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Design of two chimaeric human–rat class Alpha glutathione transferases for probing the contribution of *C*-terminal segments of protein structure to the catalytic properties*

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Two chimaeric human–rat class Alpha glutathione transferases were constructed by fusion of DNA segments derived from the plasmids pTGT2-AT and pGTB38 and expression of the corresponding proteins in *Escherichia coli*. The recombinant proteins H1R1/1 and H1R1/2 encoded by plasmids pH1R1/1 and pH1R1/2 are composed of a segment of the human class Alpha subunit 1 from the *N*-terminus to His-143 and Pro-207 respectively, followed by the complementary *C*-terminal portion of the rat class Alpha subunit 1 sequence. Compared with the parental human enzyme, H1R1/1 is altered in 20 positions due to the introduction of 79 residues from the rat enzyme, while H1R1/2 is altered in five positions out of 15 in the *C*-terminal region. The design of mutant H1R1/1 is equivalent to introduction of exons 6 and 7 of the rat subunit 1 gene in place of the homologous human nucleotide sequence. The two chimaeric proteins are enzymically active with several substrates, even though the activity in most cases is somewhat decreased in comparison with the wild-type human enzyme. Inhibition studies show that the kinetic properties mimic those of the human enzyme, indicating that the *N*-terminal two-thirds of the primary structure plays the major role in governing the catalytic properties. The results of this study demonstrate that recombination of segments of primary structure between homologous enzymes may serve as a useful cassette technique for design of novel catalytically active proteins.

INTRODUCTION

Little is known about the structure of the binding sites for substrates and other ligands of glutathione transferases. The primary structure has been determined for many of the enzymes, but neither the particular amino acids nor the segments of the polypeptide chains involved in the interaction with the ligands are known (Mannervik & Danielson, 1988). A truncated form of a class Alpha glutathione transferase, lacking 12 residues from the *C*-terminus, displays decreases in both catalytic activity and binding affinity for hydrophobic *S*-substituents on the glutathione molecule (Board & Mannervik, 1991), suggesting a direct or indirect contribution of this part of the protein structure to formation of the substrate-binding site. In view of the established differences in kinetic properties between individual forms of glutathione transferase, it was considered possible to exchange a segment of one enzyme molecule with a corresponding part of a separate enzyme and, by investigating the altered functional properties, to identify structural regions that contribute to substrate binding or other interactions with ligands. Such constructions of new enzyme molecules are most likely to be successful when applied to enzymes within the same structural class of glutathione transferase, since in this case the amino acid sequence identities are usually at least 70% and the hydropathy profiles and predicted secondary structures suggest similar folding of the polypeptide chain backbone (Persson *et al.*, 1988).

In the present investigation, the human class Alpha glutathione transferase subunit 1 was modified by substituting segments in the *C*-terminus by the corresponding segments of rat subunit 1. Two such chimaeric enzymes were created in which segments of 79 and 15 amino acid residues from the rat enzyme were substituted for the corresponding segments in the human enzyme. The constructs were made by fusion of corresponding cDNA segments and expression of the chimaeric genes in *Escherichia coli*. A preliminary report on the investigation has been given (Mannervik *et al.*, 1989).

MATERIALS AND METHODS

Materials

Plasmids pTGT2-AT (Stenberg *et al.*, 1991) and pGTB38 (Pickett *et al.*, 1984) originated from the laboratories of Dr. P. G. Board (John Curtin School of Medical Research, Canberra, Australia) and Dr. C. B. Pickett (Merck Frosst Centre for Therapeutic Research, Quebec, Canada) respectively. M13mp19 DNA (Norrander *et al.*, 1983) was obtained from Boehringer–Mannheim (Mannheim, Germany), plasmid pKK223-3 (Brosius & Holy, 1984) from Pharmacia LKB Biotechnology (Uppsala, Sweden) and oligodeoxynucleotides from Operon Technologies (Alameda, CA, U.S.A.). Restriction enzymes and other DNA-modifying enzymes were from Amersham International (Amersham, Bucks., U.K.), Promega Corp. (Madison, WI, U.S.A.), Pharmacia LKB Biotechnology and Boehringer–Mannheim.

