Characterization of chicken-liver glutathione S-transferase (GST) A1-1 and A2-2 isoenzymes and their site-directed mutants heterologously expressed in *Escherichia coli*: identification of Lys-15 and Ser-208 on cGSTA1-1 as residues interacting with ethacrynic acid

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Escherichia coli-expressed chicken-liver glutathione S-transferase, cGSTA1-1, displays high ethacrynic acid (EA)-conjugating activity. Molecular modelling of cGSTA1-1 with EA in the substrate binding site reveals that the side chain of Phe-111 protrudes into the substrate binding site and possibly interacts with EA. Replacement of Phe-111 with alanine resulted in an enzyme (F111A mutant) with a 4.5-fold increase in EA-conjugating activity (9.2 mmol/min per mg), and an incremental Gibbs free energy ($\Delta\Delta G$) of 4.0 kJ/mol lower than that of the wild-type cGSTA1-1. Two other amino acid residues that possibly interact with EA are Ser-208 and Lys-15. Substitution of Ser-208 with methionine generated a cGSTA1-1(F111AS208M) double mutant that has low EA-conjugating activity (2.0 mmol/min per mg) and an incremental Gibbs free energy of +3.9 kJ/mol greater than the cGSTA1-1(F111A) single mutant. The cGSTA1-1(F111A) mutant, with an additional Lys-15-to-leucine substitution, lost 90 % of the EA-conjugating activity (0.55 mmol/min per mg). The K_m values of the cGSTA1-1(F111A) and cGSTA1-1(F111AK15L) mutants for EA are nearly identical. The wild-type cGSTA2-2 isoenzyme has a low EA-conjugating activity (0.56 mmol/min per mg). The k_{cat} of this reaction can be increased 2.5-fold by substituting Arg-15 and Glu-104 with lysine and glycine respectively. The K_m^{EA} of the cGSTA2-2(R15KE104G) double mutant is nearly identical with that of the wild-type enzyme. Another double mutant, cGSTA2-2(E104GL208S), has a K_m^{EA} that is 3.3-fold lower and a k_{cat} that is 1.8-fold higher than that of the wild-type enzyme. These results, taken together, illustrate the interactions of Lys-15 and Ser-208 on cGSTA1-1 with EA.

INTRODUCTION

Glutathione S-transferases (GSTs, EC 2.5.1.18) comprise a large group of dimeric enzymes that catalyse the conjugation of glutathione to a wide variety of electrophiles and xenobiotics [1,2]. The reactions are considered to be one of the initial steps in the biotransformation of xenobiotics [3]. Besides their detoxification roles, these dimeric proteins are also involved in the reduction of organic hydroperoxides [4], isomerization of prostaglandins [5] and the binding of non-substrate hydrophobic ligands, such as bile acids, bilirubin, a number of drugs and thyroid hormones [1,6].

GST isoenzymes can be distinguished by their physical, chemical, immunological, enzymic and structural properties. Six evolutionary classes of cytosolic GSTs have been identified, namely Alpha, Mu, Pi [7], Theta [8,9], Sigma [10,11] and Kappa [12]. A separate membrane-bound transferase, designated microsomal GST, has been described by Morgenstern et al. [13].

X-ray crystal structures have been determined for representative molecules from mammalian class Alpha, Mu and Pi GSTs [14–16]. In addition, the structure of class Theta and Sigma GSTs from insect [17] and invertebrate [18] respectively have been reported. Even though these proteins have only 20–30 % sequence identity, they share a two-domain structure. The N-terminal Domain I can be considered as the glutathione binding domain (G-site). Domain II seems to be the primary binding site for the xenobiotic substrates (H-site). Combined with mutagenesis experiments and kinetic results, residues that are important for the enzymic activities of GSTs have also been suggested [19–23].

We have previously isolated and expressed in Sf9 cells a class Alpha GST, CL3, from chicken liver (c) [24]. We have also isolated another full-length class Alpha cDNA, pGCLα-1, from a chicken-liver cDNA library [25]. In compliance with the class-based subunit nomenclature as proposed by Mannervik et al. [26], CL3 and the gene product from pGCLα-1 are redesignated as cGSTA1 and cGSTA2 respectively from here on. The cGSTA1 isoenzyme has a relatively low 1-chloro-2,4-dinitrobenzene (CDNB), but high ethacrynic acid (EA)-conjugating, activity. Careful examination of the primary sequence of cGSTA1 reveals the presence of Lys-15 and Gly-104, whereas cGSTA2 has Arg-15 and Glu-104. Arg-15 is conserved in all other known class Alpha GSTs, while Glu-104 is preserved in the majority of the sequences determined. The ionic interaction between Arg-15 and Glu-104 is unique for class Alpha GSTs.

We report here a structural model of cGSTA1-1 with EA in the H-site. The coordinates of cGSTA1-1 are based on the 3.0 Å resolution X-ray structure of this isoenzyme co-crystallized with *S*-hexylglutathione (Y.-C. Liaw and M. F. Tam, unpublished work). The EA molecule was inserted by a molecular modelling technique. This model assists us in identifying Lys-15, Phe-111

Abbreviations used: GSTs, glutathione S-transferases; c, chicken liver; h, human; CDNB, 1-chloro-2,4-dinitrobenzene; EA, ethacrynic acid; tPBO, *trans*-4-phenyl-3-buten-2-one.

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and Ser-208 as residues interacting with EA. Using site-directed mutagenesis, we generated and expressed cGSTA1-1 and cGSTA2-2 [25] mutants in *Escherichia coli*. Data from kinetic studies support our conclusion that the high EA-conjugating activity of cGSTA1-1 results from Lys-15 and substrate interaction.

