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Glutathione Transferase: New Model for Glutathione Activation

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Abstract: Glutathione transferases are enzymes of the cellular detoxification system that metabolize a vast spectrum of xenobiotic and endobiotic toxic compounds. They are homodimers or heterodimers and each monomer has an active center composed of a G-site in which glutathione (GSH) binds and an H-site for the electrophilic substrate. When GSH binds to the G-site, the pK_a value of its thiol group drops by 2.5 units; this promotes its deprotonation and, therefore, produces a strong nucleophilic thiolate that is able to react with the electrophilic substrate. The mechanism behind the deprotonation of the thiol group is still unknown. Some studies point to the fact that the GSH glutamyl α-carboxylate group is essential for GSH activation, whereas others indicate the importance of the active-center water molecules. On the basis of QM/MM calculations, we propose a mechanism of GSH activation in which a water molecule, acting as a bridge, is able to assist in the transfer of the proton from the GSH thiol group to the GSH glutamyl α-carboxylate group, after an initial GSH conformational rearrangement. We calculated the potential of mean force of this GSH structural rearrangement that would be necessary for the approach of both groups and we then performed a QM/MM ONIOM scan of water-assisted proton transfer. The overall free-energy barrier for the process is consistent with experimental studies of the enzyme kinetics.

Keywords: enzyme catalysis · glutathione · proton transfer · reaction mechanisms · transferases

Introduction

Glutathione transferases (GSTs) have been described as the most important enzymes involved in the metabolism of electrophilic compounds.[1] They are enzymes of the cellular detoxification process, a mechanism responsible for metabolizing and expelling toxic xenobiotic and endobiotic compounds from the cell.[2]

There are three different families of GSTs: cytosolic, mitochondrial, and microsomal (also known as membrane-associated proteins in eicosanoid and glutathione metabolism or MAPEG[3]). The cytosolic alpha, pi, and mu classes are abundant and the most extensively studied GSTs.[4–50]

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GSTs are ≈50000 Da proteins forming homodimers or heterodimers (both subunits have to derive from the same class of isoenzymes[5]) and each monomer has binding sites for each of the substrates, that is, a G-site for glutathione (GSH) and an H-site for the hydrophobic electrophile (Figure 1).

The G-site is only completed after dimerization, because it is located in a cleft between the N-terminal domain of

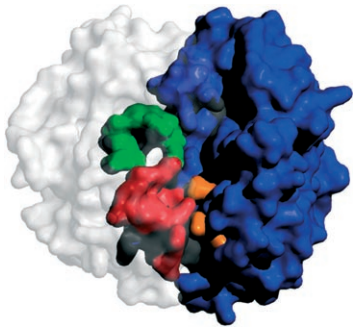

Figure 1. Wild-type enzyme GSTA1-1 monomers showing subunit 1 (white) and subunit 2 (blue). The H-site (green) and G-site (red) of subunit 1 are identified. Residues of subunit 2 that belong to the G-site of subunit 1 are also shown (orange).

one subunit and the C-terminal domain of the other. It is an essentially conserved pocket among all GST classes and shows high specificity for GSH. On the other hand, the H-site is found primarily in the C-terminal domain and its structure varies among GSTs, which allows a vast spectrum of electrophilic toxic compounds to bind to it. GST catalysis of substitution reactions can be described by Equation (1).

$$GSH + R{-}X\ (\text{electrophilic substrate}) \rightarrow GSR + H^{+} + X^{-} \quad (1)$$

The electrophilic substrate (R−X) reacts with GSH to form a less toxic and more soluble compound (GSR). The product release, controlled by the C-terminal region, is the rate-limiting process of the catalytic cycle of substrates that react quickly.[12] In the first step of the mechanism, GSH is activated into its anionic thiolate form, in order to become a strong nucleophile and react with the electrophilic substrate. When GSH binds to the G-site, the pK_a value of the thiol group drops from 9.2 to about 6.2–6.6 pH units,[15] which promotes deprotonation of the GSH. The nature of the residue that receives the proton from the thiol group, thereby behaving like a base, is still unknown. We briefly review here the more popular hypotheses for GSH activation that can be found in the literature and that are crucial to better understand our own proposal.

Active-center tyrosine: In the active center of alpha-, mu-, and pi-class GSTs, the hydroxy group of a conserved tyrosine (in the theta class of GSTs, the tyrosine is replaced by a serine residue[51]) is capable of establishing a hydrogen bond with the glutathione sulfur atom[16–18] (Figure 2).

