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Concerted Proton Transfer Mechanism of *Clostridium thermocellum* Ribose-5-phosphate Isomerase

Jun Wang and Weitao Yang*

Department of Chemistry, Duke University, Durham, North Carolina 27708, United States

Abstract

Ribose-5-phosphate isomerase (Rpi) catalyzes the interconversion of D-ribose-5-phosphate and D-ribulose-5-phosphate and plays an essential role in the pentose phosphate pathway and the Calvin cycle of photosynthesis. RpiB, one of the two isoforms of Rpi, is also a potential drug target for some pathogenic bacteria. *Clostridium thermocellum* ribose-5-phosphate isomerase (CtRpi), belonging to RpiB family, has recently been employed to the industrial production of rare sugars because of its fast reaction kinetics and narrow substrate specificity. It is known this enzyme adopts a proton transfer mechanism. It was suggested that the deprotonated Cys65 attracts the proton at C₂ of the substrate to initiate the isomerization reaction and this step is the rate-limiting step. However, the elaborate catalytic mechanism is still unclear. We have performed quantum mechanical/molecular mechanical simulations of this rate-limiting step of the reaction catalyzed by CtRpi with the substrate D-ribose. Our results demonstrate that the deprotonated Cys65 is not a stable reactant. Instead, our calculations revealed a concerted proton-transfer mechanism: Asp8, a highly conserved residue in the RpiB family, performs as the base to abstract the proton at Cys65 and Cys65 in turn abstracts the proton of the D-ribose simultaneously. Moreover, we found Thr67 cannot catalyze the proton transfer from O₂ to O₁ of the D-ribose alone. Water molecule(s) may assist this proton transfer with Thr67. Our findings lead to a clear understanding of the catalysis mechanism of RpiB family and should guide the experiments to increase the catalysis efficiency. This study also highlights the importance of initial protonation states of cysteines.

Keywords

QM/MM; QM/MM-MFEP; enzyme catalysis; isomerization

Introduction

Ribose-5-phosphate isomerase (Rpi; EC 5.3.1.6) catalyzes the reversible isomerization of D-ribose-5-phosphate and D-ribulose 5-phosphate. The reaction direction is determined by the concentration of the two compounds. Rpi is a key enzyme in the pentose phosphate pathway and the Calvin cycle of photosynthesis^{1,2}. In the non-oxidative branch of the pentose phosphate pathway, Rpi converts D-ribulose 5-phosphate to D-ribose-5-phosphate, which then is converted into intermediates for glycolysis and supplies the precursor for the synthesis of nucleotides. While in the Calvin cycle of photosynthesis, Rpi converts D-ribose-5-phosphate to D-ribulose-5-phosphate, which is subsequently converted to ribulose-1,5-bisphosphate to accept the carbon dioxide in the first dark reaction of photosynthesis. The importance of this reaction has been demonstrated by the experimental finding that knocking out the isomerase gene of *E.coli*³ or *S. cerevisiae*⁴ leads to severely impaired growth. Two distinct isoforms of Rpi originated from independent evolution⁵. RpiA is broadly

*To whom correspondence should be addressed. weitao.yang@duke.edu. Telephone: 919-660-1562.

distributed in most eukaryotic organisms, while RpiB exists in most prokaryotic organisms with some exceptions of lower eukaryotes. Because of its importance to bacterial growth and its different distributions in eukaryotic and prokaryotic organisms, it has attracted much attention as a potential drug target for some pathogenic bacteria including *M. tuberculosis*⁶, *T. cruzi*⁷, *C. immitis*⁸ and *L. donovani*⁹. Recently, RpiB has also been employed in the industrial production of rare sugars, which are involved in the production of low-calorie sweeteners^{10–13} and pharmaceutical compounds^{14–17}.