MATERIALS AND METHODS

Materials

Restriction enzymes and DNA-modification enzymes were obtained from New England Biolabs (Beverly, MA, U.S.A.). The oligonucleotide-directed '*in vitro*' mutagenesis kit was purchased from Amersham International (Amersham, Bucks., U.K.). The Sequenase kit was obtained from U.S. Biochemicals (Cleveland, OH, U.S.A.). [α-^{35}S]dATP for sequencing was purchased from New England Nuclear (Wilmington, DE, U.S.A.). Epoxy-activated Sepharose 6B and a prepacked Superose 12 column were obtained from Pharmacia Biotech (Uppsala, Sweden). Glutathione-linked Sepharose 6B was prepared according to Simons and Vander Jagt [27]. Substrates for enzymic assays were obtained from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, U.S.A.). All other chemicals used were reagent grade or better.

Construction and expression of wild-type and mutant cGSTA1 and cGSTA2 in *E. coli*

The Bluescript phagemids, pGCL301 [24] and pGCLa-1 [25], containing the coding region of wild-type cGSTA1 and cGSTA2 respectively, have been described previously. The inserts were subcloned into the *Bam*H1 site of an M13mp18 vector for single-strand DNA production. Oligonucleotides used in the site-directed mutagenesis experiments and the residues mutated are listed in Table 1. The amino acid residues of class Alpha GSTs are numbered according to Sinning et al. [14] with the initiator methionine as the first residue. Mutagenesis was performed as described by Taylor et al. [28]. The mutated phagemids were used as templates for PCR amplification. Primers 5 and 10 were used as sense primers for introducing a unique *Nde*1 restriction site at the location of the ATG start codon for the cGSTA1 and cGSTA2 genes respectively. Primers 6 and 11 were used as antisense primers for introducing a unique *Sal*1 restriction site for cGSTA1 and cGSTA2 respectively. PCR amplifications were carried out for 40 cycles under the following conditions: 92 °C for 1.5 min, 50 °C for 1.5 min and 72 °C for 1.5 min.

The PCR products were digested with *Nde*1 and *Sal*1. After purification on agarose gels, the DNA fragments were inserted into pBAce expression vectors [29] and the resultant plasmids were transformed into *E. coli* TG1 cells. Fragments encoding the wild-type and mutant GST isoenzymes on the pBAce vector were sequenced in their entirety [30] to ascertain that only the desired mutations had occurred during the manipulations. Expression of GST isoenzymes on pBAce plasmid under the control of *pho*A promoter has been described in detail [31].

Enzyme purification

Harvested cells were suspended in a homogenization buffer [50 mM Tris/HCl (pH 8.0)/5 mM EDTA/0.2 mM dithiothreitol/0.5 mM PMSF] with 20 % (w/v) sucrose at a concentration of 100 ml/litre of cell culture. Cells were lysed by passing through a microfluidizer (Microfluidics, Newton, MA, U.S.A.) and debris was removed by centrifugation in a Beckman 70 Ti rotor for 1 h at 50000 rev./min. The expressed proteins were loaded on to a GSH affinity column that had been equilibrated with Buffer A [10 mM Tris/HCl (pH 7.8)/1 mM EDTA/0.2 mM dithiothreitol/0.5 mM PMSF]. The proteins were eluted from the column with Buffer B [10 mM 3-cyclohexylamino-1-propanesulphonic acid (pH 11.0)/1 mM EDTA/0.2 mM dithiothreitol/500 mM NaCl/0.5 mM PMSF/5mM GSH]. Proteins after affinity-column chromatography were immediately desalted on a preparative Superose 12 gel-filtration column (Pharmacia, Uppsala, Sweden) with 150 mM potassium phosphate, pH 7.5. The protein samples were stored at −70 °C at a concentration of 1 mg/ml or higher in the presence of 5 mM GSH. The purity of the enzymes was analysed with SDS/PAGE [32]. The molecular masses of the recombinant proteins were determined by electrospray mass spectrometry [33].

Enzyme assay

GST activity was assayed by published methods [34–36] at 25 °C. The rates of all the enzymic reactions in this study were obtained by subtracting the non-enzymic from the observed reaction rates. The concentration of the purified GST was determined by measuring the absorbance at 280 nm. Protein solutions of wild-type and cGSTA1 mutants with a concentration of 0.77 mg/ml have 1 A_{280} unit [24]. The method of Gill and von Hippel [37] was used to calculate the molar absorption coefficients of cGSTA2-2 and its mutants. Protein solutions of wild-type, R15KE104G and E104GL208S mutants with a concentration of 1.56, 1.45 and 1.47 mg/ml respectively have 1 A_{280} unit.

Table 1 Primers used in molecular cloning and mutagenesis

Isoenzyme	Sequence	Mutation
cGSTA1		
Primer 1	5′-CAGCTGACGAGGCAGGGAAACTC-3′	Phe-111 → Ala
Primer 2	5′-GTATTTATCATCCATAATAGGTTTTC-3′	Ser-208 → Met
Primer 3	5′-GATCGAC TCCATCAGGCCTCTTCC-3′	Lys-15 → Leu
Primer 4	5′-GGAGAAAATCATATGGCTGCAAAACC-3′	*Nde*1 site
Primer 5	5′-TTAATTGTCCCATCTGGTAGCTCTCGTCGACCAAGAACAGC-3′	*Sal*1 site
cGSTA2		
Primer 6	5′-TGATTCCATTTTGCCTCGTGTG-3′	Arg-15 → Lys
Primer 7	5′-TCATGATTAACCCGTACAGATC-3′	Glu-104 → Gly
Primer 8	5′-CTTTTTCCTCTGAGCGTGGTTTC-3′	Leu-208 → Ser
Primer 9	5′-GATACGTGGGCATATGTCTGGGAAG-3′	*Nde*1 site
Primer 10	5′-CGCTTATCAGTCGACAGAATGAAATC-3′	*Sal*1 site

Determination of kinetic constants

K_m and V_{max} were determined as outlined by Pabst et al. [38]. Kinetic constants were determined with CDNB and EA as variable substrates and a GSH concentration constant at 1 mM. The catalytic rate constant, k_{cat}, is expressed as the maximum velocity/mol of catalytic site of GST subunit [39].

