Human Glutathione Transferase A1-1 Demonstrates Both Half-of-the-sites and All-of-the-sites Reactivity*

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A study of the kinetics of a heterodimeric variant of glutathione transferase (GST) A1-1 has led to the conclusion that, although the wild-type enzyme displays all-of-the-sites reactivity in nucleophilic aromatic substitution reactions, it demonstrates half-of-the-sites reactivity in addition reactions. The heterodimer, designed to be essentially catalytically inactive in one subunit due to a single point mutation (D101K), and the two parental homodimers were analyzed with seven different substrates, exemplifying three types of reactions catalyzed by glutathione transferases (nucleophilic aromatic substitution, addition, and double-bond isomerization reactions). Stopped-flow kinetic results suggested that the wild-type GST A1-1 behaved with half-of-the-sites reactivity in a nucleophilic aromatic substitution reaction, but steady-state kinetic analyses of the GST A1-D101K heterodimer revealed that this was presumably due to changes to the extinction coefficient of the enzyme-bound product. In contrast, steady-state kinetic analysis of the heterodimer with three different substrates of addition reactions provided evidence that the wild-type enzyme displayed half-of-the-sites reactivity in association with these reactions. The half-of-the-sites reactivity was shown not to be dependent on substrate size, the level of saturation of the enzyme with glutathione, or relative catalytic rate.

The glutathione transferases (GSTs, EC 2.5.1.18) are a family of broad specificity detoxication enzymes found ubiquitously. They achieve their function by conjugating a glutathione molecule (7). In- terestingly, Asp-101 and Arg-131 are located in the monomer C terminus, respectively, of the glutathione molecule (7). In- terestingly, Asp-101 and Arg-131 form salt links to the N and C terminus, respectively, of the glutathione molecule (7). In- terestingly, Asp-101 and Arg-131 are located in the monomer adjacent to that in which the glutathione is bound, suggesting that they could play a role in signaling between the subunits. Several mutagenic studies involving different GSTs have shown that mutation of the conserved Asp-101 residue results in an enzyme that is severely compromised in its KᵦGH (28-30).

Given that the GSTs readily form heterodimers, this provides the means to investigate the possibility of cooperativity between the subunits of GST A1-1 with a GST that is active in only one monomer. A D101K point mutant of GST A1-1 was therefore created. As expected, this single mutation impaired the binding of glutathione to the enzyme. A His-tagged variant of GST A1-1 as well as a heterodimer of the His-tagged A1 subunit and the D101K point mutant were also produced and studied with several different substrates of various sizes and characters. This allowed fundamental conclusions to be drawn about the number of reactive sites in the wild-type enzyme with a given substrate. The results show for the first time that GST A1-1 demonstrates both “half-of-the-sites” or “all-of-the-sites” (reviewed in Ref. 31) reactivity, depending on the nature of the reaction. Furthermore, it was determined that the mode of reactivity was not dependent on either substrate size or on catalytic rate.

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The abbreviations used are: GST, glutathione transferase; TNB, 1,3,5-trinitrobenzene; AD, 1-nitrobenzene; AD, 1-nitrobenzene; GSH (28–30).

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MATERIALS AND METHODS

Expression and Purification—The plasmid pET21D101K was generated by site-directed polymerase chain reaction mutagenesis of the wild-type human GST A1-1 cDNA in the pGEM vector, pGEMHisA1 (21). Primer sequences were as follows: 5′-AGGTTAGCAGAAATGTGGTG-GAAATGATC-3′ (forward, the mutagenic codon is shown in bold) and 5′-CTCTATATACATCACTGAGGCTCT-3′ (reverse). After verification of the clone by sequencing, the mutated cDNA was subcloned by NdeI and SalI restriction enzyme digestion and ligation into the pET-21a vector. The final construct contained the cDNA for human GST A1-1 with a D101K point mutation. The plasmid pET24HisA1, which is a construct of pET24a, contains the cDNA sequence of human GST A1-1 with an N-terminal hexa-His extension as an aid to purification.

