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Insights into the Catalytic Mechanism of Glutathione S-Transferase: The Lesson from *Schistosoma haematobium*

Minireview

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Summary

Glutathione S-transferases (GSTs) are involved in detoxification of xenobiotic compounds and in the biosynthesis of important metabolites. All GSTs activate glutathione (GSH) to GS^-; in many GSTs, this is accomplished by a Tyr at H-bonding distance from the sulfur of GSH. The high-resolution structure of GST from *Schistosoma haematobium* revealed that the catalytic Tyr occupies two alternative positions, one external, involving a π-cation interaction with the conserved Arg21, and the other inside the GSH binding site. The interaction with Arg21 lowers the pK_a of the catalytic Tyr10, as required for catalysis. Examination of several other GST structures revealed the presence of an external pocket that may accommodate the catalytic Tyr, and suggested that the change in conformation and acidic properties of the catalytic Tyr may be shared by other GSTs. Arginine and two other residues of the external pocket constitute a conserved structural motif, clearly identified by sequence comparison.

Introduction

Glutathione S-transferases (GSTs) are promiscuous enzymes that catalyze several reactions with various substrates. Their essential function is detoxification of xenobiotic compounds that are conjugated to glutathione and then excreted; other functions, not associated with detoxification, include repair of macromolecules oxidized by reactive oxygen species, regeneration of S-thiolated proteins, and biosynthesis of physiologically important metabolites (Armstrong, 1997; Sheehan et al., 2001). GSTs are classified on the basis of their fold, their thermodynamic and kinetic properties, the nature of the residues involved in catalysis, and the specific reaction catalyzed (Sheehan et al., 2001; Wilce and Parker, 1994). In general, GSTs catalyze the nucleophilic attack of the activated thiolate of GSH to electrophilic substrates, and an important step of the catalytic cycle is the activation of GSH to GS^-. However, the precise mechanism of this reaction is still incompletely understood. In the GSTs belonging to the classes alpha, mu, pi, and sigma, glutathione activation proceeds via the interaction with a Tyr at H-bonding distance from the sulfur of GSH; in the enzymes from other classes, the catalytic residue is either Cys or Ser (Armstrong, 1997; Sheehan et al., 2001).

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Results and Discussion

The *Schistosoma haematobium* GST

Johnson et al. (2003) solved, at 1.65 Å resolution, the 3D structure of the GST from *Schistosoma haematobium* (Sh28GST), a sigma class enzyme active toward several classical substrates and also involved in the biosynthesis of prostaglandin D_2 (PGD_2) from prostaglandin H_2 (PGH_2) (Hervé et al., 2003). PGD_2 is of vital importance to the parasite, being involved in the inhibition of the host immune response and thereby penetration into the host's tissues.

Starting from analysis of this structure, a novel mechanism for GSH activation, which may be relevant to the search for new drugs against schistosomiasis and may possibly be extended to other GSTs, is presented. The structure of Sh28GST (Johnson et al., 2003) shows that the catalytic Tyr occupies two alternative positions, one pointing toward the catalytic site ($Tyr^{in}10$) and contacting the sulfur atom of GSH (as canonical for GSTs) and the other pointing outside ($Tyr^{out}10$) with the phenolic oxygen exposed to water (Figure 1). $Tyr^{in}10$ is stabilized by contacts ($\leq$4.6 Å) with 10 amino acid residues plus GSH, if present, and $Tyr^{out}10$ with 11 different residues, including a remarkable polar interaction with Arg21. The structure of Sh28GST incubated with an excess of GSH shows that the population of $Tyr^{in}10$ is somewhat increased, but never approaches 100%. Although the Tyr^{out} conformer has not been described, examination of other GST structures reveals the existence of a cavity in the position occupied by Tyr^{out} in Sh28GST (Table 1); in the published structures this cavity contains excess electron density, interpreted as water molecules. The cavity is delimited by the amino-terminal region of the polypeptide chain, which forms three β strands and an α helix containing Arg21; this helix packs against the last α helix and is relatively close to the carboxyl terminus.

