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On the Reaction Mechanism of Class Pi Glutathione S-Transferase

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ABSTRACT **Theoretical calculations were performed to examine the ionization of the phenolic group of Tyr7 and the thiol group of glutathione in aqueous solution and in the protein class-pi glutathione S-transferase (GST-Pi). Three model systems were considered for simulations in the protein environment: the free enzyme, the complex between glutathione and the enzyme, and the complex between 1-chloro-2.4-dinitrobenzene, glutathione, and the enzyme. The structures derived from Molecular Dynamics simulations were compared with the crystallographic data available for the complex between the inhibitor S-(p-nitrobenzyl)glutathione and GST-Pi, the glutathione-bound form of GST-Pi, and the free enzyme carboxymethylated in Cys47. Free-energy perturbation techniques were used to determine the thermodynamics quantities for ionization of the phenol and thiol groups. The functional implications of Tyr7 in the activation of the glutathione thiol group are discussed in the light of present results, which in agreement with previous studies suggest that Tyr7 in un-ionized form contributes to the catalytic process of glutathione S-transferase, the thiolate anion being stabilized by hydrogen bond with Tyr7 and by interactions with hydrating water molecules. Proteins 28:530–542, 1997** © 1997 Wiley-Liss, Inc.

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INTRODUCTION

Glutathione S-transferases (GSTs) are a widely distributed family of detoxication enzymes.[1] They catalyze the nucleophilic attack of the sulfur atom of glutathione on electrophilic groups of a variety of hydrophobic compounds. Five different classes of mammalian soluble GSTs (alpha, mu, pi, and theta) have been described, each one with different substrate specificity.[2–4] The structural characterization of representative enzymes of each class has revealed differences in the active sites,[5–13] even though they are believed to share a similar catalytic mechanism. The first step in the catalytic process is the conversion of the thiol group of glutathione to thiolate. The increased reactivity of the ionized thiol facilitates the attack to the electrophilic center of the hydrophobic substrate, leading to formation of a glutathione-lipophilic compound conjugate with increased solubility.[14] How glutathione is activated by the enzyme is still unclear. The three-dimensional structures of class pi, mu, and alpha GSTs complexed with different inhibitors show that the only residue in close proximity to the sulfur atom of glutathione is a tyrosine (Tyr7 in GST-Pi). Mutation of Tyr7, or of its equivalent in the alpha and mu classes, to phenylalanine results in a considerable reduction (although not complete elimination) of enzymatic activity.[15–19] Several mechanisms have been proposed for thiol activation. Tyr7 has been suggested to act as a general base in the form of tyrosinate accepting the proton released by the glutathione thiol.[20,21] Because there is no positively charged residue that could interact with Tyr7 to stabilize the reactive ionized hydroxyl, the electrostatic potential at the active site has been proposed to reduce the effective pK_a of the tyrosine. The helix dipole of the αA helix has also been suggested to have a relevant contribution in lowering pK_a.[22] Theoretical studies[23] have also indicated that the interaction of Thr13 with the aromatic ring of Tyr6 can stabilize the tyrosinate anion in class-mu GSTs.

The high-resolution structure of the S-(*p*-nitrobenzyl)glutathione pi-GST complex[9] provides detailed information of the active site including the exact location of the hydrophobic site and water structure about the active pocket. These water molecules were suggested to play a key role in the reaction mechanism of GST. Inspection of the active site revealed that this region of glutathione was quite well hy-

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drated, in contrast with Tyr7, which was poorly hydrated. In fact, three water molecules were located near the sulfur atom of glutathione, whereas no water molecule was found near Tyr7. The recent determination of the glutathione-GST-Pi complex (A. Párraga, I. García-Sáez, S. Walsh, T.J. Mantle, and M. Coll, in preparation) and of a chemically modified unliganded enzyme structure (C. Vega, S. Walsh, T.J. Mantle, and M. Coll, in preparation) has provided additional insights into the structure of the active site of GST and in particular into the hydration pattern of GST at different putative stages of the reaction.* Such information combined with state-of-the-art molecular dynamics and quantum mechanical ab initio calculations has been used to put forward a model of the reaction mechanism of p_i-class GSTs.

METHODS

Crystallographic Methods

The three-dimensional crystal structure of the binary complex between mouse liver GST-Pi and its substrate glutathione (GSH) has been determined by molecular replacement and refined to 2.4 Å resolution and a final *R* factor of 19.4%. The three-dimensional structure of the Cys47-carboxymethylated unliganded enzyme has been determined by molecular replacement and refined to 1.9 Å resolution and a final *R* factor of 19.5%. A detailed crystallographic analysis of both structures will be published elsewere.

Computational Methods

Building-up of the simulation systems

Thermodynamic quantities for the ionization of Tyr7 and glutathione in water and in the protein under physiologic conditions were determined using molecular dynamics coupled with free-energy perturbation theory (MD-FEP). The three-dimensional structure[9] of the enzyme complexed with S-(*p*-nitrobenzyl)glutathione at 1.8 Å resolution was used as starting structure for simulations of the protein.

Three model systems were considered in simulations for the protein environment: the free enzyme (GST), the complex between glutathione and the enzyme (GST-GSH), and the complex between 1-chloro-2,4-dinitrobenzene, glutathione, and the enzyme (GST-GSH-CDNB). All of them were modeled from the crystallographic structure,[9] which was neutralized by adding two chloride ions placed in regions of strong positive electrostatic potential (near Lys81 and Lys290). One of the chloride ions was removed in simulations of the GST-GSH and GST-GSH-CDNB systems to maintain charge neutrality. Most of the 151 crystallographic water molecules are located near charged groups, far away from the active site, but 34 are less than 14 Å from the sulfur atom of glutathione. These latter 34 water molecules were removed from the starting structure to avoid a biased description of the active site hydration. Subsequently, the model system was rehydrated by centering a sphere of 23 Å of TIP3P[24] water molecules about the sulfur atom of GSH or the oxygen atom of Tyr7. All water molecules whose oxygen atoms were less than 2.2 Å from any residue of the protein were removed. The final model systems (more than 7,000 atoms) consisted of the dimeric protein (418 residues) alone, or complexed with glutathione or with glutathione plus 1-chloro-2,4-dinitrobenzene, one or two chloride counterions, and 953 water molecules, which include 117 crystallographic water molecules.