The presence of the His tag has been shown previously to have no effect on the catalytic activity of the enzyme (21), so the homodimer is hereafter simply referred to as GST A1-1. The heterodimer GST A1-D101K was expressed in E. coli BL21(DE3) cells as described previously (21), except that bacterial cells were grown for 4 h after induction before harvesting.

The heterodimer was partially separated from the D101K homodimer (hereafter simply referred to as D101K) and GST A1-1 by using a HiTrap SP cation exchange column (Amersham Pharmacia Biotech, Uppsala, Sweden) that had been previously equilibrated with 20 mM sodium phosphate buffer, pH 7.0. Three GST species were then resolved using a 0.0–0.8 M NaCl gradient. Fractions containing enzyme were identified during all stages of purification by assaying for GST activity with 1-chloro-2,4-dinitrobenzene (CDNB) using experimental conditions described previously (22). Pure GST A1-1 homodimer was isolated in its absence of glycerol. The D101K heterodimer was therefore expressed separately, and yields of the D101K heterodimer were low. The D101K mutant was therefore expressed separately, and the presence of the His tag has been shown previously to have no effect on the catalytic activity of the enzyme (21), so the homodimer is hereafter simply referred to as GST A1-1. The heterodimer GST A1-D101K was expressed in E. coli BL21(DE3) cells as described previously (21), except that bacterial cells were grown for 4 h after induction before harvesting.

Both the purified homodimers were stored at −80 °C in the absence of glycerol.

Fractions from the cation chromatography containing the heterodimer were further purified by immobilized metal affinity chromatography (Amersham Pharmacia Biotech) charged with Ni2+ ions. Separation was achieved by a 50–400 mM imidazole gradient in the presence of 20 mM sodium phosphate, 0.5 M NaCl, pH 7.4. Fractions containing pure heterodimer were pooled and desalted using PD-10 columns (Amersham Pharmacia Biotech) into 20 mM sodium phosphate, pH 7.0. The desalted heterodimer was then stored at −80 °C in the presence of 10% glycerol.

Both SDS-polyacrylamide gel electrophoresis and isoelectric focusing (pH 3.5–9.5) were used to confirm enzyme purity. Concentrations of the purified enzymes were calculated from the absorbance at 280 nm using an extinction coefficient of 24,700 M−1 cm−1. The molecular mass for each subunit was taken to be 25,500 Da.

Stopped-flow Experiments—The pre-steady-state reaction burst in the GST-catalyzed conjugation of CDNB and glutathione was measured using a stopped-flow spectrophotometer (Applied Photophysics Ltd., Leatherhead, United Kingdom). Reactions were performed at 5 °C in 0.1 M sodium phosphate buffer at several pH values. Volumes of 75–100 μl were injected from each syringe, to achieve the following final concentrations. GST A1-1 (at a subunit concentration of 10–20 μM) and 0.8 mM CDNB were rapidly mixed with 0.1–4 mM glutathione (GSH). Alternatively, GST A1-1 incubated with a constant concentration of 0.5 mM GSH was rapidly mixed with 0.1–0.8 mM CDNB. Product formation was monitored at 340 nm, and the data were analyzed using software supplied with the stopped-flow apparatus. The observed rate constant ($k_{obs}$) for the pre-steady-state formation of the glutathione-dinitrophenyl (GS-DNP) conjugate, the amplitude of the burst ($ΔA_{max}$), and the initial rate of the steady-state phase ($v$), were determined using a single exponential function combined with a linear function. The amount of product formed per subunit enzyme (mol/mol) was calculated by dividing the $ΔA_{max}$ by the enzyme concentration and the extinction coefficient of the GS-DNP conjugate (9600 M−1 cm−1).

Meisenheimer Complex Formation—The formation of the $\alpha$-complex between GSH and TNB was monitored using difference spectroscopy, as described previously (22, 33). The enzymes (20 μM) were titrated with 0.05, 0.1, and 0.2 mM TNB in 0.1 M sodium phosphate, pH 6.5, at 30 °C in the presence of 5 mM GSH. Spectra were recorded between 400 and 600 nm with a maximum of absorbance at 450 nm. Formation constants and extinction coefficients were calculated by non-linear regression analysis of data from two separate experiments.