To investigate whether the peculiar Tyr^{out} conformer seen in Sh28GST is uniquely due to the presence of tyrosine, we solved the structure of the site-directed mutant Tyr10-Phe (Y10F-Sh28GST) expressed in *Escherichia coli* (kindly provided by M. Hervé, J. Fontaine, and F. Trottein, Lille, France). The interaction between the aromatic ring of Phe10 and Arg21 is clearly evident in the 2.0 Å structure of the Y10F mutant, showing that the conformer $Phe^{out}10$ is substantially populated despite exposure of the nonpolar Phe to water. The GSH conjugation and the prostaglandin D_2 synthase activities of this mutant are of course very low to absent (M. Hervé et al., personal communication), confirming the crucial role of Tyr10 in catalysis.

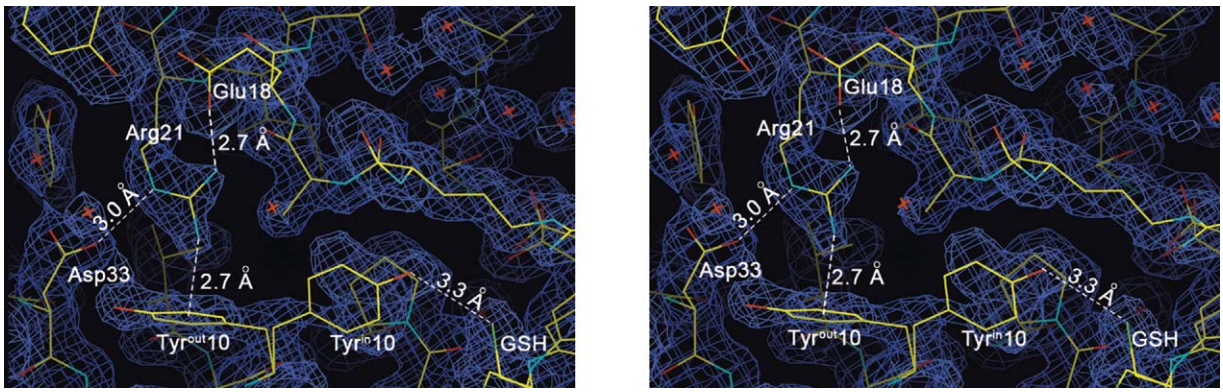

Figure 1. Stereo View of the Electron Density ($2F_o - F_c$ at 1σ; Blue Contours) Showing the Double Conformation of Tyr10 in Sh28GST

Tyr^{in}10 contacts the sulfur atom of GSH by H bonding (3.3 Å). Tyr^{out}10, exposed to water, is stabilized by the π-cation (2.7 Å) interaction with the conserved Arg21, which is held in the proper stereochemistry by Asp33 and Glu18.

A New Functional Role for π-Cation Interaction in Enzymes

Electrostatic interactions between electropositive groups and the electron-rich π-clouds of aromatic rings are not uncommon in proteins and are thought to contribute significantly to the stability of folded proteins (Gallivan and Dougherty, 1999). However, only in rare instances have such interactions been implicated in catalysis; for example, π-cation interactions were shown to be important in substrate and/or transition state recognition at the active site of some enzymes (Zacharias and Dougherty, 2002). Two different geometries are possible: the more common stacked arrangement and the T-shaped (or on-face H-bond) one. Ab initio calculations indicate that, in the gas phase, interaction of the guanidinium group with benzene is stronger in the T-shaped geometry ($\Delta G° = -10.6$ kcal/mol) with respect to the stacked configuration ($\Delta G° = -4$ kcal/mol) (Gallivan and Dougherty, 1999). However, in proteins and when the cation is an amino acid residue containing an sp^2-hybridized nitrogen atom, the stacked geometry is more frequent than the perpendicular H-bond geometry by a 25:1 ratio (Ma and Dougherty, 1997).