The model systems were partitioned into a mobile and a rigid region. The former includes all the protein residues containing at least one atom within 14 Å of the hydroxyl group of Tyr7 or the sulfur atom of glutathione, as well as all the 836 noncrystallographic water molecules, whereas the rest of the atoms define the rigid part. Both mobile and rigid regions were allowed to relax in energy minimization calculations performed at the beginning of the simulation, but only the mobile region was free to move in molecular dynamics simulations.

Simulations in water were performed using phenol and ethanethiol as model compounds of tyrosine and glutathione. A similar protocol was used as for the protein models discussed above, which allows a direct comparison of the results. Thus, the solute was placed in a sphere of about 18 Å centered on the hydroxyl oxygen of phenol or the sulfur atom of ethanethiol. The final systems consisted of one solute and about 770 TIP3P water molecules. After initial energy minimization, molecular dynamics simulations were performed. No counterions or restrictions to the movement of atoms were necessary in these simulations.

Set-up of the simulation systems

Model systems for the protein were energy minimized in a sequential way. First, all the water (both TIP3P and crystallographic) molecules and counterions were optimized for 600 cycles of steepest descent, followed by 200 cycles of conjugate gradient. Subsequently, the whole (both mobile and rigid regions) system was relaxed for 500 cycles of conjugate gradient. The rigid part was fixed for the rest of the simulations, whereas the mobile part was further optimized for 2,000 cycles of conjugate gradient. These partially optimized structures were the starting systems for molecular dynamics simulations, which were performed considering the interactions in the whole system, but with atom movement only allowed in the mobile region.

Heating and equilibration of the systems were performed slowly and in a sequential way to avoid

*Coordinates for these structures have been deposited in the Brookhaven Protein Data Bank.

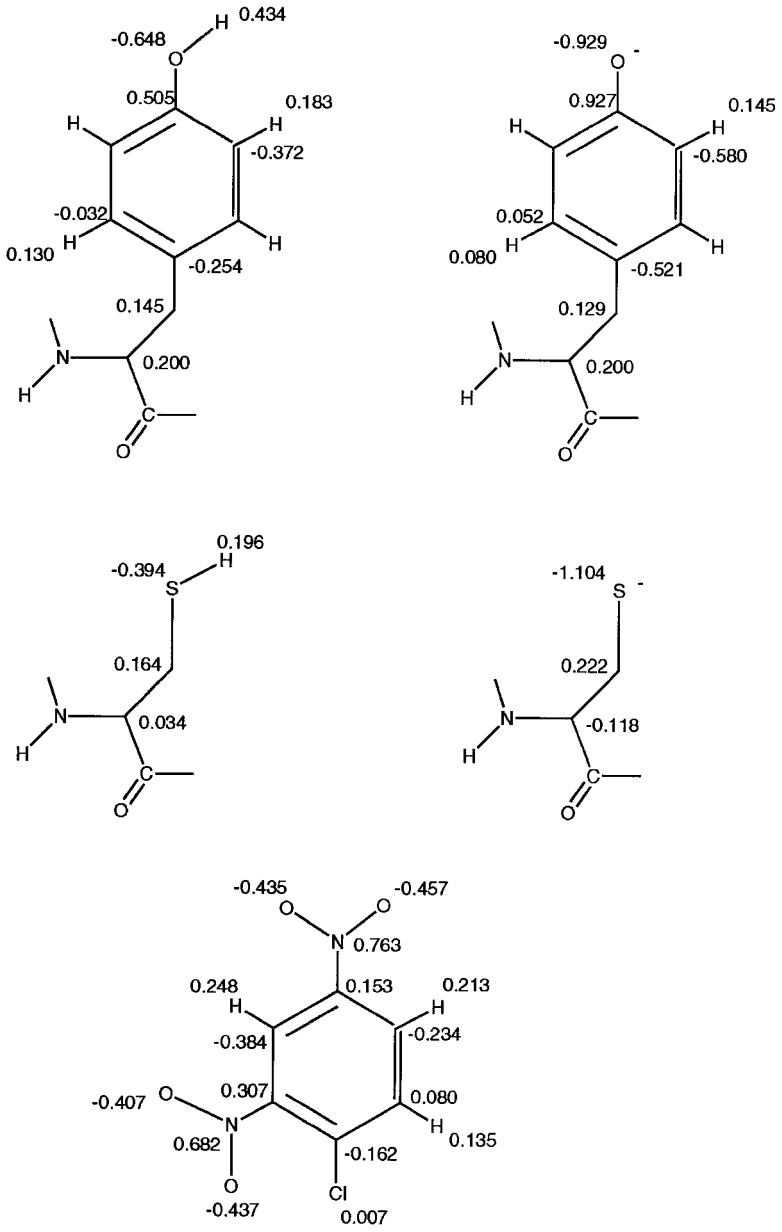

Fig. 1. Atomic partial charges used in simulations for neutral and anionic states of the phenol ring in Tyr7 and the tiol side chain in glutathione and for 1-chloro-2,4-dinitrobenzene.

artifactual structural distortions. First, water molecules and counterions were heated at 298 K over 5 ps, and then the global system was also heated to 298 K over 5 ps. The structures were then equilibrated at 298 K for 40 ps of molecular dynamics at constant temperature. The equilibrated structures were the starting systems for free-energy perturbation (FEP) studies. The AMBER/OPLS force field[25–28] was used for the entire system but Tyr7, glutathione, and 1-chloro-2,4-dinitrobenzene, whose charge distribution was represented by partial atomic charges (Fig. 1) determined by fitting classical and quantum mechanical molecular electrostatic potentials following the standard procedure.[29,30] The quantum mechanical electrostatic potential was determined rigorously from the RHF wave function using the 6 − 31 + G(d) basis set.[31] SHAKE[32] was used to maintain all the bonds at their equilibrium distances, which allowed an integration step of 2 ps. A nonbonded cutoff of 12 Å was used to reduce the number of nonbonded pairs.

