ADVANCES IN ENZYMOLOGY

AND RELATED AREAS OF MOLECULAR BIOLOGY

Founded by F. F. NORD

Edited by ALTON MEISTER

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LIST OF CONTRIBUTORS

JAMES M. ANGELASTRO, Department of Biochemistry and Molecular Biology, University of Florida College of Medicine Health Science Center, Gainesville, Florida 32610-0245.

RICHARD N. ARMSTRONG, Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742.

DAVID P. HAJJAR, Department of Biochemistry, Cornell University Medical College, New York, New York 10021.

RICHARD W. HANSON, Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio 41106.

TIMOTHY M. PALMER, Departments of Medicine and Pharmacology, Duke University Medical Center, Durham, North Carolina 27710.

YASHOMATI M. PATEL, Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106.

JORAM PIATIGORSKY, Laboratory of Molecular and Developmental Biology, National Eye Institute, National Institutes of Health, Bethesda, Maryland 20892.

DANIEL L. PURICH, Department of Biochemistry and Molecular Biology, University of Florida College of Medicine Health Science Center, Gainesville, Florida 32610-0245.

CHRISTINA M. SAX, Laboratory of Molecular and Developmental Biology, National Eye Institute, National Institutes of Health, Bethesda, Maryland 20892.

GARY L. STILES, Departments of Medicine and Pharmacology, Duke University Medical Center, Durham, North Carolina 27710.

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CONTENTS

GLUTATHIONE S-TRANSFERASES: STRUCTURE AND MECHANISM OF AN ARCHETYPICAL DETOXICATION ENZYME

By RICHARD N. ARMSTRONG, *Department of Chemistry and Biochemistry, University of Maryland, College Park, MD*

CONTENTS

I. Introduction—A Historical Perspective

In 1961 Booth, Boyland, and Sims (1) and Combes and Stakelum (2) independently described an enzymatic activity in the cytosolic

fraction of homogenized liver that catalyzed the addition of glutathione (GSH) to 1,2-dichloro-4-nitrobenzene. In the three decades hence it became apparent that the group of proteins responsible for this activity, the glutathione S-transferases (EC 2.5.1.18), is crucial to the effective metabolism and detoxication of electrophilic xenobiotics in vertebrates. The enzymes also appear to play the same role in plants, insects, and aerobic microorganisms. Each decade since their discovery has marked a significant advance in the understanding of the structure and function of the GSH transferases. In the early 1970s, the enzymological investigations of the GSH transferases began in earnest with the pioneering work of Jakoby, Arias, Ketterer, Mannervik, and numerous others. Several enzymes were purified to homogeneity. Among the notable advances made in this decade was the elucidation of the dimeric quaternary structure of the cytosolic enzymes, the realization that there were numerous subunit types with differing but overlapping substrate specificities and the discovery that the organic anion-binding proteins of liver (ligandins) were, in fact, GSH transferases. The reader interested in a historical perspective of this period may consult a number excellent review articles on the subject (3–5).

The availability of highly purified isoenzymes from which both sequence information and substrate specificities could be obtained led Mannervik and coworkers (6) to propose the first classification of various isoenzymes into groups or classes, namely *alpha*, *mu* and *pi*. At this same time the Pickett, Tu, Board, Taylor, Pearson, and Listowsky laboratories, as well as others, reported sequences, deduced from cDNA clones, for several isoenzymes. The full-length sequences suggested interspecies familial relationships between groups of isoenzymes fully consistent with at least three gene families. The cDNA clones allowed investigations of the regulation of gene expression to be initiated, facilitated the elucidation of genomic sequences, and led to the construction of efficient bacterial expression systems. Several laboratories also reported the preparation of diffraction-quality single crystals of various isoenzymes. Much of this progress during the 1980s has been reviewed recently (7–11). Readers interested in a recent summary of the progress made in understanding the regulation of GSH transferase gene expression may wish to consult the review of Rushmore and Pickett (11).

The recent confluence of the ability to manipulate the structure

of the proteins by site-directed and modular mutagenesis and the solution of the three-dimensional structures of isoenzymes from each of three classes of GSH transferases has challenged us to refine our understanding of the relationship between the structures of these enzymes and their functional properties. It is unfortunately true that neither the elegant and detailed structural pictures provided by X-ray crystallography nor the simple catalytic activity of a particular isoenzyme or site-specific mutant can provide unambiguous insight into questions of structure-function. For this reason, a truly useful understanding of these relationships can be achieved only through rigorous mechanistic analysis of the connection between protein structure and catalytic function. This article reviews the advances made in the mechanistic and structural enzymology of the cytosolic GSH transferases during the last four or five years, with particular emphasis on investigations that attempt to elucidate the mechanistic basis of structure-function relationships. It concludes with some thoughts on the evolution of catalytic diversity in the GSH transferases.

II. Background

A. THE MULTIGENE FAMILIES OF GSH TRANSFERASES

Glutathione S-transferases are found in all vertebrates, many plants, insects, and aerobic bacteria. The soluble, cytosolic enzymes are invariably dimeric proteins consisting of identical or closely related subunits with molecular masses of about 25 KDa. The primary structures, largely deduced from cDNA clones, of over 70 GSH transferase subunits have been reported. Sequence comparisons suggest that the enzymes can be grouped into at least four classes, *alpha, mu, pi* (6), and *theta* (12), which correspond to multigene families that comprise a superfamily of genes. Intraclass protein sequence identities are generally quite high, in the range of 65–80%, while interclass sequence comparisons show much lower identity—25–35%. A squid GSH transferase, which is 42–44% identical in sequence to the lens S-crystallins of cephalopods, exhibits relatively low (<35%) identity to the four principal classes mentioned above and may represent yet another class of cytosolic enzyme (13). A number of sequence alignments have been published for the principal classes (8, 13–15) based on the primary structures alone. More

accurate sequence alignments based on the three-dimensional structures are described below. Heterodimers composed of subunits of the same gene class are commonly isolated. However, interclass heterodimers have not been observed, suggesting a very strong familial recognition among subunit types.

