Determinants of specificity for aflatoxin B₁-8,9-epoxide in Alpha-class glutathione S-transferases

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We have used homology modelling, based on the crystal structure of the human glutathione S-transferase (GST) A1-1, to obtain the three-dimensional structures of rat GSTA3 and rat GSTA5 subunits bound to *S*-aflatoxinyl–glutathione. The resulting models highlight two residues, at positions 208 and 108, that could be important for determining, either directly or indirectly, substrate specificity for aflatoxin-*exo*-8,9-epoxide among the Alpha-class GSTs. Residues at these positions were mutated in human GSTA1-1 (Met-208, Leu-108), rat GSTA3-3 (Glu-208, His-108) and rat GSTA5-5 (Asp-208, Tyr-108): in the active rat GSTA5-5 to those in the inactive GSTA1-1; and in the inactive

human GSTA1-1 and rat GSTA3-3 to those in the active rat GSTA5-5. These studies show clearly that, in all three GSTs, an aspartate residue at position 208 is a prerequisite for high activity in aflatoxin-*exo*-8,9-epoxide conjugation, although this alone is not sufficient; other residues in the vicinity, particularly residues 103–112, are important, perhaps for the optimal orientation of the aflatoxin-*exo*-8,9-epoxide in the active site for catalysis to occur.

Key words: 1-chloro-2,4-dinitrobenzene, ethacrynic acid, homology modelling, mutagenesis.

INTRODUCTION

Aflatoxin B₁ (AFB) is a naturally occurring carcinogenic mycotoxin which is one of number of difuranocoumarin toxins produced by *Aspergillus parasiticus* and *Aspergillus flavus* [1]. These micro-organisms can grow on poorly stored food crops, such as cereals and nuts, causing spoiling of the crop by synthesis of aflatoxins as secondary metabolites. Exposure of humans to AFB has been implicated in liver, lung and colon cancers, and in kwashiorkor and Reye's syndrome [2,3]. The risk of AFB-induced cancer is increased by hepatitis B virus infection [4], and mice transgenic for hepatitis B virus show increased levels of cytochrome *P*-450 activities which correlate with elevated tumour formation in response to dietary AFB [5].

The toxicity of AFB is substantially affected by its metabolism by cytochromes *P*-450, and in humans at least five different cytochromes *P*-450, including 1A2, 2A6, 2B7, 3A3 and 3A4, catalyse chemical modifications of this mycotoxin [6]. Biotransformation of AFB by cytochromes *P*-450 produces a range of hydroxylated (aflatoxin M₁, aflatoxin Q₁) and *O*-demethylated (aflatoxin P₁) metabolites, but perhaps the most important reaction is the epoxidation of the terminal furan ring to give the reactive metabolite AFB-8,9-epoxide (AFBO) [7]. Two stereoisomers, AFB-*exo*-8,9-epoxide and AFB-*endo*-8,9-epoxide, are formed [8a]; both are strong electrophiles, although the *endo* epoxide appears to be only a minor product in the enzymic and chemical epoxidation of aflatoxin [8b]. AFBO reacts with nucleophilic centres in macromolecules, particularly DNA [9], attacking the N⁷ atom of guanine to form an 8,9-dihydro-2-(guan-7-yl)-3-hydroxy-aflatoxin adduct [10]. This adduct can spontaneously

depurinate to leave an apurinic site, producing G → T transversions and G → A transitions. AFB–DNA adducts have been shown to produce mutations in several genes, including *ras* [11–13]. Primary hepatomas in humans, the aetiology of which is believed to involve exposure to aflatoxins, have been found to exhibit high incidences of a specific mutation in the *p53* gene [14,15].

The reactive AFB epoxides have a relatively short half-life in the cell. Both *exo*- and *endo*-epoxides hydrolyse spontaneously to AFB-8,9-dihydrodiol. At physiological pH, this exists in a dialdehydic phenolate form, which can produce Schiff bases with amino groups in proteins [16], a reaction that may be involved in the cytotoxicity of AFB; it is reduced to a dialcohol by an aldehyde reductase [17]. In addition, AFBO reacts with the thiol group of glutathione to form a conjugate in a reaction catalysed by some glutathione S-transferases (GSTs).