When the tyrosine is substituted by a phenylalanine, the catalytic activity is dramatically reduced,[19–21] so it has been suggested that tyrosine, behaving like a base, could receive the proton from the GSH thiol group, thereby activating it.[22]

In a later study, the tyrosine pK_a value of the wild-type enzyme GSTA1-1 was found to be 8.1, which is 2 units below the value for free tyrosine in solution, and it was noted that addition of GSH to the GST increases the pK_a value of the tyrosine by 1 unit.[23]

Since the optimal pH value for GST activity ($\approx$7.4) is below the above-mentioned tyrosine pK_a value, tyrosine should be protonated and, therefore, should not behave like a base but rather like a hydrogen-bond donor[23] to stabilize the deprotonated form of the GSH thiolate.

GSH glutamyl α-carboxylate group: The glutamyl α-carboxylate group has been found to be essential for catalysis.[11,24,25,52,53] When a decarboxylated analogue of glutathione, 4-aminobutyric acid–Cys–Gly (dGSH), is used as the substrate for the wild-type enzyme GSTA1-1, the catalytic activity drops 15000-fold and the pK_a value of the thiol group increases from 6.7 to 9.2 pH units.[25] The same studies also demonstrated that the T68E mutation, and to a lesser extent the T68D mutation, restored part of the catalytic activity (dGSH thiol group pK_a value of 8.2 for the T68E mutant). Why these mutations have such a great influence on catalysis is still unclear. In the wild-type enzyme, the threonine 68 side chain and the backbone establish hydrogen bonds with the α-carboxylate group of the GSH glutamate. It was, therefore, proposed that the mutation of threonine 68 to a glutamate would add a carboxylate group capable of taking the place of the α-carboxylate group of the GSH glutamate, which is absent in dGSH. However, in a recently obtained crystallographic structure of the GSTA1-1 T68E mutant complexed with an S-substituted dGSH, glutamate 68 is not facing the substrate and a chloride ion is taking the place of the α-carboxylate group of the GSH glutamate. The presence of the chloride ion emphasizes the propensity for that region to bind and stabilize negative charges and, thus, provides indirect evidence that the presence of the carboxylate group of glutamate 68 in that pocket would be energetically favorable. The exact reason why the chloride ion takes the place of the glutamate is unclear.[26]

Nevertheless, the proposal that the GSH glutamyl α-carboxylate group is able to accept the proton of the thiol group should be taken into account.

Active-center water molecule: Supported by some crystallographic structures of cytosolic GSTs, it has been suggested that water molecules in the active center could assist the proton release and extrusion.[6,28]

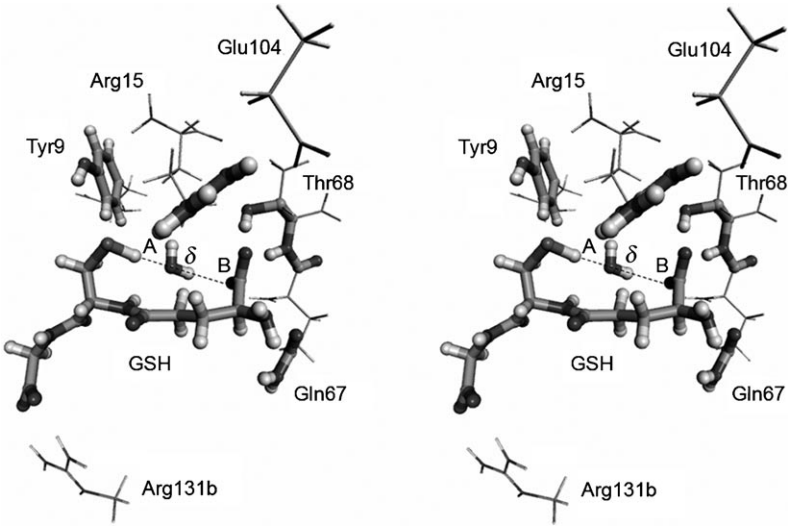

Figure 2. Stereoview of the G-site model, later used to perform the QM/MM calculations. The high layer (78 atoms) is represented by sticks; the low layer is represented by thin tubes. Also shown are atoms A and B; these define distance δ, which was taken as the reaction coordinate.