In Sh28GST, we observed that the out-conformer is populated not only in the wild-type but also in the Y10F site-directed mutant (see above). The geometry is always T-shaped due to a network of interactions of Arg21 (with Asp33 and Glu18) that forces the NH1 to be perpendicular to the aromatic ring of Tyr10 or Phe10 in the external configuration (Figure 1). In wild-type Sh28GST and in Y10F-Sh28GST, the distance between NH1 of Arg21 and the centroid of the aromatic ring is ~2.7 Å, consistent with a strong π-cation interaction between the NH1 and the π-electron cloud. Interaction with the positively charged Arg side chain is expected to favor ionization of Tyr by 2–3 kcal/mol, thus lowering its pK_a by at least 2 pH units.

In order to test the role of the double conformation of the catalytic Tyr in Sh28GST, the wild-type enzyme and its site-directed mutant were titrated spectroscopically in the pH range 6–11, in the absence of GSH (Figure 2). The UV difference spectrum is diagnostic of the ionization of the phenol ring of Tyr residues and has been compared with that of phenol. The wild-type enzyme displays a distinct transition around neutrality ($pK_a = 7.2$) whose amplitude is compatible with one Tyr residue per GST monomer, and a second one in the alkaline range ($pK_a \geq 9.6$). Y10F-Sh28GST displays a single spectroscopic transition ($pK_a \approx 9.6$). These experiments imply that the catalytic Tyr of Sh28GST can be confidently assigned a $pK_a = 7.2$ in the absence of GSH. Because this low pK_a should result from the weighted average of Tyr^{in} and Tyr^{out}, the pK_a of the latter must be somewhat lower, although we are unable

Table 1. Volume and Area of the Tyr^{out}10 Cavity in Sh28GST and the Homologous Cavities for Two Other GSTs

Protein (PDB Code)	N_mth[a]	Area[b]	Volume[c]	Reference
S. haematobium (1OE7)	1	100.16	86.48	Johnson et al. (2003)
H. sapiens GST P1-1 (16GS)	1	101.00	87.37	Oakley et al. (1998)
H. sapiens GST A1-1 (1GUH)	0	76.69	49.93	Sinning et al. (1993)
O. sloani (2GSQ)	2	110.41	68.34	Ji et al. (1996)
H. sapiens GST M2-2 (1HNA)	0	75.53	49.50	Raghunathan et al. (1994)

Volume and internal surface area of the Tyr^{out}10 cavity of Sh28GST and the homologous water-filled cavities of representative GSTs from various classes as calculated with the program Cast-p using a probe radius of 1.0 Å (Liang et al., 1998). The pockets are selected considering the presence of the NH_2 of the conserved Arg and the C-α and C-β of the catalytic Tyr.

[a] N_mth: The number of mouth openings for the pocket.

[b] Area: The molecular surface area of the cavity in Å^2.

[c] Volume: The volume enclosed in the molecular surface area of the cavity in Å^3. The volume of the pocket for Sh28GST (PDB code 1OE7) was calculated removing the Tyr^{out}10 conformer.

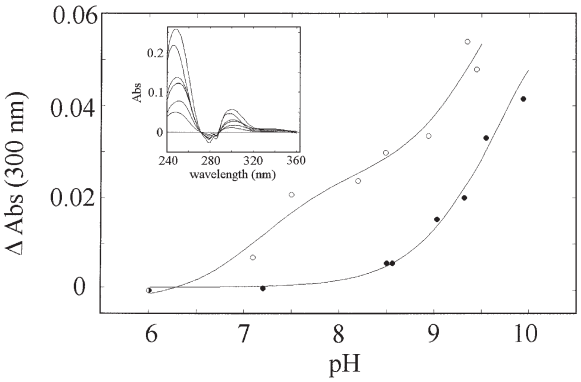

Figure 2. Spectroscopic Acid-Base Titration of Sh28GST and Its Site-Directed Mutant Y10F

Wild-type Sh28GST or Y10F mutant were diluted to a final concentration of 30 μM (per monomer) in a buffer solution containing 50 mM sodium phosphate plus 50 mM boric acid adjusted to the desired pH with NaOH. Absolute absorbance spectra were recorded on a Hewlett Packard 8453 spectrophotometer (Palo Alto, CA), and the difference spectra were calculated using the spectrum recorded at pH = 6.0 as a reference (see inset for the difference spectra of wild-type Sh28GST). The differential absorbance readings at 300 nm are plotted as a function of pH for wild-type Sh28GST (open circles) and the Y10F mutant (closed circles); lines are drawn according to the least squares fit of the experimental data to one (Y10F) or two (wild-type) transitions assigned to the ionizable groups with the pK_as reported in the text.

