Ser<sup>11</sup> in rat glutathione transferase T2-2 is important for stabilization of the reactive enzyme-bound glutathione thiolate in the reaction with 1-menaphthyl sulfate. The S11A mutation increased the pK<sub>a</sub> value for the pH dependence of the rate constant for pre-steady-state product formation, from 5.7 to 7.9. This pH dependence is proposed to reflect titration of enzyme-bound glutathione thiol. Further, the mutation lowered the k<sub>cat</sub> value but not because of the impaired stabilization of the glutathione thiolate. In fact, several steps on the reaction pathway were affected by the S11A mutation, and the cause of the decreased k<sub>cat</sub> for the mutant was found to be a slower product release. The data presented here contradict the hypothesis that glutathione transferase T2-2 could act as a sulfatase that is not dependent on Ser<sup>11</sup> for the catalytic activity, as proposed for the corresponding human enzyme (Tan, K.-L., Chelvanayagam, G., Parker, M. W., and Board, P. G. (1996) Biochem. J. 319, 315–321; Rossjohn, J., McKinstry, W. J., Oakley, A. J., Verger, D., Flanagan, J., Chelvanayagam, G., Tan, K.-L., Board, P. G., and Parker, M. W. (1998) Structure 6, 309–322). On the contrary, Ser<sup>11</sup> governs both chemical and physical steps of the catalyzed reaction.

Glutathione transferases (GSTs)<sup>1</sup> are enzymes that catalyze the conjugation of the nucleophilic sulphydryl group of glutathione (GSH) to a large number of different electrophiles (for reviews see Refs. 1 and 2). GSTs occur in most organisms, and mammalian GSTs have been divided into different classes based on sequence similarity (3). The GSTs studied most extensively are those from classes Alpha, Mu, and Pi. These GSTs stabilize the thiolate form of GSH, which is the reactive species, by making a hydrogen bond from the hydroxyl group of a Tyr residue in the N-terminal region to the deprotonated sulphydryl group, thereby facilitating catalysis (see Ref. 4 for review on the catalytic mechanism of GSTs). Subsequent to the identifications of Theta class GSTs (5–7), it was found that the human GST T2-2 of this class has a Ser residue, and not a Tyr, within hydrogen bonding distance from the enzyme-bound GSH thiolate (8, 9). Mutation of the Ser into an Ala residue abolished the enzyme activity with cumene hydroperoxide and ethacrynic acid, as expected. However, the activity remained or even increased with 1-menaphthyl sulfate (MS) (8), the substrate originally used to detect the rat ortholog of human GST T2-2 (5). In addition, the human enzyme was found to catalyze the reaction with thiols other than GSH provided that the unreactive GSH analog S-methylglutathione was present. In the crystallographic analysis of human GST T2-2 a sulfate-binding pocket unique among the GSTs was discovered (9), and because the leaving group in the MS reaction is a sulfate ion the available experimental data led to the hypothesis that GST T2-2 could act as a sulfatase in the presence of GSH or GSH analogs (8, 9).

The proposed menaphthyl carbonium ion derived from MS would be able to react with any thiol group and not exclusively with the enzyme-bound GSH thiolate.

However, recent work on the rat ortholog of GST T2-2 (rGST T2-2) indicated that the Ser residue may in fact contribute to the catalysis of the MS reaction (10). To investigate the possibility that Ser<sup>11</sup> is involved in stabilization of rGST T2-2-bound GSH thiolate in the reaction with MS, as well as with other electrophiles, and to test the sulfatase hypothesis, we have made the S11A mutation in the rat GST T2-2. The mutant enzyme was characterized with regard to steady-state as well as pre-steady-state kinetics, and the results were compared with those of the wild type enzyme.

**EXPERIMENTAL PROCEDURES**

S-(1-Menaphthyl)glutathione (MSG) was synthesized essentially as described by Hyde and Young (11). MS was a kind gift from Dr. Brian Gillham, synthesized as described by Clapp and Young (12). S-Propylglutathione was synthesized essentially as described by Vince et al. (13). All other chemicals used were of high purity and obtained from commercial sources. Enzymes for recombinant DNA work were purchased from Roche Molecular Biochemicals and MBI Fermentas (Vilnius, Lithuania).