Sepharose 6B was from Pharmacia LKB Biotechnology. *S*-Hexyl glutathione was synthesized according to method A as described by Vince *et al.* (1971). Δ^5-Androstene-3,17-dione was generously provided by Dr. Paul Talalay (Johns Hopkins University, Baltimore, MD, U.S.A.). 4-Hydroxypentadecenal was generously provided by Dr. H. Esterbauer (Institut für Biochemie, Universität Graz, Graz, Austria). All other chemicals used were of the highest quality commercially available.

Nomenclature of the recombinant glutathione transferases

The designations for the class Alpha glutathione transferase subunits of the human and the rat transferases used here are hA1 and rA1 respectively, and the corresponding dimeric enzyme

* This paper is dedicated to Professor Doriano Cavallini, University of Rome, Italy, in honour of his 75th birthday.
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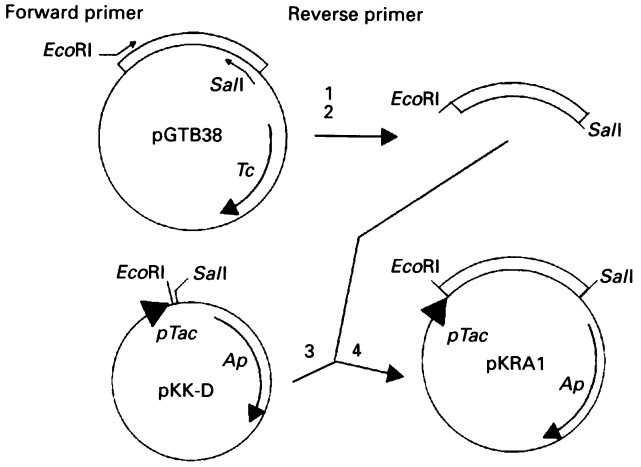

Fig. 1. Schematic diagram showing the construction of the expression plasmid pKRA1

Key: *Ap*, ampicillin-resistance gene; *Tc*, tetracycline-resistance gene; *pTac*, isopropyl β-D-thiogalactopyranoside-inducible *Tac* promoter. The unfilled segment represents the DNA sequence encoding the rat glutathione transferase subunit rA1. Key to arrows: 1, amplification of the coding part of rA1 cDNA; (2) isolation and digestion with *Eco*RI and *Sal*I of the product from the polymerase chain reaction; (3) digestion of pKK-D with *Eco*RI and *Sal*I; (4) ligation of vector and amplified DNA.

proteins are referred to as hA1-1 (also denoted as GST2-type 1, GSTε and $GSTB_1B_1$; see Mannervik & Danielson, 1988) and rA1-1 (GSTYaYa).

Construction of plasmid for expression of recombinant class Alpha rat glutathione transferase A1-1 in *E. coli*

The construction of the expression plasmid pKRA1 is outlined in Fig. 1 and described in detail below.

The complete coding sequence of plasmid pGTB38 was amplified by the polymerase chain reaction. Primers were designed for introduction of restriction sites allowing direct subcloning (Table 1). The cDNA encoding rat glutathione transferase subunit rA1 in pGTB38 was amplified during 30 cycles (2 min at 54 °C, 3 min at 72 °C and 1 min at 94 °C) in a Thermal Cycler (Perkin–Elmer Cetus, Emeryville, CA, U.S.A.), resulting in a fragment containing the entire coding part of the cDNA flanked by an *Eco*RI restriction site in the 5′ end and a *Sal*I restriction site in the 3′ end (Table 1).

The amplified fragment was ligated to the expression vector pKK-D, a derivative of pKK223-3 obtained by deletion of a fragment located between the *Acc*I restriction sites at position 651 and 2246 in the vector. The expression plasmid thus constructed was denoted pKRA1 (Fig. 1).