Kinetic Studies—The kinetic parameters for the two homodimers and the heterodimer were determined with CDNB, 3,4-dichloro-1-nitrobenzene (DCNB), $\Delta^3$-androsten-3,17-dione (AD), nonenal, 4-nitrocin-

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nalamaldehyde (NCA), and benzylisothiocyanate (benzyl-ITC) in essentially the same manner as described previously (22, 32, 34–37), although some changes to electrophile and glutathione concentration were made. Glutathione was used at concentrations of: 0.05–1.5 mM with 2 mM CDNB, 0.025–1 mM with 0.8 mM DCNB, 0.025–1 mM with 0.2 mM AD, 0.05–2 mM with 0.1 mM nonenal, 0.01–2 mM with 0.4 mM NCA, and 0.05–1 mM with 0.4 mM benzyl-ITC. Electrophile concentrations were varied as follows: 0.1–2 mM CDNB with 2.5 mM GSH, 0.2–0.8 mM DCNB with 5 mM GSH, 0.5–150 μM AD with 2 mM GSH, 0.13–0.3 mM nonenal with 1 mM GSH, 0.05–0.4 mM NCA with 2.5 mM GSH, and 0.02–0.4 mM benzyl-ITC with 1 mM GSH. The AD reaction was performed in 50 mM Tris buffer, pH 8, and the DCNB reaction was performed in 0.1 M sodium phosphate buffer, pH 8, whereas all other reactions were conducted in 0.1 M sodium phosphate buffer, pH 6.5.

Data were analyzed by non-linear regression using the software package GraphPad Prism® (GraphPad Software, Inc. San Diego, CA). The Michaelis-Menten equation was fit to the saturation curves of GST A1-1, the heterodimer, and the theoretically predicted results for the heterodimer, whereas the second degree rate equation $v = (\frac{[S]}{K_M} + \frac{1}{K_S} + \frac{1}{v_{max}}[S])^{-1}$ (dashed line) was fitted to data generated by D101K. In order to determine catalytic efficiency ($k_{cat}/K_M$) for GST A1-1, the heterodimer and the theoretical curve, the equation $v = \frac{(V_{max}/K_M)[S]}{[S] + (1/K_M)[S]}$ was fit to the data.

RESULTS

Expression and Purification—Enzymes were expressed and purified either using a single step of cation exchange chromatography, in the case of the two homodimers, or cation exchange chromatography followed by Ni2+-immobilized metal affinity chromatography for the heterodimer. Both SDS-polyacrylamide gel electrophoresis and isoelectric focusing confirmed that the three enzyme species were pure (data not shown). Final yields were calculated to be 35 mg/liter for GST A1-1, 66 mg/liter for the D101K homodimer, and 15 mg/liter for GST A1-D101K. Specific activities ± standard errors (μmol/min/mg) were calculated for each enzyme with CDNB and were found to be 100 ± 10 for GST A1-1, 0.61 ± 0.02 for the D101K homodimer, and 50 ± 1 for GST A1-D101K. For comparison, the average of the specific activities of the two homodimers was 50 μmol/min/mg, suggesting that there was no cooperativity between the subunits of the heterodimer.

Stopped-flow Analysis of Pre-steady-state Product Formation with GST A1-1—The amplitude of the pre-steady-state product formation burst in the reaction of CDNB and glutathione was measured in order to investigate the stoichiometry of binding of CDNB to wild-type GST A1-1. The suitability of the hyperbolic function used for data analysis can be seen in Fig. 1, where a representative reaction trace and the fitted function are shown. The experiments were conducted at several pH values, with...
Maximum burst amplitude (and varying the concentration of CDNB (Fig. 2). Therefore achieved by saturating the enzyme with glutathione (data not shown). A more accurate determination of the maximum reactivity was performed at a constant concentration of CDNB (data not shown). Analogous results were obtained (data not shown). Results from a representative curve, at pH 7.5, are therefore depicted in Fig. 2. This pH was chosen in order to maximize the burst phase rate constant (k_{db}; 330 s^{-1}) relative to the steady-state rate of product formation (11 s^{-1}), allowing accurate estimation of the concentration of enzyme active sites (38). Due to the low concentrations of glutathione compatible with measurements on the stopped-flow apparatus when the reaction was performed at a constant concentration of CDNB (data not shown). A more accurate determination of the maximum product formation burst amplitude per enzyme subunit was therefore achieved by saturating the enzyme with glutathione and varying the concentration of CDNB (Fig. 2).