Model construction

The coordinates of CL3-3 (Y.-C. Liaw and M. F. Tam, unpublished work) and human (h) GSTA1-1 [14] were used as templates in the model constructions. The CDNB and EA molecules were assembled by the Builder Program (QUANTA). The EA molecule can assume multiple configurations. The conformation that has maximum interactions with the pocket and confirmed by structural study [40] was adopted in the present study. The initial position of CDNB and EA in the binding pocket were aligned according to the coordinates of *S*-benzyl–glutathione [14] and EA–glutathione [40] complex respectively. The molecules were then adjusted manually to minimize the steric hindrance and maximize possible interactions. The final models were derived by applying the CHARMM energy-minimization process with the CHARMM/QUANTA program package [41].

RESULTS AND DISCUSSION

Structural model of cGSTA1-1

Among class Alpha GSTs, X-ray crystallographic structure has been reported solely for hGSTA1-1 [14,40]. The primary structure of hGSTA1-1 and the two chick liver class Alpha GSTs reported in this work are listed in Figure 1 for comparison. The amino acid sequence of these three isoenzymes can be aligned without any gap using the multiple-sequence alignment algorithm of Feng and Doolittle [42]. The sequence identity between cGSTA1-1 and hGSTA1-1 is 64 %. Including conservative substitutions, the similarity between these two molecules is 74 %. The sequence identity between cGSTA2-2 and hGSTA1-1 is 65 %. The similarity between these two molecules is 76 %, if chemically similar substitutions are included.

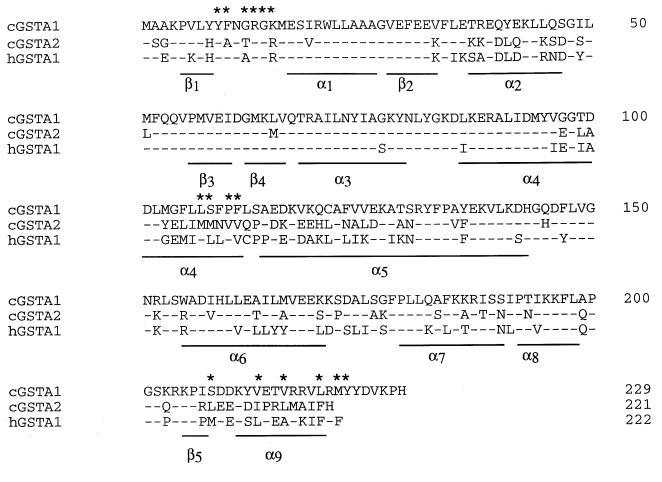

Figure 1 Comparison of the deduced amino acid sequences of cGSTA1-1, cGSTA2-2 and hGSTA1-1

The amino acid residues of class-Alpha GSTs are numbered according to Sinning et al. [14], with the initiator methionine as the first residue. The amino acid sequence is given as a single-letter code. Dashes (–) indicate identity with amino acid residues of cGSTA1-1. An * denotes H-site residues on cGSTA1-1. The secondary structure of class-Alpha GSTs are underlined and specified.

The structure of cGSTA1-1, as determined by X-ray crystallography, resembles the overall folding pattern of class-Alpha GST [14] and will be published elsewhere (Y.-C. Liaw and M. F. Tam, unpublished work). Models of cGSTA1-1 with EA or CDNB in the active site are depicted in Figure 2. The corresponding models of hGSTA1-1 [14,40] are also included for comparison.

The H-site of cGSTA1-1 is composed of the loop connecting β1 and α1 (Tyr-9, Phe-10, Gly-12, Arg-13, Gly-14 and Lys-15), the C-terminus of α4 (Leu-107, Ser-108, Pro-110 and Phe-111) and residues on (Val-213, Val-216 and Leu-220), and proximal to (Ser-208, Met-222 and Tyr-223), α9. The top of the H-site is made up of Pro-110, Phe-111, Ser-208 and Val-213. The left wall is hydrophobic and consists of Met-222, Val-216, Leu-220 and Phe-10. Gly-12, the backbone of Arg-13, and Gly-14 line the bottom of the pocket. Lys-15, Leu-107 and Ser-108 form the right wall of the H-site. Tyr-9 is located between the H- and G-site, whereas Tyr-223 covers part of the entrance to the pocket.

The top of the H-site on hGSTA1-1 [14] is made up of Pro-110, Val-111, Met-208 and Leu-213. The presence of Val-111 and Met-208, instead of Phe-111 and Ser-208, as in cGSTA1-1, gives the human enzyme a more open structure. The left wall of the H-site is composed of Phe-222, Ala-216, Phe-220 and Phe-10. Compared with the corresponding residues on cGSTA1-1, these amino acid side chains occupy more space and press in towards the H-site. On the right wall of the H-site, the human enzyme has a leucine and arginine instead of serine and lysine at residue 108 and 15 respectively. The orientation of Arg-15 is fixed by forming a salt bridge with Glu-104. Conversely, Glu-104 is replaced with glycine in the chicken enzyme, and Lys-15 exhibits a certain degree of flexibility by not forming a salt-bridge or H-bond with any residue.

The model of cGSTA1-1 with CDNB in the H-site is presented in Figure 2(A). Possible interactions between cGSTA1-1 and CDNB are: Ser-208 and Lys-15 interacting with the *para*- and *ortho*-nitro group of CDNB respectively; and Tyr-223 might be interacting with the chloro atom of CDNB.

Two out of the three residues mentioned above possibly interact with EA in our model (Figure 2C). Ser-208 and Lys-15 interact with the phenoxy and keto oxygen atom of EA respectively. The electrons on the aromatic Phe-111 side chain probably interact repulsively with the chloride atom of EA. In addition, Ser-208 might form a weak hydrogen bond with the chloro atom of EA. To test the accuracy of the model, mutants were generated for cGSTA1-1 and the kinetic properties were determined.