Although RpiA and RpiB have little similarity in their sequences and structures, they both prefer proton-transfer mechanism to hydride-transfer mechanism, which is adopted by D-xylose isomerase¹⁸ and L-rhamnose isomerase¹⁹. Many crystal structures of Rpis have been determined.^{1,7,8,20–27} Among these known Rpis, *Clostridium thermocellum* Rpi (CtRpi) is most promising for industrial production of rare sugars because of its fast reaction kinetics and narrow substrate specificity. According to the sequence alignment and structural analysis, its catalysis mechanism was proposed as shown in Scheme 1²⁶. In the first and rate-limiting step, the C₂ proton is abstracted by the general base residue Cys65. Then Thr67 protonates the O₁ to generate an enediol and abstracts the proton from O₂ to regenerate an enolate. Finally this enolate is reprotonated at C₁ by Cys65 to form the product. This mechanism is proposed for most RpiBs, except *M. tuberculosis* Rpi, in which a glutamine acid occupying the same position as Cys65 acts as the base²⁸. The multiple sequence alignment indicates Cys65 is conserved in most of the RpiBs except *M. tuberculosis* Rpi. The importance of Cys65 has been demonstrated by the mutagenesis experiment that the enzyme become inactive if it is replaced by an alanine.²⁹ In some other RpiBs, the residue Thr67 is replaced by a serine. It should be pointed out that the steps of the open and closure of sugar ring is omitted in Scheme 1 because these steps are not the rate-limiting step and the sugar ring is open in crystal structure.

Though the proton-transfer mechanism is well known for the aldose-ketose isomerization, key issues for the CtRpi catalysis reaction are not clear. First, Cys65 is proposed to be deprotonated in Scheme 1 because only its deprotonated state can act as a base to abstract the C₂ proton. However the normal pK_a of the side chain of cysteine is greater than 7. The model pK_a of cysteine is set as 9.0 in PROPKA^{30–33}. The predicted pK_a of Cys65 by PROPKA using the structure of the complex of CtRpi and the ribose is even larger than 10. This indicates that the side chain of cysteine favors protonated state, especially when it is fully buried inside the complex. Second, Asp8 is another conserved residue in RpiBs. In Scheme 1, the important role of Asp8 is to form a hydrogen-bond with the hydroxyl group at C₃ of substrates, which helps the recognition and binding of substrates. It has been recently reported that the enzyme totally loses its ability to catalyze the conversion of D-psicose to D-allose if Asp8 is replaced by an alanine.²⁹ Given that this replacement does not change the structure of the active site significantly, this replacement should reduce the affinity of the enzyme and the substrate greatly. However, the mutant enzyme should have more or less activity because Asp8 is not a catalytic residue in Scheme 1. This leads to very interesting questions: does Asp8 take part in the chemical catalytic reaction and how? To our knowledge, there has been no reported computational investigation of this system.

To address these questions, we carried out accurate quantum mechanical/molecular mechanical (QM/MM) simulations on the rate-limiting step, *i.e.* the abstraction of the C₂ proton. Our results confirm that the deprotonated side-chain of Cys65 is not a stable reactant state. A concerted proton transfer for the abstraction of the C₂ proton is shown to be viable. In this mechanism, Asp8 deprotonates Cys65, which in turn simultaneously deprotonates C₂ of the substrate. The calculated free energy barrier of this mechanism agrees well with the experimental reaction rate. The computed reaction pathway demonstrates how the proton transfers from O₂ to O₁ and the critical role of an additional water molecule.

