Higher flexibility of Glu-172 explains the unusual stereospecificity of Glyoxalase I

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Abstract

Despite many studies during the latest two decades, the reason for the unusual stereospecificity of glyoxalase I (GlxI) is still unknown. This metalloenzyme converts both enantiomers of its natural substrate to only one enantiomer of its product. In addition, GlxI catalyzes reactions involving some substrate and product analogues with a similar stereospecificity as its natural substrate reaction. For example, the enzyme exchanges the pro-$S$, but not the pro-$R$ hydroxymethyl proton of glutathiohydroxyacetone (HOC-SG) with a deuterium from $\mathrm{D}_2\mathrm{O}$. To find some clues to the unusual stereospecificity of GlxI, we have studied the stereospecific proton exchange of the hydroxymethyl proton of HOC-SG by this enzyme. We employed density functional theory and molecular dynamics (MD) simulations to study the proton exchange mechanism and origin of the stereospecificity. The results show that a rigid cluster model with the same flexibility for the two active-site glutamate residues cannot explain the unusual stereospecificity of GlxI. However, using a cluster model with full flexibility of Glu-172 or a bigger model with the entire glutamates, extending the backbone into the neighboring residues, the results showed that there is no way for HOC-SG to exchange its protons if the alcoholic proton is directed towards Glu-99. However, if the hydroxymethyl proton instead is directed towards the more flexible Glu-172, we find a catalytic reaction mechanism for the exchange of the $\text{H}_5$ proton by a deuterium, in accordance with experimental findings. Thus, our results indicate that the special stereospecificity of GlxI is caused by the more flexible environment of Glu-172 compared to that of Glu-99. This higher flexibility of Glu-172 is also confirmed by MD simulations. We propose a reaction mechanism for the stereospecific proton exchange of the hydroxymethyl proton of HOC-SG by GlxI with an overall energy barrier of 15 kcal/mol.

Keywords: Stereospecificity, Glyoxalase I, Metalloenzyme, Glutathiohydroxyacetone, Proton Exchange Mechanism, DFT, QM-cluster Calculations, Molecular Dynamics Simulations
1. Introduction

Glyoxalase I (EC 4.4.1.5, lactoylglutathione lyase; GlxI) is a metalloenzyme present in most organisms from bacteria to human. It is one of the two members of the glyoxalase system. The system consists of two enzymes, GlxI and glyoxalase II (GlxII). These enzymes detoxify methylglyoxal (MG) that is produced in normal cell metabolism. This system is vital for organisms, because MG is a highly toxic metabolite. It is a reactive dicarbonyl compound endogenously produced primarily as a by-product of glycolysis, but also as a product of ketone body metabolism, of threonine catabolism, and in the degradation of glycated proteins. The glyoxalase system has been implicated in chemoresistance in some human tumors and it has therefore been suggested to use GlxI inhibitors as anticancer drugs.

GlxI catalyzes the conversion of hemithioacetals of MG and a variety of aromatic and aliphatic \(\alpha\)-ketoaldehydes to \(\alpha\)-hydroxythioesters and after that, GlxII splits the produced lactoylglutathione into glutathione and D-lactate (Scheme 1). The hemiacetal substrates of GlxI are formed from a non-enzymatic reaction of a thiol (either glutathione, H-SG, or trypanothione, depending on the organism, e.g. H-SG for human GlxI) and MG. Interestingly, GlxI converts both enantiomers of the substrate into the same S-D-lactoylglutathione product.

![Scheme 1. The glyoxalase pathway.](image)

For catalytic activity, GlxI requires a divalent metal ion, which varies depending on the organism. It is Zn(II) for human GlxI. However, GlxI of *Escherichia coli* shows its maximal catalytic activity in presence of Ni(II), whereas it is inactive in presence of Zn(II) and has a reduced activity with Co(II), Cd(II) and Mn(II). Aronsson et al. showed that GlxI from both human and yeast contains...
a Zn(II) ion in a stoichiometry of about 1 mole per mole of enzyme subunit and that removal of the Zn(II) ion gave an inactive enzyme.\textsuperscript{11}

Crystal structures of human GlxI show that the active-site Zn(II) ion is coordinated by His-126, Gln-33, Glu-99, Glu-172 and two water molecules.\textsuperscript{12} It is generally accepted that the Glu-172 or Glu-99 residue of the active site initiates the catalytic reaction of GlxI by abstraction the H1 atom from the S- or R-substrate, respectively (the naming of the atoms is shown in Scheme 2). This proton abstraction generates an enediolate intermediate, a key structure in the reaction pathway\textsuperscript{12–15} (cf. Scheme 2). However, experiments show that the proton is always added to the \textit{si} face of C2 and not to its \textit{re} face, resulting in only one enantiomer of the product.\textsuperscript{5} This shows that the two active-site glutamate residues play different roles in the reaction, probably owing to their different environment in the enzyme (see reference\textsuperscript{16} for a detailed description of the reaction mechanism).

