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Computational modeling of the rate limiting step in low molecular weight protein tyrosine phosphatase

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Abstract Hydrolysis of the phosphoenzyme intermediate is the second and rate limiting step of the reaction catalyzed by the protein tyrosine phosphatases (PTPs). The cysteinyl phosphate thioester bond is cleaved by nucleophilic displacement where an active site water molecule attacks the phosphorus atom. Starting from the crystal structure of the low molecular weight PTP, we study the energetics of this reaction utilizing the empirical valence bond method in combination with molecular dynamics and free energy perturbation simulations. The reactions of the wild-type as well as the D129A and C17S mutants are modeled. For the D129A mutant, which lacks the general acid/base residue Asp-129, an alternative reaction mechanism is proposed. The calculated activation barriers are in all cases in good agreement with experimental reaction rates. The present results together with earlier computational and experimental work now provide a detailed picture of the complete reaction mechanism in many PTPs. The key role played by the structurally invariant signature motif in stabilizing a double negative charge is reflected by its control of the energetics of both transition states and the reaction intermediate.

Key words: Protein tyrosine phosphatase; Catalysis; Reaction free energy profile; Molecular dynamic; Free energy perturbation; Empirical valence bond method

1. Introduction

Protein tyrosine phosphatases (PTPs) are ubiquitous enzymes that catalyze the dephosphorylation reaction of specific phosphotyrosine residues in proteins. This is an important signaling mechanism in cellular processes such as cell growth, differentiation, proliferation and T-cell activation [1,2,3]. Unlike the protein phosphatases acting on phosphoserine and phosphothreonine substrates, PTPs do not require metals in the active site in order to accomplish catalysis. Instead, the characteristic phosphate binding loop motif (C-X_5-R-S/T) common to all PTPs is the structural basis for efficient lowering of the activation energy in these enzymes. The main chain nitrogens of the loop residues in combination with the guanidinium group of the arginine side chain coordinate the oxygens of the phosphate group during binding and catalysis. The two-step reaction catalyzed by the PTPs is initiated by a nucleophilic attack by the active site cysteine on the phosphorus atom of the bound substrate. The thiolate form of the nucleophile is found to be stabilized by the hydroxyl group of the Ser/Thr residue in the signature motif [4,5]. As the axial P-O bond is cleaved and the leaving group oxygen is protonated by a closely positioned aspartic acid, the phosphate group becomes covalently bound to the cysteine residue via a thioester linkage. A double displacement mechanism involving a covalent intermediate was first proposed by Saini, who found overall retention of stereochemistry at the phosphorus atom [6]. The existence of a phosphorylated cysteine in the active site was later confirmed by ^{31}P-NMR and ^{32}P-labelled phosphoenzyme trapping in combination with mutagenesis experiments [7,8,9]. Recently, the phosphoenzyme intermediate of a PTP1B mutant was trapped and its crystal structure determined [10]. The thiophosphate ester is hydrolyzed by a water molecule in the second step of the reaction. The reacting water molecule is believed to be activated by the same aspartic residue that previously acted as general acid. Nucleophilic displacement at the cysteinylphosphate intermediate results in release of inorganic phosphate as the final product. Hydrolysis of the phosphoenzyme intermediate has been found to be the rate limiting step for most substrates in a number of protein tyrosine phosphatases [11–13].

A well-characterized mutant of the PTPs is the replacement of the catalytic aspartic acid by an alanine. This mutation considerably decreases the reaction rate of all PTPs, but to different extents. In the PTP1B D181A mutant, the rate is decreased by a factor of 10^5 [14], whereas in the low molecular weight PTP (low M_r PTP), the corresponding mutation (D129A) only causes a decrease of a factor 2000–3000 compared to the wild-type enzyme in both steps of the reaction [15].