A recent report (16) suggests that a soluble 13-KDa protein isolated from rat liver by S-hexylglutathione affinity chromatography, which is related to the human lymphokine macrophage migration inhibitory factor (MIF), has a detectable GSH transferase activity and an N-terminal sequence that is similar to isoenzyme 3-3 of rat GSH transferase. Although the sequence similarity is significant, residues that are conserved in most GSH transferases and which have been demonstrated to be important for catalytic activity (tyrosine 6 of isoenzyme 3-3 for example) are not conserved in the 13-KDa protein. The significance of the GSH transferase activity of the 13 KDa protein is not clear.

In addition to the large number of cytosolic isoenzymes, there are at least two distinct microsomal forms of GSH transferase. One appears to be a general catalyst with a relatively broad substrate specificity (17), and the other is a leukotriene C_4 synthase, specific for addition of GSH to leukotriene A_4 (18). Neither enzyme appears to resemble the cytosolic enzymes with respect to sequence. This is particularly true for the microsomal enzyme involved in xenobiotic metabolism extensively studied by Morgenstern, DePierre and their coworkers (see 19 and 20 for reviews). This enzyme appears to exist as a trimer of identical 17-KDa subunits that bear no obvious sequence resemblance to the cytosolic enzymes. Interestingly, the first 103 residues of the 154-residue microsomal enzyme exhibit a statistically significant sequence similarity to the β subunit of the nitrogenase molybdenum-iron protein from *Clostridium pasteurianm* (21). Whether this similarity is of any structural, biological, or evolutionary significance remains to be seen. At this writing, only the amino terminal sequence of the leukotriene C_4 synthase has been reported, so its relationship to other proteins or GSH transferases is not clear. However the physical properties of the polypeptide purified from the membrane suggest that it is a dimer of identical 18-KDa subunits. The role of peptidoleukotrienes in bronchial asthma make this enzyme an obvious target for the development of antiasthma drugs (11).

To the uninitiated the nomenclature of the cytosolic GSH transferases must seem arcane in the extreme. The only GSH transferase with an unambiguous designation is the microsomal leukotriene C_4 synthase, named appropriately after the reaction it catalyzes. In the early days, for lack of any alternative, individual laboratories devised their own nomenclatures for the cytosolic enzymes for which specific electrophilic substrates were difficult to define. The first systematic nomenclature, which simply designated subunit types by Arabic numerals and holoenzymes by the appropriate binary combination of those numerals (e.g., isoenzyme 3-3, 3-4, and 4-4), was introduced in 1984 (22). The system was widely but not uniformly adopted. More recently, a system has been proposed for the human isoenzymes which recognizes not only different subunit types but the gene family with which they are associated as well (23). Although this nomenclature is readily transferable to other species, it has not yet been generally adapted in this respect. For this reason the 1992 (23) nomenclature will be used for the human enzymes and the 1984 nomenclature (22) will be used for all others in this article.

B. CATALYSIS AND DETOXICATION

The GSH transferases catalyze the addition of GSH to molecules that have electrophilic functional groups. The generic reaction is shown in equation 1.

$$GSH + RX \rightarrow GSR + XH \qquad (1)$$

There is substantial evidence that the peptide moiety of the product acts as a signal for the export of glutathione conjugates from cells by an ATP-dependent GSR pump (24). The reaction also represents the first step in the formation of mercapturic acid conjugates of xenobiotic and endogenous electrophiles. The cytosolic enzymes from vertebrates generally exhibit a broad spectrum of selectivity toward both the topology of the electrophilic substrate and the type of electrophilic functional group. However certain isoenzymes or groups of isoenzymes are particularly efficient at catalyzing specific reaction types. For example, the class *mu* isoenzymes are often very efficient at catalyzing reactions with substrates containing oxirane or α,β-unsaturated carbonyl groups. Inasmuch as these functional groups occur in endogenous compounds such as leukotriene A_4, cholesterol

epoxide, dopaquinone, and 4-hydroxyalkenals, it can be imagined that these or related compounds provided part of the evolutionary pressure for the diversification of a variety of isoenzymes. In this regard, it is interesting to note that the class *alpha* isoenzyme 8-8 from rat has very high catalytic activity towards 4-hydroxyalkenals which are natural products of lipid peroxidation (25). The microsomal enzyme has been proposed to participate in the metabolism of phospholipid hydroperoxides (26). Furthermore, the microsomal enzyme appears to have a high activity, compared to the cytosolic enzymes, towards polyhalogenated hydrocarbons such as chlorotrifluoroethylene (27) which are relatively recent additions to the biosphere.

With the exception of leukotriene C_4 synthase, the so-called natural substrates for the GSH transferases have remained an enigma and largely a matter of speculation. The existence of a large number of isoenzymes with different catalytic properties perhaps suggests that these enzymes may have originally evolved to catalyze a number of relatively specific transformations with endogenous substrates. Their role in the metabolism and detoxication of xenobiotics may be the result of a fortuitous recruitment for that more general purpose. Alternatively, a compelling case can be made that the natural substrates are, in fact, xenobiotic molecules or their metabolites. This particular view has been argued most eloquently by Jakoby (28, 29). Perhaps the most convincing evidence for this point of view is not enzymological but genetic. The fact that the expression of the genes encoding the cytosolic GSH transferases is regulated by xenobiotic signals (9, 11) strongly suggests that the enzymes' primary role is to respond to chemical insult.

The GSH transferases are active in the metabolism and detoxication of many carcinogens. Many cancer chemotherapeutic compounds are alkylating agents that are mutagenic or carcinogenic. Thus the GSH transferases, which help protect normal cells from alkylating agents, may also, in principle, contribute to the resistance of neoplastic tissue to chemotherapeutic agents. The enzymes are, therefore, potential targets for the development of specific inhibitors that may be useful as adjuvant chemotherapeutics. The issue of drug resistance and possible strategies for its prevention has been reviewed (30). The reader also interested in the very complex issues of acquired drug resistance and the possible participation of GSH

transferases is referred to the work by Cowan, Hayes, Tew, Wolf and others, which can be found in reference 31. More recent leading references on this topic can be found in Rushmore and Pickett (11).