The cDNA encoding rat subunit rA1 was further subcloned from pKRA1 into M13mp19 for dideoxynucleotide sequencing (Sanger *et al.*, 1977), and the absence of unwanted mutations caused by the *Taq* DNA polymerase reaction was confirmed.

Construction of plasmids encoding chimaeric glutathione transferase subunits

Two plasmids termed pH1R1/1 and pH1R1/2, capable of expressing the human–rat chimaeric enzymes termed H1R1/1 and H1R1/2, were constructed by replacing 240 bp and 46 bp respectively of the coding sequence in the 3′ end of the human class Alpha subunit hA1 cDNA in pTGT2-AT with the homologous cDNA derived from pGTB38 encoding rat glutathione transferase subunit rA1. The chimaeric enzyme encoded by pH1R1/1 contains 143 residues derived from the *N*-terminal two-thirds of the human glutathione transferase subunit hA1 and 79 *C*-terminal residues from the rat glutathione transferase subunit rA1. The expression product of pH1R1/2 consists of the human subunit hA1 from the *N*-terminus to Pro-207 and the last 15 residues of the corresponding part of rat subunit rA1. The steps taken to assemble pH1R1/1 and pH1R1/2 are shown schematically in Fig. 2 and are described in detail below.

The plasmid pGTB38 was subjected to complete *Eco*RI digestion, followed by partial digestion with *Nco*I (Fig. 2). Fragments of 3805 bp and 3999 bp, extending from the *Eco*RI site to the two *Nco*I sites closest to the 3′ end of the insert, both containing the tetracycline-resistance gene and the origin of replication, were isolated by agarose gel electrophoresis and adsorption to silica gel (Geneclean; Bio101, La Jolla, CA, U.S.A.). These vector fragments were ligated to DNA fragments derived from *Eco*RI and partial *Nco*I digests of pTGT2-AT to give plasmids pH1R1/1 and pH1R1/2. Competent *E. coli* JM103 cells were transformed with the plasmids, and transformants were selected for tetracycline resistance. Plasmid preparations (Maniatis *et al.*, 1982) were made from subcultures of resistant colonies; the two plasmids pH1R1/1 and pH1R1/2 were identified by restriction analysis and the structures were confirmed by complete DNA sequence analysis.

Expression and purification of recombinant class Alpha glutathione transferases

The wild-type and chimaeric glutathione transferases were expressed in *E. coli* and purified by affinity chromatography using *S*-hexylglutathione–Sepharose, essentially as described previously (Stenberg *et al.*, 1991). The purity of the enzymes was confirmed by SDS/PAGE (Laemmli, 1970) using silver staining (Blum *et al.*, 1987) as the detection method. Protein concentration was determined by the method of Peterson (1977).

Physicochemical characterization of recombinant glutathione transferases

The apparent subunit M_r of the purified enzymes was determined by SDS/PAGE (Laemmli, 1970) using glutathione transferases purified from human and rat liver (Warholm *et al.*, 1985; Ålin *et al.*, 1985*a*) as markers. The native enzyme M_r was determined using gel filtration analysis on a Superose 12 HR 10/30 column (Pharmacia LKB Biotechnology) in 10 mM-Tris/HCl, pH 7.8, and 0.5 M-NaCl at a flow rate of 0.2 ml/min. Yeast glutathione reductase (M_r 110000), BSA (M_r 68000) and chymotrypsinogen A (M_r 25000), were used as standard proteins, monitored by their absorbance at 280 nm; the glutathione transferases were detected by measuring the enzymic activity with glutathione and 1-chloro-2,4-dinitrobenzene.