In this work, we have studied the energetics of the second step of the reaction catalyzed by the low M_r PTP (for review see [16,17]), using the empirical valence bond (EVB) method in combination with molecular dynamics (MD) and free energy perturbation (FEP) simulations [18,19]. The obtained reaction free energy profile is combined with that of the first reaction step published by us elsewhere [20,21] and the calculated overall rate limiting barrier of 16 kcal/mol agrees well with the observed turn-over rate for the wild-type enzyme. An alternative reaction mechanism has been investigated for the mutant enzyme lacking the general acid/base (Asp-129 in low M_r PTP) and the effect of mutating Cys-17 is examined.

2. Materials and methods

The hydrolysis of the phosphoenzyme intermediate is described here by the EVB model. This model treats each reaction step as a transformation between different valence bond (VB) states or resonance structures. These states are modeled by separate molecular mechanics force fields or potential functions, that describe their different geometries and charge distributions. The intervening configurations of a reaction are obtained by mixing of the relevant VB states. The EVB method is easily incorporated into MD simulations where the reaction

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free energy can be mapped out by a combination of the free energy perturbation and umbrella sampling techniques as described in detail elsewhere [18,19]. By running MD/FEP/EVB simulations of a given reaction step in solution and in the solvated enzyme substrate complex, it is possible to compare the energetics of the catalyzed and uncatalyzed reactions and thus quantify the effect of the environment surrounding the reacting groups. The key feature of the EVB method is the possibility to calibrate the hamiltonian against experimental data. Calibration involves determining gas-phase energy difference as well as off-diagonal matrix elements between pairs of VB states so that the EVB potential surface obtained from the uncatalyzed simulations reproduce experimental reaction free energies and barrier heights of relevant reference reactions in solution. The same calibrated EVB hamiltonians are then used in subsequent simulations where the surrounding water is replaced by the solvated enzyme.

The VB states of the phosphoenzyme hydrolysis used here are depicted in Fig. 1. We have shown in previous work [20,21] that with a negatively charged nucleophile, the substrate of low M_r PTP is bound in its mono-anionic form, i.e. the reacting groups (Cys-12, Asp-129 and the phosphate moiety) have a total charge of -2. Consequently, the VB states modeled in this work have the protonation state as in Fig. 1 with the cysteinyl phosphate singly protonated prior to the nucleophilic attack by the incoming water molecule which is activated by the negative aspartate. For the alternative reaction mechanism where the aspartic acid is absent, the thiophosphate group has a double negative charge.

The detailed procedure of calibrating the EVB hamiltonian for phosphoryl transfer reactions in PTPs has been presented by us in [20]. For proton transfer reactions such as $\Phi_7 \rightarrow \Phi_8$, the p$K_a$ difference between donor and acceptor together with linear free energy relationships for proton transfer were used as described in [19] for calibration of the relevant EVB parameters. Calibration of nucleophilic displacement steps, such as $\Phi_5 \rightarrow \Phi_7$ (via Φ_6) was done using data from Åkerfeldt [22] on hydrolysis of phosphorothioic acids, from Bourne and Williams [23] on equilibrium constant dependence on leaving group pK_a and from Guthrie's [24] thermodynamic data on phosphoric acid derivatives. The resulting estimated energetics for the uncatalyzed reaction mechanism in water are shown in Fig. 2 (upper curve).

2.1. Simulation details

All calculations were carried out using the program Q [25] with force field parameters from the GROMOS87 potential [26]. However, bonds within the reacting fragments were replaced by Morse potentials using standard bond lengths and dissociation energies. Non-standard charges and geometries involving S-P bonding were obtained from AM1-SM2 [27] calculations. The reacting fragments were surrounded by a 16 Å sphere of SPC water in the solution simulations and by a sphere of the same size containing both protein and water in the enzyme simulations. The protein coordinates were those of bovine liver low M_r PTP in complex with sulfate ion [28] (PDB accession code 1PHR). Protein atoms outside the simulation sphere were restrained to their crystallographic coordinates and interacted only via bonds across the boundary. A non-bonded cut-off of 10 Å was used together with the local reaction field method [29] for long-range electrostatics. The water surface was subjected to radial and polarization restraints as described elsewhere [25].

The simulation protocol was essentially as in [5]. All simulations were performed at a constant temperature of 300 K with a time step of 1 fs. The perturbations were sampled using 51–55 λ-points, each λ including 2 ps equilibration and 3 ps data collection.