Mice are resistant to the toxic effects of dietary aflatoxin, but adult male rats are acutely sensitive, in spite of the fact that mouse cells show higher activating (cytochrome *P*-450) activities than those of rats [18]. Comparisons of the glutathione conjugation of AFBO [19,20] showed that the main mechanism of resistance in the mouse was the constitutive expression of an Alpha-class GST with high deactivation capacity that could conjugate AFBO with GSH. This enzyme was subsequently cloned and called mouse GST YcYc [21,22]; this transferase has now been redesignated mGSTA3-3. Sequence analysis revealed that the mouse GSTA3 subunit is highly similar to the rat GST Yc₁ subunit [23] (now designated rGSTA3 [24]), which is constitutively expressed in rats. Resistance to the acute toxicity of AFB is conferred in the normally sensitive rat at the stage of

Abbreviations used: GST, glutathione S-transferase; AFB, aflatoxin B₁; AFBO, AFB₁-8,9-epoxide; CDNB, 1-chloro-2,4-dinitrobenzene.

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aflatoxin-induced pre-neoplasia [25]. Subsequently, protection against the carcinogenic activity of an AFB-contaminated diet was demonstrated by the concurrent administration of AFB at a low level and the synthetic antioxidant ethoxyquin [26]. This response was later shown to be associated with inducible GSTs [27]. Characterization of GSTs induced by antioxidant pretreatment of F-344 rats identified a novel Alpha-class subunit, designated Yc₂ (now designated rGSTA5), having 91 % sequence identity with rGSTA3, but with considerably enhanced catalytic activity towards AFBO in heterodimeric combinations with Ya₁ and Yc₁ subunits (now redesignated rGSTA1 and rGSTA2 respectively) [24,28]. Interestingly, the rGSTA5 subunit was also overexpressed in aflatoxin-induced hepatic pre-neoplasia, consistent with the earlier observation of a mouse-like response to acutely toxic doses of AFB. The antioxidants butylated hydroxytoluene and butylated hydroxyanisole have similar GST-inducing properties to those of ethoxyquin [29]. AFBO-conjugating properties are evident in foetal and neonate F-344 rat liver, and show a marked decline during the early development of males; this occurs to a lesser extent in females during the same period [30].

Chimaeric proteins between mouse GSTA3 and rat GSTA3 show that the residues responsible for AFBO conjugation activity are found between residues 56 and 221 of the mouse A3 subunit [31]. The aim of the present work was to throw more light on the determinants of substrate specificity for AFBO among Alpha-class GSTs by investigating the contributions of individual amino acid residues.

MATERIALS AND METHODS

Bacterial strains

Strains used were as follows: (1) *Escherichia coli* RZ1032, derived from *E. coli* BW313; partial genotype only available: *lacZbd-279::Tn10 Hfr dut⁻ ung⁻*; *E. coli* JM109, F' *traD36 lacI^q D(lacZ) M15 proA⁺B⁺ e14 (McrA⁻) D(lac-proAB) thi gyrA96 (Nal^r) endA1 hsdR17 (r_k⁻ m_k⁺) relA1 supE44 recA1*; *E. coli* BL21 (DE3), F⁻ *ompT hsdS^B (r_B⁻ m_B⁻) gal dcm* (DE3) [32].