The small molecule model systems in water were energy minimized, heated, and equilibrated following a sequential protocol. First, water molecules were minimized by 1,000 cycles of steepest descent, followed by 2,000 cycles of conjugated gradient for

the whole system. The water molecules were heated to 298 K over 5 ps, and then the whole system was heated during 5 ps. The systems were equilibrated for 40 ps of molecular dynamics at constant temperature (298 K). The same simulation protocol was used as for the protein models in these MD simulations.

Free-energy calculations

FEP calculations[33,34] (see Eq. 1) were used to determine the environment effect on the acidity of Tyr7 and of the thiol group of glutathione. The difference in the free energy for ionization in water and in the protein was determined from the mutations of the functional group (hydroxyl; thiol) from neutral to anionic states. The mutation was performed using molecular dynamics in 21 double-wide sampling windows. Each window consisted of 5 ps equilibration and 5 ps averaging, leading to a total of 210 ps molecular dynamics for each simulation. SHAKE was used with an integration step of 2 ps, and a nonbonded cutoff of 12 Å was defined for the nonbonded pairs. Hysteresis was determined as half the difference between "forward" and "reverse" paths, whereas the standard deviations (SD) in the averages were computed using Eq. 2, in which the standard error in each window (SD_λ) was determined from 10 independent averages. After the mutations were completed, 40 ps of molecular dynamics was also performed for the final system to study the average properties of the mutated system.

$$\Delta G_{FEP} = \sum_{\lambda=0}^{1-\Delta\lambda} -RT \ln \langle \exp[-(E_{\lambda+\Delta\lambda} - E_\lambda)/RT] \rangle_\lambda \quad (1)$$

$$SD = \sqrt{\sum_{\lambda=1}^{N} SD_\lambda} \quad (2)$$

N: total number of windows

The use of a cutoff in the FEP calculation for processes with charge generation leads to an underestimation (in absolute terms) of the difference between free energies of solvation for neutral and anionic species. Correction of this error for simulations in water is rather straightforward because (1) it cancels when the relative acidity of two neutral species are compared, and (2) it can be reasonably well estimated by the Born expression. Unfortunately, long-range corrections are more difficult for simulations in the protein due to the anisotropy of the system and to the partition of the protein into mobile and rigid regions. Therefore, long-range contributions to the free energy for the mutation were determined for the final structure at each window using Poisson-Boltzman techniques as implemented in the Delphi computer program.[35,36] These calculations were performed using default values for dielectric response in the protein and in aqueous solution (ϵ = 2 and 80, respectively). The long-range correction in each window was added to the FEP estimate to determine the total free-energy change for the mutation.

Quantum mechanical geometry optimization

Ab initio quantum mechanical geometry optimization for different dimers was performed to assess the agreement between crystal structures and the proposed reaction mechanism. Calculations were carried out at the MP2/6−31+G(d) level, keeping the hydrogen-heteroatom bond length fixed at the optimum value found for the monomer. This is necessary to avoid proton transfer in the energy minimizations.

Calculational details

Quantum mechanical calculations were performed with Gaussian-92 DFT.[37] Electrostatic charges were determined using MOPETE/MOPFIT[38] computer programs. Classic MD and FEP simulations were performed using the AMBER-94 computer program.[28] All simulations were performed on the Cray YMP of the Centre de Supercomputació de Catalunya and on SGI and HP workstations in our laboratories.

RESULTS

Description of the Protein Models Obtained Through MD Simulations

Three systems were modeled: (1) the protein alone (GST), (2) the complex formed by glutathione bound to the protein (GST-GSH), and (3) the complex formed by 1-chloro-2,4-nitrobenzene, glutathione, and the protein (GST-GSH-CDNB). In the two former cases, structural MD models are available for Tyr7 in neutral and anionic states. The same applies for the thiol group of glutathione in the latter two model systems. Some of these models can be compared with unpublished X-ray structures to verify their accuracy, as well as to gain insight into the details of the reaction mechanism.

The general structural features of the active site in the GST-GSH-CDNB model derived from MD simulations were similar to those observed from the crystallographic data for the inhibitors S-(*p*-nitrobenzyl)glutathione or glutathione sulfonic acid bound to GST[9] (Fig. 2). Tyr7 has the same spatial orientation and is placed in an anhidrous environment. CDNB is slightly displaced with respect to the position of the aromatic moiety of S-(*p*-nitrobenzyl)glutathione, mainly resulting from interactions with the side chain of N204, which during the simulation moved slightly toward the hydrophobic subsite. The C^α-trace of the crystallographic structure fits very well with that obtained from the MD simulation, the largest deviations being found in the V33-G41 and K44-L52 areas, at helix αB and the subsequent helix 3_{10}B; in the area R100-I107 at the C-end of helix αD; and at residues G205-G207 at the C-terminus of the

