0.74 ± 0.02		

[a] n_H obtained at constant GSH and variable CDNB concentrations.
[b] n_H obtained at constant CDNB and variable GSH concentrations.
[c] n_H obtained from GSH binding experiments.

plots are concave down *versus* GSH and concave up *versus* CDNB (Fig. 3). This behavior may be a signal that the enzyme follows a steady state kinetic mechanism where a preferred pathway for the ternary complex exists (29) or, alternatively, may be due to a cooperative binding of the substrates to the enzyme as already reported for the Cys-47 mutants of GST P1–1 (30). To discriminate between these possibilities, the isothermic binding of GSH was studied. Binding of GSH to other GST classes has been easily measured by following the intrinsic fluorescence quenching of the enzyme (31), but the intrinsic fluorescence of the *Lucilia* GST is not perturbed by the addition of GSH up to a 20 mM concentration. The isothermic binding curve of GSH has been obtained by measuring the amount of GSH thiolate that is formed at the active site as function of the substrate concentration. At pH 6.5, a K_D^{GSH} of 107 μM and a Hill coefficient of about 0.90 have been calculated (Table II), indicating a nearly Michaelian behavior. Similar nonhyperbolic kinetics but noncooperative binding of substrates to the enzyme have been already described for the rat GSTs M1–1, M1–2, and A3–3 (32, 33) and explained by a steady state random sequential Bi Bi mechanism. The dissociation constant (K_D) of GSH, obtained from binding experiments, is almost 5 times lower than the $S_{0.5}$ value derived from kinetic experiments; this discrepancy may be explained by a negative effect of CDNB on GSH binding, as already observed in GST P1–1 (34), or may be the consequence of a steady state random mechanism in which $S_{0.5}$ does not correspond to K_D. Table II contains kinetic and thermodynamic data obtained with *Lucilia* GST wild type and Y113F and S9A mutants; both mutants show $S_{0.5}$ values toward GSH and CDNB and Hill coefficients comparable with that of wild type. On the other hand, the Y113F turnover number is about 3 times higher than that of the wild type, while the S9A mutant shows an extremely low specific activity (about 0.5% of that of wild type).

Rate-limiting Step in Lucilia GST-catalyzed Reaction—It is well known that the nucleophilic aromatic substitution reactions proceed via a σ-complex intermediate (35). Substitution of chlorine by the more electronegative fluorine in the CDNB molecule increases about 50-fold the second order rate constant of the spontaneous reaction with GSH, suggesting that the σ-complex formation is rate-limiting (4). The reaction catalyzed by *Lucilia* GST is insensitive to the nature of the leaving group; the k_{cat}^{FDNB} to k_{cat}^{CDNB} ratio ranges between 1.0 and 1.6 for wild type and Y113F and S9A mutants. Therefore, a physical step rather than a chemical step may be rate-limiting in this enzymatic reaction as previously observed in human GST P1–1 (24) and in rat GST M1–1 (18).

The effect of viscosity on kinetic parameters has been mainly utilized to study the rate-determining step of very efficient enzymes in which the encounter of substrates with the protein is rate-limiting (k_{cat}/K_m = 10^7 to 10^8 s^{-1} M^{-1}). A decrease of the pseudo-second order rate constant (k_{cat}/K_m) by increasing the medium viscosity should reflect the weight of diffusive events on catalysis (36). The reactions catalyzed by *Lucilia* GST, wild

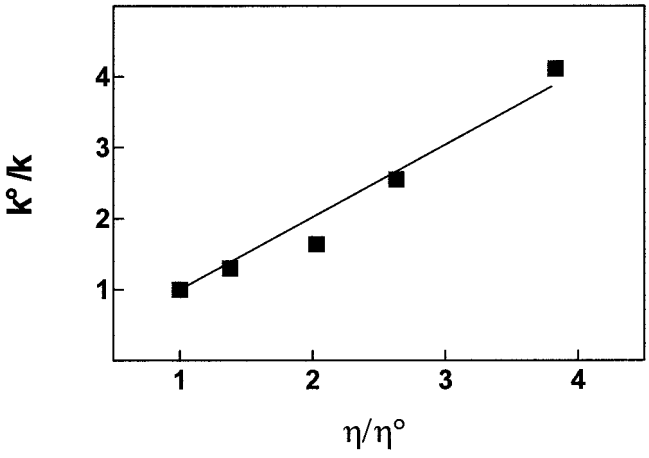

FIG. 4. **The effect of viscosity on kinetic parameters of *Lucilia* GST.** Plot of the reciprocal of the relative turnover number (k_{cat}^{o}/k_{cat}) as a function of relative viscosity (η/η^{o}) with glycerol as cosolvent. Kinetic data were obtained as reported under "Experimental Procedures." Experiments were performed in triplicate, and S.E. for each point does not exceed 8%. A slope of 1.02 is obtained from linear regression of the experimental points.