Plasmid construction and mutagenesis

An expression construct for human GSTA1-1, pGWL11, has been described [33]. In order to make rapid mutations and to express them with minimal subcloning, the fl origin was amplified

from M13 and inserted into the unique *Sph*I site in pGWL11 to form pEMGST (details of all PCR procedures and primers used are given in [34]). This allowed single-stranded DNA to be isolated as a template for the mutagenesis reactions. The genes for rat GSTA3-3 and GSTA5-5 were amplified by PCR from the original library clones λJH24 and λJH30 respectively, using oligonucleotides that included restriction enzyme recognition sequences for *Eco*RI and *Xma*I to allow insertion of the amplified genes into the expression vector pEM, to give plasmids called pEMYc₁ and pEMYc₂. In each case, the entire GST gene was sequenced using external primers, with the PRISM DyeDioxy Terminator Cycle Sequencing Kit and an Applied Biosystems Model 373A DNA sequencer (Protein and Nucleic Acid Chemistry Laboratory, University of Leicester) to give the published DNA sequences. Although pEM was an expression vector, for some of the proteins there was minimal expression from the *ptacII* promoter, so the wild-type and mutant proteins were subcloned into pET12a (Invitrogen) to give pETYc₁ and pETYc₂ (Figure 1), which gave improved expression. Mutagenesis was carried out by using a modification [34] of the Kunkel [35] procedure. Large numbers of bacterial colonies were screened concurrently for recombinant plasmids using colony hybridization [36]. The DNA was fixed to the membrane using a Stratagene UV Stratalinker and the membrane was hybridized with a sequence-specific radiolabelled probe (for details see [34]).

Expression and purification of GSTs

Expression from pEM vectors with a *ptacII* promoter was carried out in *E. coli* JM109. A single colony was used to inoculate a 50 ml overnight culture in 2 × YT (containing 60 µg/ml ampicillin), and this was used to inoculate 1 litre of prewarmed 2 × YT (60 µg/ml ampicillin). The culture was induced at A₆₀₀ = 0.6 by adding 10 ml of 100 mM isopropyl β-D-thiogalactoside and left to grow for a further 3 h before harvesting the cells by centrifugation at 4420 g for 10 min at 4 °C. Expression from pETYc₁ and pETYc₂ was carried out in *E. coli* BL21 (DE3). Expression was started with a 100 ml overnight culture of LB (60 µg/ml ampicillin), of which 50 ml was used to inoculate 1 litre of prewarmed LB (60 µg/ml ampicillin). The cells were not induced with isopropyl β-D-thiogalactoside, as the leaky expression through the promoter gave sufficient protein. The cultures were allowed to grow for 5 h and were harvested by centrifugation at 4420 g for 10 min at 4 °C.

Figure 1 Expression vectors for rat GSTs

Shown is the configuration of pET12-based expression vectors used to express rat Alpha-class GSTs.

Harvested bacterial pellets were washed with 20 ml of loading buffer (20 mM Tris, 20 mM NaCl, 0.3 g/l dithiothreitol, pH 7.2) and finally resuspended in 25 ml of loading buffer, and the cells were disrupted by French pressing twice. The cell debris was pelleted by centrifugation at 17600 *g* for 10 min at 4 °C. The soluble protein was run through a 1.5 ml column of glutathione-agarose resin (Pharmacia) that had been equilibrated with 5 column vol. of loading buffer. The column was washed with a further 5 vol. of loading buffer to remove unbound protein. The enzyme was eluted using 2 ml of elution buffer (25 mM Na₂CO₃, 20 mM NaCl, 0.3 g/l dithiothreitol, pH 10.5), the eluent being dripped into 20 mM NaHCO₃ to neutralize the buffer. The protein samples were further dialysed against 100 mM sodium phosphate buffer, pH 7.5, to remove any remaining Tris.

GST assays

The enzymes were assayed spectrophotometrically with the substrates 1-chloro-2,4-dinitrobenzene (CDNB) and ethacrynic acid, in 0.1 M phosphate buffer, pH 6.5, at 25 °C. The reaction between GSH and CDNB was monitored at 340 nm, and that between GSH and ethacrynic acid was monitored at 240 nm. The concentrations of the substrate and cofactor were varied between 0.1 mM and 1 mM. All rates were measured in duplicate and corrected for the background rate of the spontaneous reaction. Conjugation of GSH with AFBO was measured as previously described [28], using an assay in which the unstable epoxide was generated *in situ* from AFB by quail microsomes. Note that AFB is a known carcinogen in humans, and appropriate precautions should be taken in its use.