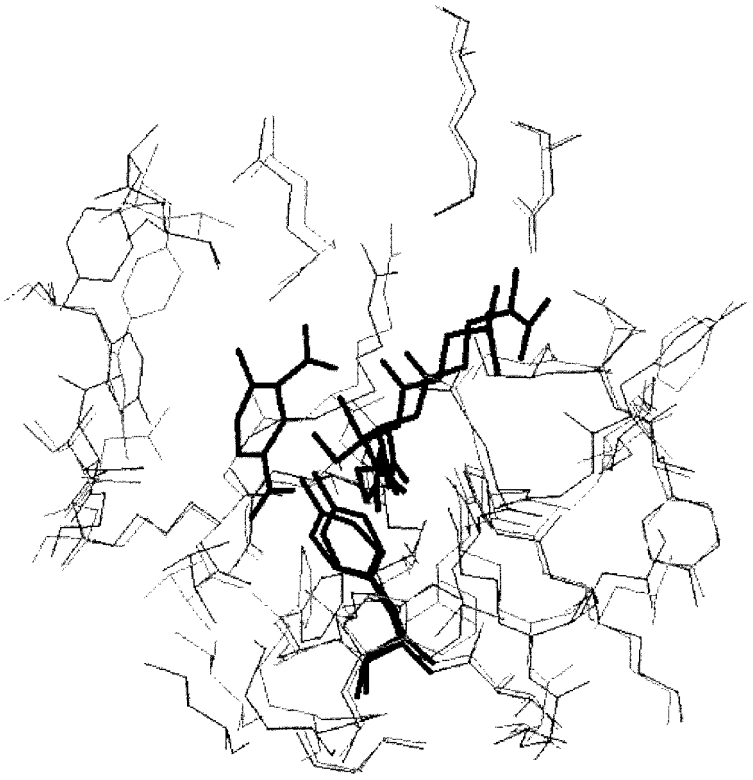

Fig. 2. Superimposition of the active site in the crystallographic structure of the GST complexed with S-(*p*-nitrobenzyl)glutathione and in the MD structure of the GST-GSH-CDNB model system. The figure collects those residues at a distance less than 9 Å from Tyr7. Glutathione, Tyr7, and 1-chloro-2,4-dinitrobenzene in black.

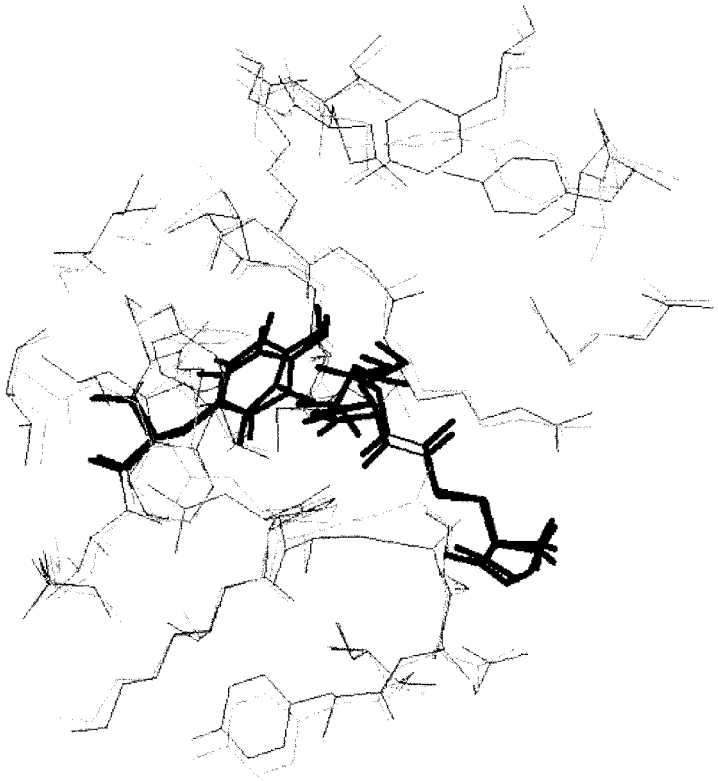

Fig. 3. Superimposition of the MD structures of the active site for the GST-GSH and GST-GSH-CDNB model systems. The figure collects those residues at a distance less than 9 Å from Tyr7. Glutathione is in black and Tyr7 in dark grey.

polypeptide chain. Very small structural changes in the active site occur on ionization of the thiol group (i.e., a small displacement of the F8 side chain appears to be an effect of the simulation rather than of the thiol group ionization). The positions of Tyr7 and CDNB remain nearly invariant. The hydroxyl group of Tyr7 interacts through a hydrogen bond with the sulfur atom of the thiolate anion. The main chain N-H group of R13 lies also in the proximity of the hydroxyl group. The sulfur atom of glutathione is surrounded, on average, by three water molecules. The negative charge of the thiol group might be partially stabilized by the guanidium moieties of R18 and particularly R13, which is about 6 Å from the sulfur atom, even though they are forming saline bridge interactions with negatively charged residues, which reduces the strength of the positive potential in the surrounding of the sulfur atom.

Inspection of MD structures for the GST-GSH system reveals that the hole created on removal of CDNB from the active site is partially filled by water molecules and by the small movement of the side chain of I35. The structure of the protein seems more flexible in the area of residues E30 to L60 and Y103 to N110, it being especially mobile at residues I35, D36, Q51, D59, and V104 (Fig. 3). The thiol group of glutathione is surrounded by three or four water molecules during the simulation. The hydroxyl group of Tyr7, which is also at hydrogen bond distance from the thiol, is mainly surrounded by one water molecule. The N-H group of R13 lies also at hydrogen bond distance from the hydroxyl group. Ionization of either glutathione of Tyr7 does not induce relevant changes in the structure or in the hydration pattern of the thiolate and hydroxyl groups. The guanidinium moieties of residues R13 and R18 range between 6 and 10 Å from either the hydroxyl group of Tyr7 or the thiol group of glutathione.