Fitting a hyperbolic function to the data in Fig. 2 indicated a maximum burst amplitude (A_{max}) of 0.37 ± 0.05 mol of product/mol of enzyme subunit, and that half A_{max} was reached at a concentration of 0.39 ± 0.12 mM CDNB. However, reactions were performed at concentrations of 0.5 mM GSH, corresponding to only ~70% saturation of the enzyme with glutathione. Correcting for the incomplete saturation with glutathione, the apparent maximum amount of product formed per subunit enzyme would be 0.52 mol/mol, indicative of half-of-the-sites reactivity of GST A1-1 with CDNB. Analogous results were obtained in similar pre-equilibrium binding experiments using TNB instead of CDNB (data not shown). However, equilibrium measurements of Meisenheimer complex formation with the three enzyme variants (see below) showed that the apparent half-of-the-sites reactivity detected by stopped-flow kinetics was artifactual. The heterodimer was not studied by stopped-flow analysis due to the relatively small amounts of the enzyme available after purification.

Meisenheimer Complex Formation—The conjugation of glutathione to CDNB proceeds through a Meisenheimer complex intermediate (Fig. 3). This complex can be mimicked by the reaction of GSTs with glutathione and TNB, which does not contain a proper leaving group. The TNB molecule is therefore reversibly conjugated with the glutathione molecule, and conjugate formation can be measured under equilibrium conditions (40). The abilities of the homodimers and the heterodimer to promote the formation of the Meisenheimer complex were compared in order to titrate the number of functional active sites within the enzymes. The results, shown in Table I and Fig. 4, are in good agreement with the previously published A_{max} and K_M values (12300 ± 400 M^{-1} cm^{-1} and 8900 ± 1400 M^{-1}, respectively) for GST A1-1 (22). The results also correspond with the specific activity results obtained with CDNB, i.e. the heterodimer exhibited an activity consistent with non-cooperativity of the monomers. Therefore, from equilibrium experiments, as opposed to pre-steady-state experiments, the wild-type enzyme displayed all-of-the-sites reactivity with CDNB and TNB. This difference in the mode of reactivity was not due to the different temperatures at which the experiments were conducted (5 °C as opposed to 30 °C), since TNB titrations of wild-type GST A1-1 at 5 °C (data not shown) were also indicative of half-of-the-sites reactivity. Accurate formation constants and extinction coefficients could not be derived for the D101K homodimer, since glutathione binding for this enzyme was so compromised that saturation could not be achieved under the available experimental conditions.

Steady-state Kinetic Studies—The activities of the homodimers and the heterodimer were further investigated under steady-state conditions with substrates exemplifying three of the different types of reactions catalyzed by GSTs. Glutathione is conjugated with CDNB and DCNB in nucleophilic aromatic substitution reactions, AD is modified by a double-bond isomerization reaction, and nonenal, NCA, and benzyl-ITC are detoxified by addition reactions (Fig. 3). Ethacrynic acid is another model substrate for addition reactions, but kinetic parameters could not be determined due to the poor catalytic activity of GST A1-1 with this substrate (data not shown).