The recombinant enzymes were also analysed by chromatography on a reverse-phase C4 h.p.l.c. column (Dynamax-300A; Rainin Instrument Inc., Woburn, MA, U.S.A.) by a modification of a procedure published previously (Ostlund Farrants *et al.*, 1987). The column was developed at a flow rate of 1 ml/min under the following conditions: 45% (v/v) acetonitrile in water for 1 min, 45 to 53% (v/v) acetonitrile in water for 5 min, and 53 to 60% (v/v) acetonitrile in water for 15 min. The mobile phase contained 0.1% (v/v) trifluoroacetic acid.

Assays of enzyme activity

Glutathione transferase activity was measured spectrophotometrically at 30 °C with five different substrates: 1-chloro-2,4-dinitrobenzene, ethacrynic acid (Habig & Jakoby, 1981), cumene hydroperoxide (Lawrence & Burk, 1976), Δ^5-androstene-3,17-dione (Benson *et al.*, 1977) and 4-hydroxypentadecenal (Ålin *et al.*, 1985*b*).

Table 1. Oligonucleotides used as primers for amplification of class Alpha rat glutathione transferase subunit rA1 cDNA

Target sequences are shown above and corresponding parts of the resulting plasmid sequences are shown below the primer structures. * indicates mismatch between target and primer sequences.

Relevant structure	Sequence
	Met Ser Gly Lys Pro Val Leu
pGTB38 sequence	5′-GTT GCT GCT ATG TCT GGG AAG CCA GTG CTT-3′
	* ** ***
Forward primer	5′-TTT GAA TTC ATG TCT GGG AAG CCA GTG CTT-3′
	EcoRI
pKRA1 sequence	5′-AGGA AACA GAA TTC ATG TCT GGG AAG CCA GTG CTT-3′
	Vector sequence
	Phe Stop
pGTB38 sequence	5′-TTT TAG $(N)_{21}$ CTT GTA ATC CAG GCT CTG ATG TTT TGC AAA-3′
	* * **
Reverse primer	3′-GAA CAT TAG GTC CGA GAC TAC CAG CTG TT-5′
	SalI
pKRA1 sequence	5′-CTT GTA ATC CAG GCT CTG ATG GTC GAC-3′

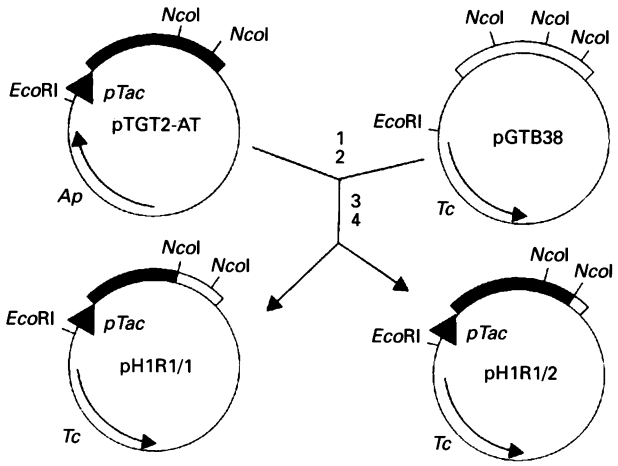

Fig. 2. Schematic diagram showing the construction of the expression plasmids pH1R1/1 and pH1R1/2 encoding human–rat chimaeric glutathione transferase subunits

Key: *Ap*, ampicillin-resistance gene; *Tc*, tetracycline-resistance gene; *pTac*, isopropyl β-D-thiogalactopyranoside-inducible *Tac* promoter. The filled segment represents the DNA sequence encoding the human glutathione transferase subunit hA1, and the unfilled segment represents the DNA sequence encoding the rat glutathione transferase subunit rA1. Key to arrows: 1, digestion of pTGT2-AT and pGTB38 with *Eco*RI and partially with *Nco*I; (2) isolation of 3805 and 3999 bp vector fragments from pGTB38; (3) ligation of vector fragments from pGTB38 and *Eco*RI/*Nco*I digest of pTGT2-AT and selection for tetracycline-resistant transformants; (4) identification of plasmid products by restriction analysis.