Experimental Details

Preparation of initial models

Because the main goal of this study is the initial protonation state of Cys65 and the role of Asp8 in the reaction, D-ribose was selected as the substrate to save the computational resource. The initial structure of the enzyme-substrate complex was taken from the crystal structure of *Clostridium thermocellum* ribose-5-phosphate isomerase B with D-ribose (PDB code 3PH3)²⁶. Generally it is hard to get a crystal structure of the enzyme-substrate complex. What is special for CtRpi is that it reversibly catalyzes the isomerization of D-ribose-5-phosphate and D-ribulose 5-phosphate. Therefore the structure of the complex can be obtained experimentally. Another possible reason is that this enzyme-substrate conformation is inactive if Cys65 is in a protonated state. Besides, the low temperature in the experiment which slows the reaction rate may also contribute to get such an enzyme-substrate complex structure. The high resolution (2.07 Å) crystal structure is a dimer with a pseudo two-fold symmetry. The two active sites locate in the crevice between the two monomers, but only one substrate was used in our calculations. Though each active site consists of the residues from both monomers, the key residues catalyzing the reaction come from the same monomer. Therefore the identity of monomer is omitted in the following discussion. All of the hydrogen atoms were added by MolProbity³⁴. The protonation states of the histidines were also determined by this web service according to their local hydrogen bond interactions with nearby residues. Three models of the active site were prepared with the same procedure and equilibrated with the same computation protocol. In model A, Cys65 and Asp8 are both deprotonated, while in model B, Cys65 is deprotonated but Asp8 is protonated. In model C, Cys65 is protonated and Asp8 is deprotonated. Because the protonated Cys65 cannot act as a base to abstract the C₂ proton, another residue is need to deprotonate Cys65. Among the residues which have the ability to deprotonate Cys65, Asp8 is the nearest one to Cys65. However, the carboxyl group of Asp8 is still 4.86 Å far away from the S proton of Cys65 in the crystal structure, indicating it is hard for Asp8 to deprotonate Cys65. Therefore, the side chain of Cys65 in model C was rotated so that it formed a hydrogen bond with Asp8. Using this model, PROPKA predicts the pK_a of Asp8 is 5.10, confirming that it is in a deprotonated state. There is a fourth possibility of both Cys65 and Asp8 being protonated. But this model is ruled out because it cannot initiate the abstraction of the C₂ proton. The resulting complexes were solvated in a periodic TIP3P³⁵ rectangular water box of 76×78×86 Å³, which resulted in systems of approximately 48600 atoms in total. The CHARMM22³⁶ force field was employed to describe the protein.

Each of the three systems was first energy minimized and then was warmed gradually with a series of restrained molecular dynamics simulations. At the beginning the position of the backbone atoms of protein were restrained by a force constant of 40 kcal/(mol×Å²). Then the force constant was reduced to 20 kcal/(mol×Å²). Finally only the C atoms were restrained by a force constant of 10 kcal/(mol×Å²). During these procedures, the D-ribose was fixed. Subsequently a 2-ns molecular dynamics simulation was performed without any restraint. Using the multiple-time step algorithm^{37,38}, the integration step sizes were 1 fs for short-range forces, 4 fs for medium-range forces, and 8 fs for long-range electrostatic forces, respectively. The PME method³⁹ was used for computing the long-range electrostatic interactions. An 8 and 12 Å dual cutoff was employed to generate the nonbonded pair list, which was updated every 16 steps. The temperature of the system was maintained at 300 K with the Berendsen thermostat.⁴⁰ The final structures of the molecular dynamics simulations were selected as the initial structures for QM/MM simulations.

QM/MM-MFEP calculation

We used the QM/MM minimum free energy path (QM/MM-MFEP) approach which has been discussed in previous publications.^{41–43} In the QM/MM-MFEP calculation, an ensemble of the MM subsystem generated by molecular dynamics simulations was used to calculate the potential mean force (PMF) surface defined on the fixed coordinates of QM subsystem. Then the geometry of QM subsystem was optimized on this PMF surface. This process was repeated until the convergence was obtained.

In this study, the QM subsystem consisted of the D-ribose and the side chains of Cys65 and Asp8. The B3LYP exchange-correlation functional^{44,45} and the 6–31+G* basis set were used to describe the QM subsystem. The boundary atoms between QM and MM subsystem including the C atoms of Cys65 and Asp8 were modeled by the pseudo-bond approach⁴⁶. The length of the molecular dynamics sampling was 40 ps in the reaction pathway driving procedure and 160 ps for performing the QM free energy perturbation and free energy gradient calculation, respectively. A dual cutoff of 10 and 15 Å was used for all QM/MM-MFEP calculations. SHAKE⁴⁷ was only applied to water molecules. The integration time steps were 1 fs for short-range forces, 4 fs for medium-range forces, and 8 fs for long-range electrostatic forces. Nudged elastic band method⁴⁸ combined with QM/MM-MFEP was used for the reaction pathway optimization. In nudged elastic band method, the complete QM degrees of freedom were used to construct a discrete reaction pathway without explicitly specifying a reaction coordinate to avoid the complication of computing the Jacobian term.

Results and discussion

Deprotonated side-chain of Cys65 is not a stable reactant state

In Scheme 1, a deprotonated Cys65 is necessary for the isomerization reaction. Therefore Cys65 in both Model A and Model B was deprotonated and adopted the conformation in the crystal structure. The difference between these two models is only the protonation state of the side-chain of Asp8. However, our calculations show that the deprotonated side-chain of Cys65 is not stable whether Asp8 is deprotonated or not.