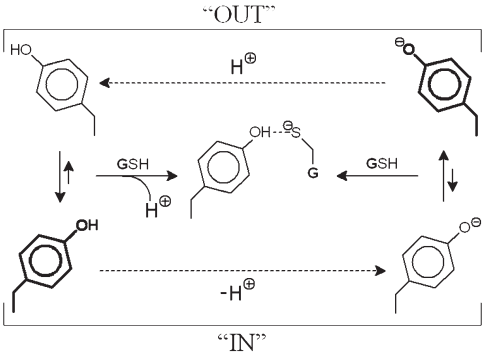

Figure 3. Dual Pathway for GSH Activation

The species in the center represents the activated GS^--enzyme complex. GSH can bind to both conformers of Tyr10 ("IN" and "OUT"), assumed to be in equilibrium. $Tyr^{in}10$ (in bold) is predominantly protonated and works as an H bond donor. $Tyr^{out}10$ (in bold) is mostly ionized and, when GSH binds, it swings in, working as a base. The preferential pathway is dictated by the apparent pK_a of the catalytic residue.

to provide an estimate. Interaction with Arg21 is suggested to be important in promoting the ionization of Tyr^{out}, but is not the only contact stabilizing this conformer.

The Mechanism of GSH Activation in Sh28GST and Its Possible Implications in the GST Superfamily

The active site Tyr of GSTs often has an unusually low pK_a (i.e., <8.5) in the absence of GSH (Ibarra et al., 2001, 2003; Dietze et al., 1996a) compared to that in solution (~10). Tentative explanations of this datum include the on-face H bond with Thr or other Tyr residues and the π-π interactions with neighboring Phe; however, these interactions only partially explain the pK_a shift (Xiao et al., 1996; Dietze et al., 1996b; Ibarra et al., 2001). In our case, the role of the π-cation interaction is expected to be similar to, but more efficient than, that of other interactions of the catalytic Tyr, as described for other GSTs. Additional evidence on the ionization state of the GST-GSH complex is as follows: (1) both the ionized and protonated states of the catalytic Tyr are detected (Dietze et al., 1996a; Ibarra et al., 2003); (2) the protonation of Tyr is dependent on the presence of GSH (Ibarra et al., 2001; Bjornestedt et al., 1995); (3) the pK_a of the bound GSH is ~6.8 (Graminsky et al., 1989) (pK_a in solution is ~9.8); and (4) the catalytic activity (k_{cat}/K_m) decreases when the pH is raised and thus the active form of GSH is unlikely to be deprotonated (Bjornestedt et al., 1995; Kolm et al., 1992).

Assuming that the two conformers are in equilibrium, we propose the hypothetical mechanism depicted in Figure 3. This suggests that Tyr10 switches alternatively toward the solvent where the π-cation bond with Arg21 lowers its pK_a and forces proton release, or toward the active site where the tyrosinate may extract the GSH proton, because of its higher pK_a. This mechanism, whereby Tyr10 works as a proton shuttle, changing its acidic properties through interaction with Arg21, to our knowledge has not been reported before.

This hypothesis may help to describe the binding mechanism of GSH in GSTs belonging to the alpha and pi classes, that is, whether GSH preferentially binds to the protonated or unprotonated GST (see Equations 2 and 3 of Armstrong, 1997). Indeed, if GSH binds with different rate constants to the Tyr^{in} and Tyr^{out} conformers of the enzyme, either path may be more prevalent than one would expect on the basis of the average pK_a of the catalytic residue. Even GSTs with much higher pK_a of the catalytic Tyr and a population of the Tyr^{out} conformer lower than in Sh28GST might still preferentially bind GSH through this conformer (Figure 3), in view of the greater accessibility of the binding site. This would mean that upon rapid mixing with substrate, only the protons bound to the fraction of enzyme presenting the protonated Tyr^{in} conformation would be released. The apparent inconsistency with the observation that proton release is synchronous with GSH binding and thiolate formation (Caccuri et al., 1999) is solved because those experiments were carried out at a pH lower than the pK_a of the catalytic Tyr, and thus even the Tyr^{out} conformation could have been partially protonated; moreover, the enzymes used may have a relatively low population of Tyr^{out}, in view of the relatively high pK_a of their catalytic Tyr.