MD structures for GST-GSH can be compared with the corresponding crystallographic structure at 2.4 Å (Fig. 4). Close agreement is found, particularly in the region around Tyr7. The largest discrepancies are in the residues E30-G41, T46-L52, and G58-L60. It is worth noting that the crystal structure reveals that there are three water molecules around the thiol group of glutathione at 2.5, 3.0, and 3.6 Å interatomic distance for monomer A and 2.8, 3.5, and 3.4 Å for monomer B. The thiol is also within hydrogen bond distance of the hydroxyl group of Tyr7 (3.3 Å). The X-ray structure also reveals no water molecules hydrating Tyr7, whereas in the MD structures only one water molecule is close to the hydroxyl group.

Analysis of the MD structures for the protein alone (GST) indicates no relevant structural differences in the active site when the protein is ligand-free or complexed with glutathione (Fig. 5). Thus, the space occupied by glutathione is filled with water molecules, and Tyr7 is almost in the same position. The hydroxyl group of Tyr7 is reasonably well hydrated. The main chain at residues I35 to G50 and G101 to T105 exhibit the largest mobility with respect to the crystallographic structures of the protein complexed to glutathione derivatives, which agrees with the large thermal factors found in the crystallographic data for the helical zone forming a wall of the active site. However, despite these local fluctuations, the simulation seems to argue against large structural changes in the active site on binding of glutathione.

Moderate changes in the active site occur on ionization of Tyr7. The hydroxyl group establishes a hydrogen bond interaction with the N-H group of R13 and is also surrounded in average by two water molecules. Furthermore, in the MD structures the Tyr7 side chain approaches by about 1–2 Å to the N-terminus of helix αA, but this displacement has a small effect in the magnitude of the interaction between the helix dipole and the Tyr7 negative charge (the interaction energy changes from 30 to 38 kJ/mol on displacement of Tyr7, assuming an effective dielectric constant equal to 4). This agrees with previous studies in which the electrostatic field generated by the helix dipole was suggested to have a moderate influence on the ionization of either the hydroxyl group of Tyr7 or the thiol group of glutathione.[7,10,39]

No X-ray data are available for the free enzyme. However, qualitative insight can be gained from comparison with the X-ray structure of the free enzyme in which the residue Cys47 has been carboxymethylated (Fig. 6). This crystallographic structure (1.9 Å resolution) suggests a large disorder in the T34-Q51 part, whereas the rest of the protein is mostly identical to the conformation of the complexed protein. These findings agree with the structural information derived from MD simulations. Indeed, only one water molecule is hydrogen bonded to Tyr7, which agrees with the MD results (about one or two water molecules in MD simulations of the nonionized structure).

Free-Energy Calculations

The free-energy profiles for mutations in water are smooth, the hysteresis and SDs being very small (Table I). The changes in free energy of solvation for mutations thiol → thiolate and phenol → phenolate in aqueous solution are −318 and −305 kJ/mol, respectively. Therefore, the thiol group is ionized more favorably than the hydroxy group by 13 kJ/mol. The delocalization of the negative charge in phenol is likely responsible for this effect, whereas for the thiol the charge is concentrated in the sulfur atom (see Fig. 1).

Simulations in the protein (Table II) are more difficult due to (1) the need to maintain a rigid part of the protein and (2) the problems arising from the use of cutoffs in an anisotropic, charged media. Nevertheless, the use of nonlinear Poisson-Boltzman correc-

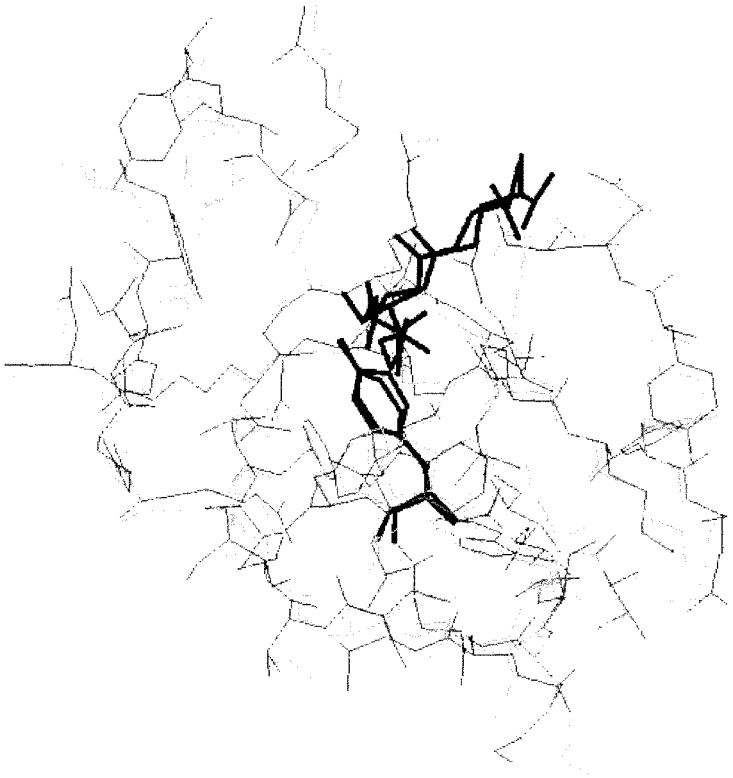

Fig. 4. Superimposition of the active site in the crystallographic and MD structures of the GST-GSH system. The figure collects those residues at a distance less than 9 Å from Tyr7. Glutathione is in black and Tyr7 in dark grey.