In Model A, the side-chain of Asp8 is also deprotonated. Figure 1 shows the structures of the QM subsystem before and after the QM/MM-MFEP optimization. In the structure before the optimization, the side-chain of Cys65 is fully buried and no strong hydrogen bond stabilizes the deprotonated S of Cys65. The distance between S of Cys65 and H_N of Gly68 is 2.46 Å, which is beyond a usual hydrogen-bond length. Moreover, the side-chain of Asp8, which also carries one negative charge, repulses the side-chain of Cys65. This repulsion results in that S of Cys65 prefers the nucleophilic attack to C₁ of the substrate to the abstraction of the proton at C₂ of the D-ribose. Therefore, the distance between S of Cys65 and C₁ of the D-ribose became from 3.17 Å in the crystal structure to 1.95 Å, that is, a covalent bond is formed between them. This demonstrates model A is not an appropriate reactant model.

In Model B, the side-chain of Asp8 is protonated. After the QM/MM-MFEP optimization, the distance between S of Cys65 and the proton at C₂ of the D-ribose is about 2.4 Å. Compared with Model A, the distance between S of Cys65 and C₁ of the D-ribose is about 2.8 Å, because of the removal of the electrostatic repulsion from Asp8. To simulate the proton transfer from C₂ of the D-ribose to the deprotonated side-chain of Cys65, the distance between S of Cys65 and the proton at C₂ of the D-ribose was used as the initial driving coordinate for the reaction pathway. The PMF profile for this proton transfer reaction after the QM/MM-MFEP optimization is plotted in Figure 2. The free energy of the reactant state is 14.6 kcal/mol higher than that of the product state and there is no significant barrier along

this reaction pathway. We doubled the simulation time, however the initial structure still stay in this local minimum. The free energies of the first five points along this pathway are very close, implying the initial structure of Model B locates in a broad local minimum, so it cannot jump out this local minimal in the limited simulation time. Therefore, the initial structure of Model B is not a stable reactant state, either.

Asp8 deprotonates the side-chain of Cys65

The above calculations demonstrate that the deprotonated side-chain of Cys65 is not a stable reactant state, either in Model A or in Model B. Therefore, in the crystal structure, the side chain of Cys65 should be protonated and the van der Waals interactions are its major interaction. If it is deprotonated, the electrostatic interaction becomes dominant, which does not favor the crystal structure. On the other hand, the side-chain of Cys65 needs to be deprotonated to abstract the proton at C₂ of the substrate. Among the nearby residues, only Asp8 has the ability to deprotonate the side-chain of Cys65. However the distance between O₂ of Asp8 and S of Cys65 is 4.86 Å in the crystal structure, implying a difficult proton transfer from Cys65 to Asp8. Therefore, when building up Model C, we rotated the side-chain of Cys65 to reduce this distance. In the optimized structure, the side-chain of Cys65 is hydrogen bonded with O₂ of Asp8 with the distance of 1.91 Å, which leads to Model C. In our Model C, the mechanism for the abstraction of the proton at C₂ of the D-ribose is a relay proton transfer shown in Scheme 2.

We first compared the free energy difference between the initial structures of Model B and Model C. A pathway connecting the two initial structures was mapped out using the distance between H of Cys65 and O₂ of Asp8 as the reaction coordinate. The PMF profile is plotted in Figure 3. The free energy of Model C is 14.8 kcal/mol lower than that of Model B. Therefore, Model C is a more appropriate reactant state than Model B. This pathway can be divided into two parts. In the first part, the side chain of deprotonated Cys65 rotates to approach to Asp8. The distance between S of Cys65 and the proton at C₂ of the D-ribose first reduces from 2.44 Å to 1.91 Å and then increases steadily. Accordingly, the free energy first decreases by 4.1 kcal/mol and then increases steadily. The free energy differences between the rotated conformations (Points 10–12) and Model B are 0.2–3.3 kcal/mol, indicating that this rotation can occur under physiological conditions. In the second part, the proton at the neutral Asp8 transfers to the side chain of the deprotonated Cys65. This is the reverse process of the deprotonation of Cys65 by Asp8. In the transition state, the distance between H of Cys65 and O₂ of Asp8 is 1.25 Å. And the distance between S of Cys65 and the proton at C₂ of the D-ribose is 2.09 Å. These distances illustrate that this is the transition state of the single proton transfer from Cys65 to Asp8. Figure 3 shows that the free energy barrier of the deprotonation of Cys65 is 20.2 kcal/mol, which is 8.3 kcal/mol larger than the free energy barrier of 11.9 kcal/mol which is calculated from the experimental k_{cat} of 11878 s⁻¹.⁴⁹ This precludes the sequential proton transfer mechanism and suggests the alternative proton transfer mechanism.

