Catalytic Mechanism of Glyoxalase I: A Theoretical Study

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Abstract: Hybrid density functional theory is used to study the catalytic mechanism of human glyoxalase I (GlxI). This zinc enzyme catalyzes the conversion of the hemithioacetal of toxic methylglyoxal and glutathione to nontoxic (*S*)-D-lactoylglutathione. GlxI can process both diastereomeric forms of the substrate, yielding the same form of the product. As a starting point for the calculations, we use a recent crystal structure of the enzyme in complex with a transition-state analogue, where it was found that the inhibitor is bound directly to the zinc by its hydroxycarbamoyl functions. It is shown that the Zn ligand Glu172 can abstract the substrate C1 proton from the *S* enantiomer of the substrate, without being displaced from the Zn ion. The calculated activation barrier is in excellent agreement with experimental rates. Analogously, the Zn ligand Glu99 can abstract the proton from the *R* form of the substrate. To account for the stereochemical findings, it is argued that the *S* and *R* reactions cannot be fully symmetric. A detailed mechanistic scheme is proposed.

I. Introduction

The glyoxalase system catalyzes the glutathione-dependent conversion of toxic methylglyoxal to D-lactate.[1] The system consists of two enzymes, glyoxalase I (GlxI) and glyoxalase II (GlxII). GlxI converts hemithioacetal (formed from methylglyoxal and glutathione) to *S*-D-lactoylglutathione, while GlxII takes the latter as a substrate and converts it to D-lactate and glutathione (Scheme 1). Increase in methylglyoxal can produce toxic effects by reacting with RNA, DNA, and proteins. The glyoxalase system has also been suggested as a target for anticancer and antimalarial drugs.[2]

Human GlxI, the subject of the present study, is a homodimer of 43 kDa and requires Zn^{2+} for its catalytic function. The enzyme shows, however, significant catalytic activity with other divalent ions, like Mg^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, and Ca^{2+}.[3] Interestingly, the *Escherichia coli* enzyme requires Ni^{2+} for catalysis (also Co^{2+}, Mn^{2+}, and Cd^{2+} show activity), but is completely inactive with Zn.[4] The X-ray crystal structures of both human[5] and *E. coli*[6] GlxI have been solved recently. The overall fold of the two enzymes is very similar, but the metal coordination at the active site differs somewhat. In the *E. coli* enzyme, the coordination around the Ni ion is close to a perfect octahedron, with four protein residues (His5, His74, Glu56, and Glu122) and two water molecules. This geometry is not changed upon substitution of the Ni ion by Co and Cd but is significantly disturbed by Zn substitution. The active site there adopts a trigonal bipyramidal coordination, which might explain the inactivation of the Zn-substituted protein.

Human GlxI has been crystallized with several inhibitors. The structure of the enzyme in complex with *S*-benzylglutathione (B-GSH) shows the Zn coordination to be square pyramidal, consisting of four protein residues (Gln33, Glu99, Glu172, and His126) and a water molecule.[5] The structure also shows the B-GSH bound in the second coordination shell. The structure with *S*-(*p*-nitrobenzyloxycarbonyl)glutathione (NBC-GSH) is very similar[7] but with an additional water molecule occupying the sixth position, completing the octahedral coordination (Figure 1A). The most interesting structure, however, is the one in complex with *S*-[*N*-hydroxy-*N*-(*p*-iodophenyl)carbamoyl]-glutathione (HIPC-GSH).[7] This is a transition-state analogue that mimics the enediolate intermediate believed to be formed in the reaction of GlxI. The substrate analogue there is bound directly to the Zn by its carbamoyl oxygens in a cis conformation (Figure 1B), displacing the two water molecules found in the other structures.

It is generally accepted that the catalytic reaction of GlxI proceeds via an enolate intermediate, resulting from a base abstracting a proton from the substrate. Cameron et al. proposed that this base is the Zn ligand Glu172, on the basis of the HIPC-GSH crystal structure[7] and mutagenesis experiments (mutation of Glu172 abolishes catalysis[8]). In the HIPC-GSH structure, Glu172 was interestingly found to be displaced from the Zn (Zn−O distance 3.3 Å) as compared to the other structures. Cameron et al. suggested that the displacement of this residue from the Zn upon substrate binding results in a pK_a shift of the carboxylate group to more match the substrate pK_a, which is

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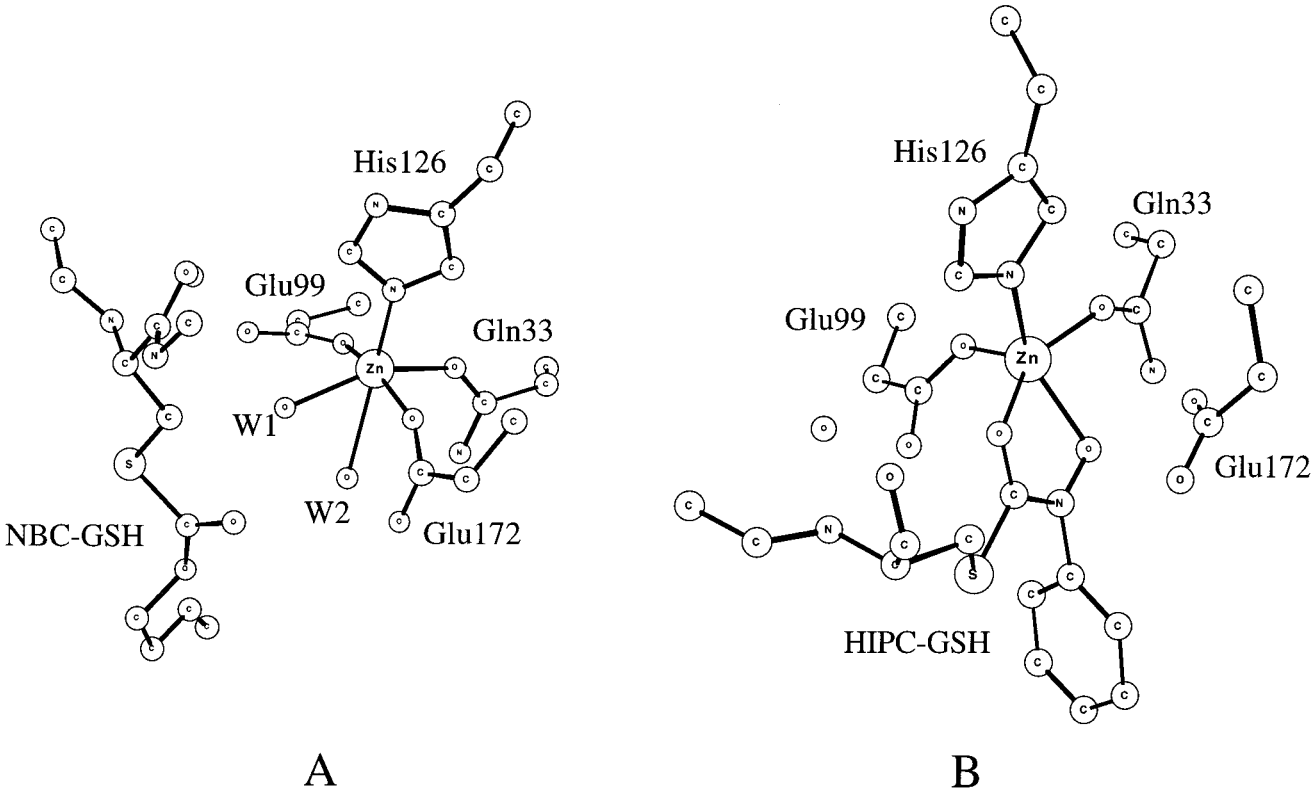
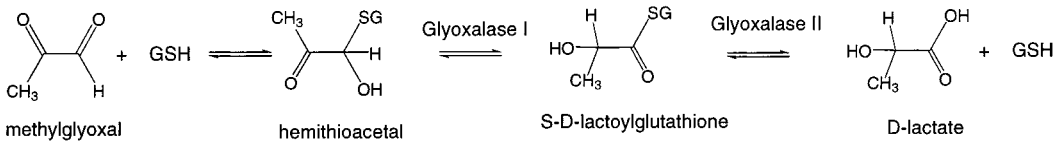