![Scheme 2](image)

Scheme 2. The initial step of the GlxI reaction with the (a) \textit{S}- and (b) \textit{R}-substrates.

GlxI can also catalyze reactions involving some substrate analogues. For example, it converts both enantiomers of the two thiohemiacetics 1 and 3 in Scheme 3 to only the \textit{S}-enantiomer of the thioesters 2 and 4, respectively.\textsuperscript{17,18} It was also demonstrated by NMR spectroscopy that the enzyme exclusively catalyzes the exchange of the pro-\textit{S}, but not the pro-\textit{R} hydroxymethyl proton of the product-analogue glutathiohydroxyacetone (HOC-SG) with a deuterium from the D\textsubscript{2}O solvent (Scheme 4). On the other hand, the enzyme also specifically mediates the exchange of the pro-\textit{S}, but not pro-\textit{R} hydroxymethyl deuterium of [\textsuperscript{2}H]-HOC-SG with a proton from H\textsubscript{2}O solvent.\textsuperscript{19} The rate constant for solvent exchange
This value is similar to the solvent exchange rate of (S)-D-lactoylglutathione (the normal product of the enzyme) by GlxI. Based on experimental results, Creighton and Hamilton concluded that a cis-enediolate intermediate is formed during the reaction of normal substrates, provided that the substrate analogues are processed in the same way as the normal substrates.

We have in a previous study used the quantum mechanical cluster (QM-cluster) approach to compare several alternative reaction mechanisms for both enantiomers of the natural substrate of human GlxI. The calculations gave support to the previously proposed mechanism involving an enediolate intermediate. However, we found that the enzyme can use the same reaction mechanism for the S and the R enantiomers of the substrate, but with exchanged roles of the two active-site glutamate residues (Glu-99 and Glu-172). From the results, we concluded that the only possibility for the stereospecificity of GlxI is differences in the electrostatic surroundings and flexibility of the glutamate residues in the active site, owing to their neighboring residues in the protein.

Thus, despite all previous studies during the latest two decades, the reason for the special stereospecificity of GlxI is still unclear. A study of the reactions of substrate analogues may give new clues to solve the old problem of GlxI stereospecificity. Therefore, we focus in this work on the stereospecific proton exchange of the hydroxymethyl proton of HOC-SG (Scheme 4). We investigate the mechanism of the enzymatic exchange of H_S by a deuterium atom and why H_R cannot be exchanged by a deuterium. We employ the QM-cluster methodology, which has been successfully used to elucidate a large number of different enzymatic reaction mechanisms. We also use molecular dynamics (MD) simulations to study the flexibility of the active site.

Scheme 3. Conversion of both isomers of thiohemiacetal 1 and 3 to only the S-enantiomer of the thioesters 2 and 4, by
2. Modeling and computational details

2.1 QM-cluster models and calculations

To study the desired reaction with QM-cluster approach, we need a model of the active site. The experimental data come from yeast GlxI, but there is no crystal structure for GlxI from this organism. Therefore, we had to use a crystal structure for GlxI from another species to model the active site. Aronsson et al. showed that GlxI from both human and yeast contains one zinc ion per active site. Therefore, we used the crystal structure of native human GlxI to model the active site (protein data bank entry 1QIN). The model consisted of the zinc atom and its first-coordination shell amino acids (Gln-33, Glu-99, Glu-172 and His-126), as well as a model of HOC-SG. The glutamates were represented by propionate, glutamine by propanamide and histidine by methyl-imidazole. The inhibitor in the crystal structure (S-[N-hydroxy-N-(p-iodophenyl)carbamoyl]glutathione) was modified to a model of HOC-SG: the para-iodophenyl group was replaced by a hydrogen atom, the N atom next to the iodophenyl group by a carbon atom and the –SG group by a –CH₂SH group. Hydrogen atoms were added manually. To maintain the overall structure of the active site, carbon atoms bound to H atoms that truncated the active-site model were fixed at their positions in the crystal structure during the geometry optimizations.

We also employed an extended QM model. In this model, the glutamine, histidine and the substrate were the same as the first model. However, the complete glutamate residues were included in the model (including the backbone). In addition, –COCH₃ and –NHCH₃ from the neighboring residues (Leu-98, Leu-100, Ile-171 and Ile-173) were also included, giving a CH₃–CONH–CHCH₂CH₂COO–
CONH–CH₃ model of each glutamate. For glutamine and histidine, the same atoms were fixed in the two models. However, for the glutamates two atoms in the ending –CH₃ groups were fixed (those corresponding to CB and N or C). We denote the two models as M1 and M2, respectively.

An investigation of the crystal structure, showed that none of the neighboring residues has any direct interaction with the active site. However, OG1 of Thr-101 makes a water-mediated (HOH-404) hydrogen bond to Glu-99. Therefore, we also extended M2 with Thr-101 and HOH-404 in a third model (M3). The threonine was modeled as isopropyl alcohol. In addition, we enlarged the model of HOC-SG by replacing –SG group by –CH₂SCH₃ in M3 (instead of –CH₂SH in M1 and M2). The same atoms were fixed in M2 and M3, and the CA atom was fixed for Thr-101. The three models are illustrated in Figure 1, marking the fixed atoms with asterisks.

