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Active-site dynamics of SpvC virulence factor from *Salmonella Typhimurium* and density functional theory study of phosphothreonine lyase catalysis

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Abstract

The newly discovered SpvC effector protein from *Salmonella typhimurium* interferes with the host immune response by dephosphorylating mitogen-activated protein kinases (MAPKs) with a β -elimination mechanism. To understand this unique phosphothreonine lyase catalysis, the dynamics of the enzyme-substrate complex of the SpvC effector is investigated with a 3.2 ns molecular dynamics simulation, which reveals that the phosphorylated peptide substrate is tightly held in the active site by a hydrogen bond network and the lysine general base is positioned for the abstraction of the alpha hydrogen. The catalysis is further modeled with density functional theory (DFT) in a truncated active-site model at the B3LYP/6-31+G(d,p) level of theory. The DFT calculations indicate the reaction proceeds via a single transition state, featuring a concerted proton abstraction from the α carbon by Lys136 and β elimination of the phosphate leaving group. Key kinetic isotopic effects are predicted based on the truncated active-site model.

I. Introduction

Cellular response to bacterial infection in both plants and animals involves a complex, highly conserved signal transduction cascade, collectively known as the mitogen-activated protein kinase (MAPK) pathway.¹ Many gram negative bacteria, such as *Shigella* and *Salmonella*, subvert this and other cellular pathways within the host cell by injection of bacterial enzymes called protein effectors using a Type III Secretion System (TTSS).^{2,3} These effectors are major components of bacterial virulence. As a result, they represent potential targets for indirect narrow spectrum antibiotics, a promising alternative to dealing with the growing problem of antibacterial resistance.^{4,5}

A subset of these effectors catalyze dephosphorylation of MAPKs into their inactive form, with examples including the acetylases found in *Yersinia pestis* and *Vibrio parahemolyticus*,^{6,7} and the recently characterized phosphothreonine lyase family found in *Shigella*,⁸ *Salmonella*,⁹ and the plant pathogen *Pseudomonas syringae*.¹⁰ The protein effector OspF found in *Shigella*, for example, dephosphorylates MAPKs such as Erk2 (extracellular signal-regulated kinase 2) and p38 in vivo, preventing histone phosphorylation and repressing

activation of a subset of NF- κ B responsive genes and subsequent production of proinflammatory cytokines.¹¹

In contrast to the more commonly seen phosphatase catalysis which cleaves the O-P bond, the mechanism of OspF has been shown to proceed by β -elimination of phosphothreonine, breaking the C β -O γ bond and forming a C β =C α double bond.^{8,9} The resulting Michael acceptor product can then react further in an irreversible fashion, permanently inactivating the MAPK. This mechanism has been confirmed in another enzyme of the so-called OspF family, namely SpvC from *Salmonella typhimurium*, showing similar substrate specificity and evidence for production of the same product.^{8,12} *Pseudomonas syringae* HopA1, a plant pathogenic effector and member of the same enzyme family has shown inactivation of MAPKs using the same mechanism.¹⁰

The β -elimination of phosphate from phosphorylated threonine and serine residues in proteins by chemical means under basic conditions is well known. In an early example, phosphoserine-containing peptides were converted to their S-ethylcysteine counterparts before sequencing by Edman degradation.¹³ More recently, the resulting dehydroalaninyl and β -methyldehydroalaninyl residues have been derivatized by Michael addition to attach affinity tags,¹⁴ or to produce adducts with enhanced properties facilitating observation by fluorescence¹⁵ or MALDI.¹⁶⁻¹⁸ In contrast to the numerous applications, evidence regarding the mechanism of the elimination reaction is scarce. The elimination in dilute hydroxide is catalyzed by group II metal ions, in the order Ba > Sr > Ca,¹⁹ and the reaction is accelerated by the addition of DMSO or ethanol.¹⁵

In contrast to the chemical process, the enzymatic β -elimination of phosphate from phosphoproteins is a novel reaction and constitutes a new means for the dephosphorylation of proteins. Of known enzymatic reactions, the most analogous is that catalyzed by threonine dehydratase, which differs in the use of a pyridoxal phosphate (PLP) cofactor.²⁰ The details of the reaction have not been elucidated, but the mechanism is assumed to be E1cB.²¹ Another PLP-dependent elimination reaction is catalyzed by threonine synthase, in which a Schiff base intermediate is formed between PLP and the substrate amine.²² Members of the OspF family utilize neither PLP nor any other cofactors, offering a further testament to the novelty of these enzymes.