type, and mutants are characterized by $k_{cat}/S_{0.5}$ values $\leq 8 \times 10^5$ s^{-1} M^{-1}, so this enzyme is far from being a "perfect" catalyst. In this case, the effect of a viscosogen on k_{cat} should indicate that the rate-limiting step of reaction is related to the product release (18) or to diffusion-controlled structural transitions of the protein (25). This may occur for *Lucilia* GST; in fact, a plot of the reciprocal of the relative catalytic constant k_{cat}^{o}/k_{cat} against the relative viscosity (η/η^{o}) gives a linear dependence with a slope of about 1.0 for *Lucilia* GST wild type (Fig. 4), 0.9 for Y113F, and 0.8 for S9A mutants. Diffusion-controlled motions of the protein were already reported to modulate the catalysis of other GST isoenzymes, and different physical events were hypothesized to influence the reaction rate: a conformational change of the ternary complex in the human GST P1–1 (25) and the product release in the rat GST M1–1 (18).

Stopped-flow Experiments—Pre-steady state kinetics of *Lucilia* GST were studied by stopped-flow experiments. As shown in Fig. 5, the early stage of catalysis displays a well defined burst phase at 340 nm, which indicates that the product accumulates before the steady state attainment. The burst phase is more evident when the temperature is lowered from 25 to 2.2 °C. At 2.2 °C and saturating substrate concentrations, a burst is observed even in the more active Y113F mutant (Fig. 5*C*). Enzyme concentrations, calculated on the basis of the burst amplitude (π) (9.9 and 9.5 μM for the Y113F mutant and wild type, respectively), are close to the protein concentration used in these experiments (11 μM). The reaction catalyzed by the *Lucilia* GST may be roughly subdivided into two steps: the formation of the enzyme-bound GSH conjugate, governed by

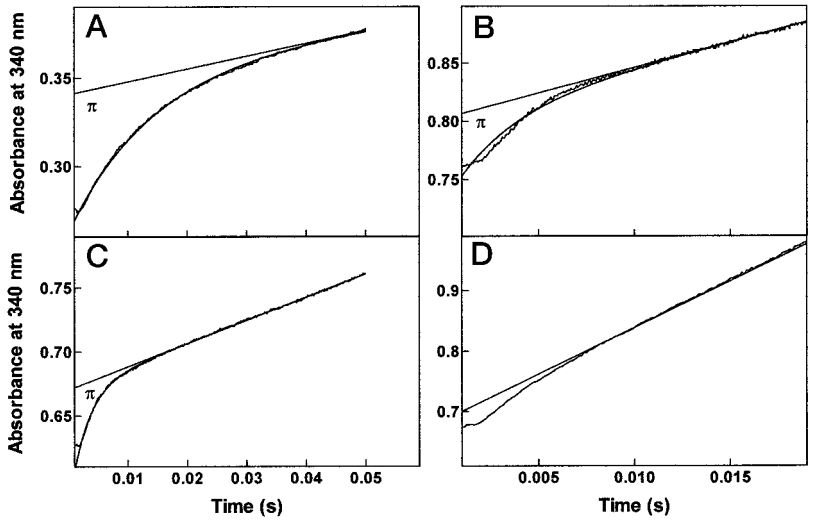

FIG. 5. **Time course of the enzymatic GS-DNB production.** Kinetics were obtained by mixing in a stopped-flow apparatus the enzyme (22 μM) in 0.1 M potassium phosphate buffer, pH 6.5, containing 20 mM GSH, with 2 mM CDNB in the same buffer. *A*, wild type *Lucilia* GST at 2.2 °C; *B*, wild type *Lucilia* GST at 15 °C; *C*, Y113F mutant at 2.2 °C; *D*, Y113F mutant at 15 °C. One representative spectrophotometric trace of six different experiments is reported in each *panel*. The *continuous lines*, representing the best fit of data to Equation 3, are superimposed on the experimental data in *panels A–C*. In *panel D*, the experimental data did not allow unequivocal determination of k_1 and k_2 by means of the fitting procedure. The *straight lines* represent the linear portion extrapolated back to the burst value (π).