From Figure 1, it can be seen that the Hᵣ and Hₛ atoms are directed towards Glu-99 and Glu-172, respectively. However, the hydroxyl proton of HOC-SG (Hₒ) can be directed towards either Glu-172 or Glu-99 (the atom names are defined in Figure 1). Therefore, we performed calculations for two different states, depending on the direction of Hₒ. We denote these two conformations Hₒto99 and Hₒto172, when Hₒ is directed towards Glu-99 and Glu-172, respectively, and they are both shown in Figure 1.
Figure 1. Optimized structures of M1 (a, b), M2 (c, d) and M3 (e, f) in the Hoto99 (a, c, e) and Hoto172 (b, d, f) conformations. HOC-SG, the Zn atom and HOH-404 are shown in a ball-and-stick representation and the amino acids by tubes. Fixed atoms are marked with asterisks.

All QM-cluster calculations were performed using the density functional B3LYP,\(^\text{26}\) implemented in the Gaussian software, versions 09\(^\text{27}\) and 16\(^\text{28}\). The structures of the reactants, transition states, intermediates and products were optimized using the 6-31+G(d) basis set for the H, C, N, O and S atoms and the LANL2DZ\(^\text{29}\) pseudo potential and basis set for the Zn ion. More accurate energies were calculated with single-point calculations on the optimized structures using the larger 6-311++G(2d,2p) basis set for all atoms. To consider the surroundings, solvation effects were evaluated at the B3LYP/6-31+G(d)/LANL2DZ level of theory by performing single-point calculations using the CPCM solvation model.\(^\text{30}\) The solvation cavity was built up using the UFF radii and the dielectric constant was set to 4. Natural bond orbital (NBO) analysis\(^\text{31,32}\) was used to calculate atomic charges.
on the optimized structures. It was performed at the same level of theory as the single-point energy calculations. Frequencies of the stationary states on the potential energy surface were calculated to obtain zero-point energies. The frequency calculations were performed at the same level of theory as the geometry optimizations. The final energy of each stationary point and all energies on the potential-surfaces were obtained by including the corresponding zero-point energy and the electrostatic part of the solvation energy as a correction to the electronic energy calculated from the higher-level single-point calculations.

2.2 MD simulations

The MD simulations were based on the same crystal structure (1QIN). The entire dimeric enzyme and all crystal water molecules were included in the calculations. However, the inhibitor was replaced by HOC-SG. The protonation states of all residues were determined from a detailed study of the hydrogen-bond pattern and the solvent accessibility. It was checked by the PROPKA software. The two subunits of the dimeric protein were treated in the same way. All Cys residues were assumed to be protonated. The active site histidine residue (His-126) was protonated on the ND1 atom, His-102 was protonated on NE2 and another histidine residue (His-115) was protonated on both ND1 and NE2. Two aspartic acid residues Asp-165 and Asp-167 were assumed to be protonated and the others were negatively charged. All Arg, Lys, and Glu residues were assumed to be charged. All protonation-states are in agreement with the predictions of PROPKA. The only exception is Glu-99, which PROPKA predicts to be protonated. However, since it is coordinated to the Zn ion, we treated it in its deprotonated form. In addition, His-126 coordinates to the Zn ion by the NE2 atom; therefore we protonated it on ND1.

All MD simulations were performed using the GPU-accelerated pmemd code of AMBER 16. The protein and HOC-SG were described with the Amber ff14SB and GAFF force fields, respectively. Water molecules were described by the TIP3P model. Two different sets of simulations were performed in this study. One involved the H0to99 conformation and the other one with the H0to172 conformation of the substrate.
For the Zn sites, restrained electrostatic potential charges were employed, fitted to electrostatic potentials calculated and sampled with the Mertz–Kollman scheme. The calculations were performed on the QM-optimized cluster models of H$_2$O$_{99}$ and H$_2$O$_{172}$ conformations, shown in Table S1 in the supporting information. The geometries were truncated from the starting crystal structure. These calculations were performed using the Turbomole software (version 7.1) at the TPSS/def2-SV(P) level of theory. The optimization process were sped up by expanding the Coulomb interactions in an auxiliary basis set, the resolution-of-identity approximation. The calculated charges are listed in Tables S1 and S2.

The metal sites were treated by a non-bonded model with restraints between the metal and the ligands. This is necessary to keep the structure of the Zn cluster intact. For the restraints, we used the metal–ligand distances observed in the crystal structure (averaged over the two subunits) and the force constants listed in Table S3. The latter were obtained from TPSS/def2-SV(P) frequency calculations of the optimized models in Figure S1, calculated by the method of Seminario and averaged over interactions of the same type.

