Heterodimers of wild-type and subunit interface mutant enzymes of glutathione S-transferase A1–1: Interactive or independent active sites?

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Abstract

Heterodimers of rat glutathione S-transferase A1–1 were formed using one wild-type subunit and one subunit with a mutation at the interface to evaluate whether the subunits are interactive or independent. Within the subunit interface, we are considering two regions of interactions: one region consists of a "hydrophobic ball and socket" with Phe 52 from one subunit as the ball and Phe 136 from the second subunit as one of the socket residues. The second region of interaction consists of Arg 69 and Glu 97 from both subunits. The heterodimers were formed after incubation in 1,6-hexanediol. Because one subunit in each pair had a His-tag, the heterodimers were purified using a nickel-nitrilotriacetic acid column. The specific activities of the heterodimer were compared with those of the two homodimers to determine whether the less active, mutant subunit communicates with the other subunit. Two of the heterodimers, wild type/R69E-His and wild type/E97Q-His, displayed specific activities much lower than that expected for independent active sites; in these cases, there are new close repulsive interactions and the low activity of one subunit is communicated to the neighboring subunit. In contrast, the other two heterodimers, wild type/R69Q-His and F136A/wild type-His, exhibited specific activities similar to those expected for independent active sites; in these heterodimers, the closest interaction is not repulsive or occurs over a much longer distance and the subunits act independently. We conclude that whether the subunits interact or are independent depends on the nature of the interactions at the subunit interface.

Keywords: glutathione S-transferase; heterodimers; interface

Glutathione S-transferases (GSTs) constitute a family of dimeric detoxification enzymes that function by conjugating glutathione (GSH) to the electrophilic center of many endogenous and xenobiotic substrates (Mannervik and Danielson 1988; Pickett and Lu 1989; Wilce and Parker 1994; Armstrong 1997). The soluble, mammalian GSTs have been divided into classes based on their sequence similarity, substrate specificity, and physical properties; multiple isozymes provide a means of catalyzing reactions with a wide variety of xenobiotic substrates. The enzymes are crystallized as dimers, with each subunit containing a complete active site: a GSH site and a hydrophobic substrate-binding site. Within a particular class of GSTs, the enzymes can form homodimers or heterodimers. These species occur in vivo and can be purified from natural sources (Mannervik and Danielson 1988). In the case of heterodimers involving one subunit from each of two isozymes, each subunit may be able to bind its preferred substrates, thus allowing for an increase in the number of substrates that can be accommodated by the dimer.

There have been conflicting results as to whether the subunits act independently or whether the activity of one

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Abbreviations and symbols: GST, glutathione S-transferase; GSH, glutathione; Ni-NTA, nickel-nitrilotriacetic acid; CDNB, 1-chloro-2,4-dinitrobenzene; wild type-His, wild-type enzyme with a six-histidine tag after the initiator methionine. Rat glutathione S-transferase, isozyme 1–1, is designated as the rGSTA1–1 isozyme in the proposed nomenclature by Hayes and Pulford (1995).

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subunit is influenced by the other subunit. Some evidence suggests that the active sites act independently. First, crystal structures have been solved in the presence of substrate or product analogs, and these structures do not show any differences between the subunits, suggesting that the subunits are independent. Second, steady-state kinetics are consistent with noncooperative active sites (Danielson and Mannervik 1985). Third, affinity labeling of GST 1–1 with 17β-iodoacetoxy-estradiol-3-sulfate results in the incorporation of 1 mole reagent/mole of enzyme dimer, concomitant with the loss of about half of the catalytic activity (Vargo and Colman 2001). On the contrary, there are also data suggesting that the active sites act cooperatively: Photoaffinity labeling of rGST 4–4 (Wang et al. 1998) or rGST 1–1 (Wang et al. 2000) with glutathionyl S-[4–(succinimidyl)-benzophenone] results in the incorporation of only 1 mole of reagent/mole of enzyme dimer, yet the enzyme loses all activity. mGST 3–3 binds 8,9-dihydro-8–(S-glutathionyl)-9-hydroxyl-aflatoxin B_1 in a ratio of 1 mole inhibitor/mole of enzyme dimer and the catalytic activity is completely inhibited (McHugh et al. 1996). Furthermore, in the case of GST pi, inactivation by 3-methyleneoxindole results in the incorporation of only 1 mole reagent per mole enzyme dimer, but there is almost complete loss of activity (Pettigrew et al. 2001).