Inhibition studies using a series of inhibitor concentrations were performed in the standard assay system containing 1 mM-glutathione and 1 mM-1-chloro-2,4-dinitrobenzene in 0.1 M-sodium phosphate, pH 6.5. The concentration of inhibitor causing a 50% decrease in enzyme activity, the IC_{50} value, was determined from plots of fractional velocity versus inhibitor concentration (Tahir & Mannervik, 1986).

RESULTS

Construction of a plasmid for prokaryotic expression of rat glutathione transferase A1-1

cDNA encoding rat glutathione transferase subunit rA1 was amplified from the plasmid pGTB38 (Pickett *et al.*, 1984) using the polymerase chain reaction (Fig. 1). The subsequent ligation of the amplified cDNA fragment into the expression vector pKK-D positioned the initiating ATG codon 10 bases downstream from the putative ribosomal-binding site (AGGA) present in the vector (Table 1). The transcription is under the control of the *Tac* promoter, inducible by addition of isopropyl β-D-thiogalactopyranoside to *E. coli lac*I^Q host cells.

Expression and physicochemical characterization of recombinant wild-type and chimaeric glutathione transferases

Four recombinant class Alpha glutathione transferases were expressed in *E. coli* and purified to homogeneity by affinity chromatography on *S*-hexylglutathione–Sepharose (Mannervik & Guthenberg, 1981). The human wild-type enzyme, transferase hA1-1 (also referred to as GST2 type 1, GSTε and GST B_1B_1), has previously been studied (Board & Pierce, 1987), and the rat wild-type enzyme, transferase rA1-1 (rat subunit 1 is also named Ya), has also been expressed in *E. coli* by Wang *et al.* (1989), an independent investigation that was published during the completion of this work. The recombinant wild-type and chimaeric proteins, transferases rA1-1, H1R1/1 and H1R1/2, were produced in adequate yields (10 mg, 20 mg and 25 mg respectively) from 3-litre batch cultures.

The purified proteins were all homogeneous, as judged by analyses in several electrophoretic and chromatographic systems. SDS/PAGE in combination with silver staining (results not shown) demonstrated single bands with mobilities corresponding to the subunits of the wild-type human and rat enzymes. Thus the apparent subunit M_r values of the chimaeric proteins were not significantly different from those reported for the parental enzymes (cf. Mannervik & Danielson, 1988).

Analysis by gel filtration on a Superose 12 HR 10/30 column demonstrated that the chimaeric proteins, like the parental rat and human enzymes, form functional dimers with an apparent M_r of approx. 45000 (results not shown).

Analysis by reverse-phase (C4 matrix) h.p.l.c. resolved the chimaeric protein H1R1/1 subunit from the parental hA1 and rA1 subunits (Fig. 3). The mobility of chimaeric H1R1/2 was not significantly different from that of subunit hA1 (Fig. 3). When chromatographed individually, each of the four recombinant proteins gave rise to a single peak, indicating homogeneity (results not shown).

Enzymic properties

The chimaeric glutathione transferases were catalytically active with all substrates tested, but the activities with 1-chloro-2,4-

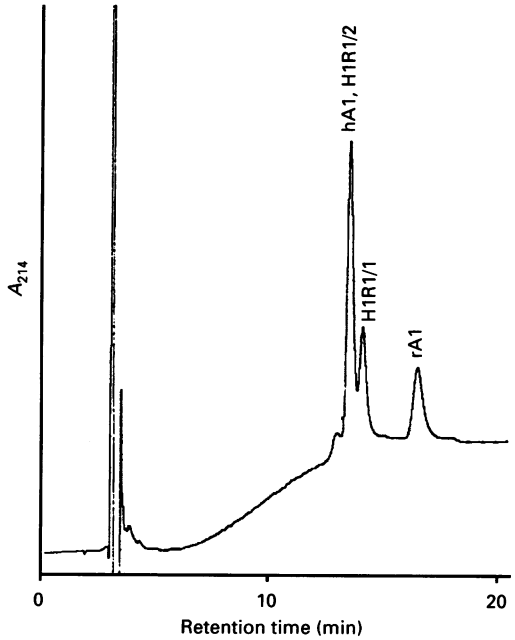

Fig. 3. Separation of recombinant glutathione transferase subunits by reverse-phase h.p.l.c.