The central issues with respect to the structural and mechanistic enzymology of the GSH transferases fall into three categories. The first is an issue common to all GSH transferases. How do the enzymes activate (or destabilize) the thiol group of GSH for nucleophilic attack? The second issue concerns the relationship between the structure of an isoenzyme and its substrate specificity. That is, what specific catalytic devices are used by particular isoenzymes to stabilize the transition states of a specific reaction type? Finally, the relationships between gene structures, the three-dimensional protein structures, and the catalytic properties of the enzymes are important for an eventual understanding of the evolution of their catalytic diversity. This article will address the progress made over the last several years in the understanding of these three principal issues.

III. Three-Dimensional Structures

A. STRUCTURE SOLUTIONS

The three-dimensional structures of a number of GSH transferases in complex with substrate (GSH), substrate analogues, or products (GSR) have been recently determined by X-ray crystallography. The size of the subunit polypeptides and the dimeric nature of the holoenzymes has thus far inhibited the use of multidimensional NMR (Nuclear Magnetic Resonance) techniques for detailed structural studies. The first diffraction-quality single crystals of a GSH transferase were described in 1987 for the *mu* class isoenzyme 3-3 from rat (32). Crystals of a number of other isoenzymes from different gene classes were subsequently reported (33–36). High-resolution three-dimensional structures of isoenzymes representing three of the principal gene classes of cytosolic GSH transferases have been solved by X-ray crystallography in the last three years. A summary of the structures that have been solved and fully refined and that are known to the author is presented in Table 1. No three-dimensional structures have been reported for a class *theta* enzyme or either of the microsomal proteins. The first solution of the three-dimensional structure of a GSH transferase was reported in 1991 (37) for a class

TABLE I
Three-dimensional Structures of GSH Transferases

Enzyme Class	Isoenzyme	In Complex With	Resolution Å	R-factor	Reference	PDB File No.
alpha	human, A1-1	S-benzylGSH	2.6	0.229	39,41	1GUH
mu	rat, 3-3	GSH	2.2	0.171	38,42	1GST
	rat, 3-3	(9S,10S)GSPhen[b]	1.8	0.160	43–45	2GST
	rat, 3-3	(9R,10R)GSPhen[b]	1.9	0.159	44,45	3GST
	rat, 3-3	GSDNB[c]	2.0	0.194	46	5GST
	rat, 3-3	GSTNB[d]	1.9	0.178	46	4GST
	rat, 3-3(Y6F)	(9S,10S)GSPhen[b]	2.3	0.174	47	—[a]
pi	porcine, *pi*	GSO_3^-[e]	2.3	0.241	37	—[a]
	human, P1-1	S-hexylGSH	2.8	0.196	40	1GSS

[a] Coordinates for these structures have been either submitted to the Brookhaven Protein Data Bank and not yet assigned a file number or they have not yet been submitted.
[b] (9S,10S)- or (9R,10R)-9-(S-glutathionyl)-10-hydroxy-9,10-dihydrophenanthrene.
[c] 1-(S-glutathionyl)-2,4-dinitrobenzene.
[d] 1-(S-glutathionyl)-2,4,6-trinitrocyclohexadienate anion.
[e] glutathione sulfonate.

pi isoenzyme from porcine lung in complex with the GSH analogue glutathione sulfonate (GSO_3^-). The first structure of a GSH transferase in complex with the physiologic substrate, GSH and was solved independently using the class *mu* isoenzyme 3-3 from rat (38). Both of these structures were solved using multiple isomorphous replacement techniques to obtain initial phase information. The solution of the first class *alpha* structure was recently completed by Jones and coworkers using molecular replacement with search fragments derived from the *pi* and *mu* structures (39).

B. QUATERNARY, TERTIARY, AND DOMAIN STRUCTURE OF A CLASS *Mu* ISOENZYME

The three-dimensional structure of the class *mu* isoenzyme 3-3 from rat liver in complex with GSH has been described in detail (38). The type 3 subunit, the structure of which is illustrated in Figure 1, is composed of two domains: domain I (residues 1–82) located in the N-terminal third of the polypeptide and domain II (residues 90–217) comprising the C-terminal two-thirds of the protein. The two domains are conjoined by a seven-residue section (residues 83–89). The tertiary structure of domain I contains a four-strand mixed β-

Figure 1. Stereoview of the α-carbon backbone of one subunit of isoenzyme 3-3 in complex with GSH. Domain I is shown in the light line. Domain II is illustrated with the bold line. The link between the two domains is shown as a dashed line. A stick representation of GSH hydrogen bonded to the hydroxyl group tyrosine 6 is shown in bold.

sheet as part of a βαβαββα structural motif. The tertiary structure of domain II contains a series of five α-helical segments and terminates in a meandering C-terminal tail. It is readily apparent from Figure 1 that most of the interactions of the polypeptide with the substrate GSH are located in domain I. Thus, domain I can be considered a GSH-binding domain. The specifics of the protein-substrate interactions will be considered below.

Two views of the structure of the dimeric holoenzyme are illustrated in Figure 2. The two subunits in the dimer contact one another primarily by interactions between domain I of one subunit and domain II of the adjacent subunit (Figure 2a). A deceptively open channel runs through the dimer and is coincident with the twofold axis relating the two subunits. Each subunit contains two long helical segments α4 and α5 that constitute the walls of the central channel. The long, crescent-shaped α5 helix is subdivided into two sections (a and b) by virtue of a proline residue at position 131. Contacts between the two subunits involve both favorable electrostatic (salt bridges and hydrogen bonds) and hydrophobic interactions. The burial of solvent-accessible surface upon dimerization of the two subunits is a relatively modest 13%. About 15% of the total hydrophobic surface is buried on dimerization. One particularly obvious hydrophobic contact between domain I of subunit A and domain II of subunit B is the intercalation of the side-chain of phenylanine 56 (domain I) of one subunit between the two helical segments (α4 and α5b) located in domain II of the other subunit where it is wedged between leucine 136, tyrosine 137, phenylalanine 140, isoleucine 98, and glutamine 102.