To drive the reaction pathway of the concerted proton transfer, the distance between S of Cys65 and the proton at C₂ of the D-ribose is used again as the initial driving coordinate. The PMF profile after the QM/MM-MFEP optimization is plotted in Figure 4. The activation free energy barrier was estimated to be 14.7 kcal/mol, in good agreement with the experimental activation free energy of 11.9 kcal/mol.⁴⁹ The structures along this pathway are shown in Figure 5. In the reactant state, the hydrogen bond distance between H of Cys65 and O₂ of Asp8 is 1.91 Å. The distance between S of Cys65 and the proton at C₂ of the D-ribose is 2.18 Å. When the bond S-H of Cys65 is stretched, the distance between the proton at C₂ of the D-ribose and S of Cys65 is ~1.9 Å, indicating a hydrogen bond formed. This hydrogen bond stabilizes the deprotonation of S of Cys65. In the transition state, the distance between H of Cys65 and O₂ of Asp8 is 1.02 Å, while the distance between O and

H of Cys65 is 2.03 Å. The proton at C₂ of the D-ribose is 1.56 Å and 1.52 Å far from S of Cys65 and C₂ of the D-ribose, respectively. In the product state, the distance between S and H of Cys65 is 2.23 Å. C₂ of the D-ribose is 2.30 Å far away from the proton transferred to S of Cys65. These distances indicate the original bonds are totally broken finally. Therefore the concerted proton-transfer mechanism is operative for the abstraction of the proton at C₂ of the D-ribose, with the calculated barrier comparable to the experimental result.

Water molecule(s) mediate the proton transfer from O₂ to O₁

It has been reported that the free energy barriers of the proton transfer from O₂ to O₁ in other aldose-ketose isomerizations are 6–10 kcal/mol^{50,51}, which are lower than the free energy barrier of the proton abstraction from C₂ (14.7 kcal/mol in Model C), implying that this proton transfer is not rate-limiting for the aldose-ketose isomerizations. Therefore we did not perform the simulations for the subsequent proton transfer from O₂ to O₁. However, we can still get some hints for this proton transfer. In Scheme 1, this proton transfer is assisted by Thr67 only. However in the crystal structure, the distance between O of Thr67 is 3.67 and 4.05 Å far from O₁ and O₂ of the substrate, respectively, indicating Thr67 is not hydrogen bonded with the D-ribose. In the product state of Model C, we found a hydrogen-bond network shown as Figure 6. Thr67 is hydrogen-bonded with the hydroxyl group at C₂ of the D-ribose and a water molecule with the distance of 1.96 Å and 1.79 Å, respectively. This water molecule is also hydrogen-bonded with O₁ with the distance of 1.55 Å. Consequently, we infer that this water molecule and Thr67 assist the proton transfer from O₂ to O₁ together, as shown in Scheme 3. We checked the reactant state to examine whether this hydrogen network exists in it. In the reactant state, only the hydrogen bond between Thr67 and the water molecule exists with the distance of 1.73 Å. The other two hydrogen bonds are not formed. The distance between Thr67 and the proton at O₂ is 2.42 Å. The distance between the water and O₁ of the D-ribose is 3.61 Å. Thus the hydrogen-bond network forms during the proton transfer process. As O₁ carries more and more negative charge during the first step, it attracts the water molecule to form this hydrogen bond network. It should be noted that this finding does not mean that only Thr67 and the water molecules can mediate the proton transfer from O₂ to O₁. This finding suggests that Thr67 is not a must for the proton transfer. Water molecule(s) may form such a hydrogen-bond network to assist this proton transfer. This agrees with the experimental results that the replacement of Thr67 by an alanine only reduce 54% catalytic activity.²⁹