A sample containing 0.5 μg each of recombinant glutathione transferases hA1-1, H1R1/1, H1R1/2 and rA1-1 was chromatographed on a reverse-phase C4 column using the conditions described in the Materials and methods section. The effluent was monitored photometrically at 214 nm.

Table 2. Specific enzymic activities for recombinant wild-type and chimaeric class Alpha glutathione transferases

Substrate	Enzyme...	Specific activity (μmol/min per mg) hA1-1	H1R1/2	H1R1/1	rA1-1
1-Chloro-2,4-dinitrobenzene		81	44	27	50
Cumene hydroperoxide		12	11	7	3
Δ^5-Androstenedione		10	6	3	4
4-Hydroxypentadecenal		0.8	0.9	0.5	0.8
Ethacrynic acid		0.2	0.1	0.1	0.1

Table 3. Inhibition parameters for recombinant wild-type and chimaeric class Alpha glutathione transferases

The IC_{50} is the inhibitor concentration giving 50% inhibition of the enzyme activity assayed at 30 °C with 1 mM-glutathione and 1 mM-1-chloro-2,4-dinitrobenzene in 0.1 M-sodium phosphate, pH 6.5.

Inhibitor	Enzyme...	IC_{50} (μM) hA1-1	H1R1/2	H1R1/1	rA1-1
S-(*p*-Bromobenzyl)-glutathione		8	17	19	50
Triethyltin bromide		5	9	3	140
Tributyltin acetate		0.03	0.03	0.08	1
Bromosulphophthalein		9	4	4	0.3

dinitrobenzene of H1R1/1 and H1R1/2 were somewhat decreased as compared with the activity of the human transferase hA1-1 (Table 2).

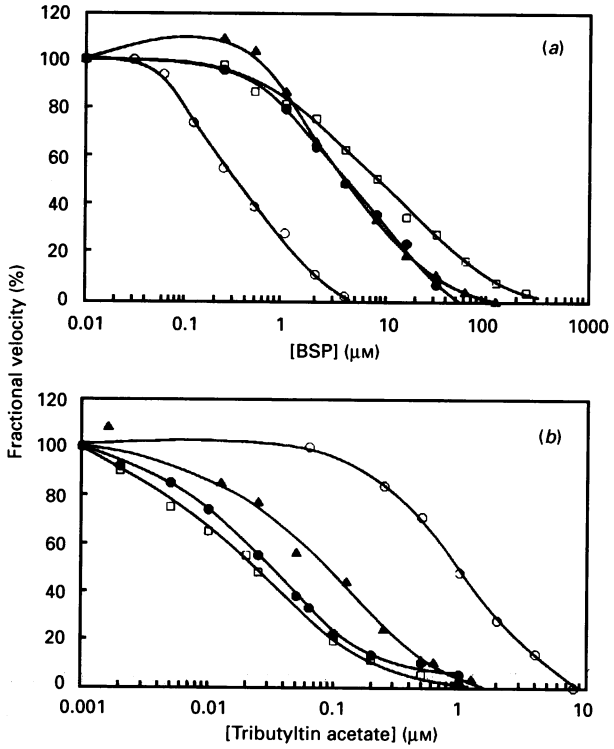

Fig. 4. Inhibition of wild-type and chimaeric recombinant glutathione transferases with bromosulphophthalein (BSP) (*a*) and tributyltin acetate (*b*)

Fractional velocity was determined with reference to uninhibited enzyme using glutathione transferases rA1-1 (○), H1R1/1 (▲), H1R1/2 (●) and hA1-1 (□). Experimental procedures are described in the Materials and methods section.