A different matter is the mechanism of activation of bound GSH, which depends on the precise position of the unique proton shared by Tyr^{in} and glutathione, that is, whether the ES Michaelis complex is more akin to GS thiolate-tyrosine or to GSH-tyrosinate (Equations 4 and 5 of Armstrong, 1997). On the basis of the isotope effect in D_2O, Armstrong and coworkers (Armstrong,

1997; Parsons and Armstrong, 1996) reported that the GS thiolate-tyrosine-type Michaelis complex is prevalent in the rat mu class GST (whose catalytic Tyr has a pK_a of ~10), although the enzyme may switch to a GSH-tyrosinate-type complex if the catalytic Tyr is fluorinated (a modification that lowers the pK_a to 7.5); thus, the physiological mechanism for GSH deprotonation would be the former. Our data do not impinge directly on this point, which critically depends on the pK_a of the Tyr^{in} conformer. However, the reactions given in Figure 3 imply that this pK_a may be significantly higher than previously expected, given that the low overall pK_a of the catalytic Tyr is assigned to the weighted average of the two conformers. Thus, if anything, our scheme would suggest that GSH preferentially binds to the deprotonated Tyr^{out} conformer, but the only proton of the ES complex is completely transferred to the Tyr^{in}, consistent with isotope effects experiments. The main reaction path would be:

$$E\_Tyr^{out}\text{-}O^- + GSH \rightarrow E\_Tyr^{out}\text{-}O^- \ldots HSG \rightarrow E\_Tyr^{in}\text{-}O^- — HSG \rightarrow E\_Tyr^{in}\text{-}OH — {}^-SG.$$

The mechanism proposed in Figure 3 qualitatively accounts for the experimental data on alpha and pi class GSTs, and is consistent with the thermodynamics of the process, assuming that $Tyr^{in}10$ has the usual pK_a of 9–10, whereas $Tyr^{out}10$ has a pK_a close to 7. According to our scheme, the two conformers $Tyr^{in}10$ and $Tyr^{out}10$ are both populated at equilibrium, even in the absence of GSH; at physiological pH Tyr^{in} is protonated, whereas Tyr^{out} is significantly unprotonated. As far as our hypothesis can be generalized, the different pK_a values reported for the catalytic Tyr, ranging between pK_a = 10 for Mu1-1 GST from rat (Xiao et al., 1996) and pK_a = 6.7 for A4-4 GST from human (Hubatsch and Mannervik, 2001), would merely reflect the equilibrium population of the two conformers that strongly favors Tyr^{in} in the former case and Tyr^{out} in the latter.

In our hypothesis, GSH may combine with both conformers and favor the translocation of the catalytic Tyr to the active site. If GSH binds to an enzyme presenting the Tyr^{in} conformation, this residue is likely to be protonated and the complex will behave like a weak diprotic acid. In this case Tyr donates an H bond, favoring the release of the hydrogen ion from GSH. If, on the other hand, GSH binds to an enzyme presenting the Tyr^{out} conformer, it promotes the switch of the tyrosinate toward the catalytic site and extraction of the GSH proton. Because experimental evidence is consistent with both mechanisms, we conclude that they are both possible, and either one may appear to prevail depending on the fraction of Tyr occupying either of the two alternative positions. Our hypothesis does not contradict any of the relevant findings, and actually reconciles many of them; in particular: (1) it explains the low apparent pK_a of the catalytic Tyr, as well as that of bound GSH; (2) it allows both protonated and unprotonated Tyr to be catalytically competent; and (3) it is not inconsistent with the decrease of the catalytic activity with increasing pH because this would stabilize the Tyr^{out} conformer and would preclude one of the two possible reaction pathways.