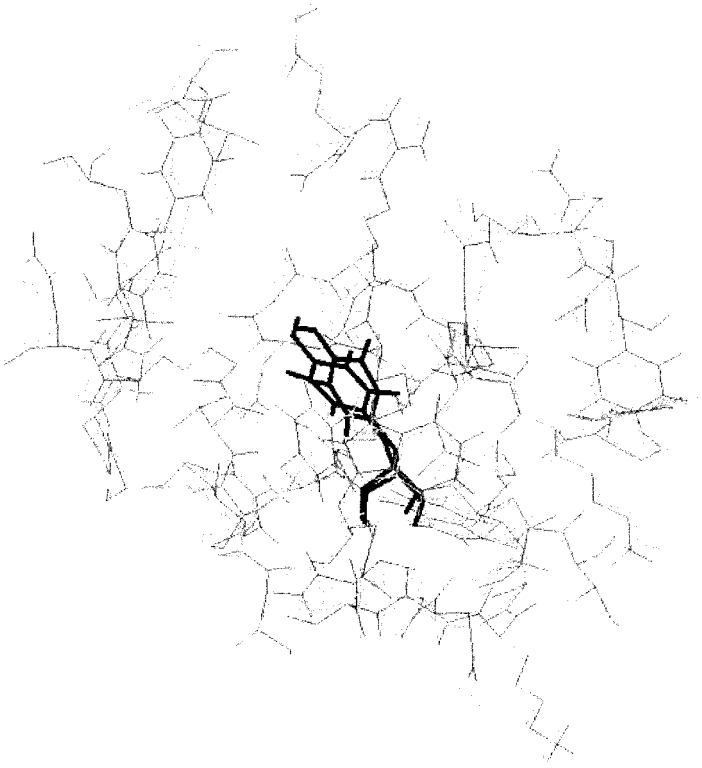

Fig. 5. Superimposition of the MD structures of the active site for the GST and GST-GSH model systems. The figure collects those residues at a distance less than 9 Å from Tyr7 (dark grey).

tions to the free energy are expected to alleviate these errors. Indeed, most of the uncertainties are expected to cancel when mutations Tyr-OH → Tyr-O^- and R-SH → R-S^- are compared, because ionization occurs in the same protein environment, and long-range effects are expected to be the same. Thus, the SDs and hysteresis in FEP simulations (Table II) are likely to be an upper limit of the error in the simulations. This suggests that even though caution is needed for a quantitative discussion of the results, qualitative conclusions can be reached with confidence considering the large magnitude of the differences in free energy found here.

Results in Table II suggest that the protein notably stabilizes ionization of Tyr7. This is quite surprising because the active site, which is buried inside the protein, is quite hydrophobic. Nevertheless, this finding agrees with results determined from Poisson-Boltzman calculations.[22] The reason for such stabilization lies in the electrostatic potential at the active site, because the hydroxyl group of Tyr7 is immersed in a positive region (Fig. 7A), which is expected to facilitate ionization. Indeed, such a region also includes the position occupied by the thiol group of glutathione. The origin of this positive electrostatic distribution cannot be attributed to any specific residue but to the whole of charged groups in the protein. This can be seen by inspection of Figure 7B, which shows the electrostatic potential at the active site determined without the contributions of residues R13 and R18 as well as of E30 and E97, which form salt bridges with the two arginines. Inspection of Figure 7 shows that exclusion of the contribution due to the positive charged side chains of the two closest arginines leads to only small changes in the electrostatic potential at the active site.

Results in Table II indicate that ionization of Tyr7 is made more difficult (by about 76 kJ/mol) on binding of glutathione. This is probably due to worse solvation of Tyr7 when the protein is complexed to glutathione. In fact, binding of glutathione greatly decreases the solvent accesible surface of Tyr7, which changes from about 20 $Å^2$ for the free enzyme to about 2 $Å^2$ for the protein-glutathione complex. Ionization of the thiol group of glutathione (−315 kJ/mol) is better stabilized by the protein than that of Tyr7 (−247 kJ/mol). This probably results, at least partially, from better hydration of glutathione, as it is suggested by the number of water molecules surrounding the thiol group in MD simulations (see above) or by the three water molecules at hydrogen bond distance from the thiol group in the crystal structure of the GST-GSH complex. Indeed, this is also stated by the solvent accessibility of the cysteine residue in glutathione, which amounts to about 25 $Å^2$ in the neutral form or even more (about 45 $Å^2$) in the ionized state. Comparison of the results in Tables I and II suggests that the acidity of the thiol is similiar in aqueous solution and in the protein. However, ionization of Tyr7 is about 58 (GST) and 134 kJ/mol (GST-GSH) more difficult in the protein than in aqueous solution.

Even though these differences have only qualitative value, they are larger than the range of expected error in the simulations. According to the results, ionization of Tyr7 in the protein conformation (the "active" one) sampled during the MD simulations is more difficult than in aqueous solution. This finding is not necessarily in disagreement with experimental pK_a measures,[20–22] because these latter values correspond to large time scales, in which conformations other than the active one can be adopted by the protein.[10,40,41] It is expected that Tyr7 becomes more exposed to the solvent in some of these conformations by being able to interchange protons more easily than in the active conformation used in MD simulations, in which Tyr7 is less accesible to the solvent. Indeed, the results argue against the existence of Tyr7 in the anionic form in the free enzyme or in the protein complexed to glutathione. This suggests that the anionic form of Tyr7 might make the binding of glutathione to the active site more difficult, which would decrease the catalytic efficiency of GST.