The impact of some aforementioned bacteria to public health is far reaching. *Shigella*, the cause of bacillary dysentery, is responsible for approximately 1.1 million deaths per year in the developing world.²³ Deadly *Salmonella* outbreaks are increasingly common in recent years. The rapid emergence of resistant strains compounds the problem. Interestingly, no eukaryotic phosphothreonine lyase has been identified, making this enzyme an attractive narrow spectrum antibiotic target. The inhibition of the pathogenic effectors differs from the many existing antibiotic paradigms and may elicit less resistance.^{4,5} Understanding the mechanistic details of this family of enzymes can help guide drug discovery and may eventually lead to new antibiotics.

The crystal structure of SpvC has recently been reported by two groups in the apo form and also for inactive mutants co-crystalized with phosphopeptides.^{9,24} These structures show a unique α/β fold likened to a cupped hand. Substrate specificity is determined by the pThr-X-pTyr motif of MAPKs, with pTyr resting in a surface groove forming hydrogen bonds with Lys160, Lys134, and Arg80. The phosphate group of pThr is engulfed by a positive pocket formed by Arg220, Arg213, Arg148, and Lys104. A loop containing Arg220 undergoes a conformational change upon substrate binding, closing the positive pocket surrounding the phosphate, as shown by comparison of apo and substrate-bound structures. The residues involved in chemical transformation have been proposed based on mutagenesis and pH kinetic

studies to be Lys136 and His106.⁹ The proposed mechanism entails Lys136 acting as a general base, abstracting a proton from C α of phosphothreonine, with His106 acting as a general acid to promote C β -O γ bond breaking and the formation of a C α =C β double bond, resulting in the β -methyldehydroalanine product. The role of Lys136 as a general base requires the deprotonated form, which is presumably stabilized by hydrogen bonding with Tyr158. His106's role as a general acid requires the protonated form, stabilized by Asp201, and is consistent with the reported pH rate profiles,⁹ but more work is needed to definitively confirm its role in catalysis. Furthermore, mechanistic information reported to date gives no insight as to whether the enzymatic elimination is concerted or stepwise. The active-site arrangement is depicted in Scheme 1.

Definitive mechanistic action and microscopic details are often difficult to establish with experiment alone and hence theoretical studies are highly desired. Here, we report molecular dynamics (MD) studies of the Michaelis complex of SpvC with a phosphopeptide strand from the activation loop of Erk5, which shed light on the active-site arrangement. Furthermore, we report a density functional theory (DFT) study of the catalysis with a truncated active-site model, which establishes the feasibility of the proposed reaction mechanism. To guide future experimental exploration, we have also calculated the kinetic isotope effects based on the truncated active-site model. This work is organized as follows. In Sec. II, theoretical methods used in both MD and DFT calculations are explained. The results are presented in Sec. III and discussed. The final section (Sec. III) concludes.

II. Methods

A. Molecular Dynamics Simulations

The starting geometry for the SpvC enzyme-substrate complex was largely based on crystal structure 2Q8Y,⁹ which consisted of an inactive K136A mutant complexed with a 13-mer phosphopeptide derived from Erk5. Erk5 is closely related to the natural substrate Erk2, containing an identical pT-E-pY motif, and has been shown to have similar binding characteristics, though with a lower catalytic activity. Our substrate model consisted of the nine amino acids that were resolved in the X-ray structure (₂₁₅YFM-pT-E-pY-VA₂₂₃). To restore the enzyme to the WT form, Lys136 was mutated back from Ala by using the mutagenesis function found in software package PyMOL (<http://www.pymol.org>), selecting a starting conformation that minimized steric clashes with surrounding residues and the substrate. Due to their proximity to the substrate, unresolved residues Ser96 and Gln97 were also restored by using coordinates from a mutant SpvC crystal structure (PDB code 2Z8P).²⁴ This proceeded by global structural alignment of the two crystal structures, followed by an additional alignment along residues 94-99 before transferring atom coordinates for the missing residues. The patched and immediately surrounding residues were then subjected to a short minimization to relieve strain and bad contacts. Unresolved N-terminal residues 1-26 were not restored, though their impact is expected to be minimal being far from the active site and substrate.