Determination of AFB binding

The dissociation constants (K_d) for AFB binding to the wild-type and mutant enzymes were determined by measuring the quenching of the tryptophan fluorescence of the protein (excitation 285 nm; emission 345 nm) which accompanies binding. Microlitre amounts of AFB (2 mg/ml) were titrated into a solution of enzyme ($> 5 \mu\text{M}$) in 0.1 M phosphate buffer, pH 7.5; the inner-filter effect was corrected for by concurrently titrating AFB into a tryptophan solution at a concentration chosen to give the same fluorescence intensity as the protein sample. The data were analysed by fitting a quadratic function to estimate K_d .

Homology modelling and ligand docking

Homology modelling was carried out using the MODELLER program [37] to predict the three-dimensional structures of the monomers of rat GSTA3 and rat GSTA5 on the basis of the X-ray crystal structure of human GSTA1-1 [38]. The models were compared with human GSTA1-1 by using BIOSYM Insight II to overlay the structures, and were also evaluated using PROCHECK [39].

A model of the aflatoxinyl-glutathione conjugate was constructed on the basis of the co-ordinates of *S*-benzyl-glutathione [from the structure of its complex with human GSTA1-1 [38], replacing the *S*-benzyl moiety with an *S*-(9-hydroxy-aflatoxin-8-yl) moiety (the co-ordinates of AFB were obtained from the Small-Molecule Database, Daresbury, Cheshire, U.K.; entry 1HMT)]. The γ -glutamyl-cysteinyl-glycine backbone was maintained in the conformation seen in the crystal structure [38]; the bond angles at sulphur were set within 0.5° of those in *S*-benzyl-glutathione, and the plane of the difuranocoumarin rings of AFB was adjusted to be approximately the same as that of the benzyl ring of the bound *S*-benzyl-glutathione [38]. The *S*-aflatoxinyl-

glutathione conjugate was initially positioned in the crystal structure of human GSTA1-1 and the models of rat GSTA3 and GSTA5 so that its glutathione moiety occupied the same position as that of *S*-benzyl-glutathione in the crystal structure of the human enzyme [38]. Each of the three models was then subjected to energy minimization using the AMBER forcefield in DISCOVER, with all hydrogen atoms included, the peptide termini capped and the pH set to 6.5.

RESULTS AND DISCUSSION

Homology modelling of rat Alpha-class GSTs

In order to identify residues that may be important in the recognition of AFBO as a substrate for Alpha-class GSTs, we modelled the reaction product, *S*-aflatoxinyl-glutathione, into the active site of the crystal structure of human GSTA1-1 [38], which shows very low activity in conjugation of AFBO, and into modelled structures of rat GSTA5-5 and GSTA3-3, which have, respectively, high and low activity against AFBO. The sequence alignment shown in Figure 2 demonstrates that the two rat GSTs are not only very similar to one another, with only 21 amino acid differences, but are both also very similar (approx. 75 % identity) to human GSTA1-1. This level of identity suggests that homology modelling of the rat enzymes on the basis of the crystal structure of the human enzyme is likely to give reliable structures. In the present study, automated homology modelling has been used to predict the structure of rat GST, using the MODELLER algorithm [37], which finds the most probable structure of a polypeptide sequence by the satisfaction of a set of spatial restraints derived from one or more known structures, rather than the manual methods that have been employed previously to generate other GST models (e.g. [40]).