Experimental data are consistent with the interpretation that Tyr7 is involved in the activation of the sulfhydyl group of glutathione, facilitating ionization in the enzyme active site.[7,15–19,42–45] Several mechanisms have been proposed for thiol activation.[10] Previous studies that revealed an unusual reactivity for the active-site tyrosine suggested that this residue can dissociate to some extent to form a tyrosinate ion at physiologic pH in the free form of the enzyme,[20–22] and thus Tyr7 may act as a general base for abstracting a proton from the GSH thiol. Alternatively, it has also been suggested that the catalytic role of Tyr7 is to donate a hydrogen bond to, and stabilize the thiolate anion of, enzyme-bound glutathione. In this case, a protonated and neutral tyrosine is required for catalytic activity. This latter catalytic role is supported by the close association observed between the negatively charged sulfonate group of glutathione sulfonate and the hydroxyl group of Tyr7 in the crystal structure of pGSTP1-1,[5] by the finding that glutathione analogs with a stable negatively charged moiety replacing the thiol group bind the enzyme 30–90-fold more tightly than does reduced glutathione[45] and by studies on the pH dependence of k_{cat}/K_m in wild-type enzyme and site-specific mutants, which suggest that the tyrosine has to be protonated to be active in catalysis.[15,19]

Present results show no enhancement in the acidity of Tyr7, which accordingly is not expected to be ionized either in the free enzyme or in the form complexed with glutathione at physiologic pH. Therefore, the relevance of Tyr7 in the enzymatic catalysis is suggested to stem from its ability to stabilize the negative charge on the sulfur atom, enhancing the

Fig. 6. Superimposition of the active site in the crystallographic structure of the GST carboxymethylated in Cys47 and in the MD structure of the GST model system. The figure collects those residues at a distance less than 9 Å from Tyr7 (dark grey).

TABLE I. Calculated Free-Energy Differences for the Mutations CH_3CH_2-SH to CH_3CH_2-S^- and C_6H_5-OH to C_6H_5-O^- in Pure Aqueous Solution[1]

Mutation	ΔG	SD	HST
Et-SH → Et-S^-	−318	±1.2	±0.4
Phe-OH → PheO^-	−305	±2.1	±0.4

[1]The values (kJ/mol) include long-range effects. The standard deviation (SD) and hysteresis (HST) accumulated during the mutation are also given.

TABLE II. Free-Energy Differences for the Mutations Cys-SH to Cys-S^- in Glutathione (GSH) and Tyr7-OH to Tyr7-O^- in the Protein (GST)[1]

Mutation	System	ΔG	SD	HST
Cys-SH → Cys-S^-	GST-GSH	−310	±31	±27
Cys-SH → Cys-S^-	GST-GLH-CDNB	−315	±35	±25
Tyr-OH → Tyr-O^-	GST	−247	±29	±16
Tyr-OH → Tyr-O^-	GST-GSH	−171	±26	±15

[1]Mutation of thiol in glutathione was performed inside the protein in absence or presence of the inhibitor 1-chloro-2,4-dinitrobenzene (CDNB). Mutation of Tyr7 was performed in the protein alone and also in presence of glutathione. The values (kJ/mol) include long-range effects. The standard deviations (SD) and hysteresis errors (HST) accumulated during the mutation are also given.

nucleophilicity of the thiol and orienting it properly for interaction with the xenobiotic molecule. This suggestion is also reinforced by two lines of empirical evidence: (1) mutation of Tyr7 to Phe reduces drastically the velocity of the reaction, but it does not suppress the catalytic activity[15–19]; (2) the three-dimensional structures of *Arabidopsis thaliana* GST[46] and *Lucilia cuprina* GST-θ[13] have revealed the absence of a tyrosine residue in the active site, even though these enzymes are fully effective. Only a serine residue appears at 4.9 (S11)[46] and 3.9 (S9)[13] Å from the thiol, respectively. These distances seem too long for a putative role as a general base equivalent to Tyr7.

Additional theoretical evidence supporting the idea that Tyr7 is not ionized arises from inspection of MD models and X-ray structures of the binary complexes. Despite the positive electrostatic potential at the active site (see above), no positively charged groups that could stabilize the generation of a negative charge in Tyr7 better than water molecules are found in close vicinity to this residue. Furthermore, the crystal structure of the Cys47-modified unliganded enzyme (see above) confirms that no positively charged group exists in the surroundings of Tyr7, in agreement with the MD models for the