The setup of the MD simulations is similar to that in our recent work. The enzyme was solvated in a periodic truncated octahedral box of TIP3P water molecules, extending at least 12 Å from the solute using the leap program in the Amber suite. The final system contained 38465 atoms. After solvation, we performed 1000 cycles of minimization, with the heavy atoms of the protein restrained towards the starting structure with a force constant of 100 kcal/mol/Å$^2$. This was followed by a 20 ps constant-volume and a 20 ps constant-pressure equilibration with the same restraints, but a force constant of 50 kcal/mol/Å$^2$. Finally, the system was equilibrated for 1 ns without any restraints, followed by a 100 ns production simulation, during which coordinates were sampled every 10 ps.

The temperature was kept constant at 300 K using Langevin dynamics with a collision frequency of 2 ps$^{-1}$. The pressure was kept constant at 1 atm using Berendsen’s weak coupling isotropic algorithm with a relaxation time of 1 ps. Long-range electrostatics were handled by particle-mesh Ewald summation with a fourth-order B spline interpolation and a tolerance of 10$^{-5}$. The cut-off radius for
Lennard–Jones interactions was set to 8 Å. All bonds involving hydrogen atoms were constrained to their equilibrium values using the SHAKE algorithm, allowing for a time step of 2 fs during the simulations.

The root-mean-square deviation (RMSD) and root mean square fluctuation (RMSF) was calculated with the AMBER *cpptraj* module, analyzing trajectories with coordinates saved every 10 ps and using the crystal structure as the reference. The reported values are averages over these 10 000 sets of coordinates.

3. Results and Discussion

Among the protons of the substrate analogue, only three of them (H_R, H_S and H_O) can directly participate in the catalytic reaction and only one of them (H_O) can be exchanged non-catalytically by deuterium (atoms names are shown in Figure 1). In other words, H_O is an alcoholic proton and can be exchanged by a deuterium from D_2O in the reaction medium. On the other hand, H_R and H_S are connected to a sp^3-carbon atom (C1) and cannot be exchanged by a solvent deuterium non-catalytically. Therefore, we suppose that H_O has been exchanged by a deuterium atom before entering the enzyme active site and after that, the enzyme exchanges H_S by this deuterium.

We used three different QM-cluster models to study the proton exchange mechanism. The first model (M1; Figure 1a and b) is the smallest one and the model of the most important residues (Glu-99 and Glu-172) in M1 are small and this makes it very rigid. Our results showed that this rigid model cannot perform the reaction (some of the proton transfers could not be performed or had too high activation energies; a full account of the M1 results are given in the Supporting Information ). Therefore, we tried to investigate the reaction mechanisms, considering the experimental fact that there is a flexible loop including residues 152–159 over the active site in the crystal structure of GlxI, which is closer to Glu-172 than to Glu-99. Therefore, we improved the model by giving more flexibility to the Glu-172 residue, by releasing the fixed atom of this residue. The results for this flexible M1 model showed that there is no way for HOC-SG to exchange its protons if the alcoholic proton is directed towards Glu-99. However, if the hydroxymethyl proton instead is directed towards the more flexible Glu-172
residue, we find a reaction path for exchange of the Hs proton by deuterium, in accordance with the experimental findings (i.e. that the product will always have the deuterium in the S position). The flexible M1 model showed that Glu-172 flexibility is important for accurately describing the reaction mechanism. This flexibility is better accounted for in the M2 and M3 models, in which the two active-site glutamate groups are extended. Therefore, we focus on the results for the M2 and M3 models, which are more reliable. The results for the rigid and flexible M1 models are described in the Supporting Information.

3.1 Results with M3 QM-cluster model

As described in the Method section and shown in Figure 1, the M2 model included the entire glutamate residues and also the backbone of the previous and following residues. We also tried a slightly larger model (M3) in which HOH-404 and Thr-101 are also added to M2. We found that models M2 and M3 gave a similar reaction path, although the energy profiles are slightly different (the barriers of M3 are lower than those of M2) indicating that the crystal water and the threonine residue do not change the reaction path but lower the energy barrier. Therefore, we will focus on the largest model (M3). The M2 optimized structures, selected distances of the optimized structures and energy profiles are described in the Supporting Information.

The optimized structures of the Re states of M3 are shown in Figure 1e and 1f. In contrast to the results obtained with models M1, the HO atom has moved from O1 to O5 of Glu-172 in the HOto172 conformation. This proton exchange stabilizes the Re state of HOto172 by 2.0 kcal/mol with respect to Re of HOto99. The abstraction of HO by Glu-172 in Re of HOto172 implies a higher basicity for Glu-172, in accordance with the proposal of Cameron et al. that the displacement of Glu-172 from the Zn ion upon substrate binding, results in a pKₘ shift of its carboxylate group to a better match that of the substrate, which is lowered by the Zn interaction.