The kinetic parameters determined for the three reaction types are shown in Table II, while representative saturation curves are displayed in Fig. 5. The kinetic constants k_{cat} and K_M could not be determined separately for DCNB, since the limited solubility of the electrophile precluded enzyme saturation. Instead, catalytic efficiency (k_{cat}/K_M) was determined from the initial reaction rate. Also shown in Table II and Fig. 5 are the predicted values for the heterodimer in the nucleophilic aromatic substitution and double-bond isomerization reactions, based on the assumption that wild-type GST A1-1 displays all-of-the-sites reactivity. These values were not calculated for the addition reactions, since it was evident that there was no significant difference between the heterodimer and wild-type GST A1-1. The k_{cat} values are apparent values in some cases, since it was not always possible to saturate the enzymes with the non-varied substrate, particularly in the case of the D101K homodimer.

The D101K mutation had a drastic effect on the catalytic ability of the mutant homodimer, as can be seen from Fig. 5. The saturation curve of this enzyme with nonenal appears sigmoidal, suggesting that positive cooperative interactions influence the kinetics of the enzyme. Data from this enzyme were therefore analyzed using a second degree rate equation (see “Materials and Methods”). In contrast, the heterodimer and wild-type GST A1-1 exhibit Michaelis-Menten kinetics, and were analyzed accordingly. It was demonstrated that the behavior of heterodimer was adequately described by Michaelis-Menten kinetics due to the small contribution of the D101K subunit to the activity of the enzyme. The phenomenon of positive cooperativity of D101K with nonenal was not investigated further, since the extremely poor activity of the enzyme in this reaction necessitated the use of large amounts of the homodimer. Elevated background absorbance levels associated with high concentrations of enzyme and substrate were prohibitive to accurate rate measurements. No marked evidence of
positive cooperativity was detected with any of the other substrates tested (Fig. 5 and data not shown).

As can be seen from Fig. 5, the reaction of the heterodimer with the different substrates examined in this study exhibited different outcomes depending on the type of reaction being catalyzed. In the nucleophilic aromatic substitution and double-bond isomerization reactions, there was no marked difference between the experimentally and theoretically determined kinetic parameters for the heterodimer (see Table II). This result is consistent with all-of-the-sites reactivity of the wild-type enzyme. Surprisingly, in the addition reactions involving nonenal, NCA, and benzyl-ITC, the heterodimer showed no significant difference in rate behavior from the GST A1-1 enzyme, indicative of a mechanism-based half-of-the-sites reactivity of wild-type GST A1-1.

### TABLE I

| Enzyme       | $K_F$ ($M^{-1}$) | $A_{\text{max}}$ ($M^{-1} cm^{-1}$) |
|--------------|-----------------|-----------------------------------|
| GST A1-1     | 7552 ± 465      | 10,510 ± 157                      |
| GST D101K    | —              | —                                 |
| Predicted    | 7509 ± 10       | 5340 ± 10                         |
| GST A1-D101K | 6485 ± 714      | 4623 ± 133                        |

$^{a}$ Absorbance at 450 nm at saturating concentrations of TNB and glutathione.

$^{b}$ Accurate values could not be determined for the D101K homodimer due to lack of saturation under the available experimental conditions.