Nearly all crystal waters were retained, except those which clashed with the restored Lys136. Protonation states of all titratable residues were carefully evaluated by their local environment and hydrogen bonding networks. This included putative catalytic residues His106 in the protonated form, and Lys136 in the deprotonated neutral form, based on the pH profile of the enzyme.⁹ Hydrogen atoms were then added using the HBUILD function in the CHARMM suite. The energy of the complex was then briefly minimized, gradually releasing harmonic constraints applied to backbone and side chain of the residues, to partially optimize the structure and remove bad contacts.

The complex was then solvated by repeated addition of a randomly rotated, pre-equilibrated 25 Å sphere of TIP3P waters,²⁵ with the origin of the sphere centered at the alpha carbon of the substrate phosphothreonine. Any waters that fell within 2.8 Å of a heavy atom were removed, before allowing the remainder to relax by 30ps of solvent molecular dynamics with protein and substrate fixed.

To model extended solvent effects, stochastic boundary conditions²⁶ were used to partition the system into three spherical regions. Atoms within a 22 Å radius from the origin are defined as the reaction zone, atoms between 22 and 25 Å as the buffer zone, and all atoms outside of 25 Å, referred to as the reservoir, are deleted from the system. Atoms in the reaction zone are governed by Newtonian dynamics on a classical potential, as provided by the CHARMM all-atom force field.²⁷ The buffer zone adds in a Langevin term, gradually scaled up at the boundary and consisting of frictional and random forces to represent the effect of extended solvent.

All minimizations and MD simulations were performed using the CHARMM suite of programs.²⁸ Non-bonded interactions were handled with an 8 Å atom-based cutoff. The SHAKE²⁹ algorithm was used to constrain bonds involving hydrogen. A time step of 1.0 fs was used for all dynamics simulations. Final structural refinement proceeded by a short period of steepest decent minimization, followed by ABNR minimization till a gradient tolerance of 0.01 kcal mol⁻¹ Å⁻¹ was reached. Dynamics simulations consisted of a 200ps heating window to bring the system to a final temperature of 300K, followed by a 500ps equilibration, and 2.5ns of data collection.

B. Truncated Active-site Models

To investigate the reaction mechanism of the SpvC phosphothreonine lyase, we have developed a truncated active-site model based on the Michaelis complex developed based on the X-ray structure, as described in the previous section. It contains the side-chains of several key active-site residues (His106, Lys136, Arg148, Arg213, Arg220), which have been demonstrated by mutagenesis experiments to be of critical importance.^{8,9} To reduce computational costs, the arginine and lysine residues were approximated by guanidinium cations and methylamine, respectively. The substrate was truncated at the two adjacent C_α-C bonds and then was capped with methyl groups, which led to a system of 72 atoms with a total charge of +2. Admittedly, the truncated active-site model does not have all the key interactions present in the enzyme active site. As a result, the calculation results are not expected to be quantitative. However, they should be sufficient provide mechanistic insights into the catalytic reaction.

We are primarily interested in the stationary points along the reaction path. All the structures were minimized using the B3LYP functional and the standard 6-31+G(d,p) basis set. Frequency calculations at the same level have been carried out to confirm the nature of these stationary points and to obtain their zero-point energies (ZPE). The intrinsic reaction coordinate (IRC) calculations³⁰ have also been performed to establish connectivity between the stationary points. To examine the dielectric effects from the solvent or the protein surrounding, more accurate single point energies were calculated with the larger 6-311+G(d,p) basis set and the polarized continuum model (PCM).³¹ All the DFT calculations were carried out using the Gaussian 03 suite of quantum chemistry programs.³²

To calculate the kinetic isotope effects (KIEs), we have used the Bigeleisen-Mayer method³³ implemented in ISOEFF,³⁴ in which the KIEs were calculated from the harmonic frequencies of both the reactant complex and transition states. Neither anharmonicity nor tunneling correction was included.

III. Results and Discussion

A. Active-site arrangement

Figure 1 illustrates the binding site of SpvC. The phosphopeptide substrate is bound along a shallow groove on the surface of the SpvC enzyme with the anionic phosphoryl group of pThr encircled by three positively charged Arg residues (Arg148, Arg213 and Arg220) and Lys104. The strong electrostatic interaction in this site, along with that in another binding site for pTyr two residues downstream, provide two anchoring points for the substrate and thus are responsible for the noted substrate specificity.