**Mutagenesis**—The expression plasmid pKRT2 encoding rGST T2-2 (14) was used as template in a mutagenic polymerase chain reaction. Primers used to insert the mutations in the cDNA were 1) 5’-GAA GAC GTA TTT CTG GCT TTC TAC CTC TAC CGC CCC AGC-3’ and 2) 5’-CTC GAG GTC GAC CGC CAG TTG GTA GAT CCT GCC AAT TG CCG AAG-3’. The triplet encoding the Ser to Ala mutation is underlined and restriction sites are italicized. A polymerase chain reaction contained approximately 5 ng of pKRT2 DNA, 0.8 μmol of each primer, 0.13 mM dNTPs, 1.5 mM MgCl<sub>2</sub>, 0.5 unit of Taq DNA polymerase and buffer as recommended by the manufacturer of the polymerase (MBI Fermentas) in a total volume of 100 μl. The reaction comprised 35 cycles of 1 min at 95 °C, 1.5 min at 55 °C, and 1 min at 72 °C, followed by 10 min at 72 °C. The products of several polymerase chain reactions were run on a 1.2% (w/v) agarose gel. A DNA fragment of the expected size, approximately 740 base pairs, was excised from the gel and purified with Gene CleanIII silica matrix (BIO 101, La Jolla, CA). The fragment was digested with EcoRI and SalI restriction enzymes and subsequently ligated to the corresponding cloning sites of the pGEM-3Zf (+) plasmid (Promega Corp., Madison, WI). These plasmids were transformed into competent Escherichia coli XL1-Blue cells (Stratagene, La Jolla, CA). The cDNA encoding the S11A mutant of
rGST T2-2 was then cloned into the expression vector pKK-D (15), and the resulting construct, named pKRT2S11A, was used to transform E. coli XL1-Blue cells. DNA sequence analysis of the cloned fragment was performed to ensure that the mutations in codon 11 were inserted and that no other mutations were present.

### Expression and Purification

Heterologous expression of wild type and mutant rGST T2-2, using the pKRT2S11A construct, and subsequent purification were performed as described previously for the wild type enzyme (14).

**Steady-state Kinetics**—All kinetic measurements were performed at 30 °C in sodium acetate (<pH 6), sodium phosphate (pH 6–8), triethanolamine hydrochloride (pH 8.5), or sodium glycine buffer (>pH 8.5). Steady-state kinetic measurements were performed on a Varian 2290 spectrophotometer.

The activity with MS was measured as described by Gillham (5), and the activities with 4-nitrobenzyl chloride (NBC) and 1-chloro-2,4-dinitrobenzene were measured as described by Habig et al. (16). The specific activity with 1-chloro-2,4-dinitrobenzene was determined in presence of 10 mM GSH and 0.50 mM of the electrophile in 0.1 M sodium phosphate, pH 6.5. The steady-state parameters $K_{cat}$, $K_{MS}$, and $K_{GSH}$ for the MS reaction were determined by varying the GSH concentration (0.010–10 mM) at a constant MS concentration (25 μM) and by varying the MS concentration (0.3–50 μM) at a constant GSH concentration (5 mM for S11A mutant and 10 mM for wild type) and then fitting the Michaelis-Menten equation to initial rate data. Figure 1 shows the reaction with NBC at 30 °C with both the S11A mutant and wild type$rGST_{T2-2}$ on a stopped flow spectrophotometer from Applied Photophysics (Leatherhead, Kent, UK). The buffers used were the same as for steady-state kinetics. The final concentrations (after the rapid mixing) of reactants and buffer in the experiments are given below. GSH solutions were neutralized with NaOH such that additions would not cause an acidification of the reaction mixture. The choice of fitting single exponentials to experimental traces was based on analysis of the residuals using the software supplied with the stopped flow spectrometer and statistical criteria (18) in the SIMFIT program (19).

The rate constant for pre-steady-state formation of product on the enzyme ($k_{cat}$) was determined by mixing 50 μM MS with 3 μM subunit preincubated with 10 mM GSH and measuring increase in absorbance at 298 nm. A single exponential function was fitted to the average of four to eight individual traces to obtain observed rate constants for pre-steady-state product formation. Owing to a high on-rate for MS, the observed rate constants can be used as estimates of the true rate constant $k_{cat}$. Three or four replicate determinations of $k_{cat}$ were made at different pH values. An apparent $k_{cat}^p$ value for $k_{cat}$ was calculated by fitting a first degree rational function to $k_{cat}$ plotted versus hydroxide ion concentration.

Pre-steady-state formation of product was also measured by mixing varying concentrations of GSH (1–130 mM) with 3 μM subunit preincubated with 50 μM MS. Observed rate constants were plotted versus GSH concentration and Equation 1, derived from general equations for pre-steady-state kinetics (20), was used to analyze the data.

$$k_{obs} = k_{cat}[GSH][K_{GSH} + [GSH]] + k_{-4} \quad \text{(Eq. 1)}$$

Pre-equilibrium binding of the product of the catalyzed reaction, MSG, to wild type$rGST_{T2-2}$ and S11A mutant was studied by mixing 1 or 5 μM subunit with different MSG concentrations (0.15–20 μM) at pH 7.5 and measuring intrinsic tryptophan fluorescence quenching. Excitation wavelength in all fluorescence experiments was 290 nm, and the emission at wavelengths ≥320 nm was detected using a cut-off filter. Observed rate constants were determined from an average of 5–14 individual traces by fitting a single exponential equation to data. Observed rate constants were plotted versus MSG concentration and analyzed by linear regression. Although the lowest MSG concentrations used in the experiments were below the subunit concentration, the data points were on a straight line. The slope was used to calculate the apparent association rate constant ($k_{obs}^{app}$) for MSG. To get a more accurate value of the MSG dissociation rate constant $k_{-4}$, the data points at low MSG concentrations were favored by weighted regression analysis (weight = $1/K_{d}$). The intercept with the y axis was interpreted as $k_{-4}$.