The active site is located at the bottom of a 19-Å-deep channel, which is defined by the so-called "*mu* loop" of domain I (residues 33–42), the α4/α5 helix-turn-helix, and the C-terminal tail. Residues in the three principal structural elements that form the channel have higher than average crystallographic temperature factors, suggesting that they may be relatively mobile so as to facilitate the approach of substrates to and the egress of products from the active site. Access to the active site is likely to be via the top of the central crevice between the two subunits (Figure 2b). The two active sites in the dimer are located about 20 Å apart as defined by the distance between the two sulfur atoms of GSH (Figure 2a). This relatively large distance is consistent with previous observations that the two active sites are independent structurally and kinetically.

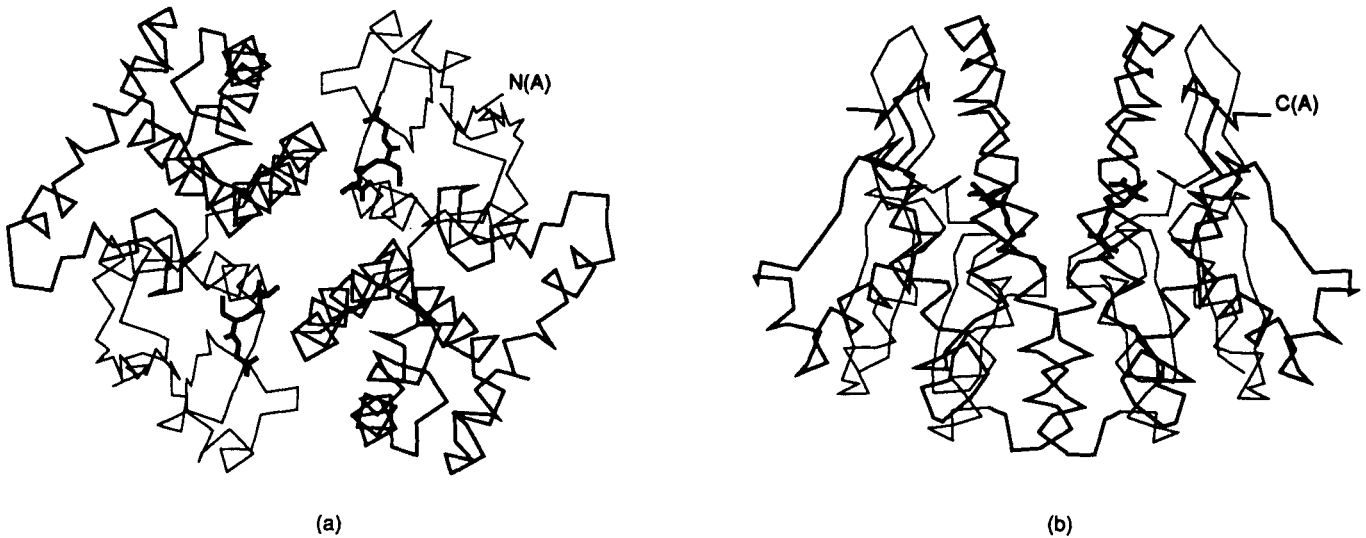


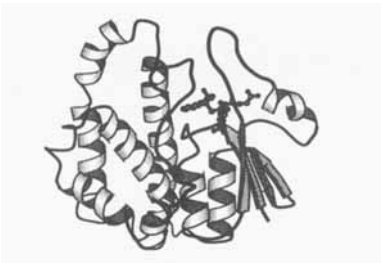
Figure 2. (a) View of the dimer of isoenzyme 3-3 down the noncrystallographic twofold axis relating the two subunits. (b) View of the dimer perpendicular to the twofold axis. Domains I and II are illustrated in the light and bold lines, respectively. A stick representation of GSH is also shown in bold.

C. STRUCTURAL COMPARISON OF THE *Alpha*, *Mu*, AND *Pi* ENZYMES

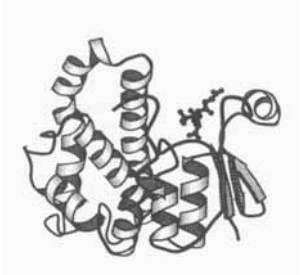
The three-dimensional structures of the class *alpha*, *mu*, and *pi* enzymes are very similar, particularly with respect to the domain structure and overall folding topology. However, the structures differ significantly in detail. A structural comparison of the *mu* and *pi* enzymes has been presented previously (38), and a more detailed comparison of all three enzymes has also been made by Jones and coworkers (39). The ribbon diagrams presented in Figure 3 illustrate the major differences in the three structures. Interestingly, the principal differences occur in regions that either define the active site or the approach to the active site. A unique structural feature of the class *mu* enzyme is the "*mu* loop" located in domain I, which forms one wall of the approach to the active site of isoenzyme 3-3. The loop is formed from a seven- or eight-residue sequence not found in the *alpha* or *pi* enzymes, as shown in Figure 4. The structures of the C-terminal sequences of the three enzyme classes are quite different beyond the α8 helix which ends at approximately residue 197 (Figure 4). The C-terminal segment of the class *alpha* enzyme is slighly longer than *mu* and *pi*. In contrast to the *mu* and *pi* structures where the C-terminal tail meanders across the active site, the *alpha* structure has an additional α-helix (α9) that snuggles down on top of the active site (Figure 3) and forms part of the xenobiotic substrate binding site. This structural element appears to block the approach to or act as a lid on the active site.

The overall topological similarity of the three enzymes is obvious from Figure 3. However, RMS deviations on α-carbon alignments of the dimers are considerable (ca. 2 Å) due to slightly different relationships between domains in the monomers and subunits in the dimers (39). Alignments of individual domains are much more precise, with RMS deviations on α-carbons of 1.3–1.5 Å for domains I and 1.2–1.7 Å for domains II. The two domains of the *mu* and *pi* enzymes appear to be more closely related to each other than either is to *alpha*. The interactions responsible for holding the subunits together in the dimer are, at first glance, quite similar from one enzyme class to the next. The slight differences in the spatial relationships between the two domains in the monomers may be the primary reason why intergene-class heterodimers are not observed. Structural comparisons of this type need to be examined in further detail.