The subunit interface of GST A1–1 has three major areas of interaction (Sinning et al. 1993), two of which we probe in this study (Fig. 1). The first region (Fig. 1B, Region I) is a hydrophobic "ball and socket" in which the "socket" includes Met 94, Phe 136, and Val 139 on one subunit and the "ball" is contributed by Phe 52 of the other subunit. Phe 52 is 3.6 Å from the closest atom of Phe 136 of the opposite subunit. The second region (Fig. 1B, Region II) consists of Arg 69 and Glu 97 from both subunits forming electrostatic interactions at the subunit interface. The guanido group of Arg 69 forms a salt link to the carboxylate group of Glu 97 of the same subunit, as well as to Glu 97 from the opposite subunit. The subunit interfaces of mu and pi GSTs also feature areas of interaction equivalent to Regions I and II of the alpha class, whereas the interactions in Region III vary more among the GST classes (Pettigrew and Colman 2001).

Previously, we considered the possibility of subunit interactions by forming heterodimers from two different isozymes (A1 and A3) within the alpha class (Wang et al. 2000). In order to further study the question of whether active sites function independently, we have now generated heterodimers in vitro, which are composed of the same subunit type (A1), one subunit of which is wild type and the other is a mutant with a single amino acid substitution at the subunit interface. The interface residues were chosen because they are the amino acids that most closely interact with the other subunit. Therefore, changes in one of these residues on one subunit may best influence the second. This is a simple system in which the subunits are matched except for a change in one amino acid at a site of interaction of the two subunits. We can thus test whether the effect of one mutation is communicated across the subunit interface. The heterodimer activity is compared with that of the homodimer species to determine whether the heterodimers have independently functioning active sites or, alternatively,

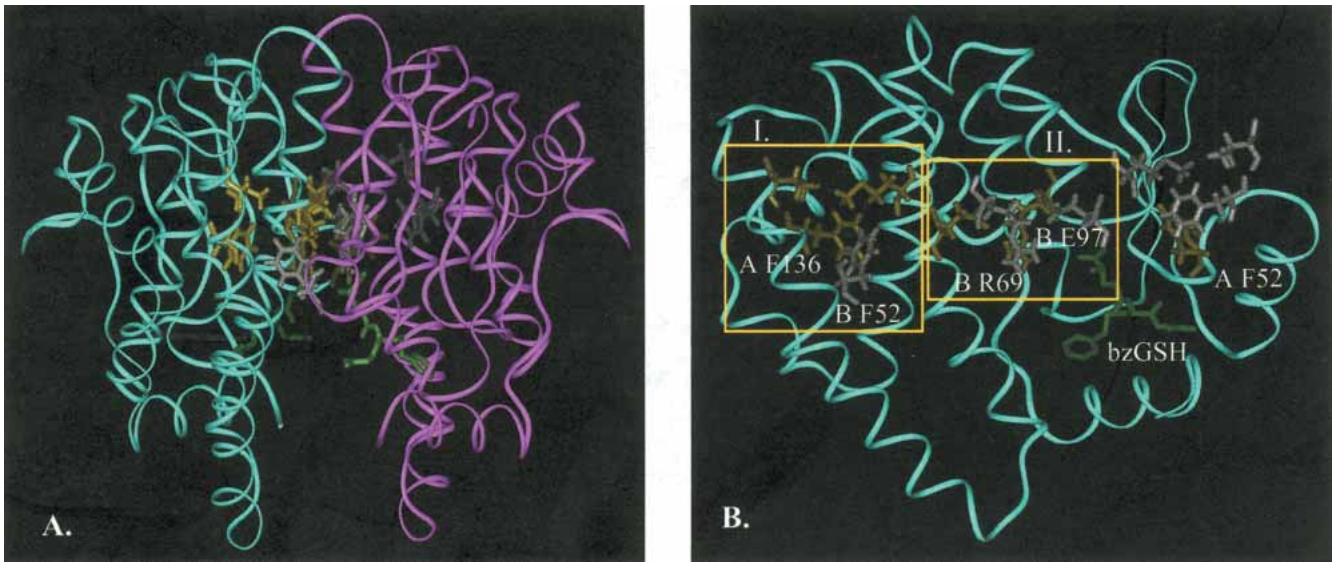

Figure 1. Model of the rat GST A1–1 showing two regions of the interface within the dimer. (*A*) The two regions of the interface being studied. Amino acids of the cyan subunit are in yellow and the amino acids from the pink subunit are in white. S-benzylglutathione (orange) is shown bound in the active site. (*B*) The hydrophobic ball-and-socket region (I) and the Arg/Glu region (II). The model in A has been rotated 90° to the left and the backbone of the pink subunit is not displayed (but would be above the page). The interface is in the plane of the paper. S-benzylglutathione (bzGSH) and the residues that were studied are labeled.

whether the activity of one subunit modifies the activity of the other.