$$k_{obs}^{app} = k_{-4} + k_{obs}^{app} K_{d}[MS] + [GSH] \quad \text{(Eq. 2)}$$

Apparent $k_{cat}^p$ values for $k_{cat}$ and $k_{cat}$ were calculated using SIMFIT. Other regression analyses were performed using the program GraphPad PRISM version 2.0 (GraphPad Software Inc., San Diego, CA). The pH dependence of kinetic parameters as well as the analysis of pre-steady-state kinetics have been described by Fersht (20).
Function of Ser11 in Glutathione Transferase T2-2

RESULTS

Mutagenesis, Expression, and Purification—The expression clone pKRT2S11A was successfully obtained as described under “Experimental Procedures.” DNA sequence analysis confirmed that no mutations other than those in codon 11 were present in the cDNA encoding rGST T2-2. The S11A mutant and wild type rGST T2-2 were expressed and purified as described previously (14).

Steady-state Kinetics with GSH as Thiol Substrate—The S11A mutation in rGST T2-2 clearly affected the steady-state kinetics with the three electrophilic substrates MS, NBC, and 1-chloro-2, 4-dinitrobenzene. The steady-state rate constant $k_{\text{cat}}$ with MS as electrophilic substrate was lowered by approximately an order of magnitude at all pH values studied (Fig. 2A and Table I). The $k_{\text{cat}}/K_m$ values did not change as much as $k_{\text{cat}}$ as a result of the mutation, because the decrease in $k_{\text{cat}}$ is counteracted by a concomitant decrease in $K_m$. The steady-state constants with NBC as electrophilic substrate were lowered in a similar manner as the values with MS as substrate (Table I). The specific activity with 1-chloro-2, 4-dinitrobenzene was decreased from 0.7 to 0.019 $\text{mol min}^{-1} \text{mg}^{-1}$ (37-fold).

The viscosity of the reaction medium was found to affect $k_{\text{cat}}$ but not $k_{\text{cat}}/K_m$ (Fig. 3). The slope of $k_{\text{cat}}/K_m$ versus the relative viscosity $\eta/\eta^0$ was calculated as 0.5 $\pm$ 0.1 for the S11A mutant, and 0.6 $\pm$ 0.1 for wild type rGST T2-2 (where $k_{\text{cat}}$ and $\eta^0$ are the values obtained in the absence of added viscosogen). Thus, both enzyme variants display fractional viscosity dependence on $k_{\text{cat}}$. Owing to the low $K_m$ value (1 $\mu$M) of rGST T2-2, the viscosity dependence of $k_{\text{cat}}/K_m$ was difficult to determine; when steady-state rates are measured at MS concentrations $<1$ $\mu$M, the total difference in absorbance is <0.0025, requiring very low enzyme concentration, which results in low precision of the data. Nonetheless, like $k_{\text{cat}}/K_m$, $k_{\text{cat}}/K_m$ seems not to be dependent on the medium viscosity. To further investigate the role of Ser11 and to identify the cause of the lowered catalytic activity, a pre-steady-state kinetic analysis was employed.

Pre-steady-state Rate Constant of Product Formation and $pK_a$ Value of the Sulphydryl Group of Enzyme-bound GSH—The pre-steady-state rate constant for product formation is denoted $k_4$ (Fig. 1) and reflects the transition from the ternary complex of enzyme and productively bound substrates to the complex between enzyme and products. This step represents the chemistry of the reaction. Observed rate constants were obtained by preincubating enzyme with one of the substrates, rapidly mixing with the other substrate in the stopped flow spectrometer, and monitoring the increase in absorbance at 298 nm associated with product formation (5). Although the actual product formation is measured, preceding steps involving binding of the second substrate will affect the observed rate constant. When enzyme preincubated with GSH is mixed with MS, the observed rate constant of product formation follows equation 3 (cf. Equation 1).

$$k_{\text{obs}} = k_6[\text{MS}]k_{\text{MS}}[\text{GSH}] + k_{\text{4}}$$

However, $K_{\text{MS}}$ is very low owing to a large rate constant for association of MS with rGST T2-2; $k_{\text{MS}} = k_1 + k_4K_m$, where $k_1$ is the on-rate, and $k_4$ is the off-rate for MS (10). Under the conditions used, $[\text{MS}] > K_{\text{MS}}$, and the hyperbolic dependence of $k_{\text{obs}}$ on MS concentration, predicted by the equation, was not observed. Further, the rate constant for the reverse chemical reaction ($k_{\text{4}}$) is negligible; hence $k_{\text{obs}} = k_6k_{\text{MS}}$. Several determinations of $k_{\text{obs}}$ were made to estimate the true rate constant $k_4$ at different pH values (Fig. 2B). As shown in the figure, the removal of the hydroxyl group of Ser11 is accompanied by a shift in the apparent $pK_a$ value of the pH dependence of $k_4$ from 5.7 in the wild type enzyme to 7.9 in the S11A mutant. However, it is clear from Fig. 2 that the decrease in $k_4$ is not responsible for the decrease in $k_{\text{cat}}$, at physiological pH values, because $k_4$ is much larger than $k_{\text{cat}}$ at pH values of $>6$.