Figure 1. X-ray crystal structures of the active site of GlxI in complex with (A) NBC-GSH and (B) HIPC-GSH.

Scheme 1. Reactions Catalyzed by the Glyoxalase System



lowered by the Zn interaction. Their suggested reaction mechanism for GlxI is depicted in Scheme 2. As can be seen from the scheme, not much is known about the steps after the initial proton transfer. In particular, it is unclear whether other residues are involved in the catalytic process or whether the Glu172 residue alone performs all the proton-transfer steps necessary for the reaction.

Recently, Åqvist and co-workers applied a combination of molecular dynamics (MD) simulations, free energy perturbation (FEP), and empirical valence bond (EVB) techniques to study the energetics of the proposed first step of the GlxI reaction, the proton transfer from the substrate C1 carbon to Glu172.[9] They concluded that the main catalytic role of the Zn ion is to electrostatically stabilize the enolate intermediate, thereby lowering the activation free energy of proton transfer. The Zn-catalyzed activation free energy was calculated to be ca. 13 kcal/mol, considerably lower than the estimated uncatalyzed activation free energy (ca. 22 kcal/mol).

GlxI possesses a remarkable feature. Using ^{1}H NMR, Landro et al. demonstrated that GlxI can process both the *S* and *R* diastereomers of the substrate, with nearly equal efficiencies, by direct nonstereospecific proton abstraction.[10] Both the *S* and *R* substrates give, however, the same chirally deuterated product, indicating stereospecific proton delivery. The authors concluded that the protein base may be positioned in such a way that it can effect proton abstraction from either diastereomer, yielding the *cis*-enediol intermediate. The active site would then reorient to render the protonated base accessible to only one face of the enediol intermediate. However, in light of the recent HIPC-GSH X-ray structure, it is clear that the proposed base (Glu172) can only abstract a proton from the *S* substrate. Cameron et al. proposed that the base in the case of the *R* substrate might be the Glu99 residue, trans to Glu172, although not displaced from Zn.[7] It is difficult to envision how the active site would reorient to make delivery of the proton to C2 possible from only one face, with the two different bases. To account for both the spectroscopic and crystallographic findings, a new mechanism needs hence to be invoked.

In the present work, we have examined the full catalytic mechanism of GlxI using the hybrid density functional theory (DFT) functional B3LYP.[11] This method has previously been successfully applied to study a number of enzyme mechanisms.[12]

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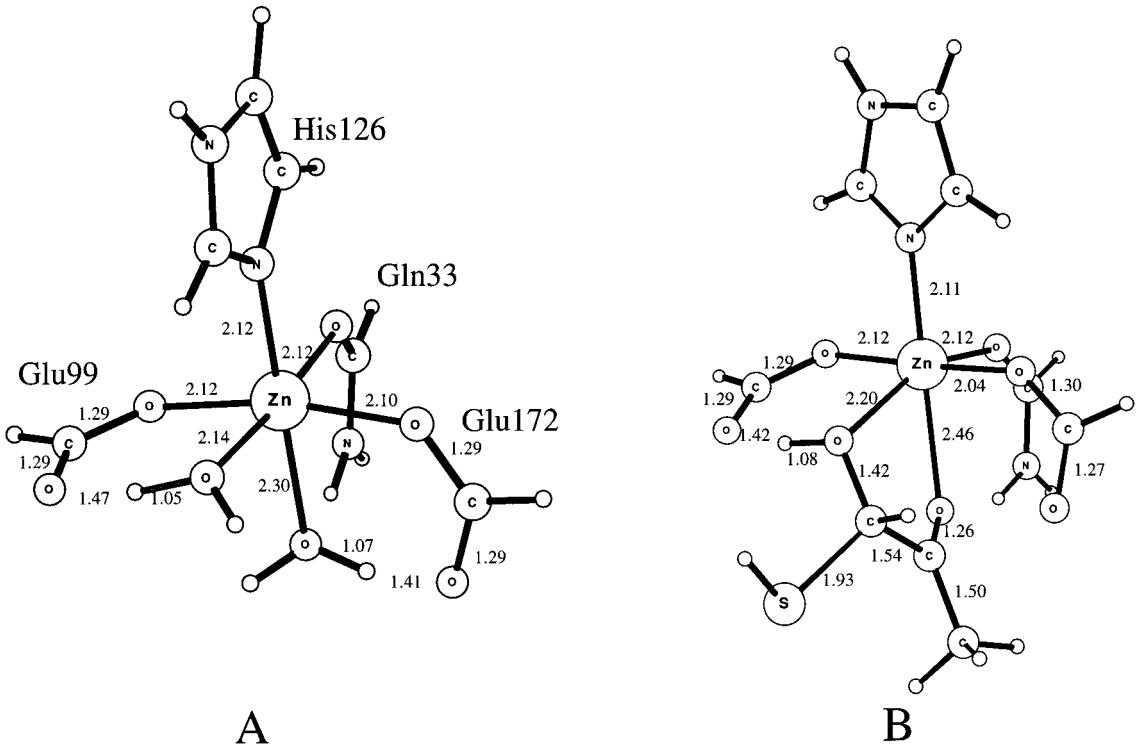
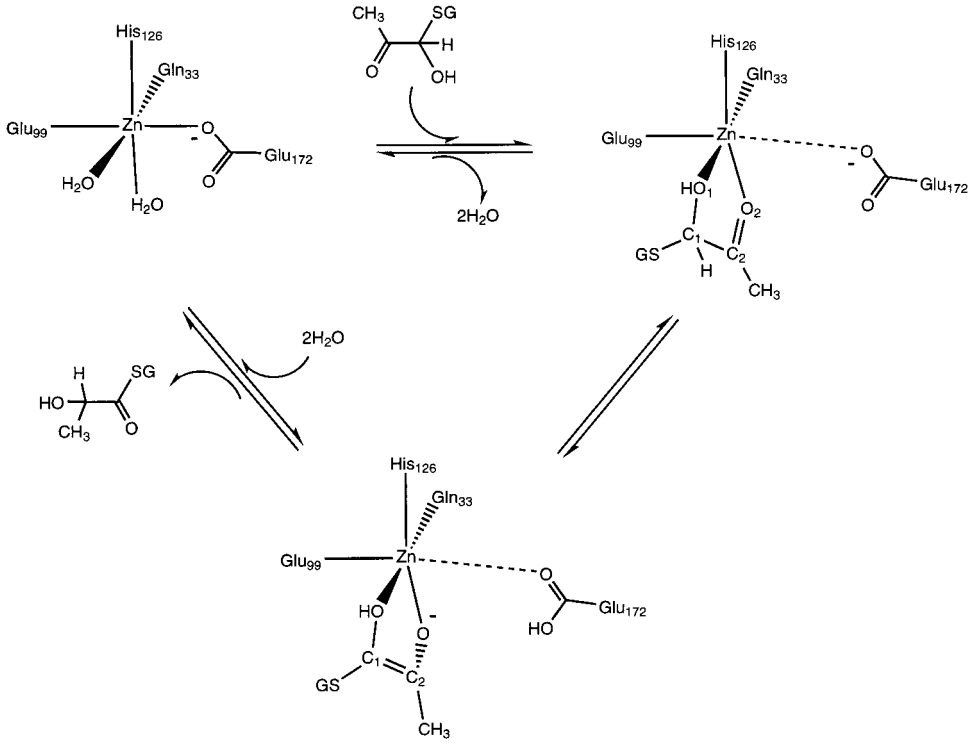