Potentiometric studies with alpha-, mu-, and pi-class GSTs indicate that the proton from the GSH thiol group is released into the surrounding solution after the formation of the binary GST–GSH complex, which seems to make it a process independent of any enzymatic reaction.[15,27]

From studies of the kinetics of GST–GSH binding, it was also suggested[15] that the process follows a multistep mechanism. First is the rate-limiting step in which the precomplex Enzyme and GSH form a more stable Enzyme*–GSH Michaelis complex. Two fast events then occur, namely GSH ionization followed by proton extrusion. However, even though the pK_a value of a buried water molecule is difficult to determine, it will be far greater than 14 (the value for pure water) because the more hydrophobic environment of the protein would not stabilize a hydronion ion as much as bulk water does. As the optimal pH value for GST activity is $\approx$7.4, this mechanism alone seems unlikely to occur. Additionally, radial-distribution-function (RDF) analysis of GSTA1-1 based on 3 ns long molecular dynamics simulations, which we have performed, point to well-defined water coordination spheres in GSH. Around one of the oxygen atoms of the glutamyl α-carboxylate group, the most suitable for reaction with the thiol, we found an extremely well-defined O–OW RDF peak for the first coordination sphere that corresponds to one water molecule. On the other hand, the first coordination sphere around the thiol group comprises more than five water molecules. Therefore, the importance of water for catalysis seems to be strongly indicated.

Our mechanistic proposal: Herein, we present a water-assisted proton-transfer mechanism that unites the suggested roles of the GSH glutamyl α-carboxylate group and the active-center water molecules in GSH activation. We propose that, after an initial conformational rearrangement of GSH, a water molecule, acting as a bridge, is able to transfer the proton from the GSH thiol group to the GSH glutamyl α-carboxylate group. A detailed theoretical study of this mechanism proposal was performed and confirmed the adequacy of the mechanism.

Methodology

Water-assisted proton-transfer mechanism: We used GSTA1-1, one of the most studied alpha-class GSTs,[4,6,8,10–12,16,17,21,23,25,26,54] as our model and divided our study into two parts.

In part 1, we calculated the energy involved in the conformational rearrangement of GSH to allow the simultaneous interaction of a water molecule with both the thiol and the glutamyl α-carboxylate groups. In fact, we first tried to perform a QM/MM scan but, due to the difficulty in obtaining optimized structures, we opted for the work described herein. To obtain the energy associated with the approach of the two GSH groups, we calculated the potential of mean force (PMF) with the umbrella sampling method. This calculation was performed at the MM level for several reasons. No bond-making or bond-breaking processes were occurring and, therefore, a QM methodology was not strictly necessary. Steric strain plays a significant role in conformational rearrangements and such strain is better captured by the full enzyme environment with its mechanical tensions explicitly considered; the use of an MM methodology allows the sampling of the conformational space.

In the second part of our work, we calculated the energy necessary to actually transfer the proton. By starting from a final PMF calculation structure, a G-site active-center ONIOM model was built. We then performed a scan of the approach of the water proton to the most suitable oxygen atom of the GSH glutamyl α-carboxylate group and we observed that the thiol group proton was simultaneously transferred to the water molecule.

A full description of parts 1 and 2 follows. Basically, these form our mechanistic proposal of GSH activation.

Part 1—Conformational rearrangement of GSH:

Molecular dynamics: The crystallographic structure of GSTA1-1 complexed with GSH was obtained from the Protein Data Bank[55] (file code 1PKW). A water molecule was later added and placed between the GSH thiol and the glutamyl α-carboxylate groups.

The gamma glutamyl group of GSH had to be parameterized as there are no parameters in the AMBER99 force field for this species.[56,57] The dihedral values, angles, bonds, and van der Waals parameters were based on the AMBER99 force field. Atomic point charges were calculated with the Gaussian software package, by following the methodology used in the AMBER99 force field of fitting the HF/6-31G*-generated electrostatic potential to atomic point charges by using the ESP (RESP) algorithm.

All of the molecular dynamics simulations and subsequent analyses were carried out by using the Gromacs software package conjugated with the Amber99 force field.[56–59] The enzyme models were solvated with $\approx$17000 single-point-charge water molecules[60] and then submitted to 100 steps of steepest descent energy minimization to remove bad contacts between the solvent and the protein. Subsequently, the system was equilibrated for 200 ps with the protein atoms restrained by weak harmonic constraints to allow for the structural relaxation of the water models. 26 (13+13) production simulations of 150 ps were performed with time steps of 0.002 ps and with the trajectories saved at 1 ps intervals.