Figure 2 displays the minimized geometry of the active site of the Michaelis complex. In particular, the N_{ζ} atom of Lys136 is close to the H_{α} atom of pThr for proton abstraction. The Lys136 base is loosely held in place by two hydrogen bonds between its two hydrogen atoms, $H_{\zeta 1}$ and $H_{\zeta 2}$, and the backbone oxygen atoms of substrate residues Met (O_{Met}) and Glu (O_{Glu}), as well the enzyme hydroxyl oxygen of Tyr158 (O_H). In addition, the H_{ϵ} atom of His106 forms a hydrogen bond with the bridging oxygen O_y of pThr, setting the stage for protonation of the phosphate leaving group, while its H_{δ} is stabilized by $O_{\delta 2}$ of Asp201 via a hydrogen bond.

The MD simulation began from the minimized geometry and consisted of 700 ps of heating and equilibration, at which point the RMSD stabilized (See Fig. 3), and the final 2.5 ns of simulation data was used to calculate bond distances and their fluctuations. Selected key distances are summarized in Table 1. It is easy to see that the positive pocket surrounding the phosphoryl group formed by Arg148, Arg213, Arg220, and Lys104, was quite stable during the simulation, as evidenced by the corresponding short hydrogen bond distances in Table 1. The dynamics as shown in Fig. 4 also suggests that the hydrogen bond between the H_{ϵ} atom of His106 and the bridging oxygen (O_y) of pThr is reasonably stable, although larger fluctuations are present. The basicity of the His106 side chain is enhanced by its interaction with Asp201, evidenced by a strong hydrogen bond between H_{δ} of His106 and $O_{\delta 2}$ of Asp201, also shown in Fig. 4. This is vital if His106 is to serve as a general acid and transfer H_{ϵ} to the leaving phosphate group. More mechanistic aspects are discussed below, but this model supports His106's important role in both substrate binding and catalysis.

On the other hand, two possible conformations for Lys136 were observed as illustrated in Figs. 5 and 6. The primary conformation corresponds closely to the minimized structure shown in Fig. 2. The secondary conformation occurs before 0.25 ns where Lys136 loses hydrogen bonds with the backbone carbonyl oxygen atoms O_{Tyr} and O_{Glu} , swinging under the peptide substrate to associate with the hydroxyl oxygen of Thr156 (O_H). This is clearly reflected in the corresponding distances shown in Fig. 6. Interestingly, the N_{ζ} - H_{α} distance is shown in Fig. 6 to stay relatively constant during this switch, making proton abstraction from the alpha carbon a possibility in either conformation. Lys136 is also partially exposed to solvent in our model, but the solvation might be replaced by tighter protein-protein interaction between SpvC and its MAPK substrate.

An important issue in the proposed catalytic mechanism is the acidity of the H_{α} atom, as no Schiff base is involved in the phosphothreonine lyase reaction. Instead, it was proposed that the enzyme utilizes active-site hydrogen bonds to reduce the pK_a of H_{α} in pThr.²⁴ Indeed, our MD simulations revealed a very strong hydrogen bond between the amine group of Lys104 and the carbonyl oxygen of substrate pThr, as evidenced by the $H_{\zeta 2}$ - O_{pThr} distance of 1.86 ± 0.15 Å. This is consistent with the experimental observation that mutation of Lys104 inactivates the enzyme.^{9,24} The pThr backbone oxygen makes an additional hydrogen bond interaction with the hydroxyl group of Tyr158 ($r(H_H-O_{pThr}) = 2.06 \pm 0.26$ Å). Finally, the alpha hydrogen may be further acidified by hydrogen bonds between $H_{\zeta 1}/H_{\zeta 2}$ of Lys136 and the backbone

oxygen (O_{Met}) of the adjacent Met residue, as shown in Fig. 6. It is conceivable that these electrostatic interactions facilitate the unusual proton abstraction by the nucleophilic Lys136.

B. Catalytic mechanism

The optimized structure of the reactant complex (RC) in the truncated active-site model is given in Fig. 7, and the key geometric parameters are labeled in the figure. The hydrogen bond between the imidazole H_ϵ of His106 and the bridge oxygen O_γ of pThr is 1.62 Å, which is in reasonably good agreement with the crystallographic distance between N_δ and O_γ of 2.90 Å, and shorter than the MD distance of 2.22 ± 0.26 Å. The distance between the methylamine nitrogen (N_ζ) and the proton (H_α) that has been proposed to transfer from the substrate to Lys136 during the reaction, is 2.67 Å, comparable to the MD distance of 2.81 ± 0.26 Å. In addition, the phosphate group is tightly bound with the guanidinium groups representing the positively arginine crown, closely resembling the X-ray structure and the MD results discussed above. Thus, this structure was used as the initial structure for subsequent optimizations.