Figure 2. Optimized structure of the GlxI active site with (A) two water molecules and (B) hemithioacetal substrate bound.

Scheme 2. Reaction Mechanism Proposed by Cameron et al.[7]



The inherent accuracy of the B3LYP method can be estimated from benchmark tests, in which the average error in the atomization energies of the G2 set, consisting of 55 small first- and second-row molecules, is found to be 2.2 kcal/mol.[13] The average deviation for bond lengths is less than 0.02 Å, and for angles and dihedral angles less than 1°.

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II. Computational Details

The calculations reported in the present study were carried out with the density functional theory (DFT) functional B3LYP[11], as implemented in the Gaussian94 program package.[14] Geometries were optimized with the ECP double-ζ basis set LANL2DZ. Based on these geometries, more accurate energies were calculated with a larger basis set, 6-311+G(2d,2p). This is a triple-ζ basis set with one diffuse function and two polarization functions on each atom. Vibrational frequencies were calculated at the LANL2DZ level.[15] From the

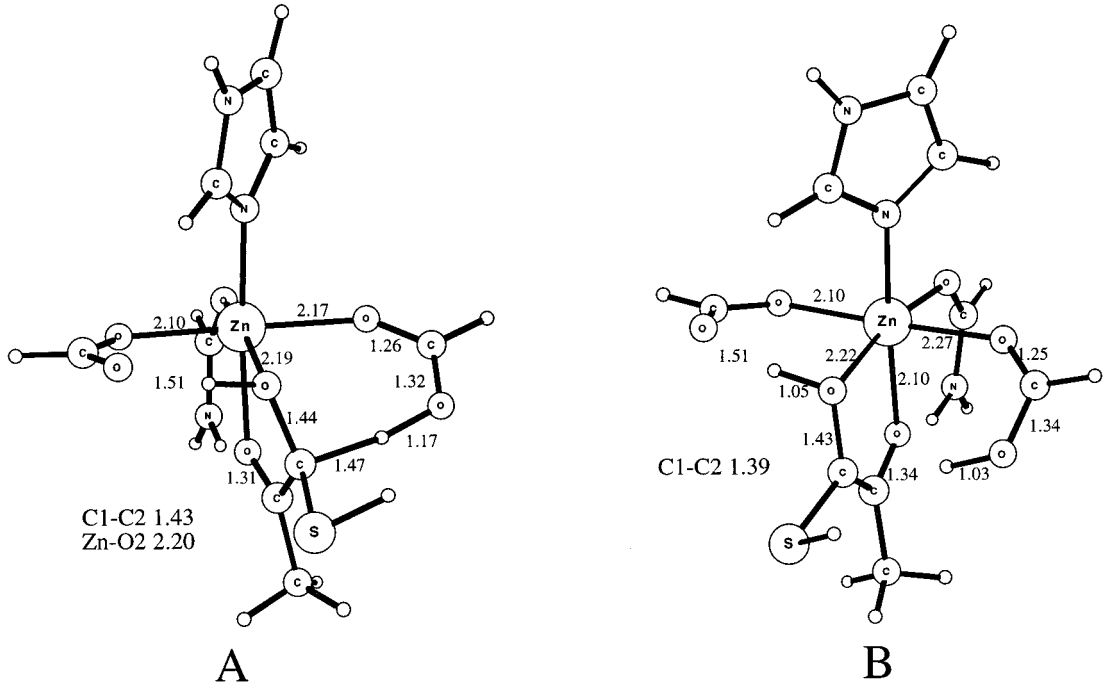

Figure 3. Optimized structure for (A) the transition state (TS1) for the initial proton-transfer step from C1 to Glu172 and (B) the resulting enediolate intermediate.

frequency calculations, zero-point energies (ZPE) and entropy effects were extracted. Vibrational frequencies also provided a control that the stationary points localized are true ones, with no imaginary frequencies for minima and only one imaginary frequency for transition states.

Solvent effects on the energies were calculated by use of the conductorlike solvation model COSMO[16] with the standard dielectric constant of 4. The effects on the reaction energies were found to be rather small (less than 4 kcal/mol). In increasing the size of the basis set from LANL2DZ to 6-311+G(2d,2p), the relative energies were somewhat affected, on the order of 4 kcal/mol.

The zero-point and the entropy effects on the relative energies were found to be <2 kcal/mol.

The energies discussed in the present paper include all the effects mentioned above: basis set corrections, solvent, entropy, and zero-point vibrational effects.

III. Results and Discussion

a. Active-Site Model. When studying a reaction mechanism at the level of theory used in the present study, it is essential to use the smallest models possible of the active site in order to limit the computational time and thereby to be able to test a number of pathways. The models should, however, reflect the basic chemical features of the system correctly.