In all simulations, periodic boundary conditions were used. The temperature and pressure were maintained as constant by using Berendsen temperature coupling and pressure coupling (parameters: $\tau_T = 0.1$ ps, $T_{ref} = 300$ K, $P_{ref} = 1$ bar).[61] The particle-mesh Ewald (PME)[62] method was applied to compute electrostatic interactions, with a cut-off of 1.0 nm. In terms of van der Waals interactions, a twin-range cut-off with a neighbor-list cut-off of 1.0 nm and a van der Waals cut-off of 1.0 nm was used.

Potential of mean force calculations: The above-mentioned simulations were performed in order to calculate the PMF associated with the approach of the thiol group to the α-carboxylate group. The PMF represents the free-energy change as a function of a coordinate of the system. In our study, distance δ between atoms A and B (see Figure 2) was taken as the reaction coordinate. It should also be mentioned that the position of the water molecule was constrained in the reaction-coordinate pathway.

The PMF calculus was performed by following the umbrella sampling method.[63] In the umbrella sampling method, a series of simulation windows is performed along a reaction coordinate and each window is restrained by imposing a harmonic umbrella biasing potential, $U'(\delta)$, as defined by Equation (2), in which κ is the force constant.

$$U'(\delta) = {}^1/_2\kappa(\delta-\delta_0)^2 \quad (2)$$

Distance δ between the hydrogen (atom A) and oxygen (atom B) atoms (Figure 2) was steadily decreased in each window by 0.04 Å. After the last δ value, the reverse process was also performed. The force constant was calibrated to allow overlapping of the windows along the reaction coordinate ($K = 50$–125 kcal mol^{-1}). A total of 7 forward and 7 backward 150 ps windows were performed, which resulted in an overall total of 2100 ps production simulations.

The unbiased probability distribution of δ values, in both the forward and backward directions, was used to calculate the free energy associated with the approach of the thiol group to the glutamyl α-carboxylate group by the constant-temperature weighted histogram analysis method (WHAM).[64] The WHAM method allows the calculation of the PMF by computation of the unbiased distribution function as a weighted sum over the individual biased distributions of each window.

Part 2—Proton transfer study in GSH:

ONIOM model: We built a G-site model from a final PMF calculation structure. This model included all of the atoms critical to catalysis, including the water molecule positioned between the thiol and the glutamyl α-carboxylate groups (143 atoms in total). In order to optimize the computation time, we resorted to the ONIOM[65–67] method, which allows a division of the model into different theoretical levels. This method has recently been used in the catalytic study of important enzymes with excellent results.[68–73]

Figure 2 shows the G-site active-center ONIOM model that was used. The high layer includes GSH, the water molecule, and the atoms that directly interact with GSH and that could have an important role in the proton transfer (78 atoms). DFT with the B3LYP functional[74,75] and the 6-31G(d) basis set, as implemented in Gaussian 03,[76] was used in geometry optimization. The low layer includes the rest of the model atoms that complete the G-site pocket. This layer was treated with the semiempirical PM3MM method.[77,78] Hydrogen atoms were used as link atoms at the truncated bonds.

Energy calculations: A QM/MM ONIOM scan of the approach of the water proton to the most suitable oxygen atom of the GSH glutamyl α-carboxylate group was performed. Approximate structures of the three stationary points (reagents (R), transition state (TS), and product (P)) were taken from the scan. The stationary points were later freely optimized and their nature was confirmed by frequency calculations.

After obtaining the three stationary points, we recalculated the energy of the entire model with a higher theoretical level. We performed single-point calculations by using DFT with the B3LYP functional for the entire system and the 6-311++G(2d,2p) basis set, as implemented in Gaussian 03. A continuum model was used as an approximation of the effect of the whole-protein environment, as described in previous studies.[79–81] We chose the C-PCM model[82,83] with a dielectric constant of 4, which is normally in conformity with the experimental protein data. Zero-point corrections and thermal and entropic effects were also added to obtain the final energy values ($T = 310.15$ K, $P = 1$ bar).