Results

Expression and purification of wild-type and mutant glutathione S-transferase A1

The plasmids were transformed into *Escherichia coli* and the cells grown and induced for expression of GST. The enzymes without a His tag were purified using an S-hexylglutathione agarose affinity column. F136A was eluted using buffer containing 2.5 mM S-hexylglutathione, whereas F52A was eluted earlier, primarily with 10 mM TRIS buffer (pH 7.8) containing 0.2 M NaCl. The behavior of F52A is probably a result of the decreased affinity of F52A for GSH. The enzymes that contain a His tag were purified using a Ni-NTA column. All of the enzymes were purified to homogeneity, yielding a single peptide by N-terminal protein sequencing and a single band of molecular weight ~25 kD by SDS-PAGE.

Formation and separation of homo- and heterodimers

The three potential species (two homodimers and one heterodimer) were separated using a Ni-NTA column equilibrated with 10 mM TRIS buffer (pH 7.8) containing 0.2 M NaCl. As a control, the first trial was conducted with wild-type enzyme and wild-type enzyme with a His tag. This would evaluate whether the method for heterodimer formation and purification was effective. The presence of zero, one, or two His tags on the enzyme dimer should allow the separation of the three species. The results for this experiment are shown in Figure 2A. Peak I, eluted with equilibration buffer, represents the wild-type homodimer (neither subunit has a His tag), peak II represents the heterodimer peak (one subunit contains a His tag and the other does not), and peak III contains the wild-type homodimer in which both subunits contain a His tag. The composition of the peaks was confirmed in each case using N-terminal sequencing, and the results for the heterodimer peak are in Table 1. As a further example, Figure 2B shows the elution of a wild-type/R69Q-His heterodimer experiment. Again, peak I represents the wild-type homodimer with no His tags, peak II is the wild-type/R69Q-His heterodimer (sequence shown in Table 1), and Peak III is the R69Q-His tagged homodimer. Figure 2C shows an elution in which the wild-type enzyme contains the His tag and the mutant enzyme (F136A) does not. The peaks follow the two previous examples, and the amino acid sequence of the heterodimer is in Table 1. The amino acids in cycles 8–15 for the subunit with the His tag are the same as in cycles 1–7 of the subunit with no His tag. The yield for each residue (in picomoles) was averaged for cycles 8–15 and the yields for peptides

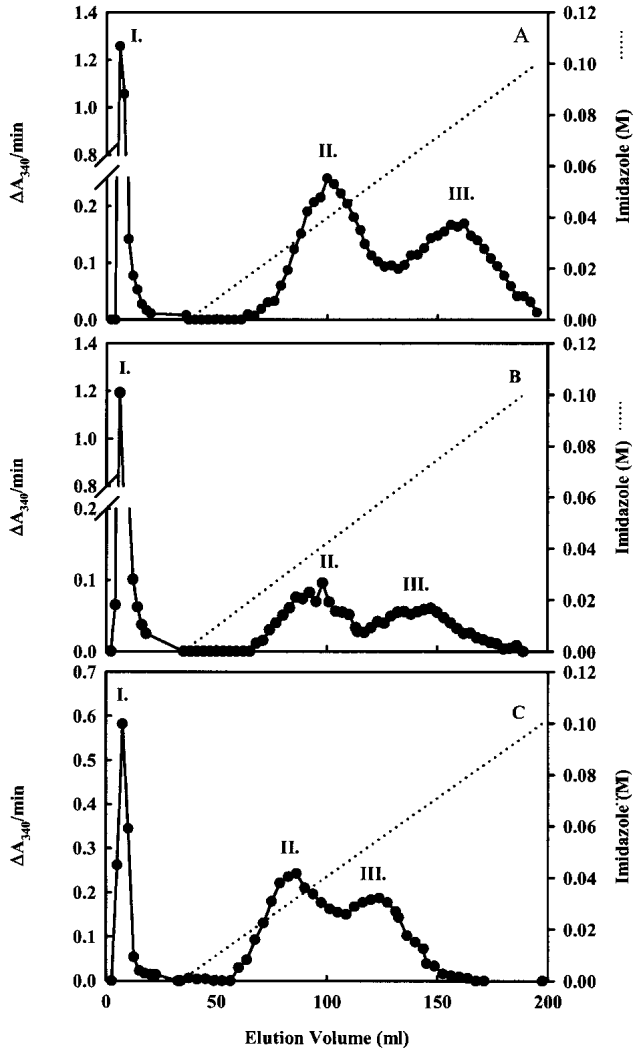

Figure 2. (*A*) Wild-type/wild-type-His elution from the Ni-NTA column at 4°C after incubation and dialysis. ΔA_{340}/min (in 30 μL) was measured for each fraction using the standard assay. (*B*) Wild-type/R69Q-His elution from the Ni-NTA column at 4°C after incubation and dialysis. ΔA_{340}/min (in 30 μL) was measured for each fraction using the standard assay. (*C*) F136A/wild-type-His elution from the Ni-NTA column at 4°C after incubation and dialysis. ΔA_{340}/min (in 30 μL) was measured for each fraction using the standard assay.

one and two were compared. (The first seven cycles were not used because the yield for PTH-His is very low as compared with other residues.) The two subunits are present in approximately equimolar quantities (Table 1), demonstrating that this peak only contains heterodimer.