The pre-steady-state formation of product was also measured by preincubating the enzyme with MS and mixing rapidly with GSH. The observed rate constant increased with GSH concentration (Fig. 4) as described by Equation 1. At high GSH concentrations, $k_{\text{obs}}$ approaches the rate constant for product formation $k_6$, which was calculated as 131 $\pm$ 18 $s^{-1}$ for wild type rGST T2-2 and 43 $\pm$ 5 $s^{-1}$ for the S11A mutant at pH 7.5 at 30 °C. At low GSH concentrations $K_{\text{GSH}}$ is $>1$ [GSH], and the slope of the curve approaches $k_m/K_{\text{GSH}}$. The parameter $k_m/K_{\text{GSH}}$ is the gradient at [GSH] = 0 in Fig. 4. This expression is related to $k_{\text{app}}$, the apparent association rate constant for GSH, as follows.

$$k_m/K_{\text{GSH}} = k_{\text{app}}k_6(k_1 + k_4)$$

For wild type rGST T2-2, $k_{\text{app}}$ was determined as 6.0 $\pm$ 0.6 $\text{mm}^{-1} \text{s}^{-1}$, and $k_{\text{app}}/K_m$ was 3.7 $\pm$ 0.6 $\text{mm}^{-1} \text{s}^{-1}$ at 30 °C. That $k_{\text{app}}$ and $k_{\text{app}}/K_m$ are similar is expected because for wild type rGST T2-2 was determined as 6 $s^{-1}$ at 30 °C; hence, $k_{\text{app}}$ $> k_3$ and $k_{\text{app}}/K_m$ $> k_{\text{app}}$. The value of $k_{\text{app}}$ is much less than a rate constant for a diffusion-controlled association. This was explained by a fast pre-equilibrium occurring before binding of GSH ($k_2$ and $k_3$ in Fig. 1); giving an apparent rate constant $k_{\text{app}} = k_2(k_3 + k_4)(10, 21)$. $k_{\text{app}}$ for wild type rGST T2-2 was measured by time-resolved fluorescence quenching upon binding of GSH. For the S11A mutant, however, this approach did not give useful experimental traces and $k_{\text{app}}$ could not be determined separately. $k_{\text{app}}$ for wild type rGST T2-2 was determined as 0.9 $\pm$ 0.1 $\text{mm}^{-1} \text{s}^{-1}$ (Fig. 4).

Pre-equilibrium Binding and Dissociation of the Reaction Product MSG—For wild type rGST T2-2, product release was previously found to be the dominant rate-contributing step at 15 °C (10). To investigate the influence of Ser11 on dissociation of product, pre-equilibrium MSG binding experiments with both wild type rGST T2-2 and the S11A mutant per-
The Michaelis-Menten equation was fitted to data in order to determine previously published; Ref. 10) for 30 °C by omitting the step rGST T2-2 in Fig. 1 was adjusted (as compared with that analyzed as single exponentials. Also, the reaction scheme for lower temperatures but disappears as the temperature is monitored in the stopped flow spectrometer as time-resolved increase in fluorescence of the enzyme, because MSG quenches the fluorescence more than does GSH. A single exponential equation was fitted to experimental traces to obtain observed rate constants. As for wild type rGST T2-2, \( k_{\text{obs}} \) for the mutant decreased with increasing GSH concentration (Fig. 6A), indicating a slow step followed by a fast one (cf. Ref. 20). At pH 7.5, \( k_{\text{app}} \) for the S11A mutant was calculated as 0.06 ± 0.05 s\(^{-1}\) and \( k_{\text{app}} \) as approximately 60 s\(^{-1}\) using Equation 2. Thus, \( k_{\text{app}} \) for the S11A mutant has decreased compared with the value for wild type rGST T2-2, which was determined as 1.3 ± 0.2 s\(^{-1}\).