ALPHA



MU



PI

Figure 3. Comparison of the three-dimensional structures of class *alpha*, *mu*, and *pi* isoenzymes. Ribbon diagrams were created from the coordinates of the 2.6-Å structure of class *alpha* human A1-1 isoenzyme in complex with S-benzylglutathione (39), the 1.8-Å structure of rat class *mu* isoenzyme 3-3 in complex with (9S,10S)-9-(S-glutathionyl)-10-hydroxy-9,10-dihydrophenanthrene (43–45), and the 2.0-Å-resolution structure of pig class *pi* isoenzyme in complex with glutathione sulfonate (37) using the program MOLSCRIPT (48).

```
                                                                  <----mu loop--->
α 1   A E K P K L H Y F N A R G R M E S T R W L L A A A G V E F E E K F I K S - - - - - - -
μ 1     P M I L G Y W N V R G L T H P I R L L L E Y T D S S Y E E K R Y A M G D A P D Y D
π 1   P P Y T I T Y F P V R G R C E A M R M L L A D Q D Q S W K E E V V T M - - - - - - -
        <-----β1---->           <------α1------->       <----β2--->

α 37  A E D L D K L R N D G Y L M F Q Q V P M V E I D G M K L V Q T R A I L N Y I A S K Y N
μ 42  R S Q W L N E K F K L G L D F P N L P Y L I D G S R K I T Q S N A I M R Y L A R K H H
π 36  - E T W P P L K P S - - C L F R Q L P K F Q D G D L T L Y Q S N A I L R H L G R S F G
       <--α2->                          <--β3->    <--β4->  <--------α3--------->

α 80  L Y G K D I K E R A L I D M Y I E G I A D L G E M I L L L P V C P P E E K D A K L A L
μ 85  L C G E T E E E R I R A D I V E N Q V M D N R M Q L I M L C Y N P D - - F E K Q K P E
π 76  L Y G K D Q K E A A L V D M V N D G V E D L R C K Y A T L I Y T N - - - Y E A G K E K
            <----------------------α4---------------------->          <--------α5a-

α 123 I K E K I K N R Y F P A F E K V L K S H G - - Q D Y L V G N K L S R A D I H L V E L L
μ 126 F L K T I P E - K M K L Y S E F L G - - K - - R P W F A G D K V T Y V D F L A L D I L
π 116 Y V K E L P E - H L K P F E T L L S Q N Q G G Q A F V V G S Q I S F A D Y N L L D L L
      -->    <----------α5b---------->                              <------------α6---

α 164 Y Y V E E L D S S L I S S F P L L K A L K T R I S N L P T V K K F L Q P G S P R K P P
μ 164 D Q Y H I F E P K C L D A F P N L K D F L A R F E G L K K I S A Y M K C S R Y L S T P
π 158 R Y H Q V L N P S C L D A F P L L S A Y V A R L S A R P K I K A F L A S P E H V N R P
      ---------->              <---------α7---------->   <-----α8---->
            <------α9------>
α 207 M D E K S L E E A R K I F R F
μ 207 I F S K L A Q W S N K
π 201 I N G N G K Q
```

It has been pointed out by Reinemer et al. (37) and Sinning et al. (39) that domain I of the GSH transferases has a fold similar to that of thioredoxin, glutaredoxin, and glutathione peroxidase. Even though the GSH binding sites of the later two proteins have not been unequivocally determined, models suggest that GSH is probably bound in the same general region as described for the GSH transferases (39). Domains I of the GSH transferases therefore appear to resemble a canonical GSH binding domain that occurs in other GSH-dependent proteins. In contrast, the three-dimensional structure of domain II bears no striking resemblance to any other known protein. Huber and coworkers have pointed out that there is some similarity of the folding topology of domain II with that of human annexin V (37).

IV. Glutathione Binding and Catalysis

A. THE GSH BINDING SITE

Glutathione is bound in an extended conformation at one end of the four-strand β-sheet of domain I. The three-dimensional structure of the class *mu* enzyme in complex with GSH reveals that the peptide is anchored by 13 electrostatic interactions (Figure 5), such that the backbone of the peptide faces the crevice between the two subunits and the sulfur points to the subunit to which it is bound. The only potential hydrogen bonding group on the peptide that does not interact directly with the protein is the amide carbonyl oxygen of the γ-glutamyl residue. There are two unusual structural features of interaction between the peptide and the protein worthy of note. Both involve the α-amino group of the γ-glutamyl residue of GSH. They include an electrostatic interaction with the carboxylate of aspartate 105 of the opposite subunit and a hydrogen bond between OE1 of glutamine 71 and the α-amino group. The former residue is the only

Figure 4. Sequence alignments of the class *alpha*, *mu*, and *pi* enzymes whose structures are illustrated in Figure 3. The alignment is as described in Sinning et al. (39) and is based on the three-dimensional structures of the three proteins. Note that the sequence numbering of all enzymes in the figure are residue numbers of the protein product and not codon numbers of the gene sequence. The positions of the secondary structural elements of the class *mu* isoenzyme 3-3 are illustrated below the sequence alignment. Locations of the structural elements unique to the class *mu* (*mu* loop) and class *alpha* (helix α9) enzymes are shown in bold above the sequence alignment.

intersubunit electrostatic interaction involving GSH, while the latter residue displays unusual polypeptide torsional angles that lie outside the Ramachandran-allowed regions. The main-chain NH and the side-chain hydroxyl of the next residue, serine 72, contribute hydrogen bonds to the oxygens of the α-carboxylate of the γ-glutamyl residue. Serine 72 is located at the N-terminal end of the α3 helix so that the α-carboxylate of the γ-glutamyl group is on the axis of and within 4 Å of the end of the helix (Figure 6). The dipole of the α3 helix, therefore, appears to make a significant electrostatic contribution to the binding of GSH.

The primary interaction between the class *mu* enzyme and the sulfur of GSH is a relatively short (3.2-Å) hydrogen bond to the side-chain hydroxyl group of tyrosine 6. The sulfur atom lies in the plane of the aromatic ring of tyrosine 6 and is in van der Waals contact with CE1 of that residue as well as CD and CG2 from the side-chain of leucine 12. These two residues effectively shield one hemisphere of the sulfur from contact with solvent. The remainder of the coordination sphere of the sulfur is occupied by one or two water molecules. The role of these interactions in catalysis are discussed in more detail below.