The models obtained in this way for rat GSTA3-3 and GSTA5-5 structures are shown as MOLSCRIPT [41] representations in

	10	20	30	40	50
human A1	MAEKPKLHYFNARGRMESTRWLLAAAGVEFEKFIKSAEDLDKLRNDGYL				
Rat A3	.PG..V....DG.....PI.....Q.L.TRD..AR.....S.>				
Rat A5	.PG..V....DG.....PI.....N.L.TRD..AR.....S.S.>				
	60	70	80	90	100
human A1	MFQQVPMVEIDGMKLVQTRAILNYIASKYNLYGKDIKERALIDMYIEGIA				
Rat A3	T.....M.....A..V.>				
Rat A5	..E.....K.....T.....M.....A..V.>				
	110	120	130	140	150
human A1	DLGEMILLLPVCPPEEKDAKLALIKEIKRNYFPAPFEKVLKSHGQDYLVG				
Rat A3	..D.IV.HY.YI..G..E.S..K..D.AR.....>				
Rat A5	..EL.V.YY.YM..G..E.S..K..D.AR.....Y.....>				
	160	170	180	190	200
human A1	NKLSRADIHVELLYVEELDSSLISSFPLLKALKTRISNLPVTKKFLQP				
Rat A3	.R.....VY..QV..H....P.ALAN.....R..V.....>				
Rat A5	VS.....H...M.PGIVDN.....R..V.....>				
	210	220			
human A1	GSPRKPPMDEKSLSEARKIFRF				
Rat A3	..Q...LE...CV.S.V...>				
Rat A5	..Q...FD...CV.S.K...>				

Figure 2 Sequence alignments

Alignments of the amino acid sequences of human GSTA1-1, rat GSTA3-3 and rat GSTA5-5 are shown. The alignments are structure-based alignments produced by the MODELLER program [37].

Figure 3 Modelled structures of rat GSTs

Modelled structures of rat GSTA3-3 (left) and rat GSTA5-5 (right), drawn using the MOLSCRIPT program [41]. See the text for details.

Figure 3. As expected, the backbones of the predicted structures are extremely similar to the crystal structure, although the C-terminal α -helix characteristic of Alpha-class GSTs is two residues shorter in the rat proteins than in human GSTA1-1. There are other minor but significant differences; for example, the salt bridge between Arg-15 and Glu-104 seen in human GSTA1-1 is missing in the model of rat GSTA5-5, where residue 104 is Leu, but is present in that of rat GSTA3-3. The quality of the models of the two rat GSTs was evaluated using PROCHECK [39]. It was found that 92.2% of the residues of rat GSTA5-5 and 91.8% of those of rat GSTA3-3 are in the most favoured regions of the Ramachandran diagram, with the remaining 8% being in additionally allowed regions, and with no residues in disallowed regions. The total quality G-factor was -0.34 for rat GSTA5-5, and -0.32 for rat GSTA3-3; acceptable values of the G-factor in PROCHECK are between 0 and -0.5 .

As described in the Materials and methods section, *S*-aflatoxynyl-glutathione was modelled into the active site of human GSTA1-1 by superimposing the glutathione moiety of the conjugate over the glutathione of the *S*-benzyl-glutathione present in the original crystal structure [38], followed by energy minimization. It was found to be particularly important to set the bond angle at the sulphur atom equal to that seen in *S*-benzyl-glutathione bound to the enzyme [38]. Any significant changes in this angle led to serious steric clashes with Phe-10, a residue whose side-chain conformation differs between the apo-enzyme and the *S*-benzyl-glutathione-enzyme complex ([38,42]; C. S. Allardyce, L. Y. Lian, P. C. E. Moody and G. C. K. Roberts, unpublished work) and which may play a part in the correct orientation of glutathione in the active site. Mouse GSTA3-3 has been reported to bind an AFB-GSH conjugate with a stoichiometry of one ligand per dimer [43]. No structure is available of a complex between the conjugate and a GST, although from modelling it is possible to accommodate the conjugate within the active site of one subunit (the present work; C. S. Allardyce, L. Y. Lian, P. C. E. Moody and G. C. K. Roberts, unpublished work). The reported stoichiometry may reflect negative co-operativity or blockage of the active site on one subunit when the site on the other subunit is occupied by AFB-GSH. Some evidence for the latter possibility comes from the observation that steroid affinity labels modify either Cys-111

Figure 4 Active site of human GSTA1-1

The Figure is based on the X-ray crystallographic structure [37]. Some of the hydrophobic residues around the active site, and the ligand, *S*-benzyl-glutathione (GSB), are shown. The Figure was generated using the INSIGHT program (MSI, San Diego, CA, U.S.A.).

or Cys-17 in rat GSTA1-1 in a mutually exclusive manner, with the modification of one cysteine in one subunit preventing the oxidation of the same residue on the other subunit [44].