In our calculations, only the first ligation shell of the Zn was included. This is a reasonable approximation because no other residues have been reported to be directly involved in catalysis. Furthermore, polarization effects from second-shell residues can be argued to be relatively small. For instance, mutation of the

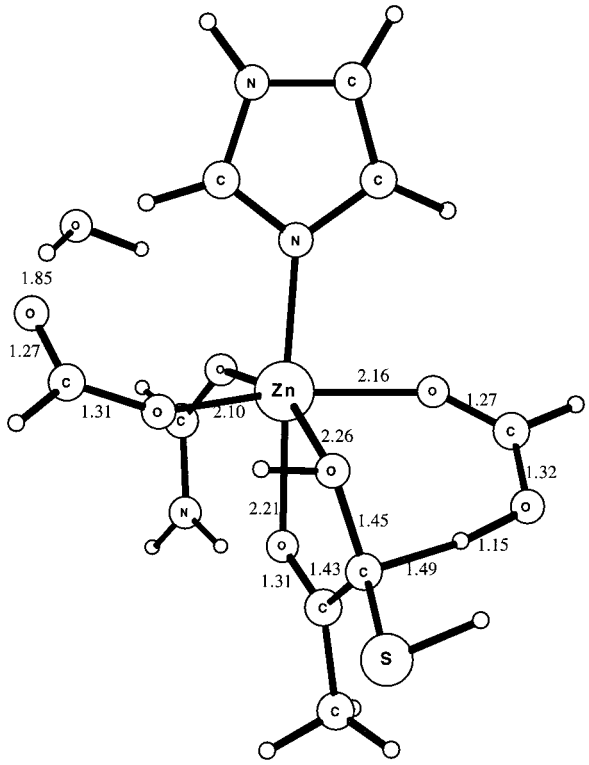

Figure 4. Optimized transition-state structure (TS1) for the initial proton-transfer step for C1 to Glu172, with Glu99 forming a different hydrogen bond.

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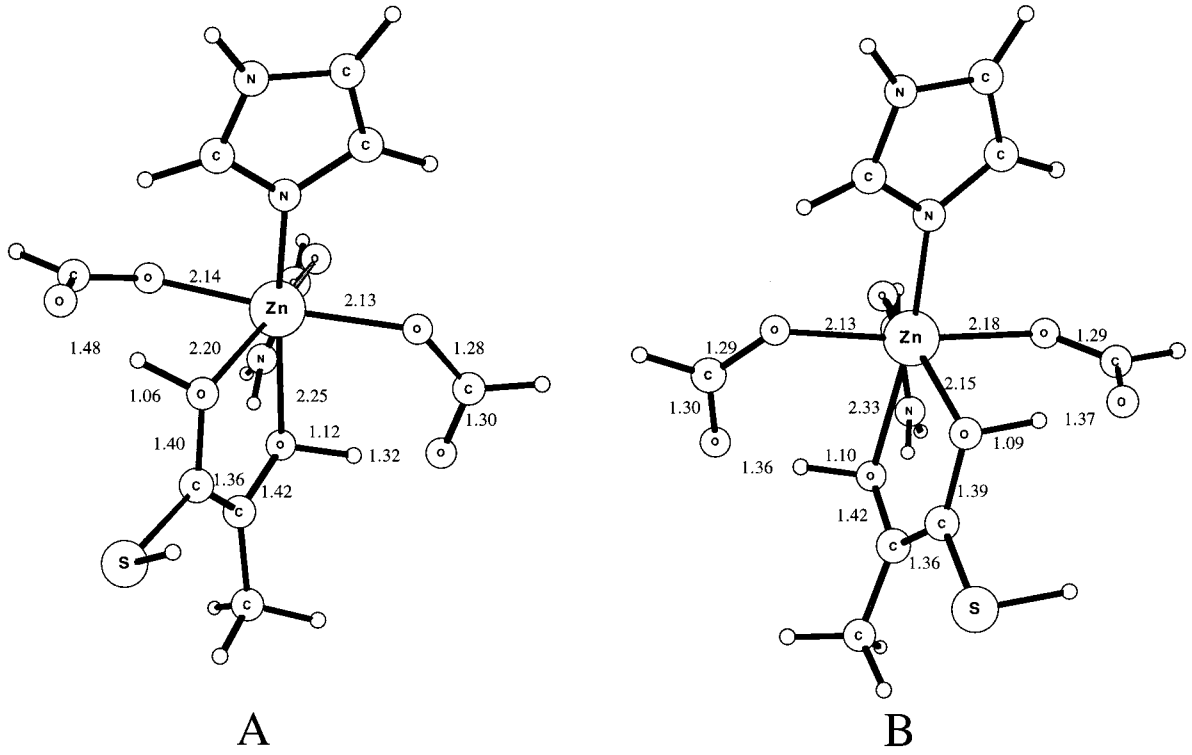

Figure 5. Optimized structure of the *cis*-enediol intermediate. The two isoenergetic structures show the carboxylates hydrogen binding to the substrate in two different ways.

active-site Met157 residue, which is conserved among several GlxI sequences, to a glutamine yielded retained activity.[17] The activity for the M157H and M157A variants decreased modestly by a factor of 5, which corresponds to an energetic effect of less than 1 kcal/mol. We believe, hence, that the error made by using dielectric cavity methods to model the protein environment, assuming homogeneous surrounding, is rather small and should not affect the conclusions drawn about the catalytic mechanism.

The Zn ligands were modeled as follows: histidine by imidazole, glutamates by formates, and glutamine by formamide. For the hemithioacetal substrate, we chose to truncate the molecule at the sulfur of the glutathione (see Figure 2).

In Figure 2, we display the optimized structure of the active-site model with two water molecules (A) or with the substrate (B) bound. We see that the Zn is in both cases in a stable octahedral coordination, in good general agreement with both the NBC-GSH and the HIPC-GSH structures (Figure 1). There is, however, one disagreement. The calculations do not confirm the dissociation of Glu172 upon substrate binding. Clearly, the displacement is not caused by an electronic effect. One likely explanation to the crystallographic observation could be that the bulky benzo substituent of the HIPC-GSH inhibitor simply does not fit in the active site when bound directly to Zn, causing the Glu172 displacement by direct or indirect steric effects. However, due to the size of the model used in our calculations, we cannot completely rule out the possibility that binding of substrate induces a similar steric disturbance at the active site.

As mentioned earlier, the displacement of Glu172 was argued to cause an increase of the pK_a of that residue to match the pK_a of the C1 proton of the substrate, hence making the proton-transfer possible between these groups. But, as we shall see below, the calculated activation energy for the proton transfer is very feasible, without the need to displace Glu172.

Figure 2 shows another interesting feature of the GlxI active site. The carboxylates form short hydrogen bonds (d_{OO} near 2.5 Å) to the water molecules and to the hydroxyl group of the substrate. This is in line with the observation that enzymes that abstract an α-proton commonly harbor this kind of short hydrogen bonds.[18] Because of the symmetry between Glu172 and Glu99, the short hydrogen bond between the substrate and Glu99 (Figure 2B) could equally well be formed to the Glu172 residue. This would not have any implication on the energetics of the reaction.