The GSH binding sites of the class *alpha*, *mu*, and *pi* enzymes display considerable similarity particularly with respect to some of the more unusual features of the site. Of the 8 to 10 residues that are involved in electrostatic interactions between each enzyme and GSH, 3 are identical and 6 are conserved in sequence and in function in all three structures (Table 2). Among the most interesting conserved interactions are those between the glutamine residue (Q66, Q71, or Q62) which has unusual torsional angles, the adjacent serine or threonine residue (T67, S72, or S63), and the aspartate from the opposite subunit (D100[B], D105[B], or D96[B]) in the *alpha*, *mu*, and *pi* enzymes, respectively. Of especial interest is the conserved interaction with the active site tyrosine. Jones and coworkers (39, 41) have also pointed out that the conserved glycine five residues to the C-terminal side of the active site tyrosine are crucial to maintain an unusual conformation of that residue which positions the peptide carbonyl oxygen away from the hydroxyl group of the tyrosine. A more usual (nonglycine) peptide conformation would result in a hydrogen bond between the peptide carbonyl oxygen and the hydroxyl group of the tyrosine, an interaction potentially deleterious to catalysis.

TABLE 2
Comparison of GSH Binding Sites of the Class *alpha*, *mu*, and *pi* Enzymes

Participating Residues in Class			Interaction with GSH
alpha	*mu*	*pi*	
Y8-OH	Y6-OH	Y7-OH	Cys-SG
	W7-NE1		Cys-O
R14-NE			Cys-SG
		R13-NH2	γ-Glu-OA1
	R42-NH2		Gly-O
	W45-NE1	W38-NE1	Gly-O
R44-NH2	K49-NZ	K42-NZ	Gly-OXT
	N58-OD		Gly-N, Cys-N
		Q49-NE	γ-Glu-O
V54-O, N	L59-O	L50-O, N	Cys-N, O
Q66-OE	Q71-OE	Q62-OE	γ-Glu-N
T67-OG, N	S72-OG, N	S63-OG, N	γ-Glu-OA1, OA2
D100(B)-OD1, OD2	D105(B)-OD1, OD2	D96(B)-OD1, OD2	γ-Glu-N
R130(B)-NH1, NH2			Gly-O, OXT

Residues with equivalent positions both in sequence and function are shown on the same line. Sequence numbering for all enzymes is derived from the protein sequence.

There are a number of notable differences between the GSH binding sites in the three structures (Table 2). These are particularly prominent in the structure of the class *alpha* enzyme where there is an additional, catalytically important electrostatic interaction between the sulfur of GSH and the side-chain of arginine 14 (39, 41, 50). The equivalent residue in the *pi* enzyme (R13) interacts with the carboxylate of the γ-Glu residue of GSH (37, 40), while the corresponding residue in the *mu* structure is a leucine (L12, Figure 4), which makes van der Waals contact with the sulfur of GSH (38). Furthermore, a second side-chain from domain II (R130) of the *alpha* enzyme appears to participate in the binding of GSH. This residue, located in the middle of the α5 helix, is not present in the *mu* and *pi* structures (Figure 4). A unique feature of the class *mu* GSH binding site is the participation of the indolyl nitrogen of tryptophan 7 in a hydrogen bond with the cysteinyl carbonyl oxygen of GSH (Figure 5).

Preliminary site-specific mutagenesis experiments have provided confirmatory evidence for the involvement of a number of protein side-chains in the GSH binding sites that are revealed in the crystal structures (51–54). However, with the exception of the active-site

tyrosine residue, no serious quantitative analysis of the contribution of the various interactions to the binding energy or catalysis has been carried out. This remains a fertile area for investigation.

B. ROLE OF THE CONSERVED TYROSINE IN CATALYSIS

Several years ago, evidence began to accumulate that GSH was bound at the active site of GSH transferase as the thiolate anion (e.g., GS^-). The pH dependence of k_{cat}/K_m^{CDNB} at saturating concentrations of GSH (Figure 7) suggested that the pK_a for the ionization of enzyme-bound GSH ($E{\cdot}GSH \rightleftharpoons E{\cdot}GS^- + H^+$) was in the neighborhood of 6.2 to 6.7, a value considerably less than the pK_a of 9.0 for GSH in aqueous solution (55). Perhaps more convincing was the spectroscopic observation of the thiolate anion in the active sites of binary complexes of isoenzymes 3-3 and 4-4 with GSH at neutral pH (42, 56). Moreover, the enzyme was shown to bind anionic glutathione analogues more tightly than GSH, suggesting that the preferential ionization state of the substrate on the enzyme surface is the thiolate (56).

The nature of the electrostatic field that stabilizes the thiolate (or destabilizes the thiol) in the active site is one of the central issues concerning the catalytic mechanism of the enzyme. Initial sugges-

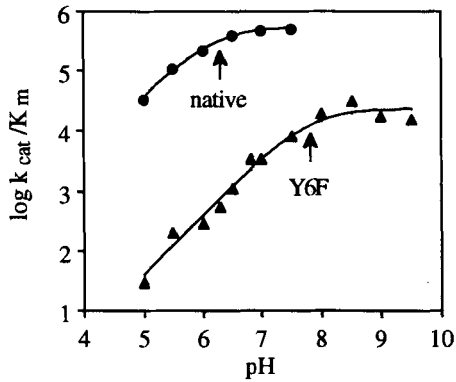


Figure 7. Dependence of k_{cat}/K_m^{CDNB} on pH for native isoenzyme 3-3 (●) and the Y6F mutant (▲). Apparent pK_as for E·GSH and E^{Y6F}·GSH are 6.2 and 7.8, respectively.

tions by several laboratories that a histidine might be involved in the catalytic mechanism, perhaps in a manner analogous to the active site of papain, proved incorrect. Site-specific mutagenesis of the histidine residues of both class *alpha* and *mu* enzymes has provided definitive evidence that a histidine residue was not involved in catalysis (57, 58). However, one histidine may serve an important structural function. For example, histidine 14 of the class *mu* isoenzyme is a conserved, buried residue located near the active site at the N-terminal end of the α1 helix (38). As a consequence, this residue exhibits an anomalously low pK_a (58). Conservative replacement of histidine 14 does not seriously impair catalysis (58, 59). However, not surprisingly, less conservative substitutions appear to cause a rather substantial destabilization of the protein structure (59). There seems to be a structural but not a catalytic imperative for the conservation of this residue in the class *mu* isoenzymes.