\( k_{\text{app}} \) determined in the displacement experiments is the apparent rate constant for all steps between the enzyme-product and the enzyme-GSH complexes. Thus, several rate constants in which MSG is displaced by GSH and binding of GSH were monitored as time-resolved increase in fluorescence of the enzyme. Because MSG quenches the fluorescence more than does GSH. A single exponential equation was fitted to experimental traces to obtain observed rate constants. As for wild type rGST T2-2, \( k_{\text{obs}} \) for the mutant decreased with increasing GSH concentration (Fig. 6A), indicating a slow step followed by a fast one (cf. Ref. 20). At pH 7.5, \( k_{\text{app}} \) for the S11A mutant was calculated as 0.06 ± 0.05 s\(^{-1}\) and \( k_{\text{app}} \) as approximately 60 s\(^{-1}\) using Equation 2. Thus, \( k_{\text{app}} \) for the S11A mutant has decreased compared with the value for wild type rGST T2-2, which was determined as 1.3 ± 0.2 s\(^{-1}\).

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GSH on wild type rGST T2–2 (●) and the S11A mutant (○). In the experiments, 1 μM subunit was mixed with MSG of different concentrations at 30 °C at pH 7.5, and the decrease in intrinsic tryptophan fluorescence was monitored. The inset is a magnification of the graph in the region of the lowest MSG concentrations.

FIG. 5. Observed rate constants for binding of MSG to wild type rGST T2–2 (●) and the S11A mutant (○). In the experiments, 1 μM subunit was mixed with MSG of different concentrations at 30 °C at pH 7.5, and the decrease in intrinsic tryptophan fluorescence was monitored. The inset is a magnification of the graph in the region of the lowest MSG concentrations.

FIG. 6. Observed rate constants for displacement of MSG by GSH on wild type rGST T2–2 (●) and the S11A mutant (○) at different GSH concentrations, pH 7.5 (A) and at different pH values in presence of 60 μM GSH for the S11A mutant (B). In B, kcat values (×) for the S11A mutant are included. The experiments were conducted at 30 °C. Rate constants were obtained by measuring increase in intrinsic tryptophan fluorescence, resulting from the replacement of enzyme-bound MSG with GSH, as described under "Experimental Procedures." The best fit curves in A were obtained by fitting Equation 2 to data. The data are weighted to favor the points obtained at high GSH concentrations (weight × 1/7).

mm GSH, a value identical to kcat. Fig. 6B shows that the observed rate constants obtained with 60 mm GSH follow kcat at different pH values for the S11A mutant. For wild type rGST T2–2, kcat was found to decrease below kcat as the pH was raised (10). Thus, it is clear that the step that limits kcat in the S11A mutant is not k5 (Fig. 1) as for wild type rGST T2–2. Instead, for the S11A mutant, kcat appears to be limited by a step on the reaction pathway (the same step as limits kcat).

Steady-state Kinetics with 2-Mercaptoethanol as Alternative Thiol Substrate—In the presence of S-methylglutathione or S-propylglutathione, both wild type rGST T2–2 and the S11A mutant were able to catalyze the reaction between 2-mercaptoethanol and MS. It was found that the enzymatic activity is dependent on the concentration of the S-alkylglutathione cofactor as well as the concentrations of the substrates MS and S. The x-ray structure of rGST T2–2, as cofactor

| Enzyme                  | kcat (s–1) | K0.5 (M) | Km (M) | Kmh (M) |
|-------------------------|------------|----------|--------|----------|
| S-Methylglutathione     |            |          |        |          |
| Wild type rGST T2–2    | 0.076 ± 0.020 | 11 ± 4 | 6 ± 2  |          |
| S11A mutant            | 0.011 ± 0.001 | 4 ± 1   | 2.0 ± 0.5 |        |
| S-Propylglutathione     |            |          |        |          |
| Wild type rGST T2–2    | 0.022 ± 0.012 | 5.5 ± 1.6 | 12 ± 3 | 4.7 ± 1.1 |
| S11A mutant            | 0.0034 ± 0.0006 | 1.3 ± 0.3 | 1.3 ± 0.3 | 4.2 ± 0.8 |

*For wild type rGST T2–2, kcat with S-methylglutathione as cofactor was determined by varying the cofactor at constant concentrations of 2-mercaptoethanol (30 μM) and MS (45 μM). Owing to the high Km value for S-methylglutathione, the kcat value obtained when 2-mercaptoethanol was varied (0.044 ± 0.006 s–1) was underestimated. kcat for the S11A mutant with S-methylglutathione as cofactor was calculated as the mean of the respective kcat values obtained in the experiments used to determine K0.5 for S-methylglutathione and Km for 2-mercaptoethanol. kcat with S-propylglutathione as cofactor was calculated as the mean of the respective kcat values obtained in the experiments used to determine K0.5 for S-propylglutathione, and the Km values for the two substrates.