Finally, Figure 2 also shows that the amino group of the glutamine forms a hydrogen bond to the water trans to the histidine (or the carbonyl oxygen of the substrate), contributing to the overall stability of the octahedral coordination.

b. Proton Abstraction from C1. The first step in the catalytic mechanism of GlxI is proposed to be a proton transfer (PT) from substrate C1 to Glu172. From Figure 2B we can see that the $O_{\epsilon 2}$ oxygen of Glu172 is in a perfect position to abstract the substrate C1 proton (without the need to dissociate the glutamate). Figure 3A shows the optimized transition-state structure (hereafter called TS1) for this reaction. The calculated barrier is 14.4 kcal/mol, in excellent agreement with the known reaction rate ($k_{cat} = 1500\ s^{-1}$, corresponding to an activation barrier of ∼14 kcal/mol) and with the barrier calculated by Åqvist and co-workers (∼13 kcal/mol) using the EVB method.[9] The structure has a single imaginary frequency of 530 cm^{-1}. The critical C−H distance at TS1 is 1.47 Å and the H−O distance is 1.17 Å. From these results, we can conclude that Glu172 is perfectly able to effect the initial proton abstraction step without the need to displace it from the Zn. It appears that the Zn ligation is sufficient to reduce the pK_a of the substrate and make the proton transfer feasible.

Upon proton transfer, the substrate forms an enediolate intermediate. We find this intermediate to be quite high in

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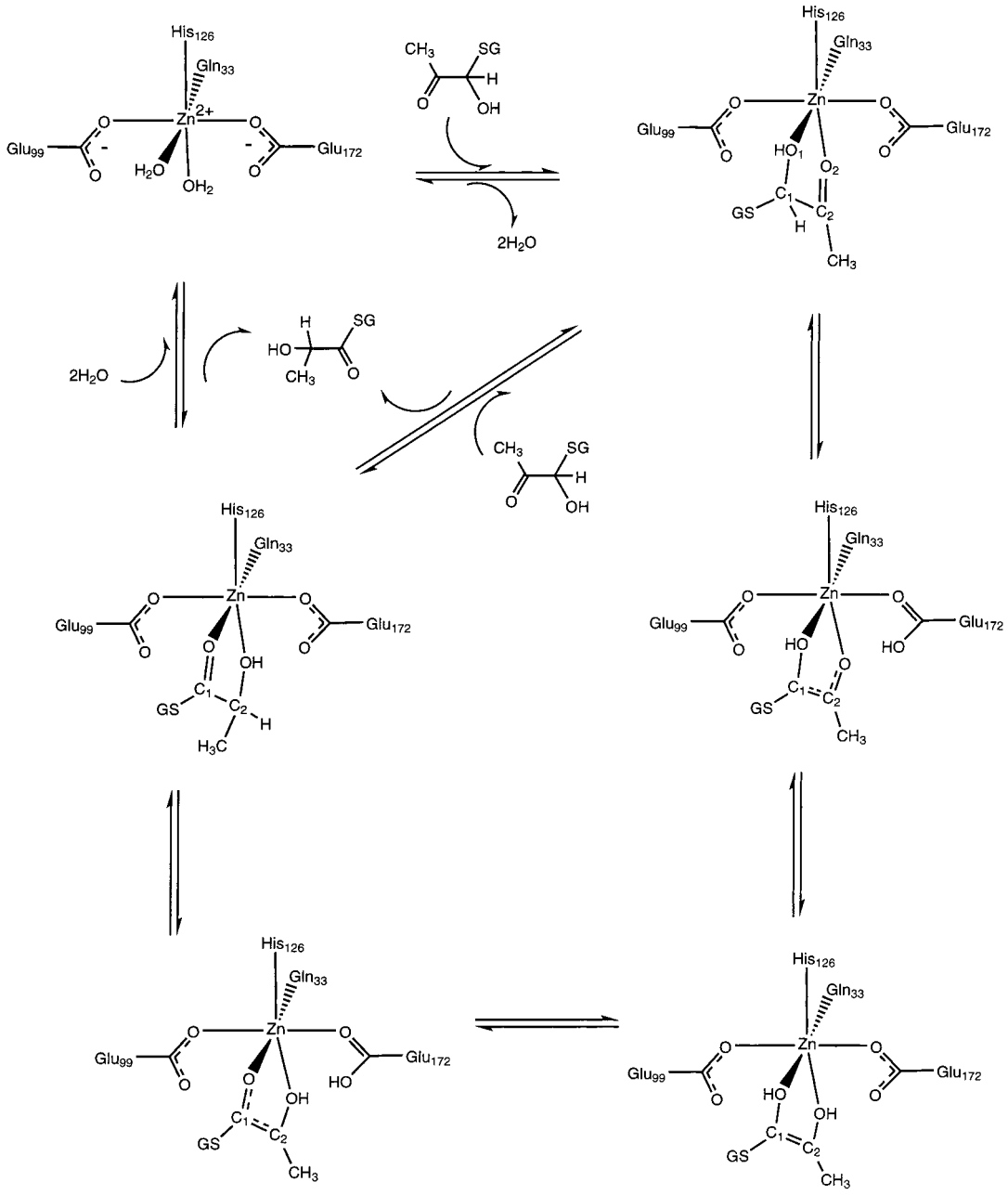
Scheme 3. Proposed Reaction Mechanism for the *S* Enantiomer of the Substrate



energy, only 1.8 kcal/mol lower than TS1, i.e., 12.6 kcal/mol higher than the reactant (optimized structure of the enediolate intermediate is shown in Figure 3B). This is different from the EVB results,[9] where it was found that the step is nearly thermoneutral. It was argued that by reducing the endothermicity of the reaction step (in enzyme as compared to water solution), the associated activation barrier is lowered, as a consequence of the linear free energy relationship correlation between the two. In our calculations, the reaction step is endothermic by 12.6 kcal/mol, and still the activation barrier is the relatively low 14.4 kcal/mol. The discrepancy in the enolate energy between our calculations and the EVB ones could originate from problems in the semiempirical EVB parametrization of the metal site. Another factor that could contribute is our treatment of the surrounding as a homogeneous solution, even though there are no indications of important outside residues from mutation experiments (see above), and there is no significant charge separation as indicated by the small computed dielectric effects.

To test this further, we performed a simple protein solvation investigation, in which we embedded the optimized active-site structures in a point charge electrostatic representation of the protein environment.[19]When the DFT structures, which were optimized without constraints, were docked into the protein, a few atoms from the surrounding (essentially from the neutral residues Gly125 and Phe162) collide with the optimized atoms,

(19) These calculations were performed with the MEAD program. The dielectric constants used were the standard 4 and 80 for protein and water environments, respectively. The details of the procedure are described at length in refs 20−22.