$^{c}$ Predicted values for the heterodimer were derived from non-linear regression analysis of the average of the GST A1-1 and GST D101K curves.
actions with CDNB, TNB, DCNB, and AD, this was not due to
what lower than the theoretically predicted results in the re-
mined heterodimer saturation curves were consistently some-
caused by changes in the extinction coefficients of the glutathi-
state and equilibrium experiments, was presumably an artifact
of-the-sites reactivity toward TNB. Hence, the apparent half-site
modimer, implying that the wild-type enzyme must display all-
was in agreement with the results from TNB-binding studies
 типе enzyme displayed all-of-the-sites reactivity. This conclusion
heterodimer corresponded to the mean of the activities of the two
measurements with CDNB demonstrated that the activity of the
of the Alpha class enzyme GST A1-1 has now been investigated
40), suggesting that GST A1-1 may be unusual in having half-
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| Enzyme | $k_{app}$ elec | $K_M^{app}$ | $k_{eq}$ | $K_M^{GSH}$ | $k_{cat}/K_M^{GSH}$ |
|--------|--------------|-------------|----------|--------------|-------------------|
| CDNB   |              |             |          |              |                   |
| A1-1   | $100 \pm 10$ | $1.2 \pm 0.3$ | $84 \pm 9$ | $62 \pm 1$   | $0.15 \pm 0.01$    |
| Predicted | $54 \pm 1$   | $1.2 \pm 0.1$ | $42 \pm 1$  | $32 \pm 1$  | $0.16 \pm 0.01$ |
| A1-D101K | $43 \pm 6$  | $1.2 \pm 0.3$ | $35 \pm 4$  | $26 \pm 1$  | $0.14 \pm 0.01$ |
| DCNB   |              |             |          |              |                   |
| A1-1   | $112 \pm 8$  | $0.065 \pm 0.001$ | $0.045 \pm 0.004$ | $1.5 \pm 0.1$ |
| Predicted | $58 \pm 1$ | $0.023 \pm 0.001$ | $0.047 \pm 0.001$ | $0.70 \pm 0.01$ |
| A1-D101K | $34 \pm 3$ | $0.021 \pm 0.001$ | $0.042 \pm 0.001$ | $0.50 \pm 0.04$ |
| AD     |              |             |          |              |                   |
| A1-1   | $22 \pm 1$   | $0.05 \pm 0.01$ | $500 \pm 40$ | $22 \pm 1$ | $0.06 \pm 0.01$ |
| Predicted | $13 \pm 1$ | $0.06 \pm 0.01$ | $230 \pm 10$ | $13 \pm 1$ | $0.12 \pm 0.01$ |
| A1-D101K | $10 \pm 1$ | $0.08 \pm 0.02$ | $150 \pm 20$ | $6.5 \pm 0.4$ | $0.90 \pm 0.01$ |
| Nonenal |              |             |          |              |                   |
| A1-1   | $0.23 \pm 0.01$  | $0.08 \pm 0.01$ | $2.9 \pm 0.3$ | $1.5 \pm 0.01$ | $0.25 \pm 0.05$ |
| Predicted | $0.20 \pm 0.01$ | $0.18 \pm 0.01$ | $1.1 \pm 0.1$  | $0.10 \pm 0.01$ | $0.40 \pm 0.01$ |
| A1-D101K | $0.25 \pm 0.02$ | $0.09 \pm 0.02$ | $2.9 \pm 0.4$  | $0.16 \pm 0.01$ | $0.28 \pm 0.05$ |
| NCA    |              |             |          |              |                   |
| A1-1   | $0.33 \pm 0.05$  | $0.27 \pm 0.08$ | $1.2 \pm 0.2$ | $0.29 \pm 0.01$ | $0.24 \pm 0.03$ |
| Predicted | $0.36 \pm 0.03$ | $0.26 \pm 0.02$ | $1.2 \pm 0.1$  | $0.28 \pm 0.05$ | $1.2 \pm 0.01$ |
| A1-D101K | $0.35 \pm 0.04$ | $0.30 \pm 0.07$ | $1.2 \pm 0.1$  | $0.26 \pm 0.02$ | $0.22 \pm 0.05$ |
| Benzyl-ITC |              |             |          |              |                   |
| A1-1   | $2.1 \pm 0.1$  | $0.12 \pm 0.01$ | $18 \pm 1$  | $1.6 \pm 0.1$ | $0.16 \pm 0.02$ |
| Predicted | $NC^a$  | $NC^a$ | $NC^a$ | $NC^a$ | $NC^a$ |
| A1-D101K | $2.1 \pm 0.1$ | $0.09 \pm 0.02$ | $22 \pm 1$  | $1.8 \pm 0.1$ | $0.19 \pm 0.03$ |

Some $k_{cat}$ values were only apparent values, because it was not always possible to saturate the enzymes with the second substrate.

Predicted values were derived from non-linear regression analysis of the average of the A1-1 and D101K curves.

Accurate values were not able to be determined due to lack of saturation under the available experimental conditions.

NC, values were not calculated.