Stabilization of the GSH Thiolate—The x-ray structure of human GST T2–2 shows that Ser11 is within hydrogen bonding distance from the GSH sulfur (9). Owing to the high identity (78%) in primary structure between the human and the rat enzymes, it is clear that their tertiary structures are also similar. We propose that the shift in pK0 value of the pre-steady-state rate constant for product formation kcat from 5.7 to 7.9 (Fig. 2B) illustrates the stabilizing effect of Ser11 on the GSH thiolate at equilibrium. The hydrogen bond between Ser11 and the thiolate thus corresponds to approximately 13 kJ mol–1 at 30 °C. However, the S11A mutant still stabilizes the GSH thiolate by lowering the pK0 by 1.3 pH units compared with that in free solution (pK0, 9.2). The equilibrium stabilization of the GSH thiolate may be important in vivo when rGST T2–2 is not operating under steady-state conditions but is mainly present as an enzyme charged with GSH and ready to inactivate potentially harmful electrophiles at low concentrations.

Product Release—Several experimental results suggest that product release is rate-limiting for the reaction catalyzed by the S11A mutant, as it is for wild type rGST T2–2. First, the MSG binding experiments show that the affinity for the product increases as a result of the mutation, and the off-rates obtained are close to the corresponding kcat values for wild type rGST T2–2 and the S11A mutant, respectively (Fig. 5 and Table I). The decrease of Km values (Table I) is in line with a lowering of the rate of a late step (kcat) in the catalyzed reaction. The displacement experiments (Fig. 6) corroborate the fact that product release is rate-limiting by showing that a step identical in rate to kcat is present between enzyme-product and enzyme-GSH complex (the burst experiments rule out the possibility

### DISCUSSION

The present results clearly show that the Ser11 residue contributes to the catalytic mechanism of rGST T2–2. The pre-

### TABLE II

| Enzyme                  | kcat (s–1) | K0.5 (M) | Km (M) | Kmh (M) |
|-------------------------|------------|----------|--------|----------|
| S-Methylglutathione     |            |          |        |          |
| Wild type rGST T2–2    | 0.076 ± 0.020 | 11 ± 4 | 6 ± 2  |          |
| S11A mutant            | 0.011 ± 0.001 | 4 ± 1   | 2.0 ± 0.5 |        |
| S-Propylglutathione     |            |          |        |          |
| Wild type rGST T2–2    | 0.022 ± 0.012 | 5.5 ± 1.6 | 12 ± 3 | 4.7 ± 1.1 |
| S11A mutant            | 0.0034 ± 0.0006 | 1.3 ± 0.3 | 1.3 ± 0.3 | 4.2 ± 0.8 |
that binding of GSH is slow). Also, the dependence of the steady-state kinetics on medium viscosity (Fig. 3) supports product release as a rate-limiting step.

However, the fractional viscosity dependence of $k_{\text{cat}}$ (slope = 0.5–0.6) suggests that product dissociation is limited by conformational transitions involving movement of regions of the protein in the surrounding solution, for the S11A mutant as well as for wild type rGST T2-2. The crystallographic analysis of human GST T2-2 (9) gives a structural basis for such a rate-contributing conformational change. The crystal structure displays an active site closed by two C-terminal $\alpha$-helices connected by a loop (9). These most probably have to be dislocated, completely or partially, before dissociation of product is possible. This opening of the binding site is probably also the explanation of the fact that binding of neither GSH nor MSG to rGST T2-2 occurs at a diffusion-controlled rate. The fast pre-equilibria ($k_{\text{on}}$, $k_{\text{off}}$, and $k_{\text{on}}, k_{\text{off}}$) are included in the reaction scheme (Fig. 1) to explain the slow binding (10, 21).

The increased activation energy for product release displayed by the S11A mutant, resulting in a lower $k_{\text{cat}}$, may be a side effect of a catalytically impaired enzyme but may also reflect additional functions of Ser$^{11}$ in catalysis. For example, after the product forming step, Ser$^{11}$ could make unfavorable interactions with the product(s), facilitating their dissociation from the enzyme.

The pH dependence of $k_{\text{cat}}$ for the S11A mutant displays two $pK_a$ values, at pH 6.0 and 10.4, respectively (Fig. 2A). Given that product release is rate-limiting at neutral and basic pH values, the upper $pK_a$ may reflect deprotonation of a lysine residue (e.g. Lys$^4$; Ref. 9) that makes a hydrogen bond with the GSH moiety of the product. Loss of the positive charge at high pH values would favor dissociation of product and hence lead to an increased $k_{\text{cat}}$. The pH dependence of $k_{\text{obs}}$ for MSG displacement by GSH is similar to that of $k_{\text{cat}}$ (Fig. 6B) and probably results from the same deprotonation. The $pK_a$ at 6.0, however, most likely reflects protonation of active site GSH thiolate. At acidic pH values, as $k_{4}$ becomes rate contributing, the lower tail of the pH dependence of $k_{4}$ affects the pH dependence of $k_{\text{cat}}$ (Fig. 2). Hence, this gives an apparent $pK_a$ value of 6.0 for enzyme-bound GSH thiol (as reflected in $k_{\text{cat}}$), whereas the true value would be 7.9 (as reflected in $k_{4}$).