All sets of enzymes chosen for the heterodimer experiments exhibited three similar peaks. The only exception was that of F52A and wild type-His. No heterodimer was observed in the mixture of F52A and wild type-His under any conditions used. This result may be due to local changes of the subunit interface of the F52A enzyme, which cause a decrease in the ability of the enzyme to dimerize. In a separate study, we have compared the dissociation constant of

Table 1. *N-terminal sequencing results for the heterodimer peaks*

	Wild type/wild type-His		Wild type/R69Q-His		F136A/wild type-His	
Cycle	Peptide 1 (pmole)	Peptide 2 (pmole)	Peptide 1 (pmole)	Peptide 2 (pmole)	Peptide 1 (pmole)	Peptide 2 (pmole)
1	S (70.24)	M (95.39)	S (59.14)	M (108.36)	S (224.3)	M (410.6)
2	G (60.51)	H (33.41)	G (54.58)	H (28.71)	G (153.8)	H (129.0)
3	K (78.37)	H (32.17)	K (87.06)	H (59.63)	K (208.3)	H (159.6)
4	P (45.95)	H (44.53)	P (49.17)	H (58.39)	P (143.4)	H (220.3)
5	V (45.04)	H (35.26)	V (49.56)	H (60.62)	V (130.0)	H (170.3)
6	L (57.17)	H (45.36)	L (60.36)	H (60.61)	L (116.0)	H (116.4)
7	H (49.05)	H (49.05)	H (82.12)	H (82.12)	H (205.8)	H (205.8)
8	Y (49.00)	S (42.01)	Y (52.29)	S (54.67)	Y (105.9)	S (105.1)
9	F (46.56)	G (35.75)	F (48.74)	G (49.28)	F (116.6)	G (120.0)
10	N (42.72)	K (61.64)	N (46.50)	K (79.87)	N (92.3)	K (169.1)
11	A (48.99)	P (28.73)	A (58.00)	P (42.02)	A (134.7)	P (101.1)
12	R (29.44)	V (28.32)	R (38.55)	V (42.55)	R (76.9)	V (105.1)
13	G (20.69)	L (50.16)	G (30.31)	L (65.89)	G (54.4)	L (143.4)
14	R (33.43)	H (13.27)	R (39.29)	H (16.93)	R (86.9)	H (41.8)
15	M (28.63)	Y (31.99)	M (45.74)	Y (45.84)	M (91.3)	Y (100.3)
Ave. pmole (cycles 8–15)[a]	37.43	36.48	44.93	49.63	94.9	110.7

[a] Aliquots of different size were used in determining the sequence of the peptides. Thus the picomoles of heterodimer do not represent the amount of heterodimer recovered in these experiments.

dimer to monomer of F52A and wild-type enzymes. Both form dimers, but the K_d for the F52A enzyme is about 10 times that of wild type (Vargo et al. 2004).

Heterodimer characterization

After all of the pools were concentrated and dialyzed into 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM EDTA, the protein concentration was determined using the Bradford assay, with wild-type GST1–1 as the standard protein. The molecular mass was determined using analytical ultracentrifugation and, as an example, the wild-type/R69Q-His heterodimer has an average molecular mass of 48.2 kD, close to the molecular weight of a dimeric GST. The specific activity of each pool was measured using the standard enzymatic assay. The results are listed in Table 2, along with a theoretical value for a heterodimer that has independently functioning active sites. The theoretical activity for "independent active sites" is the average of the two homodimer specific activities. F136A or R69Q paired with wild type also have independently functioning active sites because the resulting activity of the heterodimer pool is very close to the theoretical value for independently functioning active sites. In contrast, the pairing of R69E or E97Q with wild-type subunits results in a heterodimer that has a specific activity much lower than that expected for independently functioning active sites (Table 2, lines 2,3). Therefore, in these heterodimers, one subunit affects the activity of the other subunit, indicating the existence of subunit interaction.

Discussion

In this study, heterodimers were formed between wild-type subunits and those with mutations at the subunit interface.