Given the considerable difference in the size of the hydrophobic moiety between the *S*-benzyl-glutathione in the crystal structure of human GSTA1-1 [38] and *S*-aflatoxynyl-glutathione, we expected the main clashes between ligand and protein in the modelled *S*-aflatoxynyl-glutathione complexes to be in the region of the hydrophobic binding site (H-site) furthest from the glutathione. This part of the active site (see Figure 4) is lined by the side chains of residues 104–111, residue 208 and residues of the C-terminal helix (212–222). In the minimized model of the *S*-

Table 1 Catalytic activities determined for human GSTA1-1, rat GSTA3-3 and rat GSTA5-5 and their mutants

Specific activities towards CDNB and ethacrynic acid were determined at 25 °C in 200 mM NaPO₄, pH 6.5, containing 2 mM glutathione and either 1 mM CDNB or 200 µM ethacrynic acid. AFBO conjugation was measured as previously described [22]. WT, wild-type protein; nd, not determined.

Enzyme	Residue		Specific activity		
	108	208	CDNB (µmol/min per mg)	Ethacrynic acid (µmol/min per mg)	AFBO (nmol/min per mg)
Human GSTA1-1					
WT	Leu	Met	50.66 ± 2.19	nd	0.006
L108Y	Tyr	Met	7.84 ± 0.61	nd	0.003
M208D	Leu	Asp	4.78 ± 0.50	nd	0.005
L108Y/M208D	Tyr	Asp	16.27 ± 0.80	nd	0.001
Rat GSTA3-3					
WT	His	Glu	8.91 ± 0.38	0.65 ± 0.07	0.01
H108Y	Tyr	Glu	4.30 ± 0.51	1.56 ± 0.08	0.01
E208D	His	Asp	7.76 ± 0.17	1.23 ± 0.58	0.01
H108Y/E208D	Tyr	Asp	4.07 ± 0.10	0.39 ± 0.04	0.30
Rat GSTA5-5					
WT	Tyr	Asp	4.86 ± 0.21	0.26 ± 0.14	13.20
Y108L	Leu	Asp	8.82 ± 0.15	0.49 ± 0.40	0.32
D208M	Tyr	Met	0.14 ± 0.01	0.09 ± 0.04	< 0.01
Y108L/D208M	Leu	Met	0.56 ± 0.10	0.17 ± 0.08	< 0.01

aflatoxynyl–glutathione complex with human GSTA1-1, unfavourable interactions between the ligand and the protein were indeed found to involve Met-208 in particular, and also Leu-107 and residues 212, 213 and 216 in the C-terminal helix. Since the C-terminal helix has a degree of flexibility ([38,42]; C. S. Allardice, L. Y. Lian, P. C. E. Moody and G. C. K. Roberts, unpublished work), and might be expected to be able to accommodate the binding of different substrates, we concentrated our attention on residues 107 and 208.

The model of the complex of rat GSTA3-3 showed steric clashes at Leu-107, Glu-208 and Tyr-111, while inspection of the rat GSTA5-5 model revealed the same contacts with Leu-107 and Tyr-111; notably, however, Asp-208 was accommodated without bad contacts with the ligand. This modelling thus suggests the hypothesis that the low activity seen with rat GSTA3-3 and human GSTA1-1 might be due in part to interference with AFBO binding in the active site arising primarily from interactions with residue 208, whose side chain is larger in the less active enzymes (human GSTA1-1, Met; rat GSTA3-3, Glu) than in the more active rat GSTA5-5 (Asp), while the charge is conserved in both rat enzymes.