**DISCUSSION**

The phenomenon of cooperativity between the two subunits of the Alpha class enzyme GST A1-1 has now been investigated with a heterodimer that is essentially catalytically inactive in one subunit. This work was initiated by pre-steady-state studies of GST A1-1 with CDNB and glutathione, in which the wild-type enzyme displayed an apparent half-of-the-sites reactivity. This result contrasted with the additivity of kinetic properties of GST subunits demonstrated previously (12, 19–21, 41). Furthermore, an earlier study of the formation of the Meisenheimer complex with GST A1-1 implied that this enzy-

m displays half-of-the-sites reactivity with TNB. The $A_{max}$ for GST A1-1 with TNB was found to be $−12,300 \, \text{M}^{−1} \, \text{cm}^{−1}$ (22), compared with 21,200–27,000 M$^{−1}$ cm$^{−1}$ for other GSTs (35, 40), suggesting that GST A1-1 may be unusual in having half-of-the-sites reactivity with CDNB and TNB.

In order to shed further light on these phenomena, a het-

erodimer was produced and compared with the wild-type enzyme in various steady-state experiments. Initial specific activity measurements with CDNB demonstrated that the activity of the heterodimer corresponded to the mean of the activities of the two parental homodimers, consistent with the theory that the wild-type enzyme displayed all-of-the-sites reactivity. This conclusion was in agreement with the results from TNB-binding studies with the heterodimer (Fig. 4). The active sites of the heterodimer were saturated at half the maximal value of the GST A1-1 homodimer, implying that the wild-type enzyme must display all-
of-the-sites reactivity toward TNB. Hence, the apparent half-site-
saturation of GST A1-1 with CDNB and TNB, seen in pre-steady-
state and equilibrium experiments, was presumably an artifact caused by changes in the extinction coefficients of the glutathione-trinitrobenzene and GS-DNP conjugates upon binding to the active site of the enzyme.

It should be noted that, although the experimentally deter-

mined heterodimer saturation curves were consistently some-
what lower than the theoretically predicted results in the re-
actions with CDNB, TNB, DCNB, and AD, this was not due to
underestimation of the concentration of the heterodimer. The saturation curves of the heterodimer and GST A1-1 were es-

sentially identical in addition reactions (Fig. 5 and Table II), suggesting that the D101K mutation has a slight effect on the activity of the wild-type subunit of the heterodimer only in reactions that are catalyzed with all-of-the-sites reactivity.

AD was the largest of the substrates used in this study and provided evidence that the half-of-the-sites reactivity detected with nonenal, NCA, and benzyl-ITC was mechanism-based and not a function of substrate size. The nucleophilic aromatic substitution reactions of CDNB, and the closely related electrophile DCNB, demonstrate that a low overall catalytic rate or incomplete saturation of the enzyme by substrate are also not sufficient grounds for half-of-the-sites reactivity with GST A1-1. As can be seen from Fig. 3, the two molecules are quite similar, but the substitution of an NO$_2$ group by CI renders DCNB 3 orders of magnitude less reactive than CDNB. It was not possible to achieve saturation with DCNB under the avail-
able experimental conditions, but the results shown in Fig. 5B are qualitatively similar to those obtained with CDNB and demonstrate that there is a marked difference between the catalytic parameters of wild-type GST A1-1 and the heterodimer.

Additionally, despite the 10-fold greater catalytic efficiency of GST A1-1 with benzyl-ITC as opposed to nonenal or NCA, there was no noticeable difference between the catalytic par-

ameters of GST A1-1 and the heterodimer with these three substrates (Fig. 5D and Table II). Thus, the mode of reactivity of the enzyme (whether it is half-of-the-sites or all-of-the-sites) is not simply a function of catalytic rate. Neither is it a function of incomplete saturation of the enzyme by glutathione, since performing the AD reaction with a glutathione concentration 5-fold lower than the $K_M^{GSH}$ (0.01 mM as opposed to 0.05 mM) gave qualitatively similar results to those shown in Fig. 5C (data not shown). Furthermore, the mode of reactivity dis-
played by GST A1-1 was independent of the concentration of glutathione used when measuring kinetic parameters (Fig. 5).
In striking contrast to the results seen in the nucleophilic aromatic substitution and isomerization reactions, GST A1-1 displayed half-of-the-sites reactivity (also known as total negative cooperativity) in the addition of glutathione to nonenal, NCA, and benzyl-ITC. This was evidenced by the essentially identical saturation curves of the GST A1-1 homodimer and the heterodimer with nonenal (Fig. 5D and Table II). The predicted curve for the heterodimer (dashed line) was calculated from the average of the curves of GST A1-1 and GST D101K and is shown in panels A–C. Curves have been adjusted to account for non-enzymatic background reactions, and the data have been fitted by non-linear regression using the program GraphPad Prism®.