The glutathione peroxidase activity of the chimaeric enzymes assayed with cumene hydroperoxide was approximately the same as that of the human parental enzyme (Table 2). The activities with the two alkene substrates, 4-hydroxypentadecenal and ethacrynic acid, were similar to those of both the human and the rat wild-type transferases (Table 2). Only in the case of Δ^5-androstenedione did the chimaeric enzymes indicate greater similarity with the rat than with the human enzyme.

In the analysis of the effects of inhibitors on enzyme activity, it was found that the inhibition curves of both chimaeric enzymes were similar to those of the human class Alpha transferase (Table 3 and Fig. 4). The inhibitors chosen for analysis were those for which significant differences had previously been established between the human and rat class Alpha transferases used (Mannervik & Danielson, 1988).

DISCUSSION

The results of the present investigation show that the chimaeric class Alpha human–rat glutathione transferases are catalytically active. The chimaera H1R1/1 consists of residues 1–143 of the human class Alpha glutathione transferase subunit 1 linked to residues 144–222 of the corresponding rat subunit 1. This chimaeric enzyme can be considered to be a mutated form of the human transferase in which 20 amino acids in the *C*-terminal one-third of the primary structure have been altered (Table 4). This construct corresponds to > 90% overall sequence identity with the parental human sequence, a value similar to those obtained in the comparison of naturally occurring isoenzymes within the same class of glutathione transferases. Most replace-

Table 4. Amino acid residue exchanges in chimaeric glutathione transferase subunits compared with the human glutathione transferase subunit A1

Residue position	Subunit hA1	H1R1/1	H1R1/2
152	K	R	
154	S	T	
156	A	V	
161	V	L	
165	Y	L	
170	L	F	
172	S	A	
175	I	L	
176	S	T	
184	L	F	
186	T	S	
190	N	S	
193	T	N	
203	P	Q	
207	P	A	
210	E	A	A
212	S	Q	Q
213	L	I	I
219	I	V	V
221	R	K	K

ments are conservative at the amino acid level (Table 4), but one position involves the loss of a negatively charged residue (Glu-210 → Ala), resulting in an alteration of the electrophoretic mobility of the native chimaeric proteins (results not shown).

When looked upon as a mutation of the rat enzyme, the chimaeric protein H1R1/1 corresponds to 34 changes in the *N*-terminal two-thirds of the rat enzyme. Even with this number of amino acid changes, the chimaeric enzyme is 85% identical in sequence to rat glutathione transferase A1-1, which can be compared with 68% sequence identity between rat subunits 1 and 2, two naturally occurring class Alpha structures. Another rat class Alpha subunit, subunit 8, has only approx. 60% sequence identity with subunits 1 and 2 (Ålin *et al.*, 1989).

The chimaeric enzymes have the physical properties expected, including the apparent subunit M_r, a dimeric structure and positive reaction with polyclonal antisera directed against the parental human and rat glutathione transferases.

Substrates that clearly distinguish between the parental human and rat glutathione transferases are difficult to find (see Mannervik & Danielson, 1988), and the substrate specificities of the chimaeric proteins are therefore similar to those of both the human and the rat enzyme (Table 2). However, inhibitors are known that give a clear discrimination. Thus inhibition studies, involving four different inhibitors (Table 3 and Fig. 4), showed that the chimaeric enzymes are highly similar to their human counterpart glutathione transferase hA1-1. This result suggests that none of the 20 amino acids that differ between the *C*-terminal one-third of the parental human and rat enzymes contribute directly to the inhibition characteristics of the parental human enzyme. More specifically, this indicates that the *N*-terminal two-thirds of the primary structure contains the sequence mainly responsible for binding of the inhibitors tested. It also implies that the amino acid residues forming the site needed for glutathione binding should be located in this region of the primary structure and not in the C-terminal one-third. This conclusion is based on the fact that three of the inhibitors [*S*-(*p*-bromobenzyl)glutathione, triethyltin bromide and tributyltin acetate] are competitive inhibitors of the glutathione transferases (Jakobson *et al.*, 1979; Tipping *et al.*, 1979).