Wild-type and cGSTA1-1 mutants

Wild-type cGSTA1-1, a mutant with single amino acid substitution (F111A) and mutants with double amino acid substitutions (F111AS208M, F111AS208A and F111AK15L) were expressed in *E. coli* as determinants for their specificity and catalytic efficiency towards EA. All the enzymes displayed strong affinity with the glutathione affinity column. Glutathione (5 mM) alone at neutral-to-slightly-basic conditions (pH 8.0) could not elute the enzymes from the affinity column. *S*-Hexylglutathione (5 mM) could facilitate the elution of enzymes from the glutathione affinity column, but it was retained on the enzymes and could not be removed effectively by dialysis or gel-filtration chromatography (results not shown). Therefore the enzymes

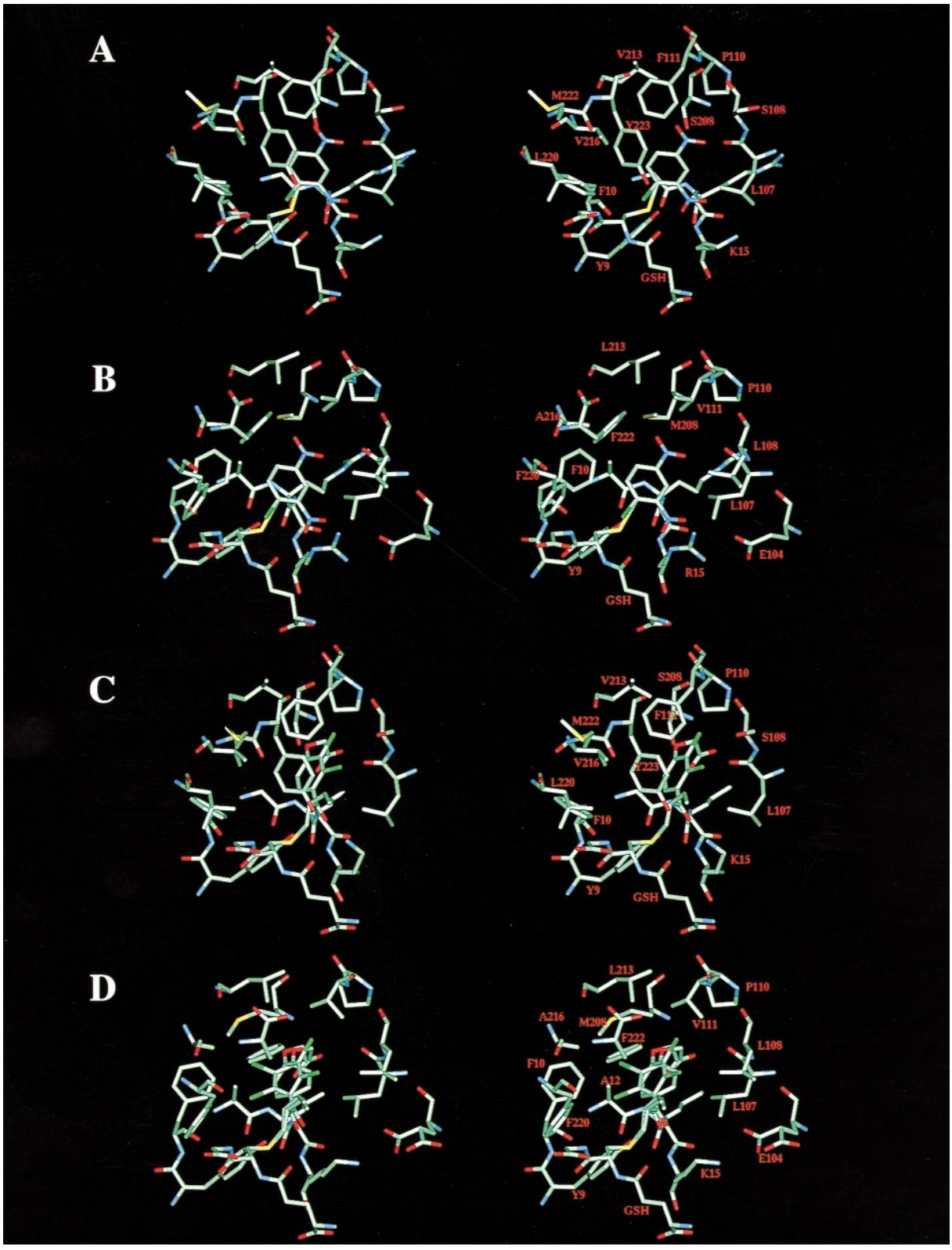

Figure 2 Stereo views of the H-site of cGSTA1-1 and hGSTA1-1 complexed with CDNB and EA

Models of the docked structure of cGSTA1-1 with CDNB and EA are presented in panels (**A**) and (**C**) respectively. The corresponding model of hGSTA1-1 with CDNB and the structure of the same enzyme with EA [40] are depicted in (**B**) and (**D**) respectively. Atoms are coloured by atom type: grey, carbon; blue, nitrogen; red, oxygen; green, chlorine; yellow, sulphur.

Table 2 Catalytic activities of recombinant cGSTA1-1, cGSTA2-2 and mutants

Results are means ± S.E.M. of duplicates of at least three experiments; CuOOH, cumene hydroperoxide.