Starting from the Re state, there are three possible proton transfers for the HOto99 conformation (HO and HR to Glu-99 and HS to Glu-172; these are represented by arrows in Scheme 5a) but only two possibility for HOto172 (HR to Glu-99 and HS to Glu-172; these are represented by arrows in Scheme
5b). Our calculations showed that H$_O$ cannot move to Glu-99 in the first step for the H$_O$to99 conformation (the proton transfer shown by a red arrow in Scheme 5a). In conclusion, transferring H$_O$ to the glutamates cannot be the first step in the proton exchange mechanism.

On the other hand, the other four proton-transfer reactions turned out to be possible (theses are shown by blue arrows in Scheme 5. We denote the corresponding products of these proton transfers as **IM1**. The optimized structures of the **IM1** states and the energy barriers for production of these states are shown in Figure 2 and 3, respectively. For the H$_O$to99 conformation we found that the height of the energy profiles of the proton abstractions shown in Scheme 5a depends on the direction of H$_O$ (cf. Figure 3 for the profiles). The energy barrier is high (23.8 kcal/mol) when H$_O$ and the abstracted proton are on the same side of the O1–C1–C2–O2 plane (H$_R$ to Glu-99; the proton transfer shown by a dark blue arrow in Scheme 5a), whereas it is low (10.2 kcal/mol) when they are on opposite sides (H$_S$ to Glu-172; the proton transfer shown by a light blue arrow in Scheme 5a). However, for the H$_O$to172 conformation of M3 the barriers of these reactions are almost the same. As shown in Figure 3, the energy barrier of H$_S$ to Glu-172 (the proton transfer shown by a dark blue arrow in Scheme 5b) and H$_R$ to Glu-99 (the proton transfer shown by a light blue arrow in Scheme 5a) are 14.9 and 15.1 kcal/mol, respectively.

Thus, the barrier for the transfer of H$_R$ to Glu-99 in H$_O$to172 is higher than the barrier of H$_S$ to Glu-172 in H$_O$to99 (15.1 vs. 10.2 kcal/mol; the barriers corresponding to the transfers shown by the light blue arrows in Scheme 5). The reason for this is that Glu-172 is already protonated in Re of H$_O$to172 (cf. Figure 1f) and this gives a negative charge on the substrate. Therefore, abstraction of the second proton (H$_R$) by Glu-99 will impose a double negative charge to the substrate, which increases the energy. In addition, the abstracting base in the H$_O$to99 conformation is Glu-172, which is a stronger
base than Glu-99. In contrast, the barrier of H₉ to Glu-99 in the H₀to99 conformation is higher than the barrier of H₅ to Glu-172 in H₀to172 (23.8 vs. 14.9 kcal/mol; the barriers corresponding to the transfers shown by the dark blue arrows in Scheme 5). This, again confirms the higher basicity of Glu-172, which is due to its different environment inside the enzyme. The different environments of the glutamates will be discussed in the MD subsection.

Previous calculations have suggested that the role of the Zn ion in the reaction of the normal substrate was to electrostatically stabilize the enediolate intermediate, thereby lowering the energy of proton transfer and or by stabilizing the developing negative charge on O2 of the enediolate intermediate.¹⁶,⁵⁷ The ion plays the same role in the present reaction. When going from Re to IM1, the single C1–C2 bond is shortened from 1.53 to 1.38 Å and the double C2=O2 bond is elongated from 1.22 to 1.29 Å (cf. Table S5 and S6) in both conformations. In addition, the increased negative charge on O2 (from −0.61 in Re to −0.83 in IM1 of H₀to99 and from −0.61 in Re to −0.81 in IM1 of HOto172) leads to a stronger coordination of O2 to the Zn atom (the Zn–O2 distances are 2.28 and ~2.07 Å for Re and IM1, respectively).
Figure 2. **IM1** states for model M3. (a) Hᵣ to Glu-99 in Hₒ99 (b) Hₛ to Glu-172 in Hₒ99 (c) Hᵣ to Glu-99 in Hₒ172 (d) Hₛ to Glu-172 in the Hₒ172 conformation. Selected distances are shown in Å.

Figure 3. Calculated energy profiles of transferring Hᵣ to Glu-99 and Hₛ to Glu-172 in model M3. The energy profiles were obtained from relaxed potential-energy scans with a single fixed distance.

Considering the barriers in Figure 3, we discard the high-energy proton abstraction (Hᵣ by Glu-99; the proton transfer shown by a dark blue arrow in Scheme 5a) and concentrate on the low energy one (Hₛ by Glu-172; the proton transfer shown by a light blue arrow in Scheme 5a) in Hₒ99 but we study both possible proton abstractions in the Hₒ172 conformation (Hₛ by Glu-172 and Hᵣ by Glu-99).