Table 2. *Enzyme pairs used to form heterodimers and the resulting relative activity[a] of the three species*

Enzyme 1 (No His tag)	Enzyme 2 (His tag)	Activity of non-His tagged homodimer	Activity of His-tagged homodimer	Theoretical activity of heterodimer[b] (independent sites)	Experimental activity of heterodimer
Wild type	R69Q-His	1	0.35	0.68	0.62
Wild type	R69E-His	1	0.12	0.56	**0.21**
Wild type	E97Q-His	1	0.27	0.64	**0.36**
F136A	wild type-His	0.67[c]	1	0.84	0.89

[a] Activity for each species is normalized to the activity of the wild-type species for each particular experiment. A typical specific activity for wild type is 51 μmole/min/mg.
[b] Theoretical activity of heterodimer is the average of the two homodimer species.
[c] It should be noted that the activity of this non-His-tagged homodimer is lower relative to the others because this is the only mutant non-His-tagged homodimer.

The heterodimers provide an approach to evaluate whether the subunit active sites of GST A1–1 act independently or whether the subunits influence one another. The heterodimers were formed by incubating the individually purified enzymes in 15% or 25% 1,6-hexanediol and then dialyzing a mixture of the two types of subunits to remove the hexanediol and allow for random reassociation of the subunits. GSTs exist in equilibrium between monomer and dimer and the 1,6-hexanediol probably acts by binding preferentially to the monomeric species when formed (increasing the population of monomeric species). Most likely it binds mainly in the interface region, where the hydrophobic portions of the 1,6-hexanediol can bind to the now exposed hydrophobic regions of the monomer. Proteins have been crystallized in organic solvents, including hexanediol, and it was found that each solvent binds in a particular manner based on steric and chemical properties (Mattos and Ringe 2001). The three species were purified using a Ni-NTA column and the identification of the heterodimer was confirmed using N-terminal sequencing.

We were successful in generating heterodimers between wild-type subunits and those with mutations at the subunit interface. The targets for mutation were based on the regions of interaction in the crystal structure of GST A1–1 (Sinning et al. 1993). The subunit interface interactions typically consist of a hydrophobic ball-and-socket region and an electrostatic Arg/Glu region. Mutations were made to residues in these conserved regions in order to study subunit cooperativity. These residues were chosen because they are the residues that are closest to the neighboring subunit.

In the case of the wild-type and R69Q-His heterodimer, the theoretical value for the activity of the heterodimer and the experimentally measured activity are similar (Table 2, line 1). These two subunits are acting independently. Pairing the R69Q subunit with a wild-type subunit removes one charge. As can be seen from Figure 3, the closest amino acids in the heterodimer that have the same charge are ~5.5 Å apart (between the two Glu 97s); because this single repulsive interaction occurs at a relatively large distance, the effect of the mutation on one subunit is not communicated to its partner, resulting in independently functioning active sites. The kinetic characteristics of the F136A homodimer are very close to that of wild type. The GSH K_m and CDNB K_m are very similar to that of wild type; however, the specific activity is lower for F136A than for wild type. This mutation is in the hydrophobic ball-and-socket region and results in the elimination of a hydrophobic interaction between Phe 52 in one subunit and Phe 136 on the other subunit. The experimentally determined specific activity for the F136A/wild-type-His heterodimer also agrees well with the theoretical value for independently functioning active sites.

In contrast, the experimental value for specific activity of the wild-type/R69E-His heterodimer is much lower than

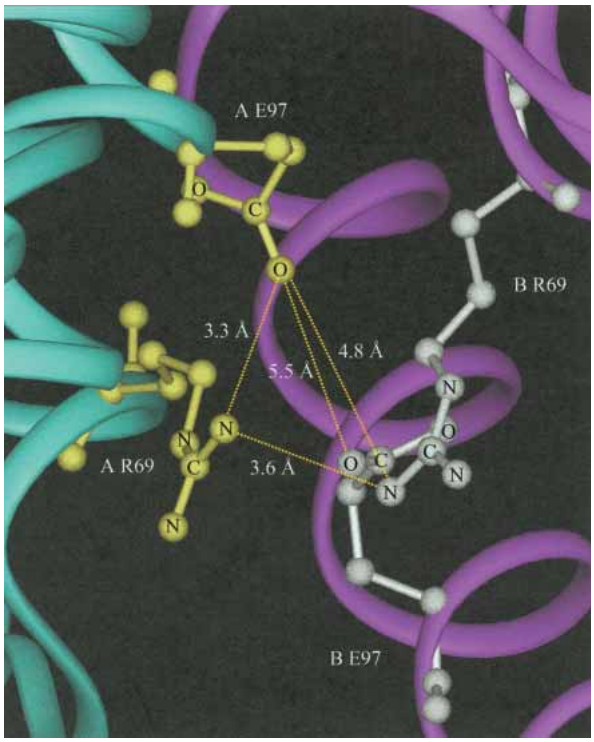

Figure 3. A model of the wild-type rGSTA1–1 interface showing Arg 69 and Glu 97 from both subunits. The distances shown are between A R69 and B R69 (3.6 Å), between A E97 and A R69 (3.3 Å), between A E97 and B R69 (4.8 Å), and between A E97 and B E97 (5.5 Å).