	Specific activity (μmol/min per mg)			
Isoenzyme	CDNB	EA	tPBO	CuOOH
cGSTA1-1	1.81 ± 0.04	2.04 ± 0.03	0.49 ± 0.01	0.112 ± 0.003
cGSTA1-1(F111A)	5.13 ± 0.03	9.21 ± 0.05	0.04 ± 0.01	0.101 ± 0.006
cGSTA1-1(F111AS208M)	5.06 ± 0.03	1.99 ± 0.02	0.010 ± 0.001	0.156 ± 0.002
cGSTA1-1(F111AK15L)	2.24 ± 0.01	0.80 ± 0.03	0.034 ± 0.002	0.044 ± 0.001
cGSTA2-2	32.16 ± 0.20	0.56 ± 0.03	0.026 ± 0.001	5.46 ± 0.16
cGSTA2-2(R15KE104G)	4.01 ± 0.02	1.10 ± 0.02	0.033 ± 0.001	1.50 ± 0.05
cGSTA2-2(E104GL208S)	26.16 ± 0.14	1.00 ± 0.06	0.028 ± 0.001	3.86 ± 0.08

were eluted from the column with glutathione under basic conditions (pH 11). Routinely, we obtained 25–30 mg of purified protein/litre of cell culture.

The N-terminal amino acid sequences of the wild-type cGSTA1-1 and mutants were confirmed by Edman degradation and matched that of the published result (AAKPVLYFNG) [24]. The initiator methionine was removed effectively by the *E. coli* methionine aminopeptidase. The molecular mass of each purified enzyme was determined by electrospray mass spectroscopy (results not shown). The results indicate that besides the initiator methionine removal, the enzymes were not modified and the correct mutants were expressed and isolated.

The specific activities of cGSTA1-1 and mutants with CDNB, EA, *trans*-4-phenyl-3-buten-2-one (tPBO) and cumene hydroperoxide as second substrates are listed in Table 2. The wild-type cGSTA1-1 has a comparatively low CDNB activity (1.81 ± 0.04 mmol/min per mg) but high EA-conjugating activity (2.04 mmol/min per mg). We have previously expressed cGSTA1-1 in Sf9 cells using a baculovirus system [24]. The proteins were purified with an *S*-hexylglutathione column that eluted with *S*-hexylglutathione at neutral pH. The protein thus prepared has an N-terminal acetylated alanine (M. F. Tam, unpublished work). In the present study, the *E. coli*-expressed cGSTA1-1 had an unmodified alanine at the N-terminus. With the exception of cumene hydroperoxide, the bacterial-expressed cGSTA1-1 had a lower specific activity than the Sf9-expressed GST with all the substrates tested. This decrease in activity was not due to the purification method. Purification of the *E. coli*-expressed proteins with *S*-hexylglutathione at neutral pH did not improve the activity of the enzymes (results not shown). Whether the observed decrease in activity is due to the N-terminal acetylation or protein folding remains to be investigated.

In our structural model of cGSTA1-1, Phe-111 is located on the top of the H-site with the side chain protruding into the middle of the substrate binding pocket (Figure 2, A and C). We removed the bulky phenyl ring and replaced it with a methyl group by a Phe-111-to-alanine mutation. We obtained a 2.8- and 4.5-fold increase in CDNB- and EA-conjugating activity respectively by this substitution. However, the peroxidase activity of the mutant was identical with that of the wild-type enzyme, whereas a 12-fold decrease in activity was observed with tPBO as the second substrate.

The kinetic parameter of the wild-type enzyme, and mutants with CDNB and EA as substrates are listed in Table 3. The cGSTA1-1(F111A) mutant has a 3- and 2.4-fold increase in the K_m and k_{cat} respectively of the CDNB conjugation reaction. Consequently, the specificity constants (k_{cat}/K_m) between the wild-type and cGSTA1-1(F111A) mutant are similar. With EA as the second substrate, the cGSTA1-1(F111A) mutant has a 4- and 17-fold increase in K_m and k_{cat} over the wild-type enzyme. The drastic increase in k_{cat} resulted in an F111A mutant that has a 4.8-fold enhancement in the specificity constant over that of the wild-type isoenzyme. Our structural model suggests an unfavourable interaction of the phenyl π electrons with the chloro atom on EA. Removal of the phenyl ring probably stabilizes the intermediate of the reaction. The calculated incremental Gibbs free energy for transition-state stabilization of the F111A mutant relative to the wild-type enzyme is 4 kJ/mol for the EA-conjugating reaction.

Since the enzymic activity of the cGSTA1-1(F111A) mutant is

Table 3 Kinetic parameters of recombinant cGSTA1-1, cGSTA2-2 and mutants

Results are means ± S.E.M. of duplicates of at least three experiments.

	CDNB			EA		
Isoenzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
cGSTA1-1	915 ± 20	1.44 ± 0.04	0.0016	28.0 ± 0.47	0.35 ± 0.004	0.012
cGSTA1-1(F111A)	2708 ± 21	3.44 ± 0.08	0.0013	108.2 ± 2	6.11 ± 0.03	0.057
cGSTA1-1(F111AS208M)	3102 ± 26	3.66 ± 0.04	0.0012	118.5 ± 4.6	1.47 ± 0.06	0.012
cGSTA1-1(F111AK15L)	3808 ± 212	2.18 ± 0.16	0.0006	87.9 ± 5.9	0.55 ± 0.02	0.006
cGSTA2-2	2757 ± 73	26.53 ± 0.92	0.0096	42.9 ± 3.2	0.18 ± 0.005	0.004
cGSTA2-2(R15KE104G)	4380 ± 60	5.68 ± 0.06	0.0013	39.8 ± 1.4	0.44 ± 0.01	0.011
cGSTA2-2(E104GL208S)	2240 ± 42	14.75 ± 0.28	0.0066	13.1 ± 1.5	0.32 ± 0.02	0.024

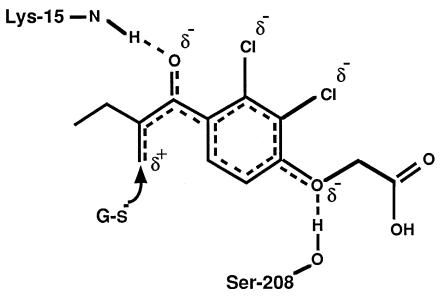

Figure 3 Possible electrophilic interaction of Lys-15 and Ser-208 on cGSTA1-1 with EA

G-s$^-$, the thiolate anion of glutathione.

significantly higher than that of the wild-type isoenzyme, we substituted amino acids on cGSTA1-1(F111A) to generate double mutants for kinetic studies. We targeted Ser-208 and Lys-15 for site-directed mutagenesis, as proposed by our model.