Results and Discussion

Water-assisted proton-transfer mechanism: As mentioned above, our GSH-activation mechanism can be divided into two steps: the GSH structural rearrangement that allows for a water molecule to interact simultaneously with the thiol and the glutamyl α-carboxylate groups, followed by the actual water-assisted proton transfer.

Figure 3 shows the energy barrier obtained for these two events, the PMF curve of the GSH structural rearrangement, and the QM/MM scan of the proton transfer.

The PMF curve obtained has a minimal hysteresis; this refutes the possibility of systematic error and emphasizes the accuracy of the calculations. In plot 1, only the PMF curve that results from the sum of the data from all of the forward and backward processes is shown. The PMF calculation demonstrates that the bent GSH, with a water molecule bridging both active groups, is actually more stable than the initial open GSH conformation. In fact, this conformational rearrangement has a difference in free energy (ΔG) of -1.62 kcal mol^{-1}.

The QM/MM scan of the actual proton transfer points to an energy barrier that is consistent with what would be expected for an enzymatic reaction.

Figure 4 shows the free energies of the three stationary structures R, TS (difference in free energy for the formation of the TS, $\Delta G^{\ddagger} = 13.39$ kcal mol^{-1}), and P, after free optimization and correction for zero-point energy and thermal and entropic effects. This is an endergonic reaction (overall difference in free energy of the reaction, $\Delta G_r = 3.19$ kcal mol^{-1}), which gives rise to a strong nucleophile (sulfur partial charge of -0.778) that is prone to react with the electro-

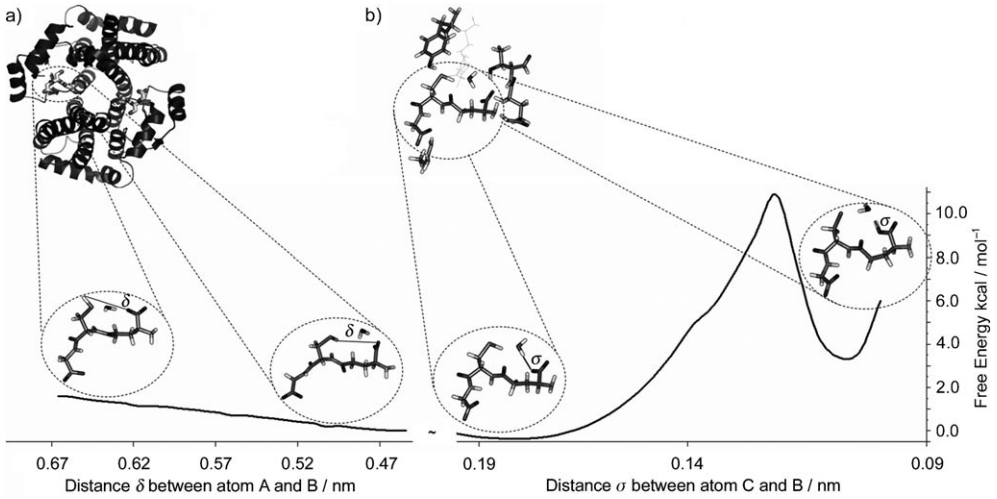

Figure 3. Water-assisted proton-transfer mechanism. a) Calculated free energy versus the SH–COO distance δ. The curve represents the sum of all of the data obtained from the PMF forward and backward processes. The initial and final PMF GSH structures from GSTA1-1 (top left-hand side) are shown. b) Potential energy surface of the water-assisted proton transfer. The distance σ, between atom C (the water proton) and atom B (the GSH glutamyl α-carboxylate oxygen atom) decreased at each scan point. The initial and final structures from the G-site model (top left-hand side) are shown.

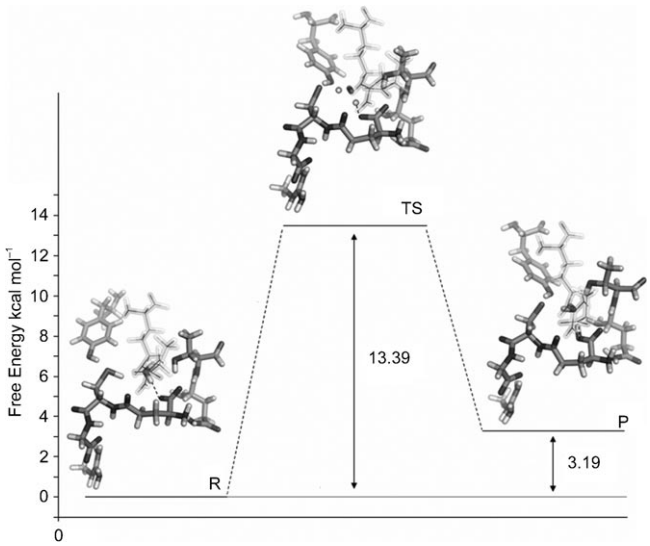

Figure 4. Water-assisted proton-transfer free energies of the three stationary points, the reagent (R), transition state (TS), and product (P). The distance σ, between the water proton and the glutamyl α-carboxylate oxygen atom, is also indicated.