It remained for the solution of the three-dimensional structure of a GSH transferase to provide insight into the nature of the active-site residues involved in catalysis. Huber and coworkers were the first to provide evidence that the conserved tyrosine located near the N-terminal of the protein was probably involved in catalysis (37). The hydroxyl group of tyrosine 7 in the class *pi* enzyme from pig was found to be hydrogen bonded to one of the sulfonate oxygens of glutathione sulfonate (GSO_3^-) bound at the active site. The structure of the class *mu* isoenzyme 3-3 from rat, in complex with GSH, provides further evidence that the tyrosine is involved in catalysis. The hydroxyl group of the active-site tyrosine (Y6) is directly hydrogen bonded (3.2 Å) to the sulfur of the bound peptide (38, 42). The mechanistic implication of this structural observation, taken in the context that GSH is bound to the enzyme as the thiolate (42, 55, 56), is that the hydroxyl group of tyrosine 6 donates a hydrogen bond to stabilize the thiolate anion (eg. Y6—OH---$^-$SG).

Mechanistic analysis of the Y6F mutant of isoenzyme 3-3 supports this point of view. Removal of the hydroxyl group causes a loss of the difference absorption band at 240 nm associated with the thiolate anion of bound GS^- and results in a shift of the pK_a of bound GSH from 6.2 in the native E·GSH complex to about 7.8 in the mutant as illustrated in Figure 7 (42). This shift in pK_a corresponds to a stabilization energy of -2.2 kcal/mol provided to the thiolate by the hydroxyl group of tyrosine 6. *Ab initio* calculations on a model (the

$CH_3C_6H_4O$—H---$^-SCH_3$ dimer) of this interaction suggests that the stabilization energy that can be realized in the gas phase from such a hydrogen bond is almost −20 kcal/mol (60). The electrostatic shielding by the protein and the dielectric of the active site would obviously decrease the actual stabilization by a considerable amount. The contribution of the hydroxyl group of the conserved tyrosine residue of *alpha* and *pi* class isoenzymes has also been investigated with similar results (51, 53, 61). Moreover, preliminary indications suggest that the tyrosine of the class *alpha* enzyme may have somewhat less of an influence on catalysis than seen in the *mu* and *pi* enzymes due to the presence of the additional electrostatic interaction with arginine 14 (50, 62).

The pH-rate profiles of k_{cat}/K_m^{GSH}, which reflect ionizations of groups on the uncomplexed enzyme and $GSH_{(aq)}$, can, in principle, reveal information about the ionization of the tyrosine or other residues in the active site of the enzyme that participate in catalysis. Data from pH-rate profiles obtained in this manner have been interpreted to suggest that the tyrosine must be protonated to be active in catalysis (51, 63). Since it is not possible to assign the position of a proton based solely on the magnitude of an equilibrium constant (pK_a), it remains possible that, in the free enzyme, the tyrosine is partially ionized, whereas in the E·GS$^-$ complex it is protonated. In fact, the data reported in these two studies (51, 63) could also be interpreted to suggest that the pK_a of the hydroxyl group in the free enzyme is significantly lower than that of a normal tyrosine, perhaps as low as 7. That is to say, the ascending limbs of the pH vs. k_{cat}/K_m^{GSH} profiles may reflect the pK_a of the tyrosine hydroxyl group in the free enzyme. This is not unreasonable since the hydroxyl group of the tyrosine is expected to experience an electrostatic field in the active site similar to that of the sulfur of bound GSH. It should also be remembered that sulfur of $GSH_{(aq)}$ is predominantly protonated at neutral pH, so there is no obvious penalty to pay in binding to an active site with an ionized tyrosine as illustrated in equation 2. Such a scenario would be consistent with the early report by Jakoby that there is no detectable release of protons upon binding of GSH to the enzyme (4). This point bears further investigation by spectroscopic techniques capable of directly determining the pK_a of the active-site tyrosine in the uncomplexed enzymes.

$$E—Y^6—O^- + GSH_{(aq)} \rightleftharpoons E—Y^6—O—H---^-SG \qquad (2)$$

C. OTHER ELECTROSTATIC AND SOLVATION EFFECTS IN CATALYSIS

Normal Brønsted behavior of a thiolate nucleophile would predict that increasing the basicity of the anion would lead to higher reactivity of the anion in reactions where $\beta_{nuc} > 0$. A comparison of the limiting values of k_{cat}/K_m^{CDNB} at high pH (Figure 7) suggests that the reactivity of the more basic thiolate ($E^{Y6F}\cdot GS^-$, $pK_a = 7.8$) is actually less than that of the native enzyme ($pK_a = 6.2$). Thus the effect of the hydroxyl group of tyrosine is more complex than just lowering the pK_a of GSH. The crystal structure of the Y6F mutant is virtually identical to that of the native enzyme (38, 47), suggesting that the anomalous Brønsted behavior is not due to a conformational change in the active site of the mutant. The edge of the tyrosine ring, along with the side-chain of leucine 12, effectively shields one hemisphere of the sulfur of GS^- from solvent. Desolvation of the nucleophile would be expected to increase its reactivity and is probably an important consideration in catalysis. Perhaps the hydroxyl group of tyrosine 6 acts as a surrogate solvent molecule that is replaced by a real one in the mutant. Kinetic solvent isotope effects have been interpreted to suggest that a change in solvation of the thiolate occurs in proceeding from E·GS- to the transition state for nucleophilic aromatic substitution reactions (64).

The apparent pK_a of GSH in complex with the Y6F mutant of isoenzyme 3-3 remains significantly lower than that of $GSH_{(aq)}$—7.8 as compared to 9.0 (42). This fact alone suggests that other electrostatic features of the active site may promote the ionization of bound GSH. The possibility that dipoles from secondary structural elements such as α-helices contribute to the electrostatics has been considered (55). The structural evidence suggests that there may be some contribution from the α1 helix, the N-terminal end of which points toward the sulfur of GSH and the hydroxyl group of tyrosine 6 (37, 38, 42) as illustrated in Figure 6. However, in the class *mu* structure, the sulfur of GSH is located about 8 Å from the end of the α1 helix and off the helix axis so that the magnitude of the effect of the helix dipole is not clear and needs further investigation.