In the Hₒ99 conformation of M3, after moving Hₛ to Glu-172 (cf. Figure 2b for the resulting **IM1**), the next possible proton transfer is to move Hₒ to O₄ of Glu-99 (the straight arrow in Scheme 6). Our
results show that this transfer is not possible, because the proton returns to the starting point after releasing any bond constraints. We also tried to directly move H$_S$ to O1 in the IM1 (the curved arrow in Scheme 6). However, we found that this transfer is also not possible. Thus, we did not find any possible reaction path for the H$_{Oto99}$ conformation. The same result was obtained also with model M2.

As was mentioned before, according to the barriers summarized in Figure 3, we study the reactions from both IM1 states of the H$_{Oto172}$ conformation (the structures shown in Figure 2c and 2d; It is notable that H$_O$ has returned from Glu-172 to O1 in both IM1 states of this conformation). In the second IM1 of H$_{Oto172}$ (IM1 of H$_S$ to Glu-172; Figure 2d) we moved H$_O$ to O5 of Glu-172 (the transfer shown by a red arrow in Scheme 7), but H$_S$ returned to C1, giving the Re state of H$_{Oto172}$. Thus, there is no reaction path from the second IM1 of H$_{Oto172}$.

In the first IM1 of H$_{Oto172}$ (IM1 of H$_R$ to Glu-99; Figure 2c), we moved H$_O$ to Glu-172, but it returned to the starting point on O1 (the transfer shown by a blue arrow in Scheme 8a). However, we found a path from the first IM1 when we tried to directly transfer H$_R$ to O1 (the transfer shown by a green arrow in Scheme 8a). In this transfer, H$_O$ moved simultaneously to Glu-172, producing IM2 (cf. Figure 4a or Scheme 8b). Finally, we transferred H$_O$ to C1 in IM2 (the transfer shown by a green
arrow in Scheme 8b) producing Pr (Figure 4b). The energy profile for the reaction path (moving first H_R to Glu-99, then H_R to O1 and finally H_O to C1) in the H_O to172 conformation is shown in Figure 5. This is the only reaction path found for both models M2 and M3. It exchanges H_S, but not H_R, with H_O (which may be exchanged by a deuterium before entering the active site). Exchanging H_S by a deuterium is in accordance with the experimental observation.\textsuperscript{19} These findings are also in line with the results of Landro et al.,\textsuperscript{5} which showed by \textsuperscript{1}HNMR analysis a non-stereospecific proton abstraction by the glutamates, but a stereospecific proton delivery to the si face of C2 (the face which is directed to Glu-172). Our results show that both glutamates can abstract protons (H_S or H_R), but deuterium can only be added by Glu-172 to only one face of C1.

\begin{center}
\textbf{Scheme 8.} The proton transfers (a) from the second IM1 and (b) IM2 of H_O to172.
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We could not find any reaction path from the H_O to99 conformation in the QM-cluster models. In fact, the H_O to99 conformation of the reactant does not seem to be stable in the active site of the enzyme. In a detailed discussion in our previous study,\textsuperscript{16} based on the experimental observation that the binding an inhibitor displaces Glu-172 but not Glu-99 from the active-site Zn ion\textsuperscript{12} and theoretical results of Richter and Krauss,\textsuperscript{13} which showed that if the two glutamate ligands are free to move during the optimization, a protonated Glu-172 will dissociate from the zinc ion, we concluded that in the crystal structure, Glu-172 abstracted a proton from the substrate and dissociated from Zn. Our current results show that this partially unstable conformation (H_O to99) does not lead to any reaction path.
3.1.1 Comparison of the results of the various QM-cluster models

The results coming from the flexible M1 model (discussed in the Supporting Information) show that Glu-172 flexibility needs to be accounted for in order to accurately describe the reaction mechanism. This is one of the most important results in this study. However, M1 is very small and the optimized geometries in the absence of any constraint in Glu-172 might not be accurate. To check the accuracy of the geometries obtained with the flexible M1 model, we compared some selected distances of three states of this model (Re of H$_{Oto172}$, IM1 of H$_{R}$ to Glu-99 in H$_{Oto172}$, and Pr; these state are the common states of the models present in reaction profile on Figure 5) with the same states in M2 and M3 in Error! Reference source not found.. In addition, we superimposed the corresponding states
of M1 and M3 (cf. Figure 6). According to Table 1, there is no significant difference between the distances of the various states in different cluster models. According to the superimposed structures in Figure 6 the IM1 and Pr states of M1 and M3 fit nicely, whereas the carboxylate groups of both of the glutamates have somewhat different positions in Re and IM1' (we use IM1' for the reactant state in which H0 is moved on Glu-172, because in Re of H0to172 conformation of M2 and M3 H0 has moved from O1 to O5 of Glu-172, giving directly the IM1’ state). According to the results summarized in Error! Reference source not found. and Figure 6, we can conclude that the geometries and the important prediction from the flexible M1 (the higher flexibility of Glu-172) are reasonable. In addition, the flexibility of Glu-172 with respect to Glu-99 is confirmed by the MD simulations, discussed in the next subsection.

Table 1. Selected distances of Re, IM1 of Hr to Glu-99 in H0to172, and Pr in the flexible M1, M2 and M3.