Only one transition state (TS) was found and it features a concerted reaction mechanism. As shown in Fig. 7, the C_β - O_γ bond at TS is largely broken, with a bond length of 2.13 Å, and the proton is almost transferred from N_δ of the imidazole to O_γ , which results in the dianion of inorganic phosphate. In the mean time, the C_α - H_α bond is elongated to 1.24 Å, while the distance between H_α and N_ζ of the methylamine decreases to 1.64 Å, signaling the proton in flight. The reaction coordinate, which has an imaginary frequency of $338i\text{ cm}^{-1}$, is a combination of proton transfer and C-O bond stretch. The predicted kinetic isotope effects (KIEs) are listed in Table 2 and they reflect the involvement of various atoms in the reaction path. Note that the hydrogen KIEs may be underestimated because no tunneling is taken into consideration.

The concerted pathway observed in the truncated active-site model discounts the possibility of two other alternative mechanisms. In the so-called E1cB mechanism, the deprotonation takes place first to form a carbanion intermediate, which subsequently eliminates the phosphate leaving group. The E1 mechanism, on the other hand, creates a carbocation first due to elimination, followed by deprotonation. Since there is no clear candidate to stabilize the charge associated with these two mechanisms, they are considered unlikely for the enzymatic reaction. Transition states for the E1cB and E1 mechanisms were not found in our studies.

The intrinsic reaction coordinate (IRC) method was used to yield the product complex (PC) shown in Fig. 7, in which the scissile C-O bond is clearly cleaved and the proton transfers completed. As shown in Table 3, the free energy barrier for this reaction is 17.33 kcal/mol in the gas phase, which is close to experimental barrier heights suggested by k_{cat} for several substrate/enzyme combinations. It should be noted that the backbone carbonyl group is not polarized by the hydrogen bond to Lys104 in the truncated active-site model, which might contribute to the barrier. The barrier increases somewhat when solvation contributions obtained using the PCM model are added in, indicating strong solvent effects. Although several key residues have been included in the truncated active-site model, it might still be insufficient to correctly represent the enzymatic environment. It would be highly desirable to carry out quantum mechanical/molecular mechanical studies in order to elucidate how the enzyme catalyzes the reaction.

IV. Conclusions

The OspF family of protein effectors differs significantly from the more well-known phosphatases in that they cleave the C-O bond instead of the O-P bond. It has been argued that there are important structural differences in the active sites of the two types of enzymes. In phosphothreonine lyases, such as SpvC, the phosphate moiety of the substrate is completely

insulated from solvent. As a result, no hydrolysis is possible. On the other hand, water molecules are allowed to access the active site and they can thus be activated to hydrolyze the phosphorylated intermediate. The completely different dephosphorylation mechanisms are associated with different transition states. As such, commonly used transition-state analogs for phosphatases, such as vanadate, are not effective in inhibiting phosphothreonine lyases.¹²

Our MD simulations of the Michaelis complex of SpvC strongly support the notion that Lys136 is the base, based on its proximity to the acidic alpha hydrogen. This observation is consistent with the observation that mutation of the conserved lysine at this critical position (136 in SpvC and 134 in OspF) completely abolishes the catalytic activity of the effector.^{8,24} In addition, His106 is shown in our MD simulations to donate a hydrogen bond to the bridge oxygen, thus setting the stage for the protonation of the phosphate leaving group. This is also consistent with mutagenesis experiments.^{8,24} Finally, there exists a network of hydrogen bonds that polarize backbone carbonyl oxygen atoms, which may contribute to the lowered pK_a of H_α .

These structural determinants for the enzymatic reaction are confirmed by the DFT reaction path, which features a concerted transition state in which the proton abstraction and C-O bond cleavage occur concurrently. We believe that the concerted mechanism should hold for the enzymatic system as well, but more detailed quantum mechanical/molecular mechanical studies are needed to confirm this hypothesis. To facilitate future comparison with experimental investigations, we have also computed kinetic isotope effects (KIEs) for several key atoms.

To summarize, the computational studies reported here provide detailed information on the interaction of active-site residues with the substrate and important insights into the novel β -elimination mechanism operating in the OspF family of effectors, thus laying the foundation for future studies of this potentially important drug target.

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Scheme 1.

Arrangement of active-site residues and their interaction with the substrate.