A recent crystallographic study of a murine GST A4-4 also suggested total negative cooperativity in the reaction with 4-hydroxynonenal (24). It was noted that, although glutathione was bound at both active sites of the dimer, the conjugate of glutathione and 4-hydroxynonenal was only found in one subunit. The “active” status was proposed to switch between the two subunits by means of a relay of interacting residues in a flip-flop mechanism (42). Residues implicated in this relay were Arg-15, which is essential for the activity of mGST A4-4 with 4-hydroxynonenal (24, 43), and Arg-69, which is part of the dimer interface (7). However, a flip-flop mechanism cannot readily explain the half-of-the-sites reactivity obtained in the present study, since the GST A1-D101K heterodimer, in which the mutated subunit has essentially lost its ability to bind glutathione, displays the same activity as the wild-type GST A1-1 homodimer. Hence, the two subunits that are active in GST A1-1 are presumably randomly alternating in the catalysis of the addition reaction.

Other recent evidence of cooperativity in the GSTs includes a study of temperature-mediated changes in hGST P1–1. At temperatures below 25 °C, this enzyme exhibited negative cooperativity with respect to glutathione binding, whereas at temperatures above 35 °C, the enzyme demonstrated positive cooperative interactions (44). The change in cooperativity was believed to be due to conformational changes in the enzyme structure caused by the thermal fluctuations. Based on a comparison of the Meisenheimer complex formation with GST A1-1 at 5 °C and 30 °C, the half-of-the-sites reactivity detected with this enzyme does not appear to be temperature-mediated. Negative cooperativity and half-of-the-sites reactivity has also been proposed for the human Theta class GST, GST T2-2 (23), but...
these results need to be taken with some caution due to the possibility of alteration of extinction coefficients in the active site environment. These investigators postulated the existence of half-of-the-sites reactivity noted in previous reports was associated with a shift in a tetramer-dimer equilibrium or binding of an enzyme reactivity (42, 51) is an unusual phenomenon, with the corollary that enzymes displaying both half-of-the-sites and all-of-the-sites reactivity are rarer still. Often the half-of-the-sites reactivity (42) is an unusual phenomenon, with the corollary that enzymes displaying both half-of-the-sites and all-of-the-sites reactivity are rarer still. Often the half-of-the-sites reactivity noted in previous reports was associated with a change in the quaternary structure of the enzyme (such as a shift in a tetramer-dimer equilibrium) or binding of an enzyme inhibitor or activator (52–57). In contrast, GST A1-1 does not exist in a monomeric state (58, 59) and was shown in this study to exhibit different modes of active-site reactivity without the addition of inhibitors or substrate analogs. The factor(s) that determine the mode of action of this enzyme therefore remain to be elucidated. In addition, the evolutionary significance of the ability of GST A1-1 to switch between half-of-the-sites and all-of-the-sites reactivity is intriguing, particularly as it would appear most efficient to catalyze detoxication reactions with all-of-the-sites reactivity.

In conclusion, the issue of cooperativity or non-cooperativity in the GSTs is not so clear-cut as had been previously assumed. In the present study, it is demonstrated that one and the same enzyme could function either with half-of-the-sites or all-of-the-sites reactivity, depending on the reaction mechanism. Furthermore, half-of-the-sites reactivity was shown not to be a simple function of substrate size, catalytic rate, or the level of saturation of the enzyme by glutathione. It remains to be seen exactly what the governing principles are to determine which of the different modes of reactivity are used, and how common this phenomenon is among other enzymes.

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