Studies of a *C*-terminally truncated human glutathione transferase A1-1 have also indicated that glutathione binding is not dependent directly on the *C*-terminal part of the primary structure (Board & Mannervik, 1991). On the other hand, this protein segment appeared to contribute to the hydrophobic substrate-binding site. In combination with the results of the present investigation, it would appear that this contribution is largely indirect, since the specificity of interaction with inhibitors is governed by regions distinct from the *C*-terminus (Table 3). One possibility is that this peptide segment is required for proper folding of the protein. Nevertheless, the photoaffinity labelling experiments of Hoesch & Boyer (1989) suggest proximity between an *S*-substituent of glutathione and the *C*-terminal amino acid residues, even if this interaction does not contribute significantly to specificity. The chimaeric transferase H1R1/2 differs from the truncated human transferase only by containing Asp and Ala rather than His and Gly in positions 209 and 210 respectively, and by containing the additional 12 amino acids of the rat transferase *C*-terminus rather than being terminated at residue 210. The chimaeric enzyme is almost fully active (Table 2), whereas the truncated form displays < 1% of the activity of the wild-type (Board & Mannervik, 1991). Thus the presence of the *C*-terminus is important for high catalytic activity, but its structure has little significance for the specificity of the interaction with the ligand with the active site.

It may appear paradoxical that glutathione transferases H1R1/1 and H1R1/2 differ only marginally in specific activities from the parental rat enzyme rA1-1 (Table 2), while some of the IC_{50} values differ by more than an order of magnitude (Table 3), in spite of the assumption that both substrates and inhibitors (with the possible exception of bromosulphophthalein) bind to the active site. The explanation for this apparent discrepancy may be that IC_{50} values basically estimate binding interactions, whereas specific activities approach $V_{max.}$ values and reflect the kinetic component of catalysis.

One of the objectives in the design of the chimaeric enzymes was to test the hypothesis that recombination events equivalent to substitution of one or several exons in the genetic material could have contributed to the molecular evolution and diversification of glutathione transferases (Mannervik, 1985; Mannervik *et al.*, 1985, 1990). Such genetic events could consist of exon shuffling or gene conversion resulting in a new gene, in which a new sequence of exons has been created. The intron/exon organization of the rat glutathione transferase 1 gene has been established (Telakowski-Hopkins *et al.*, 1986), and corresponding human class Alpha genes appear to be similarly organized (P. G. Board, unpublished work). Thus the design of the chimaeric transferase H1R1/1 is such that the gene product is encoded by human cDNA corresponding to exons 2, 3, 4 and 5, while exons 6 and 7 originate from the rat sequence. Therefore, even if our approach disregards the contribution of the variety of regulatory genetic elements and possible effects of intervening sequences in the genomic DNA, the experiment still lends support to the proposal that recombinations could have contributed to the evolution of glutathione transferases. It could be argued that the change of the DNA in the present investigation may represent a 'neutral' mutation of the human enzyme, since the enzymic properties did not change significantly. However, the mutation may also be looked upon as an alteration of a rat sequence in which exons 2–5 of the rat sequence gene have been altered. From this point of view, the properties of a human glutathione transferase have been conferred to the rat enzyme.

A second objective in planning the construction of the chimaeric glutathione transferases was to test the possibility that

artificial enzymes with new catalytic properties could be created by modification of naturally occurring enzymes. The results obtained demonstrate that shifting segments of primary structure between homologous enzymes may give rise to functionally active proteins and consequently could serve as a cassette technique for rational design in protein engineering. Such studies have now been carried out on class Alpha (Mannervik *et al.*, 1989, and the present work) as well as on rat class Mu glutathione transferases 3-3 and 4-4 (Zhang & Armstrong, 1990). This approach should serve as a valuable complement to single-point site-directed mutagenesis and random mutagenesis of proteins.

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