philic substrate. The three stationary structures are shown in Figure 5.

From Figure 5, we can observe that the side chains of Arg15, Thr68, Gln67, and Arg131b are hydrogen bonded to GSH. On the other hand, Tyr9 is near the GSH thiol group but not close enough to make a significant interaction. As distance σ decreases, the thiol proton gradually gets closer to the water oxygen atom. This movement is compensated by a shortening of the hydrogen bond between Tyr9 and the nascent thiolate. At the transition state, the thiol proton, as well as the proton characterized by distance σ, is partially bonded to the water oxygen atom and Tyr9 assumes the role of stabilizing the partial negative charge in the thiol sulfur atom, which increases from −0.079 in R to −0.649 at the TS. After the TS, the GSH carboxylate group receives the proton from the water molecule and the thiol proton is transferred to the water molecule. This gives rise to the product, a strong nucleophilic GSH thiolate with a negative partial charge of −0.778. In P, amino acid Tyr9, which is even closer to the sulfur atom, is capable of establishing a strong ionic H-bond interaction with the thiolate. On the other hand, the Thr68 side chain, which was hydrogen bonded to the GSH glutamyl carboxylate oxygen atom, becomes hydrogen bonded to the water molecule. This is because the protonated carboxylate group is now neutral and cannot establish a strong charged interaction (the partial negative charge on the GSH glutamyl carboxylate oxygen atom decreases from −0.629 in R to −0.447 in P).

The role played by the water molecule: To further elucidate the role of water, we have additionally calculated the potential of mean force associated with the GSH structural rearrangement that is necessary for the thiol and the glutamyl α-carboxylate groups to interact directly, that is, without the bridging water molecule. The method used for the PMF calculation was similar to the one described in the methodology section for the water-assisted proton-transfer mechanism proposal. However, as both active groups need to be closer, a total of 13 forward and 13 backward 150 ps windows were performed, which resulted in an overall 3900 ps production simulation.

The results showed that the GSH thiol and glutamyl α-carboxylate groups approach to a distance δ of 0.2 nm is highly energy consuming ($\Delta G = 15.88\ \mathrm{kcal\,mol^{-1}}$). Subsequent to these calculations, we have built a G-site model