the pseudo-first order rate constant (k_1), which summarizes all steps from the substrate binding to the product formation, and the release of product from the active site governed by the first order rate constant (k_2), shown in Scheme I.

$$E + \text{GSH} + \text{CDNB} \underset{}{\overset{k_1}{\rightleftharpoons}} E\text{P} \overset{k_2}{\rightleftharpoons} E + \text{P}$$

SCHEME I

The rate constants k_1 and k_2 obtained from pre-steady state kinetics performed at different temperatures are reported in Table III. The free energy levels of the catalytic steps related to the rate constants k_1 and k_2 may be estimated by their temperature dependence. The Arrhenius plot gives activation energy values of about 54 and 104 kJ/mol for k_1 and k_2, respectively (Fig. 6), indicating that two very different energy barriers are involved. Temperature dependence of k_{cat} gives an activation energy value of about 96 kJ/mol, confirming that the release of product is rate-limiting. The rate constant k_2 is much more sensitive to the temperature than k_1, so the gap between the two rate constants becomes smaller as the temperature increases. Pre-steady state data suggest that the hydroxyl group of Tyr-113, which resides in α-helix 4 (15), affects negatively both rate constants k_1 and k_2; at 2.2 °C, the Y113F mutant shows a k_1 value of about 325 s^{-1} and a k_2 of about 18 s^{-1}, while at the same temperature, the wild type shows a k_1 value of about 67 s^{-1} and a k_2 of about 5 s^{-1}. This may be the consequence of additional interactions involving the hydroxyl group of Tyr-113, which will be discussed below.

TABLE III
Temperature dependence of kinetic parameters

Temperature	Wild type		Y113F	
	k_1	k_2	k_1	k_2
°C	s^{-1}	s^{-1}	s^{-1}	s^{-1}
2.2	67	5	325	18
5.6	122	14		
15.2	293	48		
24.7	415	172		

The values of the rate constants k_1 and k_2 (see Scheme I) at different temperatures were calculated from the best fit of experimental data according to Equation 3. S.E. does not exceed 5%. In the case of the Y113F mutant, it was impossible to find a unique solution to discriminate between k_1 and k_2 at a temperature higher than 2.2 °C.

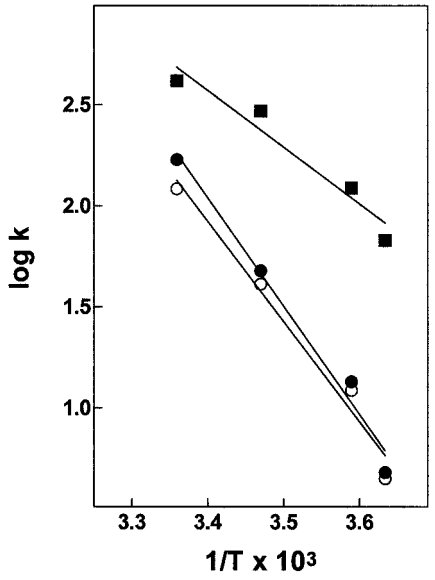

FIG. 6. **Temperature effect on kinetic parameters.** The rate of product accumulating at the active site k_1 (s^{-1}) (■), the rate of product release k_2 (s^{-1}) (●), and k_{cat} (s^{-1}) (○) were obtained, between 2.2 and 25 °C, from the best fit of the pre-steady state kinetic data according to Equation 3; for details see "Experimental Procedures." Activation energies were obtained from linear regression of data.