Tyr-111 is conserved in both rat GSTs (Val in human GSTA1-1), while Leu-107 is conserved in all three enzymes. The bad contacts observed for these conserved residues might reflect inadequacies of the model. However, they might also reflect indirect effects of nearby substitutions. It is notable that there are a number of substitutions in the region of residues 103–129 (Figure 2). In particular, residues 103, 108 and 113 are different in each of the three GSTs, and the side chain of residue 108 lies close to that of Leu-107. Multiple sequence alignments of human GSTA1-1, mouse GSTA3-3, rat GSTA3-3 and rat GSTA5-5 show that the sequence identity is highest between the aflatoxin metabolizers (mouse GSTA3-3 and rat GSTA5-5).

Comparison of catalytic activities of mutants of Alpha-class GSTs

We therefore chose to introduce mutations at positions 108 and 208 in order to investigate the origins of the different activities of the three enzymes towards AFBO. We replaced the residues in

these positions in the active rat GSTA5-5 by those in the inactive human GSTA1-1, and those in the inactive rat GSTA3-3 and human GSTA1-1 by those in the active rat GSTA5-5; in each case, the two single mutants and the double mutant were made.

The catalytic activities of the wild-type and mutant enzymes towards AFBO and the two 'control' substrates, CDNB and ethacrynic acid, are given in Table 1. Comparison of the three wild-type enzymes shows that the two rat enzymes have similar activities towards CDNB and ethacrynic acid, differing by no more than a factor of three, but markedly (1300-fold) different activities towards AFBO. The activity of GSTA5-5 towards AFBO given in Table 1 is in good agreement with that reported previously [22]. Human GSTA1-1 is more active (5–10-fold) towards CDNB than either rat enzyme, but has a very low activity towards AFBO, comparable with that of rat GSTA3-3.

Substitution of residues 108 and 208 in the human enzyme with their equivalents in rat GSTA5-5 (Leu-108 → Tyr; Met-208 → Asp) led only to a decrease in the activity of the enzyme towards AFBO, making it even less active than before. The reciprocal substitutions in GSTA5-5 (Tyr-108 → Leu; Asp-208 → Met) did, however, lead to a marked decrease in the activity of this enzyme towards AFBO, down to levels comparable with that of human GSTA1-1. The Tyr-108 → Leu mutant showed a 41-fold decrease in activity, and the Asp-208 → Met mutant and the double mutant showed even larger (≥ 1300-fold) decreases. Asp-208 is thus necessary, but not sufficient, for high catalytic activity towards AFBO. It is clear that it is not the charge on this residue that is important in this context, since rat GSTA3-3, which has low activity towards AFBO, has a glutamate in this position, and the Glu-208 → Asp substitution in GSTA3-3 does not increase this activity. There is also no measurable effect of the His-108 → Tyr substitution, but the double mutant of GSTA3-3, in which residues 108 and 208 are both converted into their equivalents in GSTA5-5, led to a marked (30-fold) increase in activity towards AFBO.

Comparison with the activities measured for CDNB and ethacrynic acid shows that the effects of substitutions at positions 108 and 208 are generally specific for AFBO. In most cases, the

Table 2 Comparison of AFB binding and catalytic activity towards AFBO for human GSTA1-1, rat GSTA3-3 and rat GSTA5-5 and their mutants