The accumulation of numerous weak electrostatic interactions probably contributes significantly to the stabilization of the thiolate anion in the active site. For example, the sulfur of GS^- in the crystal structure of the $E\cdot GS^-$ complex lies in the plane of the aromatic ring, 3.9 Å from the ring edge as illustrated in Figure 8. This represents an

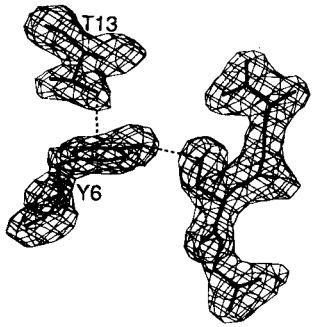


Figure 8. Electron density contoured at 1 σ for GSH, tyrosine 6, and threonine 13 from the 2.2-Å resolution structure of isoenzyme 3-3 in complex with GSH. The hydrogen bond between the hydroxyl group of tyrosine 6 and the sulfur of GS^- and the on-face hydrogen bond between the hydroxyl group of threonine 13 and the π-electron cloud of tyrosine 6 are shown as dashed lines.

electrostatically favorable interaction between the thiolate and the electropositive edge of the quadrapole of the aromatic ring (65). This interaction itself could be worth as much as -1 kcal/mol toward the stabilization of the thiolate.

More remote, second-sphere electrostatic interactions have recently been demonstrated to affect catalysis. Close examination of the structure of the active site of isoenzyme 3-3 reveals another weakly polar interaction that favors the thiolate anion. There is an on-face hydrogen bond between the hydroxyl group of threonine 13 and the π-electron cloud of tyrosine 6 as shown in Figure 8. In principle, this hydrogen bond would be anticipated to decrease the proton affinity of the hydroxyl group of tyrosine 6 and make it a better hydrogen bond donor to the thiolate, further decreasing the pK_a of the conjugate acid. In practice, this does seem to be the case. Removal of the on-face hydrogen bond in either the T13V or T13A mutants increases the pK_a of E·GSH from 6.2 to 6.9 (60). The ΔpKa of 0.7 corresponds to a -1.0 kcal/mol stabilization of the thiolate by the on-face hydrogen bond between threonine 13 and the π-cloud of tyrosine 6. *Ab initio* calculations comparing the proton affinities of the model system of a hydrogen-bonded trimer of CH_3OH/*p*-cresol/CH_3S^- (e.g., CH_3O—H---π-$CH_3C_6H_4$—O—H---$^-SCH_3$) and the same system in which the CH_3OH is moved 100 Å away, avoiding the on-face hydrogen bond, suggest the effect should be $\leq$1.5 kcal/mol, in good agreement with the experiment. Although this particular electrostatic device is not conserved in the active sites of GSH transferases, it serves as an illustration of the subtile second-sphere effects that help govern the catalytic characteristics of these enzymes.

V. Xenobiotic Substrate Specificity and Mechanism

A. LOCATION OF THE XENOBIOTIC SUBSTRATE BINDING SITE

Several hints as to the location of the xenobiotic substrate binding site began to emerge before the crystal structures of the GSH transferases had been solved. In 1989 Boyer (66) provided evidence from photoaffinity-labeling experiments that two regions of the primary structure of class *alpha* enzymes were involved in the architecture of the substrate binding site. One region was located near the middle of the sequence (residues 91–110), and the other was near the C-terminal (residues 206–218). The construction and characterization of chimeric isoenzymes led to the conclusion that both the N- and C-terminals of the class *mu* isoenzymes were located sufficiently close to the active site to influence catalysis (67). Affinity labeling with the active-site-directed reagent S-(4-bromo-2,3-dioxobutyl)glutathione provided evidence that tyrosine 115 of rat *mu* class isoenzyme 4-4 and cysteine 111 of class *alpha* isoenzyme 1-1 were located near the active sites prior to the elucidation of the related crystal structures (68, 69). Although numerous other investigations involving chemical modification, affinity labeling and site-specific mutagenesis have been performed on the GSH transferases, unlike the ones mentioned above, few have actually provided useful structural information concerning the location or composition of the xenobiotic substrate binding site. Thus far, the size of the protein has limited the applicability of NMR spectroscopy to the elucidation of structural features of the cytosolic GSH transferases. However, Rule and coworkers have used paramagnetic line broadening of protein resonances by a nitroxide-labeled product analogue to identify some of the residues near the active site of a human class *mu* enzyme (70).

The X-ray crystal structures of GSH transferases, in complex with several alkylated derivatives of GSH (GSR), have been solved recently (Table 1). The structures of these product complexes are and will continue to be of enormous value in deducing the location and topology of the xenobiotic substrate binding site. The overall structures of class *alpha*, *mu*, and *pi* enzymes suggest that three common regions compose the binding site for the electrophilic substrate. These include the floor of the active site provided by the loop that connects the first β-strand to the α1 helix and two walls contributed by one face of the α4 helix and the C-terminal tail located

in domain II. The α9 helix, which is a unique feature of the class *alpha* enzyme, forms a lid on the electrophile binding site, as shown in Figure 3. All of these regions of polypeptide were implicated, by one means or another, as part of the active site before the solution of the three-dimensional structures. The three regions are highlighted in Figure 9 for the *mu* class isoenzyme.

B. BINDING-SITE STRUCTURE AND CATALYTIC SPECIFICITY

1. Structures of Product Complexes

One of the most interesting and important issues with respect to the structures of the xenobiotic substrate binding site is what they can tell us about the substrate specificities of the enzymes. For example, the class *mu* GSH transferases catalyze the addition of GSH to

Figure 9. Stereo representation of the three general regions of the class *mu* type 3 subunit that compose the electrophilic substrate binding site. The floor of the active site (residues 7–13) and the two walls (residues 100–115 of helix α4, and residues 202–213 of the C-terminal tail) are illustrated in bold. The product diastereomer (9R,10R)-GSPhen is also shown in bold. The remainder of the structure is shown in the light line.