CONCLUSION

Kinetic and binding experiments show, for the first time in a Theta GST, that the isoenzyme purified from the sheep blowfly *L. cuprina* is able to lower the pK_a of bound GSH about 3 pH units despite the absence, in the G-site, of the crucial tyrosine found in the Alpha, Pi, and Mu class GSTs (11–13). The role played by the hydroxyl group of Ser-9 and also of Tyr-113, two residues possibly involved in the GSH activation, has been examined. Removal of the hydroxyl groups of Tyr-113 and of Ser-9 does not affect the affinity toward both GSH and CDNB. Moreover, the pK_a of the *E*·GSH complex is unchanged in the Y113F mutant, while it shifts from 6.5 to about 7.6 in the S9A mutant, a value still lower than the pK_a (9.0) of the GSH in aqueous solution. Another contributor to the lower than expected pK_a might be the influence of a helix dipole, since the sulfur atom of GSH lies directly above the N terminus of α-helix 1 (15). On the other hand, removal of the hydroxyl group of Ser-9, hydrogen-bonded to the thiol group of GSH, results in a very low turnover number (about 0.5% of that of wild type). Similarly, replacement of Tyr-6 with phenylalanine in the rat GST M1–1 (11) resulted in a very low turnover number and the pH dependence of k_{cat}/K_m^{CDNB} for the *E*·GSH complex gave a pK_a value of 7.8. Zheng and Ornstein (37), by

using *ab initio* molecular orbital calculations, found a pK_a value of 7.8 for the bound GSH when the active site tyrosine was replaced by phenylalanine. These results confirm that Ser-9, in *L. cuprina* GST, is equivalent to the conserved tyrosine residues in the G-site of other GST classes. Like Tyr-6, in the rat GST M1–1, Ser-9 may contribute to the stabilization of the GSH thiolate; however, on the basis of the pK_a value of 7.6 in the S9A mutant, a k_{cat} 6-fold lower than that of wild type is expected at pH 6.5, while a 200-fold decrease of k_{cat} is actually observed. The dramatic lowering of k_{cat} observed in the absence of Ser-9 can be explained neither on the basis of an increased pK_a of the bound GSH nor by a different thermal stability between S9A and wild type (data not shown); thus, Ser-9 seems to be mainly implicated in the correct orientation of the sulfhydryl group of GSH in the catalytic step or in the transition state stabilization. In the last few years, a great effort has been made to clarify whether the crucial tyrosine residue acts as a general base proton acceptor or as a hydrogen bond donor in the GSH activation mechanism; in the former, a relevant amount of tyrosinate is required. The functional equivalence between serine and tyrosine suggests that the tyrosinate ion idea may not be physiologically important. In fact, in the *Lucilia* GST the ionization of the key hydroxyl residue Ser-9 is not possible, and this ionization may be also not be required for the equivalent tyrosine residue in other GST classes. Recently, a consensus pattern for Theta class GSTs based on *Lucilia* GST structure has been derived and utilized to identify key residues in the polypeptide fold (38); in this study the serine (or sometimes threonine) residue near the N terminus is found in virtually all Theta class GSTs, so the present findings are probably applicable to varying degrees to all of the family members.

Stopped-flow experiments show that the reaction catalyzed by the *Lucilia* GST is limited by the product release and allows calculation of the rate constants for the formation and release of product, k_1 and k_2. At about 2 °C a "burst" phase is clearly visible even with the more active Y113F mutant, suggesting that the Tyr-113 mutation does not change the rate-limiting step of the reaction. Despite the very low interclass GST sequence identity, which is less than 20% in the C-terminal domain (where Tyr-113 is located), there are remarkable analogies between the ancient Theta class GST and the mammalian class isoenzymes. Tyr-113 is equivalent to Tyr-115 of rat GST M1–1 and to Tyr-108 of human GST P1–1, and in these isoenzymes the hydrogen bonding interactions of the tyrosine hydroxyl group with electrophilic substrate binding site (H-site) residues or substrate, reduce the flexibility of the H-site, and affect the catalysis (18–20). Removal of the OH group of Tyr-113 in the *Lucilia* GST Y113F mutant favors the rate constants k_1 and k_2 of two different catalytic steps. This may be explained by observing that Tyr-113 resides on α-helix 4, which contributes to the H-site, and that the hydroxyl group of Tyr-113 hydrogen bonds directly to the hydroxyl group of Tyr-105 (15); this would reduce the flexibility of the hydrophobic binding region and raise the energy barriers for both the formation and release of the GSH conjugate. The negative role in catalysis of a conserved residue is a quite unusual finding, but we must stress that it is observed in an aromatic substitution reaction; other GST-catalyzed reactions like epoxide ring opening or Michael addition could require the assistance of this residue as recently observed for the rat GST M1–1 with phenanthrene 9,10-oxide (18) and for the human GST P1–1 with ethacrynic acid (19).

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