Ser-208 on cGSTA1-1(F111A) was replaced by methionine. The catalytic activities of the cGSTA1-1(F111AS208M) double mutant are listed in Table 2. In general, the CDNB-conjugating activity of the double mutant was similar to the F111A mutant, but significantly higher than the wild-type enzyme. The peroxidase activity of the mutant and wild-type enzyme were similar. The EA- and tPBO-conjugating activities of the double mutant were 4.6- to 4-fold lower than that of the cGSTA1-1(F111A) single mutant.

The kinetic parameters of the double mutant are listed in Table 3. The affinity of the cGSTA1-1(F111AS208M) double mutant towards CDNB and EA were nearly identical with that of the cGSTA1-1(F111A) single mutant. The k_{cat} value of this double mutant was unchanged in the CDNB conjugation reaction. However, a 4-fold reduction in the k_{cat} value of the EA-conjugating activity was observed, resulting in a double mutant that had an incremental Gibbs free energy of $+4$ kJ/mol higher than that of the F111A single mutant in this reaction. We hypothesize that during the reaction between the thiolated glutathione and EA, the π electrons originating from the methylene group partially delocalize over the carbonyl group, the phenyl ring and the phenoxy oxygen atom. Electron-withdrawing residues near the carbonyl and phenoxy groups stabilize the reaction intermediate (Figure 3). The hydroxy group of the Ser-208 side chain forms a H-bond with the phenoxy oxygen atom and stabilizes the intermediate. The methionine side chain, on the other hand, does not have a proton available for H-bonding.

A lysine residue (Lys-15) is located near the carbonyl group of EA and possibly interacts with it. We generated a cGSTA1-1(F111AK15L) double mutant to test the involvement of Lys-15 in stabilizing the intermediate. The catalytic activities and kinetic parameters of this double mutant are listed in Tables 2 and 3 respectively. The CDNB-conjugating activity of this double mutant was higher than that of the wild-type isoenzyme but substantially lower than that of the cGSTA1-1(F111A) single mutant. The EA- and tPBO-conjugating activity and the peroxidase activity of this double mutant was significantly lower than that of the wild-type isoenzyme or the single mutant.

The binding affinity of the cGSTA1-1(F111AK15L) double mutant towards EA is similar to that of cGSTA1-1(F111A). However, the k_{cat} value of the reaction is 11 times lower (Table 3). Obviously the amino group of the lysine side chain interacts with the carbonyl oxygen of EA. Lys-15 possibly also interacts with the *ortho*-nitro group of CDNB. The k_{cat} of the CDNB-conjugating reaction for this double mutant is substantially lower than the cGSTA1-1(F111A) single mutant. We have previously modified Lys-15 with diethyl pyrocarbonate [43]. The N-carbethoxylated cGSTA1-1 has a substantially lower CDNB- and EA-conjugating activity than the wild-type enzyme.

Wild-type and cGSTA2-2 mutants

To authenticate the interaction of EA with Lys-15 and Ser-208 of cGSTA1-1, we expressed and characterized wild-type and cGSTA2-2 mutants. We hypothesize that class Alpha GSTs can have a higher EA-conjugating activity by having a lysine and a serine at positions 15 and 208 respectively.

The wild-type cGSTA2 has arginine and glutamic acid at positions 15 and 104 respectively. The CDNB- (32.2 ± 0.2 mmol/min per mg), EA- (0.56 ± 0.03 mmol/min per mg), tPBO- (0.026 ± 0.001 mmol/min per mg) conjugating activities and the peroxidase activity (5.46 ± 0.16 mmol/min per mg) of this enzyme are listed in Table 2. Since the reported activities of hGSTA1-1 were determined at 37 °C [44] instead of 25 °C, a direct comparison between the activities of these two isoenzymes was not made.

We attempted to generate E104G, R15KE104G, E104GL208S and R15KE104GL208S mutants. The gene products of the single and the triple mutants were recovered as insoluble proteins after cell lysis and were excluded from this study. The CDNB (4.01 ± 0.02 mmol/min per mg) and peroxidase activity (1.50 ± 0.05 mmol/min per mg) of the cGSTA2-2(R15KE104G) mutant were drastically reduced compared with the wild-type enzyme, whereas an increase in EA (1.10 ± 0.02 mmol/min per mg)-conjugating activity was observed (Table 2).

The decrease in CDNB-conjugating activity of the cGSTA2-2(R15KE104G) double mutant was reflected in an increase in K_m^{CDNB} (4380 ± 60 μM) and a reduction in k_{cat} (5.68 ± 0.06 s^{-1}), resulting in a 7-fold decrease in specificity constant compared with the wild-type enzyme.

Our results are in close agreement with Bjornestedt et al. [22], who have replaced the Arg-15 on hGSTA1-1 with lysine. The resulting mutant has a 3-fold decrease in CDNB-conjugating activity and a 7-fold increase in K_m for GSH (from 0.1 to 0.7 mM). They suggest that the guanidinium group of Arg-15 may contribute to the electrostatic field in the active site and participate in the binding and activation of glutathione.

It is interesting to note that cGSTA2-2 has a K_m^{GSH} of 1.04 mM, 10- and 1.4-fold higher than that of the hGSTA1-1 and the hGSTA1-1(R15K) mutant respectively. The K_m^{GSH} value of the cGSTA2-2(R15KE104G) mutant is 1.82 mM. Assuming Glu-104 does not contribute significantly to GSH binding, the results suggest that the Arg-15 side chain of cGSTA2-2 does not contribute as much to GSH binding and activation as the human class Alpha isoenzyme.