Evolutionary Conservation of Arg and Its Interaction Network

Prompted by the hypothesis that the π-cation bond between $Tyr^{out}10$ and Arg21 is crucial, we further investigated the evolutionary conservation of the latter residue and its interaction network. Two hundred forty-three sequences of GSTs from different organisms are listed in the SwissProt database (Bairoch et al., 2005); because the sequence and the 3D structure of all GSTs are conserved, especially in the amino-terminal region (Sheehan et al., 2001), a comparison is justified. Of the 243 sequences, 95 are assigned to the classes alpha, mu, pi, and sigma, having Tyr as the catalytic residue (we call this group set #1); 83 to the classes beta, kappa, omega, phi, tau, theta, and zeta, having either Cys or Ser as the catalytic residue (set #2); and 65 are not assigned to any class (set #3). The analysis shows that 94% of the GSTs of set #1 (89 out of 95) present a characteristic structural motif constituted by the catalytic Tyr at position *n* followed by Glu, Gln, or His at *n + 8*, by Arg at *n + 11*, and by Asp or Glu at *n + 22* or *n + 23*, that is, ([Y] 7X [E,Q,H] 2X [R] 10-11X [E,D]). In the alpha class human GST, the functional relevance of the interaction between Arg19 and Glu31 (topologically homologous to Arg21 and Asp33 in Sh28GST) has been substantiated by mutagenesis (Lee et al., 1995), but has not been explained. The conservation of Arg at *n + 11* and its interaction network is specific for Tyr-containing GSTs, as demonstrated by the fact that only 6% of GSTs of set #2 (5 out of 83) present the extended structural motif ([Y,C,S] 7X [E,Q,H] 2X [R] 10-11X [E,D]).

The residues of the motif are identified by their putative functional and structural roles: that is, the catalytic Tyr, the Arg residue required to establish the π-cation bond, and the two residues that can interact with the Arg and stabilize its correct orientation. Their evolutionary conservation is an independent, though indirect, confirmation of their putative role. Because these four residues are located on two β strands and an α helix, their stereochemical proximity depends on the correct folding of the amino-terminal region of the protein; the short coil regions between these secondary structure elements might allow insertions or deletions between the Arg and the Glu or Asp. The structural organization of these residues is strongly conserved, as evident from comparison of human alpha class GST A1-1 (Cameron et al., 1995; Protein Data Bank (PDB) code 1GSD), sigma class GST from squid (Ji et al., 1996; PDB code 2GSQ), and human pi class GSTP1-1 (Oakley et al., 1998; PDB codes 16GSA and 16GSB), all reported in Figure 4.

As an unbiased empirical control of the statistical frequency of the ([Y] 7X [E,Q,H] 2X [R] 10-11X [E,D]) motif identified in GSTs containing a Tyr in the active site, we searched the whole SwissProt database which lists 178,171 protein sequences. The motif was found in 3750 (2.1%) sequences, a value that does not seem unreasonably high, in view of the relative frequency of these amino acids (Klapper, 1977).

It is interesting to analyze the six Tyr-containing GSTs of set #1 that do not contain the ([Y] 7X [E,Q,H] 2X [R] 10-11X [E,D]) motif. Four of the exceptions have a substitution at *n + 8*: the mu class GSTs from rabbit (*Oryctolagus cuniculus*; SwissProt code P46409) and

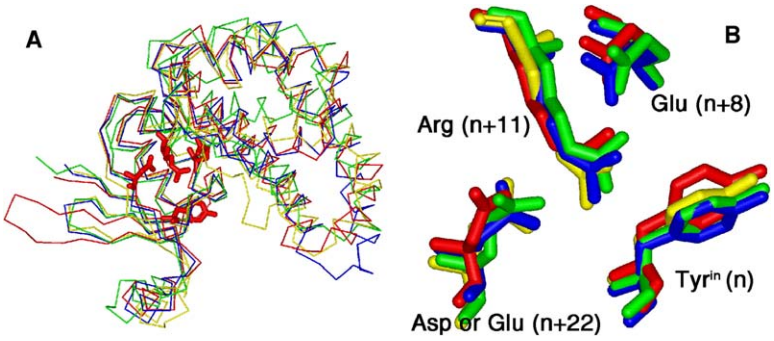

Figure 4. Comparison of the Structure of Representative GSTs

(A) Superimposition of the C-α traces of Sh28GST (red), α class GST A1-1 (green), sigma class GST (blue), and pi class GST P1-1 (yellow); PDB codes are 1OE7, 1GSD, 2GSQ, and 16GS, respectively. Tyr10 ($Tyr^{in}10$), Glu18, Arg21, and Asp33 in Sh28GST are highlighted in red.