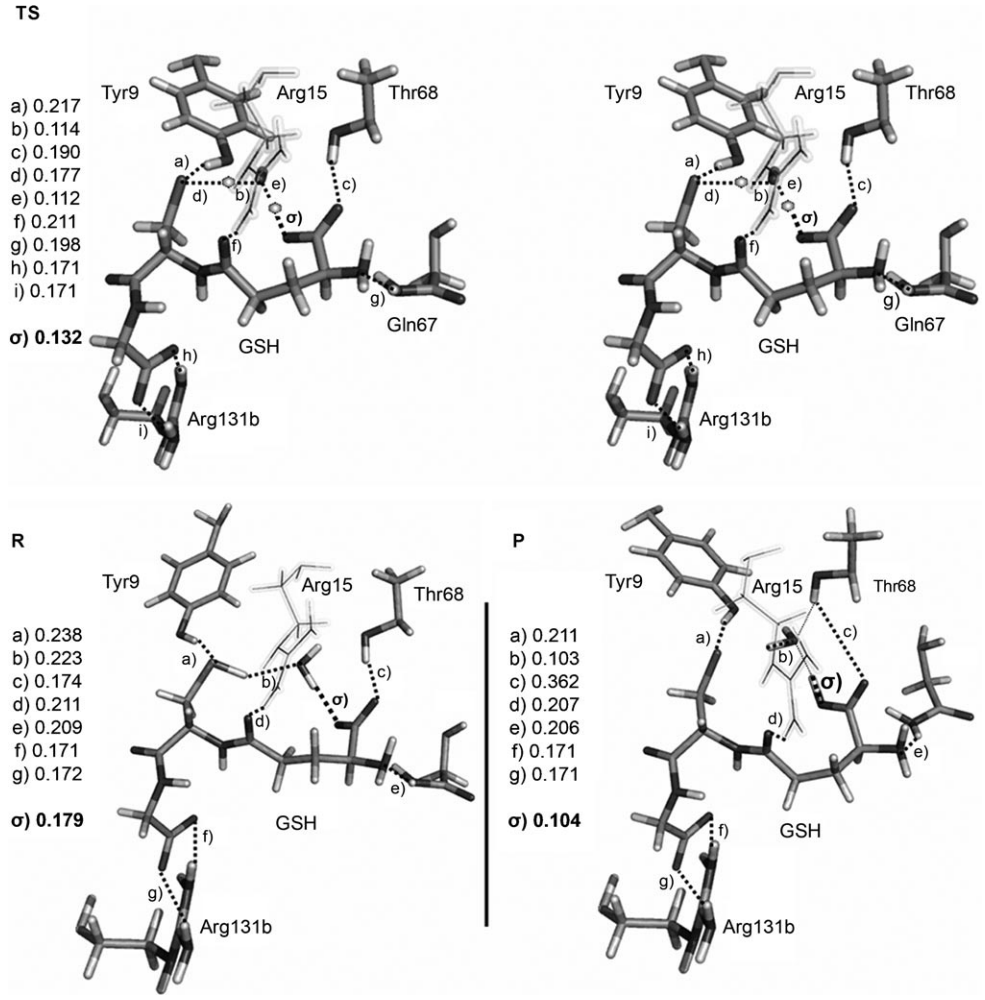

Figure 5. G-site model for highlighted amino acid side chains that directly interact with GSH. Characterization of the three stationary points of the water-assisted proton transfer, the reagent (R), transition state (TS, in stereoview), and product (P). Relevant distances [nm], together with the distance σ, are shown.

based on a final PMF structure and performed a QM/MM ONIOM scan of the direct proton transfer between the thiol and the glutamyl α-carboxylate groups. With the exclusion of the water molecule, the G-site model used was similar to the one shown in Figure 2. The energy barrier associated with this scan only was 19.44 kcal mol^{-1}. Therefore, 15.88 kcal mol^{-1} were needed for the GSH conformational rearrangement plus 19.44 kcal mol^{-1} for the actual proton transfer. This means that the total energy required for proton transfer without the assistance of a water molecule is 35.32 kcal mol^{-1}. Therefore, the importance of water for decreasing the energy barrier of GSH activation seems to be strongly indicated.

Conclusion

The water-assisted proton-transfer mechanism proposed here is in agreement with the known experimental data and seems to adequately explain the GSH activation. The initial GSH conformational rearrangement, which allows a water molecule to interact directly with the thiol and the glutamyl α-carboxylate groups, has a ΔG value of -1.62 kcal mol^{-1}. The energy barrier of the actual proton transfer (13.39 kcal mol^{-1}) is in conformity with the experimental value obtained for the GST-catalyzed conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CNDB), a common electrophilic substrate (rate of catalysis ($k_{cat} = (88 \pm 3)$ s^{-1}, $\Delta G\neq = 15.06$ kcal mol^{-1}[11]).

We have also demonstrated the fundamental role of water in effectively diminishing the GSH-activation energy barrier. The barrier to direct proton transfer between the glutamyl α-carboxylate and thiol groups, 35.32 kcal mol^{-1}, is too high for a catalyzed reaction.

The crystallographic structure we used shows a molecule of water hydrogen bonded to the GSH glutamyl α-carboxylate group but not bridging the two GSH active groups. Without the bridging water molecule, the open conformation of GSH is more stable. It is possible that a small barrier might exist to move the water molecule to the bridging posi-

tion, which results in the rearrangement of GSH from the open to the closed conformation ($\Delta G = -1.62$ kcal mol^{-1}). The potential energy surface for these rearrangements is usually very flat, with multiple minima, and is surely not rate limiting. On the other hand, the different GSH hydration pattern observed in the crystallographic structure could result from a reduced water content in the crystals. Given the energetic proximity between the two conformations, any small difference between the simulated and experimental systems may lead to a shifting towards one or the other side of the equilibrium.

Acknowledgements

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