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Zn-CB-172  4.94  7.05  5.17  5.20  4.88  4.65  4.67  4.81  4.67  4.68

M1-F refers to the Flexible M1 model.
a The extra atoms of model M3 were omitted.
b Other important results are that i) Glu-172 is a stronger base than Glu-99 and that ii) H_0to99 conformation does not led to any proton exchange reaction. Both of these results are confirmed by the results of the bigger cluster models. On the other hand, some details of the predicted reaction profile from the flexible M1 model (Figure S9) are different from those obtained with M2 and M3. Therefore, our proposed mechanism (Scheme 9) and the energy profile (Figure 5) are based on the results of the biggest model, which should be more reliable.

Figure 6. Superimposition of (a) Re on Re (b) Re on IM1' (c) IM1 on IM1 and (d) Pr on Pr of M3 and M1. M1 and M3 states are shown by tubes and ball and stick, respectively. Zn are shown by ball in both models.
From the results of the QM-cluster models, we can conclude that the proton-exchange mechanism depends on the different actions of the active-site glutamates; in particular, Glu-172 is more flexible and more basic than Glu-99. To confirm this suggestion, we studied the flexibility of the glutamate residues in MD simulations. The results are discussed in the next subsection.

3.2 MD results

We have run two 100-ns MD simulations of GlxI with the substrate analogue bound in either the H_{Oto99} or H_{Oto172} conformations. To analyze the flexibility of the two active-site glutamate residues, we calculated the mass-weighted RMSD and RMSF values (with the crystal structure as the reference) of these two residues during the simulations. The results in Table 2 show that the RMSD values of Glu-172 in both active sites (we simulated the full dimeric protein) and with the both conformations of the substrate are 2–3 times larger than those of Glu-99 (0.85–0.94 Å, compared to 0.29–0.38 Å).

The RMSF values of Glu-172 in both active sites and with the both conformations of the substrate are also larger than those of Glu-99. These show that Glu-172 is much more flexible than Glu-99. This can be understood by looking at the position of the glutamates in the crystal structure, as is shown in Figure 7. It can be seen that both residues are buried in the protein (both residues have a vanishing solvent-accessible surface area), but Glu-172 is somewhat closer to the surface of the protein and Glu-99 is closer to the center of the protein. It is quite natural that a residue nearer the surface is more flexible than a buried one.

Table 2. Mass weighted RMSD and RMSF values (in Å) of the two active-site glutamate residues for the H_{Oto99} and H_{Oto172} conformations of HOC-SG. Glu-99A and Glu-172B belongs to active site 1 and Glu-99B and Glu-172A belong to active site 2.

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<td>Active site 2</td>
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<td>Glu-99B</td>
<td>Glu-172A</td>
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To check the active site glutamate environments, we calculated RMSF of the whole enzyme in both conformations of the substrate and summarized them in Figure 8. According to the RMSF results shown in Figure 8, residues 135–145 is the part of the enzyme with highest RMSF in the H₀to99 conformation. The experimentally suggested flexible loop (residues 152–159) is not the most flexible part of the enzyme in the H₀to99 conformation. However, the average RMSF value of this loop is more than the average RMSF of the whole enzyme (11.3 vs. 9.8 Å) in the H₀to99 conformation (cf. Figure 8a). The results are somewhat different in the H₀to172 conformation. The average RMSF of the loop 152–159 is higher than the average RMSF of the whole enzyme (15.0 vs. 11.9 Å) in chain A but it is the most flexible part of chain B (cf. Figure 8b). Residues 61–66 have the highest RMSF in chain A in the simulation of the H₀to172 state. We showed the position of these flexible loops (residues 61–69, 135–145 and 152–159) along with the two glutamate residues in Figure 9. According to Figure 9, loops 135–145 and 152–159 are much closer to Glu-172 than Glu-99. However loop 61–69 are almost at a same distance from both glutamates. These show that Glu-172 environment is more flexible than Glu-99 environment.
Figure 8. The calculated RMSF values of chain A and B of GlxI (a) in Hoto99 and (b) Hoto172 conformation.
Figure 9. The flexible loops around the glutamates inside GlxI. Loop 152-159 is shown with wireframe to be distinguished from the other loops. **This figure is hard to understand. You must include the whole protein and then perhaps colour code the loops and the Glu residues.** Perhaps, this can be done already in Figure 6 (if the remaining protein is coloured gray. I enclose an attempt: 61–69 are magenta, 135–145 are yellow and 152–159 are red. Glu-99 is cyan and Glu-172 is green, as before.