would be expected for independently functioning active sites (Table 2, line 2). R69E introduces a negative charge to the interface region. Instead of Glu 97 being "balanced" by the positive charge of the arginine, in the Arg/Glu region there are now three negative charges at the interface of the heterodimer. When R69E is paired with a wild-type subunit to form the heterodimer, the mutant subunit has two negative charges, whereas the wild-type subunit has a positive and negative charge. Two of the interactions between the subunits are repulsive (between the two negatively charged glutamates of the mutant subunit and Glu 97 of the wild-type subunit); these interactions occur at distances of 4.8 Å and 5.5 Å (see Fig. 3). Within the R69E subunit there is a repulsive interaction at a distance of only 3.3 Å (between Glu 97 and glutamate at 69). Because there are several repulsive interactions between interfacial groups, the low activity of one subunit affects the other subunit. This communication may be due to a small conformational change at the interface because of the repulsion. Arg 69 is close to Thr 68 and Gln 67, which both make contacts to the bound GSH; thus, a perturbation at position 69 in the subunit interface is likely to cause some alteration near the active site, resulting in a reduction in the activity of the wild-type subunit.

The heterodimer formed between E97Q-His and wild-type subunits also has a much lower activity than expected

for independently functioning active sites. In this case, in the mutant enzyme, a negatively charged amino acid charge has been removed and replaced with a neutral amino acid. The closest interaction across a subunit is a repulsive one between the two arginines at a distance of only 3.6 Å (see Fig. 3). This interaction is not balanced by the negative charge of the glutamate, as it is in the wild-type enzyme. This repulsive interaction could also result in a local conformational change, which transmits the low activity of the E97Q subunit to the wild-type subunit, as in the wild-type/R69E-His heterodimer.

Other studies show similar results. Work with aldehyde dehydrogenase shows an interaction among subunits of the tetramer in the E487K mutant (Zhou and Weiner 2000). In the aldehyde dehydrogenase tetramer, a dimer of dimers, subunit interface residue Glu 487, when mutated to lysine, affects the properties of its dimer partner. This effect is very similar to those seen for the heterodimers involving the R69E and E97Q mutants. In a heterodimer study using GSTs, wild-type A1 subunits and subunits with mutations to Asp 101 were used to form heterodimers (Lien et al. 2001). Asp 101 forms a salt link to the α-amino group of the GSH bound in the neighboring subunit. In these heterodimers, the enzyme illustrated either cooperative or noncooperative behavior, depending on the substrate used.

Based on the examples in this study, it appears that when the closest interaction across the subunit interface is a repulsive one, the lower activity of the mutant subunit is communicated to the wild-type subunit; however, if the interaction across the subunit interface occurs at a much longer distance or is not repulsive, the subunits act independently of one another. We conclude that the nature of the interactions at the subunit interface is a determinant of whether the active sites of the two subunits are independent, or whether the activity of one subunit is influenced by the activity of the second subunit.

Materials and methods

Reagents

GSH, CDNB, and S-hexylglutathione were purchased from Sigma. Ni-NTA resin was purchased from Qiagen. 1,6-hexanediol was purchased from ACROS Organics.

Plasmids and mutagenesis

The full-length cDNA for rat glutathione S-transferase A1–1 encoded in a pKK2.7 plasmid, as described in Wang et. al. (1989) and Dietze et. al (1998), was a gift from William M. Atkins at the University of Washington. Site-directed mutagenesis was performed using the Stratagene QuikChange kit. The following oligonucleotides and their complements were used to incorporate the mutations (position of mutation is underlined):

F52A, GACGGGAATTTGATGGCTGACCAAGTGCCC;
F136A, CGGTACTTGCCTGCCGCTGAAAAGGTGTTG;
R69E, GCTGGCACAGACCGAAGCCATTCTCAAC;
R69Q, GCTGGCACAGACCCAAGCCATTCTCAAC; and
E97Q, GCCCTGATTGACATGTATTCACAGGGTATTTTAGA TCTG.

Mutations were confirmed by DNA sequencing (forward sequencing primer: 5′-GTTGACAATTAATCATCGGC and reverse sequencing primer: 5′-ATCAGACCGCTTCTGCGTTC), which was carried out at the University of Delaware Biology Core Facility using a Long Readir 4200 DNA Sequencer from LiCor, Inc. or at the Delaware Biotechnology Institute and University of Delaware Center for Agricultural Biotechnology using an ABI Prism model 377 DNA sequencer (PE Biosystems).