(B) Details of the superimposition shown in (A). Arg is kept in place by interaction with two residues of the same polypeptide chain, ready to stabilize the possible Tyr^{out} conformer (see text).

Y1 from hamster (*Cricetulus longicaudatus*; SwissProt code Q00285) have Leu and Asn at *n + 8*, respectively, whereas human GSTP1-1 (SwissProt code P09211) and GSTP1 from macaque (*Macaca mulatta*; SwissProt code Q28114) both have Ala. All other residues of the motif are conserved. The last two GSTs have a full, but probably functionally silent, ([Y] 7X [E,Q,H] 2X [R] 10-11X [E,D]) motif at significant distance from the amino terminus.

The amino-terminal residues of the sigma class GST2 from *Manduca sexta* (SwissProt code P46429) could not be aligned satisfactorily with the other GSTs. Our best guess is that the catalytic residue is Tyr8 and, if this enzyme has the external pocket at all, it is organized around Arg16 (*n + 8*) and Glu27 (*n + 19*), respectively; this could be the consequence of a three-residue deletion between positions 8 and 16, also involving one of the residues that were expected to interact with Arg16. The sigma class GST2 from *Caenorhabditis elegans* (SwissProt code O16115) presents the extended ([Y,C,S] 7X [E,Q,H] 2X [R] 10-11X [E,D]) motif and aligns nicely with the other sequences, but has a Cys in the putative catalytic position instead of a Tyr; perhaps it is misclassified.

The sequence conservation of the amino-terminal region of Tyr-containing GSTs is more extended than described above, and several other residues appear to be conserved in the region between the amino terminus and the first 25 residues or more downstream of the catalytic Tyr; for example, the second residue before the catalytic Tyr (***n – 2***) is always large and nonpolar, and the Glu or Asp at *n + 22* or *n + 23* is very often followed by a second Glu or Asp. A search for the motif ([Y] 7X [E,Q,H] 2X [R] 9-12X [E,D][E,D]) in set #1 found 85 GSTs out of 95 and only 875 proteins in the whole SwissProt database (i.e., 0.5%). However, we do not have a clear-cut functional explanation for these other residues in the catalytic mechanism of Figure 3.

Conclusions

As far as we know, there is no other example of a protein in which a Tyr in a π-cation interaction with an sp^2 nitrogen of an amino acid changes its acidic properties with functional consequences. Only in artificial "receptors" has it been demonstrated that electron-rich phenolate could bind quaternary ammonium ions more tightly than neutral ones (Ma and Dougherty, 1997). Sh28GST may be the first example of a new role for the π-cation interactions, although Nurizzo et al. (2001) noticed that a similar motif may be of significance in other proteins.

Although we cannot extend our mechanism to all Tyr-containing GSTs, we suggest that it may at least be considered when the three following conditions apply: (1) the pK_a of the catalytic Tyr is lower than the usual value of Tyr residues by more than 1 pH unit; (2) a water-filled external pocket is present; and (3) the pocket hosts Arg ***n*** **+ 11** and its interaction network. Conditions (2) and (3) can be easily verified by computer modeling if the 3D structure of the enzyme is available.

The in and out movement of Tyr10 may be relevant to the search for GST inhibitors. This is intriguing because the GSTs are detoxifying enzymes characterized by promiscuous substrate specificity, which makes it difficult to design class-specific drugs. If the swing off of the active site Tyr is involved in the detoxifying activity, as we propose, it may be profitable to orient drug search toward the pocket in which the Tyr is held by the interaction with the conserved Arg. On the other hand, if this mechanism were unique to Sh28GST, it may be a specific clue to inhibit the schistosomial enzyme with possible therapeutic implications.

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