As stated by Cameron *et al.*\(^{12}\) and as our QM-cluster results show, Glu-172 is a stronger base than Glu-99. The higher basicity of Glu-172 cannot be due to a direct induction effect from the neighboring residues because the neighboring residues of both Glu-99 and Glu-172 are similar (they are all hydrophobic residues: Leu-98, Leu-100, Ile-171 and Ile-173). The most important cause for higher basicity of Glu-172 can be that it dissociates from the Zn ion, as was suggested by Cameron *et al.*\(^{12}\) **This is not clearly mentioned above. For which states does it dissociate? This should be**
mentioned in section 3.1. In other words, dissociation from a positive ion (the Zn ion) induces more negative charge and therefore more basicity to the acetate group of Glu-172. As our MD results show, Glu-172 has a more flexible environment than Glu-99. This flexibility allows dissociation of Glu-172 and in consequence increases its basicity. We also checked this hypothesis by comparing average RMSF values of the backbone atoms of the four nearest neighbors of the glutamates (i.e. the backbone of residues 95–103 for Glu-99 and 168–176 for Glu-172). The results are summarized in Table 3. The side chains of the glutamates are connected to the backbone and the more flexible backbone allows the side chain to dissociate from the Zn ion. According to Table 3, the neighboring backbone of Glu-172 is more flexible than that of Glu-99 in both simulations and both active sites.

Table 3. Average RMSF values of backbone of 4 nearest neighbors of the glutamates.

|  | 
|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
|  | Héoto99  | Héoto172  |
|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| RMSF/Å  | 7.80  | 8.00  | 7.52  | 8.64  | 11.27  | 12.00  | 7.38  | 9.14  |

4. Conclusion

To gain some understanding of stereospecific deuterium exchange of HOC-SG by GlxI, we have performed DFT calculations on active-site QM-cluster models. With the minimal M1 model, the height of the energy barriers for proton abstraction from C1 depends on the direction of H_O: The reaction barrier is high if H_O and the abstracted proton are on the same side of the O1–C1–C2–O2 plane but it is lower if H_O and the abstracted proton are on opposite sides. However, this effect vanished in the Héoto172 conformation with the larger M3 model. The small model with the same flexibility for the two active-site glutamate residues could not explain the unusual stereospecificity of GlxI. However, if we do not constrain any atom in Glu-172 or use a bigger model, including the entire glutamate residues as well the backbone of the surrounding residues (models M2 and M3), we find a catalytic reaction mechanism for the exchange of the H_S proton by a deuterium as was observed
by experiments.\textsuperscript{19} It is possible only if the substrate H\textsubscript{O} is directed towards the more flexible Glu-172 residue, whereas no such reaction is possible if H\textsubscript{O} is directed towards Glu-99. The reason for this is that Glu-172 is more flexible and more basic than Glu-99. These suggestions were confirmed by MD simulations, which showed that Glu-172 is 2–3 times more flexible than Glu-99. This flexibility allows it to temporarily dissociate from the active-site Zn ion.

We found that models M2 and M3 gave a similar reaction path, although the energy profiles are slightly different (the barriers of M3 are lower than those of M2) indicating that the crystal water HOH-404 and the Thr-101 residue do not change the reaction path but lower the energy barrier.

Based on the DFT calculations with the largest M3 QM-cluster model, we propose the mechanism shown in Scheme 9 for the proton exchange of HOC-SG. Before binding, HOC-SG exchanges non-catalytically its alcoholic proton with a deuterium from the solvent. Next, the deuterium is abstracted by Glu-172 and H\textsubscript{R} moves to Glu-99. After that, H\textsubscript{R} is transferred from Glu-99 to O1, leading to the simultaneous transfer of the deuterium from O1 to Glu-172. Finally, the deuterium moves from Glu-172 to C1 producing the proton-exchanged product. Finally, the product dissociates from the active site, allowing the enzyme to start a new catalytic cycle. According to this mechanism, the product will always have the deuterium in the $S$ position, in accordance with the experimental findings. It is the higher flexibility of Glu-172 that explains the unusual stereospecificity of GlxI.
Scheme 9. The proposed mechanism for proton exchange of HOC-SG by GlxI.

Supporting Information Available:

MM charges, force constants and restraints used for the Zn clusters, full discussion of M1 results, selected distances of the various states in M1, M2 and M3, illustration of stationary states and energy profiles of M2, Cartesian coordinates of stationary points in the QM-cluster models.

Acknowledgements

This investigation has been supported by grants from the Swedish research council (project 2014-5540) and from the University of Kurdistan. The computations were performed on computer resources provided by the Swedish National Infrastructure for Computing (SNIC) at Lunarc at Lund University and at HPC2N at Umeå University.

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(40) Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D.; Imey, R. W.; Klein, M. L. Comparison


Synopsis

Glyoxalase I converts both enantiomers of its natural substrate to only one enantiomer of its product. To find some clues to the old problem of unusual stereospecificity of GlxI, we studied the stereospecific proton exchange of the hydroxymethyl proton of HOC-SG by GlxI. Our results indicate that the unusual stereospecificity of GlxI is caused by the more flexible environment of Glu-172 compared to that of Glu-99.
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Higher flexibility of Glu-172 explains the unusual stereospecificity of Glyoxalase I