Conclusion

We have performed QM/MM-MFEP calculations to understand the enzymatic isomerization reaction mechanism of Ctrpi with D-ribose. In previous suggested mechanism, the residue Cys65 is supposed to be deprotonated to attract the proton at C₂ of the substrate to initiate the isomerization reaction. To validate the deprotonated states of Cys65, we built two models with deprotonated or protonated Asp8, respectively, following the previous mechanism. The calculations indicate that the free energy of the initial structure with a deprotonated Cys65 is too high. Therefore we built a novel model, in which Cys65 is neutral in the reactant state and Asp8 is deprotonated. In the concerted proton transfer mechanism, the neutral Cys65 donates its proton to Asp8 and accepts the proton at C₂ of the substrate simultaneously. The calculated activation free energy of this mechanism is 14.7 kcal/mol, in good agreement with the experimental activation free energy of 11.9 kcal/mol calculated from the experimental k_{cat} of 11878 s⁻¹. This mechanism can explain the experimental observation that the mutant of Asp8 makes the enzyme lost all of its catalytic activity. This new concerted proton transfer mechanism reveals the new role of Asp8 in the isomerization reaction. It not only forms a hydrogen bond with the hydroxyl group at C₃ of the substrate to help the enzyme bind and recognize the substrate, but also initiates the isomerization

reaction by deprotonating the neutral Cys65. The neutral Cys65 acts as a relay residue to shuttle the proton at C₂ of the substrate to Asp8.

In addition, we found that Thr67 is not hydrogen-bonded with O₁ of the D-ribose in the product state. A water molecule is found to be hydrogen-bonded with Thr67 and O₁ of the D-ribose. It is possible that the proton transfer from O₂ to O₁ need the assist of water molecule(s).

This work also indicates the importance of initial protonation states of cysteine in the theoretical calculation of chemical reactions. An incorrect initial protonation state of cysteine may distort the calculated free energy profile. Therefore, initial protonation states of cysteine should be assigned with care.

The active site and the catalysis residues are highly conserved in the family of RpiBs. The residues Asp8 and Cys65 are conserved in all the known RpiBs. The only exception is that in *M. tuberculosis* Rpi, the cysteine is replaced by glutamic acid. In some RpiBs the threonine are replaced by the similar residue serine. Therefore, the mechanism we suggested for CtRpi should apply to the whole family of RpiBs. This study provides a deeper understanding to the catalysis mechanism of RpiB and will guide the mutation experiment to enhance the catalysis efficiency.

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Figure 1.
The structure of the QM subsystem before (A) and after (B) the QM/MM-MFEP optimization. Carbon atoms are in cyan; oxygen atoms are in red; hydrogen atoms are in white; sulfur atom is in yellow; nitrogen atoms are in blue.

Figure 2. Potential of mean force of the proton transfer from C₂ of the D-ribose to the deprotonated Cys65 for Model B. The x axis is the order of the conformations along the reaction pathway.

Figure 3.

Potential of mean force of the pathway from Model B to Model C. The x axis is the order of the conformations along the pathway.

Figure 4. Potential of mean force of the concerted proton transfer from C₂ of the D-ribose to Asp8 for Model C. The x axis is the order of the conformations along the reaction pathway. The structures of the reactant, transition and product states along the reaction pathway are shown in Fig. 5

Figure 5. the QM-MM/MFEP optimized structures of the reactant state, transition state and product state for the concerted proton transfer from C₂ of the D-ribose to Asp8 for Model C.

Figure 6. the hydrogen network among the D-ribose, Thr₆₇ and a water molecule in the product state for Model C.

Scheme 1.

The proton transfer mechanism for CtRpi to catalyze aldose-ketose isomerization proposed in the literature^{26,28}. The dot line represents the hydrogen bond interaction between Asp8 and the hydroxyl group at C₃. Both Models A and B follow the first step of this scheme. The difference between these two models is that Asp8 is deprotonated in Model A and protonated in Model B.

Scheme 2.

Concerted proton-transfer mechanism to abstract the proton at C₂ of the substrate, recently determined through QM/MM-MFEP calculations. The dot line represents the hydrogen bond interaction between Asp8 and the hydroxyl group at C₃. Model C follows this scheme.

Scheme 3.

Possible mechanism of the proton transfer from O₂ to O₁ of the substrate. In contrast to the second step of Scheme 1, an additional water molecule also assists this proton transfer with Thr67.