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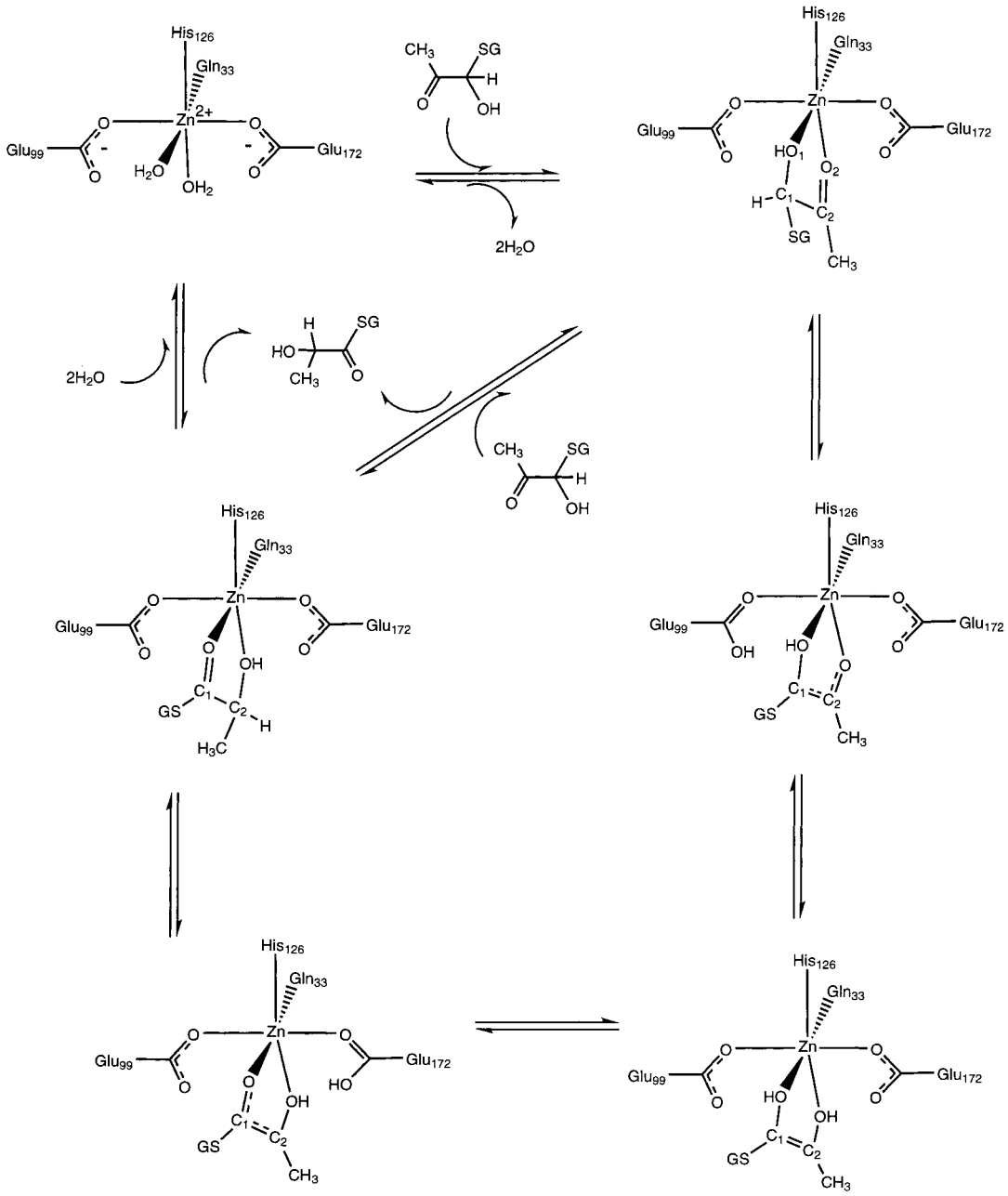
Scheme 4. Proposed Reaction Mechanism for the *R* Enantiomer of the Substrate



especially with the histidine 126 residue, which is rotated differently in the optimized strcutures compared to the X-ray structure (see Figures 1−3). If the partial charges of these atoms are set to zero in the protein solvation calculations, almost no effect (about 1 kcal/mol) on the present energetics was found due to the surrounding charges. This result is in line with previous findings in our group for other enzymes.

In going from hemithioacetal substrate (Figure 2B) to the enolate intermediate (Figure 3B), via TS1 (Figure 3A), we can trace some geometrical changes that reflect the chemistry of this step. The substrate C1−C2 single bond is shortened from 1.54 to 1.39 Å (1.43 Å at TS1) and the C2═O2 double bond is elongated from 1.26 Å to 1.34 Å (1.31 Å at TS1). As a result of this oxygen becoming more negatively charged, the Zn−O2 distance is significantly shortened, from 2.46 to 2.10 Å (2.20 Å at TS1). The opposite is seen for the distance of the Zn to the $O_{\epsilon 2}$ of Glu172: it increases from 2.04 to 2.27 Å (2.17 Å at TS1), as the carboxylate C−O bond acquires more of a double bonding nature.

As seen from Figures 2 and 3, Glu99 forms a short hydrogen bond to the substrate O1, suggesting that this residue might be a candidate for the abstraction of the second proton. This would, however, result in the wrong enantiomer of the product when the proton is delivered to C2 (detailed discussion below). We decided therefore to examine the importance of this hydrogen bond for the energetics of this step, to see whether the initial step can be performed without the hydrogen bond being present.

We made Glu99 rotate away from the substrate and let a water molecule hydrogen bind to it, preventing it from forming the hydrogen bond to the substrate. In Figure 4, we show the optimized transition-state structure for the initial PT transfer after doing these modifications. As seen, the TS structure is virtually identical to the one before. The barrier is slightly higher (less than 3 kcal/mol). The structure and the energy of the enolate intermediate are not significantly changed either (data not shown). We can hence conclude that whether the hydrogen bond between Glu99 and the substrate is there or not will not