Incorporation of a six-His tag

A six-histidine tag was incorporated at the N terminus of the protein. The His tag was incorporated using a PCR technique based on a procedure in the QIAexpressionist handbook from Qiagen. A forward primer (a 52-mer) was used that incorporates the six histidines (shown in bold after initiator methionine) and the 5′ restriction site for EcoR1 (underlined): 5′-CAGGAAACAGAATTCATG**CATCACCATCACCATCAC**TCTGGGAAGCCAGTGC. A reverse primer (a 20-mer) was used that incorporates the 3′ restriction site for HindIII (underlined): 5′-CCAAGCTTGGCTG CAGGTCG. These primers were used to amplify the wild-type GST insert. The new GST 1–1 insert, with the His tag, was digested with HindIII and EcoRI and then ligated into the original plasmid (digested with HindIII and EcoRI) using T4 DNA ligase.

Protein purification

GST A1–1 was expressed in JM105 *E. coli*. Cells were grown at 37 °C, and when $A_{600} = 0.4$, the cells were induced with 1 mM IPTG. After induction, the cells were grown for 24 h at 25°, at which time they were harvested by centrifugation at 10,000*g* for 20 min. The pellets were then frozen at −80°C. Cells were resuspended in 10 mM TRIS buffer (pH 7.8; ~50 mL for 6 L of culture), followed by sonication for 6 min using a sonicator (Ultrasonic, Inc.) at 20 kHz and 475 W. This suspension was then centrifuged for 25 min at 10,000*g*.

For those enzymes with a His tag (wild type-His, R69Q-His, R69E-His, and E97Q-His), the following procedure was used: the enzyme activity in the supernatant was assayed and applied to a Ni-NTA column (~7 mL of resin) equilibrated with 10 mM TRIS buffer (pH 7.8; at 4°C). The column was eluted first with 10 mM TRIS buffer (pH 7.8), followed by 10 mM TRIS buffer (pH 7.8) containing 0.2 M NaCl. The enzyme was eluted using a linear gradient of imidazole (0–0.5 M) in 10 mM TRIS buffer (pH 7.8) containing 0.2 M NaCl (100 mL of each buffer). The fractions exhibiting activity were pooled, concentrated, and dialyzed into 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM EDTA. The protein concentration was determined using $\varepsilon_{270nm} = 22{,}000\ M^{-1}\ cm^{-1}$ (Katusz and Colman 1991) and M_r of 25,500 per subunit (Mannervik and Danielson 1988).

For those enzymes that do not have a His tag (wild type, F52A, and F136A), a different purification method was used. These enzymes were purified using affinity chromatography on S-hexylglutathione agarose, as previously described (Wang et al. 1996). Briefly, the column was eluted with 10 mM TRIS buffer (pH 7.8), followed by 10 mM TRIS buffer (pH 7.8) containing 0.2 M NaCl

to elute any weakly bound proteins. The GST was eluted with 10 mM TRIS buffer (pH 7.8) containing 0.2 M NaCl plus 2.5 mM S-hexylglutathione and dialyzed into 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM EDTA. The purity of the enzymes was determined using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, or by N-terminal sequencing performed on an Applied Biosystems Procise Sequencing System.

Enzymatic assay

Enzyme activity was measured using a Hewlett Packard 8453 UV-VIS Spectrophotometer. As a standard assay, the formation of the conjugate of GSH (2.5 mM in assay) and CDNB (1 mM in assay) was monitored at 340 nm ($\Delta\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$) in 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM EDTA according to Habig et al. (1974). The activity is expressed as specific activity: micromole substrate/minute per milligram protein.

Formation of heterodimers

Heterodimers were formed from wild-type subunits and subunits with substitutions for amino acids in the subunit interface. To facilitate purification of the potential dimeric enzyme species, one of the enzymes has a His tag and the other enzyme does not (see Table 1). The general strategy is to mix the two enzymes (mutant and wild type), dissociate them to monomers, allow them to reassociate randomly to form three dimeric species, and then to separate the two homodimeric species from the heterodimer. To dissociate the subunits, we incubated the enzymes (1 mg of each in 2 mL) in 25% 1,6-hexanediol in 0.1 M potassium phosphate buffer (pH 7.5) for 2 h at 25°C. The enzyme activity was measured before and after incubation. In all cases, the two enzymes were incubated together, with the exception of F136A/wild type-His. F136A was incubated separately because the enzyme precipitated in 25% 1,6-hexanediol. Thus, F136A was incubated in 15% hexanediol and the wild-type-His enzyme was incubated in 25% hexanediol. At the end of the 2 h, the wild-type-His solution was diluted with buffer so that the final 1,6-hexanediol percentage was 15% and was then mixed with the F136A mixture. After incubation in 1,6-hexanediol, the mixture was dialyzed overnight into 10 mM TRIS buffer (pH 7.8) containing 0.2 M NaCl at 4°C to allow for reassociation of the subunits.