Protein	Residue		AFB binding K_d (μ M)	Specific activity with AFBO (nmol/min per mg)
	108	208		
Human GSTA1-1	Leu	Met	2.0 ± 0.5	0.006
Rat GSTA3-3 wild type	His	Glu	3.0 ± 1.0	0.01
Rat GSTA3-3 H108Y/E208D	Tyr	Asp	6.1 ± 1.9	0.30
Rat GSTA5-5 wild type	Tyr	Asp	9.0 ± 3.0	13.2
Rat GSTA5-5 Y108L/D208M	Leu	Met	6.7 ± 1.5	< 0.01

substitutions in the rat A3 and A5 subunits changed the activities towards CDNB and ethacrynic acid by less than a factor of three, while the activity towards AFBO was changed over a 1300-fold range. The only exception is the substitution of Asp-208 by Met in GSTA5-5, which led to a 35-fold decrease in CDNB-metabolizing activity (although to less than a 3-fold decrease in that towards ethacrynic acid); even in this case, the effect on AFBO activity was much greater. Interestingly, the reciprocal substitution in human GSTA1-1 (Met-208 $\rightarrow$ Asp) also led to a clear (10-fold) decrease in activity towards CDNB, although, because the wild-type GSTA1-1 has a much higher level of activity, the mutation brings its activity down only to a level comparable with that of wild-type rat GSTA5-5. In terms of their effects on activity towards CDNB, there appears to be a greater interaction between residues 108 and 208 in human GSTA1-1 than in rat GSTA5-5: in the latter, the Asp-208 $\rightarrow$ Met substitution led to a decrease in activity (15–35-fold) whether residue 108 was tyrosine or leucine, whereas in GSTA1-1 the same substitution led to a decrease in activity when residue 108 was tyrosine, but to a marked increase when residue 108 was leucine. NMR experiments are currently in progress to investigate the relationship between CDNB and these residues in the binding site.

The nature of the assay for AFBO-conjugating activity, in which the unstable substrate is generated *in situ* [22], makes it impossible to determine K_m and k_{cat} values. To obtain an indication of whether the effects of the mutations described here are primarily on substrate binding or on catalytic turnover, we have determined the equilibrium dissociation constant of AFB for the enzyme by measuring the quenching of the tryptophan fluorescence of the enzyme, due to resonance energy transfer, which accompanies AFB binding. The measured dissociation constants are given in Table 2; it is clear that the affinity for AFB is similar in all cases, and in fact the highest affinity is shown by human GSTA1-1, which has the lowest specific activity. The > 1300-fold range of specific activities towards AFBO is thus most likely to arise from differences in k_{cat} , rather than in substrate affinity, between the different enzymes [45] and their mutants. Since neither residue 108 nor residue 208 is in the immediate vicinity of the site of reaction of the epoxide with the thiol of glutathione, the effect on k_{cat} can most probably be ascribed to effects on the orientation of the substrate in the binding site.

Conclusions

The clearest conclusion from the results reported here is that, in the three GSTs studied, an aspartate residue in position 208 is a prerequisite for high activity in conjugation of AFBO. No enzyme with a glutamate or a methionine in this position shows a specific activity towards this substrate of more than 0.01 nmol/min per mg, compared with 13.2 nmol/min per mg

for rat GSTA5-5, the most active of the enzymes studied. However, an aspartate in position 208 is not sufficient for high activity towards AFBO. The effect of the side chain in this position depends upon the context, i.e. upon the other sequence differences between the three GSTs in which it has been studied. This is particularly apparent when comparing human GSTA1-1 with the rat GSTs. Thus the Asp-208 $\rightarrow$ Met substitution in rat GSTA5-5 drastically decreases the activity towards AFBO, but the reciprocal Met-208 $\rightarrow$ Asp substitution in human GSTA1-1 does not lead to any increase in activity towards this substrate. Similarly, the 'optimum' combination of Tyr-108 and Asp-208 results in activities of 13.2 nmol/min per mg in a rat GSTA5-5 'background', 0.30 nmol/min per mg in rat GSTA3-3, but only 0.001 nmol/min per mg in human GSTA1-1. A possible explanation for these observations comes from the indications that the effects of substitutions at positions 108 and 208 on activity toward AFBO arise from changes in AFBO orientation in the binding site. The exact orientation of the substrate in the active site might be expected to be sensitive to changes in a number of residues in the vicinity, perhaps particularly residues 103–112, in which there are several differences between the three GSTs studied.

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