GSH Binding—Pre-equilibrium binding of GSH could not be measured directly for the S11A mutant. However, $k_{4}/K_{GSH}^{0.5}$ decreased for the mutant. For wild type rGST T2-2, $k_{4}/K_{GSH}^{0.5}$ follows the apparent association rate constant for GSH $k_{GSH}^{\text{app}}$ (10). According to Equation 4, the reason for the decrease in $k_{4}/K_{GSH}^{0.5}$ cannot be the decrease in $k_{4}$ alone, because the factor $k_{4}/(k_{4} + k_{GSH})$ will be approximately 1 as long as $k_{4} > k_{GSH}$. The $k_{4}$ value for the mutant ($k_{4} = 43 s^{-1}$) is larger than the off-rate of GSH from wild type rGST T2-2 ($k_{\text{off}} = 6 s^{-1}$). Thus, the mutation S11A affects not only the equilibrium stabilization of enzyme-bound GSH thiolate but also the binding of GSH, either by increasing $k_{\text{off}}$ or decreasing $k_{GSH}^{\text{app}}$ or both. Structurally, Ser$^{11}$ may increase the affinity for GSH via hydrogen bonding to the thiolate (thereby decreasing $k_{\text{off}}$) or assist in proper positioning of GSH in the active site during association (thereby increasing $k_{GSH}^{\text{app}}$).

Relaxed Thiol Specificity—The finding that Ser$^{11}$ of rat GST T2-2 is indeed important in the catalysis of the MS reaction, as illustrated by the effects on different reaction steps, is crucial because it underlines the sulfatase hypothesis (8, 9). Another argument for the sulfatase hypothesis was related to the relaxed thiol specificity of human GST T2-2 induced by S-methylglutathione (8). GST is the preferred thiol substrate for GSTs, but in presence of certain GSH analogs alternative thiol substrates are accepted. This effect was first shown to exist for rat GST M2–2 (22, 23) and more recently for human GST T2-2 (8). The mechanism has not been elucidated. Principato et al. (22) proposed that S-methylglutathione, like GSH, induces a conformational change in the protein structure necessary for the catalyzed reaction to take place. When the catalytically competent conformation was established, other thiols than GSH could be used as substrate. Tan et al. (8) used a similar explanation, although they proposed that the sulfate group of MS was cleaved off in the active site of human GST T2-2 subsequent to the conformational transition induced by S-methylglutathione, without a thiolate performing a nucleophilic attack. The enzyme would hence act as a sulfatase rather than as a GST.

The kinetic data with 2-mercaptoethanol as thiol substrate obtained in this study (Table II) were found to be qualitatively similar to the data obtained using GSH (Table I), suggesting that Ser$^{11}$ plays a role in the catalyzed reaction with alternative thiols such as 2-mercaptoethanol, as well as with GSH. Also, it was found that the relaxed thiol substrate specificity induced by S-methylglutathione is not restricted to a certain electrophilic substrate nor to sulfate as leaving group, because the reaction between NBC and 2-mercaptoethanol was also catalyzed by rGST T2-2. These results do not support the sulfatase hypothesis.

What is the mechanism behind the relaxed thiol substrate specificity? The ligand-induced slow transition reaction mechanism proposed for rGST T2-2 (10) may provide an answer. According to this model, the distribution of enzyme species present at equilibrium is distinct from that observed during catalytic turnover. The closed conformation in the apo structure of human GST T2-2 is indistinguishable from the conformations of the GSH and product liganded structures (9). Upon addition of substrates, the enzyme adopts the preferred steady-state conformation. Possibly, GSH derivatives such as S-methylglutathione could also induce formation of the catalytically active form and sustain the reaction as long as alternative thiol and electrophilic substrate are available. At equilibrium the majority of enzyme molecules would return to the “noncatalytic form.” Catalysis could be accomplished either by simultaneous binding of S-methylglutathione, 2-mercaptoethanol, and MS to the active site or by a mnemonic mechanism, in which the enzyme “remembers” the active conformation after release of S-methylglutathione long enough for substrates to bind and subsequent catalysis to occur. Soluble GSTs are dimers, and a third possibility would be that S-alkylglutathione bound to one subunit activates the other subunit. Cooperativity in GSH binding has recently been shown to exist for GST P1–1 (24), and crystal structures of murine GST A4–4 revealed possible signaling across the dimer interface (25). However, studies addressing this question on other GSTs did not detect evidence for “communication” between the subunits (26, 27).