The substitutions of Arg-15 and Glu-104 with lysine and glycine respectively on cGSTA2-2 did not change the K_m value of this enzyme towards EA. However, there was nearly a 2.5-fold increase in k_{cat}. Consequently, the Gibbs free energy for transition-state stabilization of the double mutant was 2.5 kJ/mol relative to that of the wild-type enzyme in the EA-conjugating reaction.

The structure of hGSTA1-1 co-crystallized with EA has been elucidated by Cameron et al. [40]. Their model suggests that the carbonyl oxygen on EA acts as a hydrogen acceptor from the hydroxy group of Tyr-9 or the guanidinium group of Arg-15, whereas the carboxylate oxygen atoms potentially hydrogen bond with water molecules or the amino nitrogen atom of Gly-

14. We would like to point out that hGSTA1-1 has a salt bridge between Arg-15 and Glu-104, directing the arginine side chain away from the EA. The interaction of Arg-15 with EA should be minimal, and the hGSTA1-1 enzyme has a low EA-conjugating activity. Cameron et al. [40] replaced Arg-15 of hGSTA1-1 with lysine. This change should have minimum impact on the enzymic activity, since a salt bridge is still expected to form between the lysine and Glu-104. In our experiment, we replaced both Arg-15 and Glu-104 on cGSTA2-2 with lysine and glycine respectively to ensure the absence of salt bridging between these two residues and facilitated the interaction of the lysine side chain with the EA. Consequently, we obtained a mutant with a higher k_{cat} compared with that of the wild-type enzyme.

The catalytic activities of the cGSTA2-2(E104GL208S) mutant are listed in Table 2. The CDNB-conjugating (26.2 ± 0.14 μmol/min per mg) and the peroxidase activity (3.86 ± 0.08 μmol/min per mg) of this mutant was slightly lower, whereas the EA-conjugating activity (1.0 ± 0.06 μmol/min per mg) was significantly higher than that of the wild-type enzyme.

The kinetic parameters of the cGSTA2-2(E104GL208S) mutant are listed in Table 3. This mutant has a K_m^{CDNB} of 2240 mM, which is slightly lower than that of the wild-type enzyme. The result here substantiates the conclusion of Widersten et al. [45], who randomly mutated the corresponding Met-208 on hGSTA1-1 and assessed the kinetic properties with CDNB and 4-nitrobenzyl chloride as substrates. They suggested that side chains (His, Trp, Tyr) that are capable of contributing a proton to a hydrogen bond could decrease K_m^{CDNB}. A slight increase in K_m^{CDNB} was observed with a Met-208-to-alanine mutation [45]. Therefore a slight decrease in K_m^{CDNB} is expected for cGSTA2-2 upon Leu-208-to-serine substitution.

The higher EA-conjugating activity of cGSTA2-2(E104GL-208S) is due to a 3-fold decrease in K_m towards the substrate and a 1.8-fold increase in k_{cat} of the reaction. Combining these factors, the mutant has a specificity constant 6-fold higher than that of the wild-type enzyme. The property of this mutant is significantly different from that of the cGSTA2-2(R15KE104G) mutant, which has a K_m^{EA} similar to the wild-type enzyme, whereas the k_{cat} of the reaction is 2.5-fold higher. Therefore Ser-208 of the cGSTA2-2(E104GL208S) mutant contributes to the binding and possibly the activation of EA.

GST8-8 and mGSTA4-4 have arginine, methionine and proline residues at positions 15, 104 and 208 respectively. These residues are not similar to the corresponding amino acids on cGSTA1-1. Nonetheless, GST8-8 and mGSTA4-4 are noteworthy for their high EA-conjugation activity [46,47]. In addition, GST8-8 and mGSTA4-4 also have high activity towards 4-hydroxynonenal, a toxic product of lipid peroxidation [44]. The role of cGSTA1-1 in lipid peroxidation has not been vigorously investigated, but cGSTA1-1 is inactive with 4-hydroxynonenal as the second substrate (results not shown). cGSTA1-1 differs from GST8-8 by only six amino acids in the H-site. Therefore each isoenzyme, even within the same family, utilizes a unique set of amino acids for substrate interaction and catalytic function. Generalization of catalytic mechanism without structural knowledge can be prone to error.

In conclusion, we have generated and characterized wild-type cGSTA1-1 and cGSTA2-2 and their mutants, heterologously expressed in *E. coli*. We generated a cGSTA1-1 mutant with higher catalytic efficiency by substituting Phe-111 with alanine. By molecular modelling, we identified Lys-15 and Ser-208 as residues on cGSTA1-1, which interact with the carbonyl and phenoxy oxygen of EA respectively. The EA-conjugating activity of cGSTA1-1 can be diminished by substituting Lys-15 and Ser-208 with other residues. Conversely, the EA-conjugating activity of cGSTA2-2 can be improved by substituting Arg-15 and Leu-208 with lysine and serine respectively.