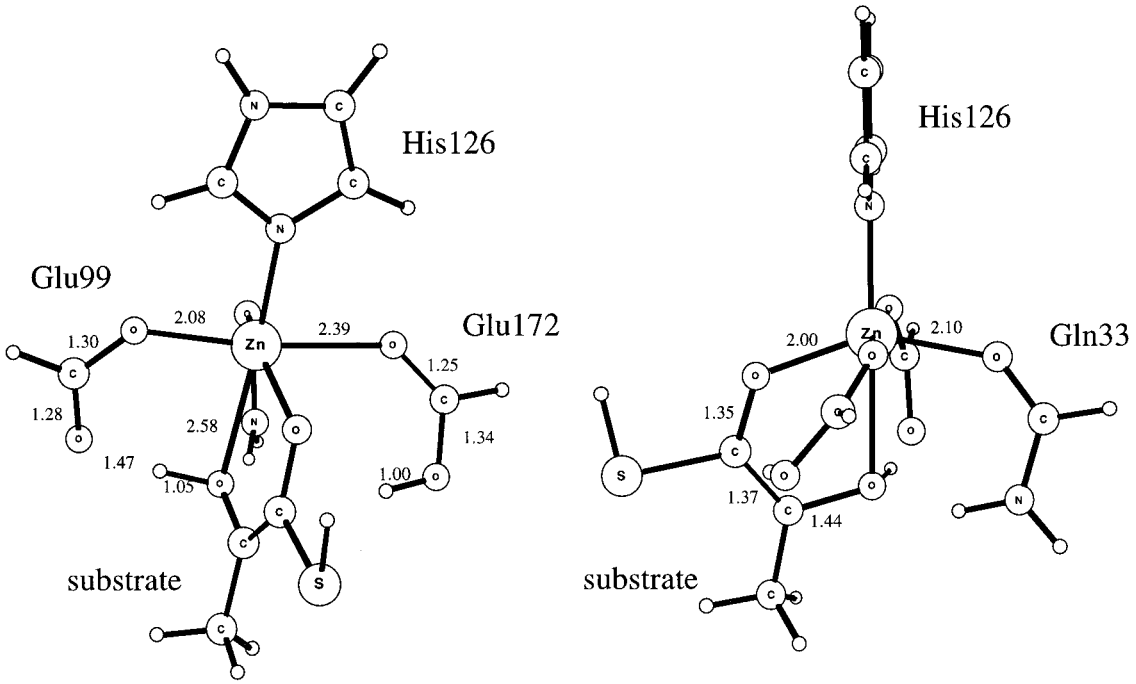


Figure 6. Optimized transition state structure (TS2) for the proton transfer from O1 to C2. For clarity, the structure is shown from two different angles.

significantly change the results presented above for the initial proton abstraction step.

c. Delivery of the First Proton. We saw in the previous subsection that the initial PT step is endothermic by 12.6 kcal/mol, i.e., the enolate intermediate is relatively high in energy. The calculations show, moreover, that this enolate is very unstable. Without or with a very low barrier, the carboxyl group of Glu172 rotates and delivers its proton to the O2 oxygen of the substrate, yielding the postulated *cis*-enediol intermediate (Figure 5A). This proton delivery step is exothermic by 5.8 kcal/mol. As seen in Figure 5A, upon delivery of the proton, the system forms another short hydrogen bond, with an O−O distance of 2.44 Å. By rotating away the carboxylic group from the substrate and reoptimizing, we can estimate the strength of this hydrogen bond to be ca. 4.6 kcal/mol. The substrate C1−C2 bond is now 1.36 Å, and the C−O distances are 1.40−1.42 Å. The zinc's distance to the O1 and O2 substrate oxygens is 2.20 and 2.25 Å, respectively.

d. Transfer of the Second Proton. After the creation of the enediol intermediate, the next steps involve the transfer of the second proton from O1 to C2 of the substrate. As seen from Figure 5A, Glu99 with its short hydrogen bond to O1 is in an ideal position to abstract the second proton. However, as pointed out before, protonation of C2 by Glu99 would result in the wrong enantiomer of the product (L-lactoylglutathione), since Glu99 can only access that face of the enediol intermediate. To obtain the correct D-form of the lactoylglutathione product, C2 has to be protonated from the opposite side. This can be achieved assuming that Glu172 performs this step. We showed above that the Glu99 hydrogen bond to O1 may or may not be there, without significantly affecting the energetics of the previous steps. In the following steps of the *S* mechanism, we assume that this hydrogen bond does not exist. In order for Glu172 to abstract the second proton from O1, it has first to break its hydrogen bond to O2. This implies an energetic cost of ca. 4.6 kcal/mol, as this was the estimated strength of this bond presented above. The same amount of energy is, however, gained back once Glu172 forms an identical hydrogen bond to

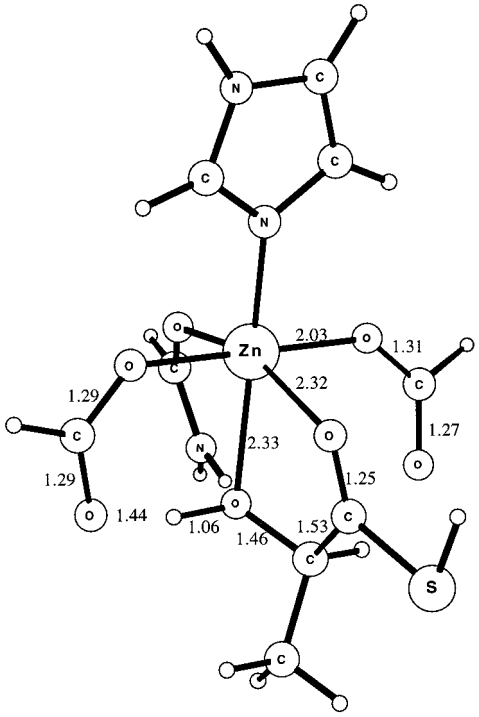


Figure 7. Optimized structure of the final product.

O1 (Figure 5B), since O1 and O2 of the *cis*-enediol intermediate are very symmetric. Note that in Figure 5, Glu99 is hydrogen-bonded to the substrate. This hydrogen bond can be omitted with no significant change on the energetics of the previous steps, as discussed earlier.

Energetically, we are hence back at the same point, and now Glu172 can abstract the second proton and deliver it to C2, giving the correct final product. These two steps turn out to occur with one transition state. The potential energy curve for the short hydrogen bond between O1 and $O_{\epsilon 2}$ of Glu172 is a single-well asymmetric potential, i.e., there is no energy minimum at which the proton is at the glutamate. The energy

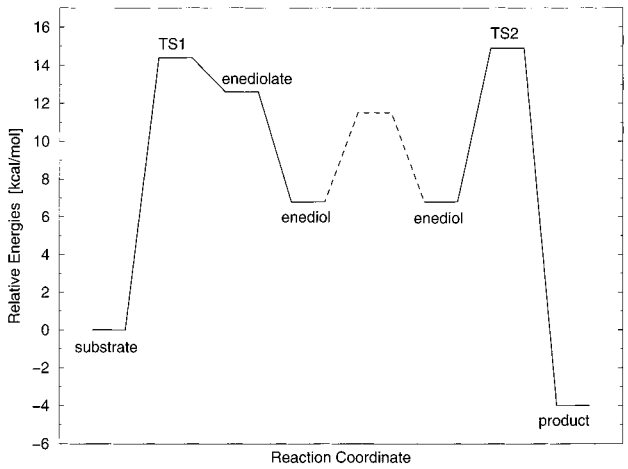


Figure 8. Calculated reaction energy profile for GlxI. Dashed line indicates the hydrogen-bonding shifting step necessary in the *S* reaction but not needed in the *R* reactions.