Implications for the Reaction Mechanism of Human GST T2-2—Given the high identity in primary structure between human and rat GST T2-2, including the residues building up the sulfate-binding pocket (9), it appears reasonable to assume that the two enzymes use a similar approach to catalyze the reaction between MS and GSH. But then why is $k_{\text{cat}}$ affected for rat but not for human GST T2-2 by the S11A mutation? First, the kinetics of human GST T2-2/S11A (8) was studied at pH 8.3 where approximately 10% of the GSH molecules in solution are already in the thiolate form. It is probable that elimination of the hydroxyl group of Ser$^{11}$ under such conditions does not decrease the rate constant for product formation on the enzyme ($k_{4}$) to a value that is lower than that for product dissociation ($k_{7}$). This would be similar to what is found for rat GST T2-2 in the present study and is most probably the reason
why the decreased stabilization of the GSH thiolate is not reflected in the steady-state kinetics of human GST T2-2. Second, the $k_{cat}$ value for human GST T2-2 (8) is lower than that for rat GST T2-2; it is actually close to $k_{cat}$ for the S11A mutant. This could be due to slower product release of the human GST T2-2 compared with the rat enzyme. The reduced rate of product release that removal of the hydroxyl group of Ser11 causes in rat GST T2-2 may therefore not apply to the already slowly dissociating human GST T2-2-MSG complex.

REFERENCES

1. Mannervik, B., and Danielsen, U. H. (1988) CRC Crit. Rev. Biochem. 23, 283–337
2. Hayes, J. D., and Pulford, D. J. (1995) Crit. Rev. Biochem. Mol. Biol. 30, 445–600
3. Mannervik, B., Ålin, P., Guthenberg, C., Jensson, H., Tahir, M. K., Warholm, M., and Jornvall, H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7202–7206
4. Armstrong, R. N. (1997) Chem. Res. Toxicol. 10, 2–18
5. Gillham, B. (1971) Biochem. J. 121, 667–672
6. Hiratsuka, A., Sebata, N., Kawashima, K., Okuda, H., Ogura, K., Watabe, T., Satoh, K., Hatayama, I., Tsuchida S., Ishikawa, T., and Sato, K. (1990) J. Biol. Chem. 265, 11973–11981
7. Meyer, D. J., Coles, B., Pembble, S. E., Gilmore, K. S., Fraser, G. M., and Ketterer, B. (1991) Biochem. J. 274, 499–414
8. Tan, K.-L., Chevanayagam, G., Parker, M. W., and Board, P. G. (1996) Biochem. J. 319, 315–321
9. Rossjohn, J., McKinstry, W. J., Oakley, A. J., Verger, D., Flanagan, J., Chevanayagam, G., Tan, K.-L., Board, P. G., and Parker, M. W. (1998) Structure 6, 309–322
10. Jemth, P., and Mannervik, B. (1999) Biochemistry 38, 9982–9991
11. Hyde, C. W., and Young, L. (1968) Biochem. J. 107, 519–522
12. Clappe, J. J., and Young, L. (1970) Biochem. J. 118, 765–771
13. Vince, R., Daluge, S., and Wadd, W. B. (1971) J. Med. Chem. 14, 402–404
14. Jemth, P., Stenberg, G., Chaga, G., and Mannervik, B. (1996) Biochem. J. 316, 131–136
15. Björnstedt, R., Widersten, M., Board, P. G., and Mannervik, B. (1992) Biochem. J. 382, 505–510
16. Hahig, W. H., Pabst, M. J., and Jakoby, W. B. (1974) J. Biol. Chem. 249, 7130–7139
17. Brouwer, A. C., and Kirsch J. F. (1982) Biochemistry 21, 1302–1307
18. Mannervik, B. (1982) Methods Enzymol. 87, 370–390
19. Bardsey, W. G., Bukhari, N. A. J., Ferguson, M. W. J., cachaza, J. A., and Burguillo, F. J. (1995) Computers Chem. 19, 75–84
20. Fersht, A. (1999) Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding, pp. 132–190, W. H. Freeman and Co., New York
21. Parsons, J. F., Xiao, G., Gilliland, G. L., and Armstrong, R. N. (1998) Biochemistry 37, 6286–6294
22. Principato, G. B., Danielson, U. H., and Mannervik, B. (1988) FEBS Lett. 231, 155–158
23. Mannervik, B., Board, P. G., Berhane, K., Björnstedt, R., Castro, V. M., Danielson, U. H., Hao, X.-Y., Kohn, R., Olin, B., Principato, G. B., Ridderström, M., Stenberg, G., and Widersten, M. (1990) in Glutathione S-Transferases and Drug Resistance (Hayes, J. D., Pickett, C. B., and Mantle, T. J., eds) pp 35–46, Taylor & Francis, London
24. Caccuci, A. M., Antonini, G., Ascencini, P., Nicol, M., Nucetelli, M., Mazzetti, A. P., Federici, G., Lo Bello, M., and Ricci, G. (1999) J. Biol. Chem. 274, 19276–19280
25. Xi, B., Singh, S. P., NANDHARI, B., Awasthi, Y. C., Zimniak, P., and Ji, X. (1999) Biochemistry 38, 11887–11894
26. Danielson, U. H., and Mannervik, B. (1985) Biochem. J. 231, 263–267
27. Gustafsson, A., and Mannervik, B. (1999) J. Mol. Biol. 288, 787–800
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