Other means of forming heterodimers were attempted. One method tried was incubation with acetonitrile (15%–30%), which worked well for wild-type enzyme; however, the acetonitrile treatment caused many of the mutant enzymes to precipitate. Another means of promoting heterodimer formation was the use of isopropanol (15%–20%). This solvent did not cause precipitation; however, no heterodimeric enzyme was recovered. Thus, treatment with 1,6-hexanediol yielded the best results.

Separation of the heterodimers

The three potential species can be separated using a Ni-NTA column. The column (1.5 ml resin) was equilibrated with 10 mM TRIS buffer (pH 7.8) containing 0.2 M NaCl (equilibration buffer). After dialysis, the mixture was loaded onto the Ni-NTA column and the column was then eluted with equilibration buffer. Fractions (1 mL) were collected throughout the column elution. The column was eluted with equilibration buffer until the activity from the homodimer (without a His tag on either subunit) was completely removed from the column (~45 mL). A linear gradient, from the equilibration buffer to equilibration buffer containing 0.1 M imidazole (100 mL of each), was then used to separate the two remaining species. The heterodimer (in which only one of the two subunits has a His tag) eluted earlier in the gradient than does the homodimer in which both subunits contain a His tag.

Postcolumn treatment

The activity of the fractions was measured using the standard enzyme assay. The regions of activity were pooled and concentrated to 1 mL using an Amicon Ultra-15 centrifugal filter device with a 10-kD molecular weight cutoff. The following procedure was used to remove the imidazole and exchange buffer: the sample was diluted with 10 mL of 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM EDTA, and then concentrated to a final volume of ~1 mL using the filter device; this dilution and concentration procedure was carried out three times. The specific activity was determined using the standard assay and the protein concentration was determined using the Bio-Rad protein assay, based on the Bradford method, using a Bio-Rad 2550 RIA plate reader with a 600-nm filter (Bradford 1976). Wild-type GST was used as the protein standard.

Evaluation of the presence of heterodimer

To test whether the "middle" pool (peak II) was the heterodimer, once it was dialyzed into 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM EDTA, we subjected a sample from the pool to N-terminal sequencing using an Applied Biosystems Procise Sequencing System. A heterodimer would have two residues in each cycle because of the presence (on one subunit) and the absence (on the other subunit) of the His tag. Once the existence of the heterodimer was confirmed, the activities of the three pools were compared to determine whether the active sites act independently (indicated by an activity that is an average of that of the two homodimers) or whether they interact with one another (indicated by an activity that is significantly different from the average value).

Molecular weight determination of the heterodimer

Analytical ultracentrifugation was used to measure the average molecular mass of the heterodimer. Using a Beckman Optima XL-A analytical ultracentrifuge, we performed sedimentation equilibrium experiments at 15,000 rpm, 17,000 rpm, and 20,000 rpm using an An-60 Ti rotor and running at a temperature of 10°C. The samples (~0.08 mg/mL) in 0.1 M potassium phosphate, 1 mM EDTA (pH 6.5), were centrifuged until equilibrium was reached (~24 h) at which time data were collected (equilibrium was confirmed by scanning at 5-h intervals.). Stepwise radial scans were performed at the particular wavelength using a step size of 0.001 cm. The resulting data were fit globally using the software package IgorPro (Wavemetrics, Inc.) as previously described (Schneider et al. 1997; Kretsinger and Schneider 2003).

Molecular modeling

Molecular modeling was conducted using the Insight II modeling package from Molecular Simulations, Inc. on an Indigo 2 work station from Silicon Graphics. The model of rat GST A1–1 was constructed as previously described (Wang et al. 1996) based on the known crystal structure of human GST A1–1 (Sinning et al. 1993) with S-benzylglutathione bound (PDB # 1GUH). The amino acid sequences of human and rat GST A1–1 are 76% identical plus

11% similar, and therefore the structure of the human enzyme provides a good basis for constructing the rat homology model. This homology model was used to produce the Insight figures and measure the distances between amino acid side chains at the subunit interface.

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Note added in proof

While this paper was undergoing review, a paper on pi class GST appeared in which a heterodimer was isolated with one wild-type subunit and one Y50A subunit (mutation at the subunit interface; Hegazy et al. 2004). The activity of the heterodimer was considerably lower than expected, suggesting that there is communication between the subunits. These results for pi class GST are consistent with our results for alpha class GST.

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