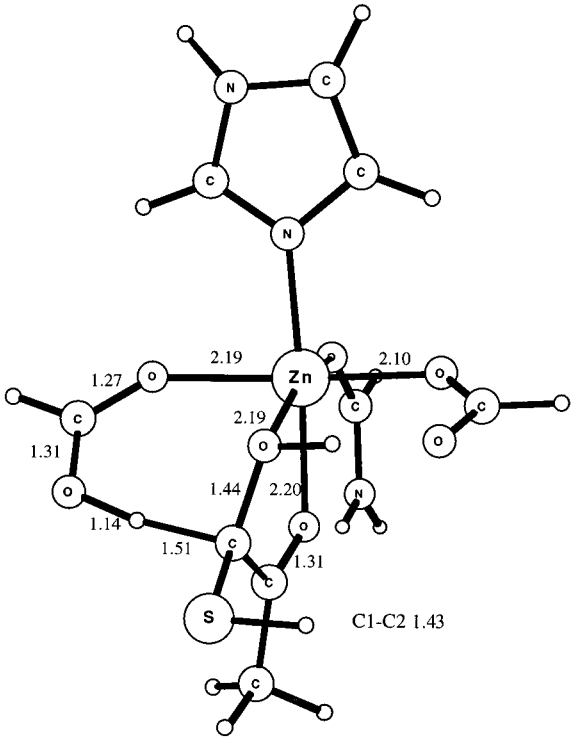


Figure 9. Optimized transition-state structure (TS1) for the initial proton-transfer step from C1 to Glu99 in the case of the *R* enantiomer of the substrate.

increases continually (without a transition state) when the proton is transferred from O1 to the carboxylate, over the short hydrogen bond. The energy cost for this is rather low, ca. 3.5 kcal/mol, as calculated by freezing the proton at a 1.00 Å distance from the $O_{\epsilon 2}$ of Glu172. The transition state is rather to break the short hydrogen bond. The optimized structure for this peculiar transition state (hereafter called TS2) is shown in Figure 6. At TS2, we see that the second proton is at Glu172 and the short hydrogen bond is broken, as the protonated glutamate is rotated away from O1 toward C2 (O1−Zn−O_{ϵ}−C dihedral angle is 39°). The barrier height is equal to the sum of the energetic costs to move the proton from O1 to Glu172 and to break the hydrogen bond (3.5 + 4.6 = 8.1 kcal/mol). TS2 has a single imaginary frequency of 160 cm^{-1} corresponding to the rotation of the glutamate about the Zn−O bond.

Once the hydrogen bond is broken, the proton will be delivered to C2 spontaneously, completing hence the catalytic cycle. From TS2, it is 18.9 kcal/mol downhill to the final product (Figure 7), making the overall step of transferring the second proton from O1 to C2 exothermic by 10.8 kcal/mol.

The full mechanism we propose for the reaction of the *S* enantiomer of the substrate is summarized in Scheme 3, and the potential energy curve is displayed in Figure 8. Important to note here is that we had to invoke that Glu172 performs a hydrogen bond swapping step in order to rationalize the experimental results and obtain the correct enantiomer of the product. It is currently unclear to us why the enzyme chooses to do this and not proceed through the other pathway, where Glu99 effects the transfer of the second proton.

e. Reactions of *R* Substrate. Let us now consider the reaction of the *R* enantiomer of the substrate. As pointed out earlier, Landro et al. showed that GlxI is nonstereospecific with respect to the proton abstraction, but it is stereospecific with respect to the proton delivery to the enediol intermediate.[10] Based on the pseudo-C_2 symmetry of the active site, Cameron et al. suggested that Glu99 can abstract the proton from the *R* enantiomer.[7] The fact that this residue was not displaced from the Zn upon substrate binding (as is the case for Glu172) was believed to speak against this. However, from the results of our calculations discussed above, we know that this displacement is not needed to perform the initial proton abstraction.

We have in the present work calculated the transition state for the PT from C1 to Glu99 for the case of the *R* enantiomer of the substrate. As seen from Figure 9, the structure of the transition state is very similar to TS1 for the *S* substrate. The barrier is slightly higher, ca. 2 kcal/mol. The structure and energy of the enediolate intermediates are also, due to the high symmetry of the active site, very similar. Of course, the symmetry between Glu172 and Glu99 is idealized in our small model of the active site. In the actual enzyme, there could be some energetic or structural differences between the *S* and *R* reactions for the initial proton-transfer step. The results of our calculations show, however, that the basic features are the same.

The first proton is delivered to O2 in a similar fashion as for the *S* substrate. In fact the resulting *cis*-enediol intermediate is the same as before (apart from the different hydrogen-bonding pattern) and is displayed in Figure 5B.

So, up to this point, the *S* and *R* reactions are perfectly symmetric. The subsequent steps must deviate from the symmetry though. A fully analogous mechanism to that of Scheme 3, in which Glu99 also effects the second proton transfer from O1 to C2, would yield the incorrect L-form of the product.

To obtain the D-lactoylglutathione product, we propose that Glu172 is involved in the *R* mechanism. That is, Glu172 abstracts the proton from the O1 and reprotonates at C2. The transition state for this is the same as TS2 of the *S* mechanism (Figure 6), where the proton first has to transfer to the glutamate and then the short hydrogen bond has to be broken. Proton delivery to C2 is then spontaneous, yielding the correct enantiomer of the product and completing the catalytic cycle. The full *R* reaction mechanism is summarized in Scheme 4. The potential energy profile is similar to that for the *S* reaction, with the hydrogen-bond-shifting step being unnecessary (Figure 8).

Although the individual reaction steps are identical to the *S* mechanism, the *R* reaction mechanism differs in one very important point. The difference is that the *R* reactions involve two bases (Glu99 and Glu172) as opposed to only one base in the *S* case (Glu172). It is currently not understood to us why the two mechanisms should diverge and break the symmetry.

This result is ultimately dictated by the fact that both the *S* and the *R* enantiomers of the substrate yield the same form of the product.[10] The symmetry has to be broken to satisfy this condition.

IV. Conclusion

This paper has discussed the catalytic mechanism of human glyoxalase I. On the basis of quantum chemical calculations and available spectroscopic, biochemical, and crystallographic information, we have proposed two mechanisms for the action of GlxI on the *S* and *R* diastereomers of the hemithioacetal substrate (summarized in Schemes 3 and 4, respectively).

The high symmetry between Glu172 and Glu99 at the active site of GlxI suggests symmetric mechanisms for the *S* and *R* mechanisms. The individual steps are, indeed, identical in both mechanisms. There is, however, one important difference. While in the *S* reactions, Glu172 performs all proton-transfer steps, Glu99 cannot do that in the *R* reactions, as this would yield the incorrect form of the product. We propose that in the *R* reactions, a second base (Glu172) is involved in the transfer of the second proton. This proposal can be tested by means of site-directed mutagenesis. Mutation of any of E99 or E172 would render the enzyme inactive toward the *R* enantiomer of the substrate.

It should be emphasized that the deviation from the symmetry between the *S* and *R* reactions is invoked only to satisfy the condition that both forms of the substrate should yield the same form of the product, as demonstrated by Landro et al.[10]

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