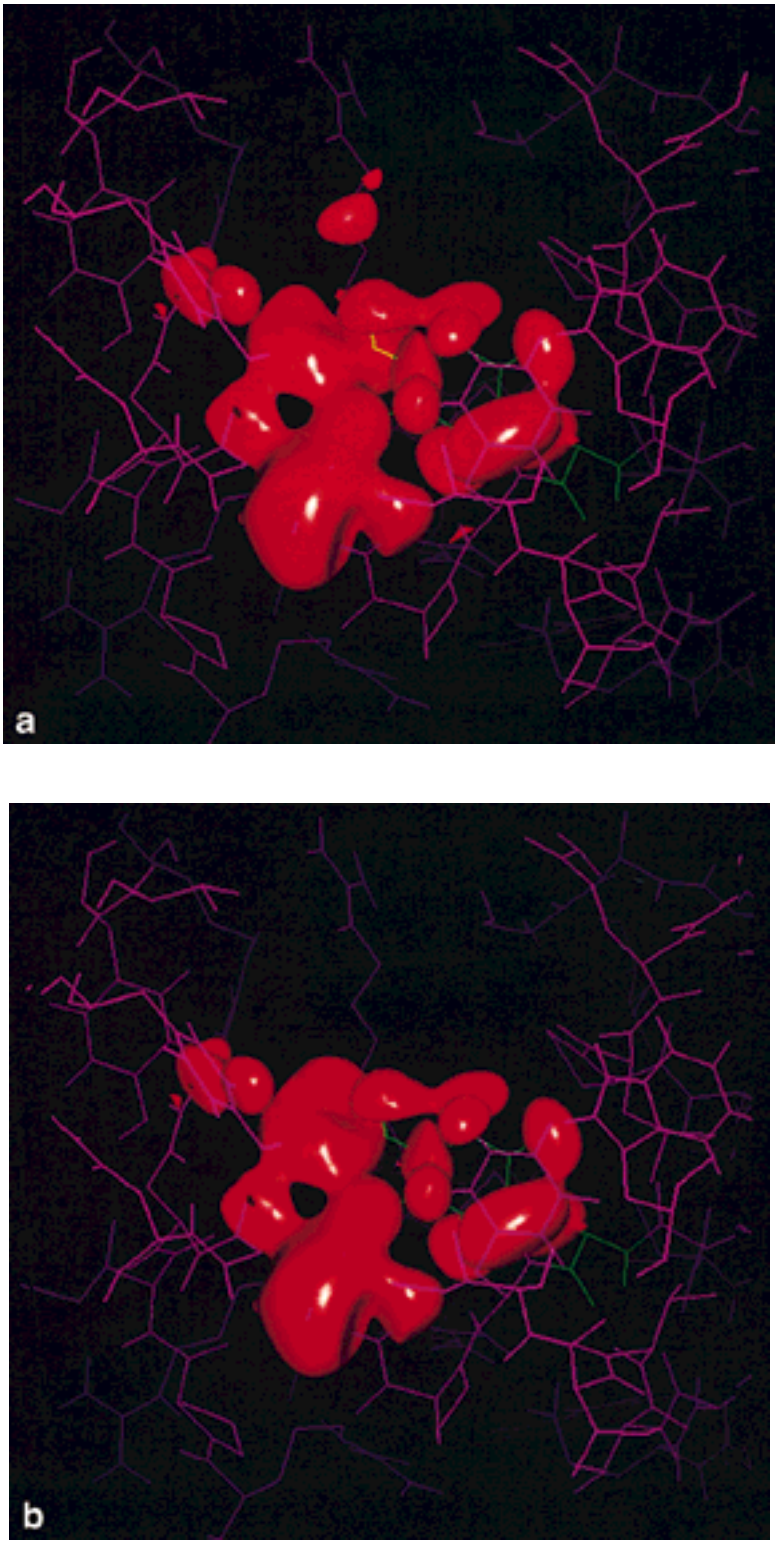

Fig. 7. Electrostatic potential in the active site determined from (**A**) all the residues or (**B**) neglecting the contributions of residues R13 and R18 (and of E30 and E97, which are forming saline bridge interactions). Contour at 38 kJ/mol is in red. Tyr7 is in green.

ligand-free form of the enzyme and with previous X-ray data of the enzyme bound to inhibitors.

Finally, indirect evidence is provided by comparison of X-ray structure hydration patterns with high level ab initio quantum mechanical data. Owing to the uncertainties in the crystallographic data and to the influence of the calculational level on the geometrial parameters, only qualitative insight can be gained from this comparison analysis. However, this approach allows us to take advantage of the large number of water molecules located precisely in the active pocket in the crystal structures of mouse liver GST-Pi.

In the crystallographic structure of the Cys47-modified unliganded GST-Pi, a single water molecule is hydrogen bonded to the hydroxyl group of Tyr7, the distance O-O being 2.7 Å, which is consistent with a polarized hydrogen bond interaction (Table III). No water molecules are found within hydrogen bond distance from the phenolic hydroxyl group in the complexes between S-(*p*-nitrobenzyl)glutathione or gluthatione with the protein. This suggests an increased hydrophobicity in the environment of this group, which difficults its ionization, as suggested by MD simulations. In the crystal structure of the S-(*p*-nitrobenzyl)glutathione-bound GST-Pi,[8] the sulfur atom of the inhibitor is stabilized by two hydrogen bonds, one with Tyr7 (distance S-O: 3.5 Å) and the other with a water molecule (distance S-O: 3.7 Å). This pattern of hydration is consistent with hydrogen bond interactions between neutral species (see Table III). The crystal structure of glutathione-bound GST shows a different hydration pattern, because up to four hydrogen bond interactions involving the thiol group are found: the hydroxyl group of Tyr7 (3.5 Å), and three water molecules, whose oxygen atoms are at 3.6, 3.0, and 2.5 Å (3.5, 3.4, and 2.8 Å in monomer B) from the sulfur atom. This situation contrasts with the poor hydration of the phenolic hydroxyl group, suggesting that the thiol group is much more hydrophilic, which partially explains the larger acidity found in MD-FEP calculations. The short distances between the sulfur atom and the surrounding water molecules suggest that the thiol group is in anionic form (see Table III). In summary, the information derived from X-ray structures and theoretical calculations suggests that Tyr7 is unionized either in the free protein or in the gluthatione-bound form, whereas the thiol group of glutathione is ionized in the complex, it being stabilized by hydrogen bond with Tyr7 and by interactions with hydrating water molecules.

Because present results support that the phenolic hydroxyl group of Tyr in unionized form contributes to the catalytic process of GST by hydrogen bonding, it can be speculated that in the activation mechanism the thiol group of glutathione in the GST-GSH complex donates a proton to a water molecule, leading to the reacting pair $R\text{-}S^- \cdots H_3O^+$, which is transiently bound by saline-bridge interactions. Formation of the $R\text{-}S^-$ anion, which can then act as a nucleophile reagent attacking the aromatic ring of the hydrophobic substrate, is achieved on release of the H_3O^+ ion to the surrounding medium. It has recently been suggested that such a proton transfer can be mediated by other functional groups of the protein[47] or even by the carboxyl groups of glutathione,[48] which might therefore be viewed not only as the substrate providing the reacting thiol group for chemical reactions but also as the proton acceptor in the catalytic mechanism.

TABLE III. Distance Between Heteroatoms in Different Hydrogen-Bonded Complexes[1]

Donor	Acceptor	Distance
H_2O	H_2O	2.9
H_2O	OH^-	2.6
H_3O^+	OH^-	2.2
H_2O	H_2S	3.8
H_2O	HS^-	3.3
H_3O^+	HS^-	2.8

[1]Values (Å) determined from geometry optimization of the dimers at the ab initio MP2/6-31 + G(d) level.

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