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REFERENCES

1 Tsuchida, S. and Sato, K. (1992) CRC Crit. Rev. Biochem. Mol. Biol. **27**, 337–384
2 Mannervik, B. and Danielson, U. H. (1988) CRC Crit. Rev. Biochem. **23**, 283–337
3 Coles, B. and Ketterer, B. (1990) CRC Crit. Rev. Biochem. Mol. Biol. **25**, 47–70
4 Tan, K. H., Meyer, D. J., Coles, B. and Ketterer, B. (1986) FEBS Lett. **207**, 231–233
5 Christ-Hazelhof, E. and Nutgeren, D. (1979) Biochim. Biophys. Acta **573**, 43–51
6 Ishigaki, S., Abramovitz, M. and Listowsky, I. (1989) Arch. Biochem. Biophys. **273**, 265–272
7 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
8 Meyer, D. J., Coles, B., Pemble, S. E., Gilmore, K. S., Fraser, G. M. and Ketterer, B. (1991) Biochem. J. **274**, 409–414
9 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
10 Harris, J., Coles, B., Meyer, D. J. and Ketterer, B. (1991) Comp. Biochem. Physiol. B **98**, 511–515
11 Meyer, D. J. and Thomas, M. (1995) Biochem. J. **311**, 739–742
12 Pemble, S. E., Wardle, A. F. and Taylor, J. B. (1996) Biochem. J. **319**, 749–754
13 Morgenstern, R., DePierre, J. W. and Jornvall, H. (1985) J. Biol. Chem. **260**, 13976–13983
14 Sinning, I., Kleywegt, G. J., Cowan, S. W., Reinemer, P., Dirr, H. W., Huber, R., Gilliland, G. L., Armstrong, R. N., Ji, X., Board, P. G., Olin, B., Mannervik, B. and Jones, T. A. (1993) J. Mol. Biol. **232**, 192–212
15 Ji, X., Zhang, P., Armstrong, R. N. and Gilliland, G. L. (1992) Biochemistry **31**, 10169–10184
16 Reinemer, P., Dirr, H. W., Ladenstein, R., Huber, R., Lo Bello, M., Federici, G. and Parker, M. W. (1992) J. Mol. Biol. **227**, 214–226
17 Wilce, M. C. J., Board, P. G., Feil, S. C. and Parker, M. W. (1995) EMBO J. **14**, 2133–2143
18 Ji, X., Von Rosenvinge, E. C., Johnson, W. W., Tomarev, S. I., Piatigorsky, J., Armstrong, R. N. and Gilliland, G. L. (1995) Biochemistry **34**, 5317–5328
19 Stenberg, G., Board, P. G. and Mannervik, B. (1991) FEBS Lett. **293**, 153–155
20 Liu, S., Zhang, P., Ji, X., Johnson, W. W., Gilliland, G. L. and Armstrong, R. N. (1992) J. Biol. Chem. **267**, 4296–4299
21 Johnson, W. W., Liu, S., Ji, X., Gilliland, G. L. and Armstrong, R. N. (1993) J. Biol. Chem. **268**, 11508–11511
22 Bjornestedt, R., Stenberg, G., Widersten, M., Board, P. G., Sinning, I., Jones, T. A. and Mannervik, B. (1995) J. Mol. Biol. **247**, 765–773
23 Nanduri, B., Hayden, J. B., Awasthi, Y. C. and Zimniak, P. (1996) Arch. Biochem. Biophys. **335**, 305–310
24 Chang, L.-H., Fan, J.-Y., Liu, L.-F., Tsai, S.-P. and Tam, M. F. (1992) Biochem. J. **281**, 545–551
25 Liu, L.-F., Wu, S.-H. and Tam, M. F. (1993) Biochim. Biophys. Acta **1216**, 332–334
26 Mannervik, B., Awasthi, Y. C., Board, P. G., Hayes, J. D., Di Ilio, C., Ketterer, B., Listowsky, I., Morgenstern, R., Muramatsu, M., Pearson, W. R., Pickett, C. B., Sato, K., Widersten, M. and Wolf, C. R. (1992) Biochem. J. **282**, 305–306
27 Simons, P. C. and Vander Jagt, D. L. (1981) Methods Enzymol. **77**, 235–237
28 Taylor, J. W., Schmidt, W., Cossick, R., Okruszek, A. and Eckstein, F. (1985) Nucleic Acids Res. **13**, 8749–8764
29 Craig, III, S. P., Yuan, L., Kuntz, D. A., McKerrow, J. H. and Wang, C. C. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 2500–2504
30 Chi, H. C., Hsieh, J. C., Hui, C. F. and Tam, M. F. (1988) Nucleic Acids Res. **16**, 10382
31 Liu, L.-F., Hong, J.-L., Tsai, S.-P., Hsieh, J.-C. and Tam, M. F. (1993) Biochem. J. **296**, 189–197
32 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
33 Yeh, H.-I., Hsieh, C.-H., Wang, L.-Y., Tsai, S.-P., Hsu, H.-Y. and Tam, M. F. (1995) Biochem. J. **308**, 69–75
34 Habig, W. H., Pabst, M. J. and Jakoby, W. B. (1974) J. Biol. Chem. **249**, 7130–7139
35 Habig, W. H., Jakoby, W. B., Mannervik, B., Guthenberg, C., Simons, P. C. and Vander Jagt, D. L. (1981) Methods Enzymol. **77**, 218–231, 231–235, 235–237
36 Lawrence, R. A. and Burk, R. F. (1976) Biochem. Biophys. Res. Commun. **71**, 952–958
37 Gill, S. C. and von Hippel, P. H. (1989) Anal. Biochem. **182**, 319–326

38 Pabst, M. J., Habig, W. H. and Jakoby, W. B. (1974) J. Biol. Chem. **249**, 7140–7150
39 Segel, I. H. (1975) Enzyme Kinetics, John Wiley and Sons, New York
40 Cameron, A. D., Sinning, I., L'Hermite, G., Olin, B., Board, P. G., Mannervik, B. and Jones, T. A. (1995) Structure **3**, 717–727
41 Brooks, B., Bruccoleri, R., Olafson, B., States, D. J., Swaminathan, S. and Karplus, M. (1983) J. Comp. Chem. **4**, 187–217
42 Feng, D. F. and Doolittle, R. F. (1987) J. Mol. Evol. **25**, 351–360
43 Chang, L.-H. and Tam, M. F. (1993) Eur. J. Biochem. **211**, 805–811
44 Hayes, J. D. and Pulford, D. J. (1995) CRC Crit. Rev. Biochem. Mol. Biol. **30**, 445–600
45 Widersten, M., Bjornestedt, R. and Mannervik, B. (1994) Biochemistry **33**, 11717–11723
46 Stenberg, G., Ridderstrom, M., Engstrom, Å., Pemble, S. E. and Mannervik, B. (1992) Biochem. J. **284**, 313–319
47 Zimniak, P., Singhal, S. S., Srivastava, S. K., Awasthi, S., Sharnam, R., Hayden, J. B. and Awasthi, Y. C. (1994) J. Biol. Chem. **269**, 992–1000

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