Fig. 1. Binding pocket for the phosphorylated peptide (green) on the surface of SpvC. The phosphoryl group of pThr is surrounded by positive residues such as Arg148, Arg213, Arg220, and Lys104.

Fig. 2. Active-site arrangement of key residues in the Michaelis complex. The hydrogen bonds are illustrated in dashed lines.

Fig. 3.
RMSD of the 3.2 ns MD simulation.

Fig. 4.
Fluctuation of selected key distances involving the His106 residue.

Fig. 5. Overlay of snapshots representing the two conformations observed in the MD simulation. The distance between N_{ζ} and H_{α} , as indicated by dashed lines, remains largely unchanged in the two conformations.

Fig. 6.
Fluctuation of selected key distances involving N_{ζ} (upper panel) and $H_{\zeta 1}/H_{\zeta 2}$ (lower panel) of Lys136.

Fig. 7. Structures of the stationary points along the β -elimination reaction path obtained at the B3LYP/6-31+G(d,p) level of theory. The hydrogen bonds are highlighted by red dashed lines, while the reaction coordinates are labeled in black dashed lines.

Table 1

Selected key distances and their average fluctuations in the Michaelis complex obtained from the MD simulation.

Residue	Bond	distance (Å)	
		<i>primary</i>	<i>secondary</i>
Lys136	N _ζ -- H _α (pThr)	2.81 ± 0.26	2.78 ± 0.23
	N _ζ -- C _α (pThr)	3.69 ± 0.23	3.64 ± 0.20
	N _ζ -- C _γ (pThr)	5.24 ± 0.35	3.69 ± 0.23
	N _ζ -- O _{Met}	3.63 ± 0.34	4.65 ± 0.35
	N _ζ -- O _{Glu}	3.79 ± 0.38	6.00 ± 0.35
	N _ζ -- O _H (Tyr158)	3.57 ± 0.31	4.66 ± 0.26
	N _ζ -- O _γ (Thr156)	5.53 ± 0.35	3.12 ± 0.35
His106	H _ε -- O _γ (pThr)	2.20 ± 0.26	-
	H _δ -- O _{δ2} (Asp201)	1.62 ± 0.08	-
Arg148	H ₁₁ -- O ₃ (pThr)	1.65 ± 0.08	-
	H ₁₂ -- O ₂ (pThr)	1.67 ± 0.09	-
Arg213	H ₂₁ -- O ₂ (pThr)	1.69 ± 0.09	-
	H ₂₂ -- O ₁ (pThr)	1.66 ± 0.09	-
Arg220	H ₃₁ -- O ₁ (pThr)	1.71 ± 0.10	-
	H ₃₂ -- O ₃ (pThr)	1.71 ± 0.10	-
Lys104	N _ζ -- O ₁ (pThr)	2.63 ± 0.07	-
	N _ζ -- O _γ (pThr)	3.27 ± 0.17	-
	N _ζ -- O _{pThr}	2.81 ± 0.12	-
	H _{C3} -- O ₁ (pThr)	1.61 ± 0.08	-
	H _{C3} -- O _γ (pThr)	2.73 ± 0.19	-
	H _{C2} -- O _{pThr}	1.86 ± 0.15	-
Tyr158			
	H _H -- O _{pThr}	2.06 ± 0.26	-

Table 2

Calculated kinetic isotope effects from the truncated active-site model

Residue	Atom	KIE
pThr	H _{α}	3.119
	C _{α}	1.006
	C _{β}	1.031
	O _{γ}	1.016
His106	H _{ϵ}	1.184

Table 3

Free energies (kcal/mol) of the RC, TS and PC for the truncated active-site model at the B3LYP/6-31+G(d,p) level of theory. The dielectric effects of water and protein are included with PCM by using the dielectric constants of 80 and 5, respectively. The experimental barrier heights for several substrate/enzyme combinations⁹ are also listed for comparison.

	RC	TS	PC
$\Delta G_{(\text{gas})}$	0.00	17.33	-23.40
$\Delta G_{(\text{protein})}$	0.00	24.34	-13.22
$\Delta G_{(\text{water})}$	0.00	26.09	-11.18
$\Delta G^{\text{exp.}}_{(\text{Erk5/OspF})}$	0.00	18.24	-
$\Delta G^{\text{exp.}}_{(\text{Erk2/OspF})}$	0.00	17.65	-
$\Delta G^{\text{exp.}}_{(\text{Erk5/SpvC})}$	0.00	16.04	-