3. Results

The resulting free energy profiles from the EVB/FEP/MD calculations using phenyl phosphate as the substrate are summarized in Fig. 2. As can be seen, all steps of the reaction are significantly catalyzed by the enzyme compared to the uncatalyzed reference reaction in water. In particular, the activation barrier of the rate limiting step, formation of the second penta-coordinated high-energy structure ($\Phi_5 \rightarrow \Phi_6$), is lowered by as much as 15 kcal/mol. Since concerted bond breaking and leaving group protonation was found to be considerably favored over a stepwise mechanism in the first part of the reaction [20], the analogous concerted pathway was also modeled here. In [20], it was found that the protein environment cannot stabilize a negative ligand in the active site outside the phosphate binding loop, which would also be the case for a stepwise proton transfer to Asp-129 and a subsequent in-line attack of a hydroxide ion.

The complete reaction in which the phosphate group is effectively transferred from phenol to a water molecule is exothermic with a change in standard free energy of -3 kcal/mol

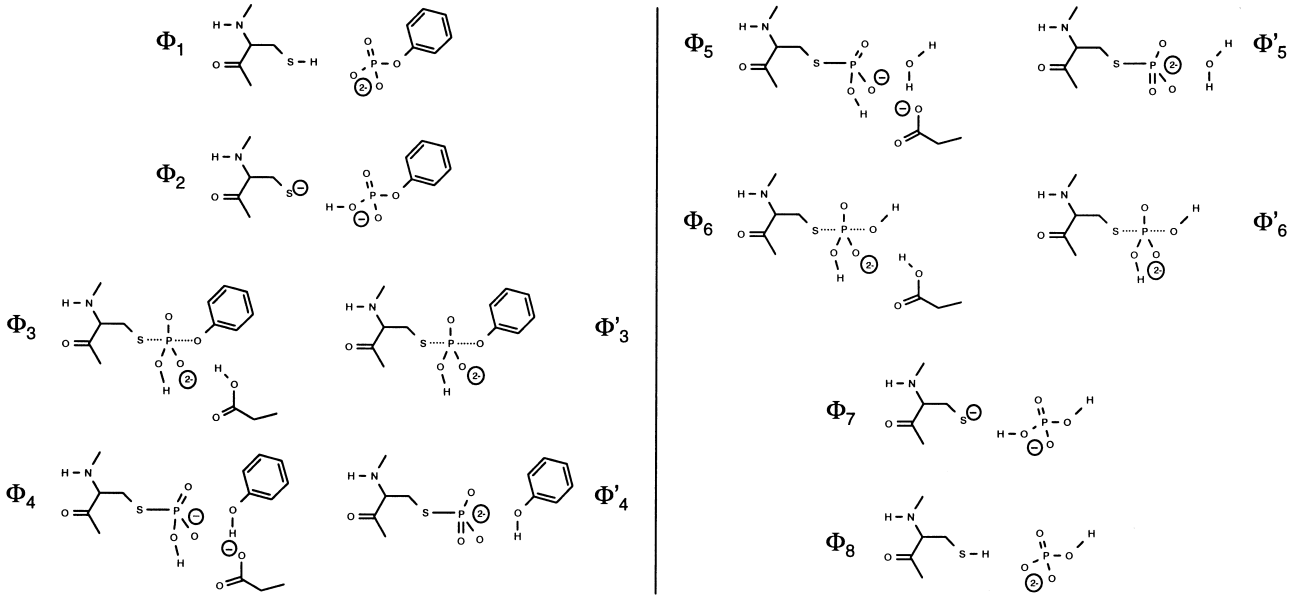

Fig. 1. VB states used in the EVB calculations of the complete reaction mechanism catalyzed by the low M_r PTP. The details of step 1 (Φ_1–Φ_4) have been presented elsewhere [20,21]. Φ'_3–Φ'_6 represent the alternative reaction pathway that may be used by mutants lacking the general acid Asp-129. The formal charges of the reacting fragments are indicated.

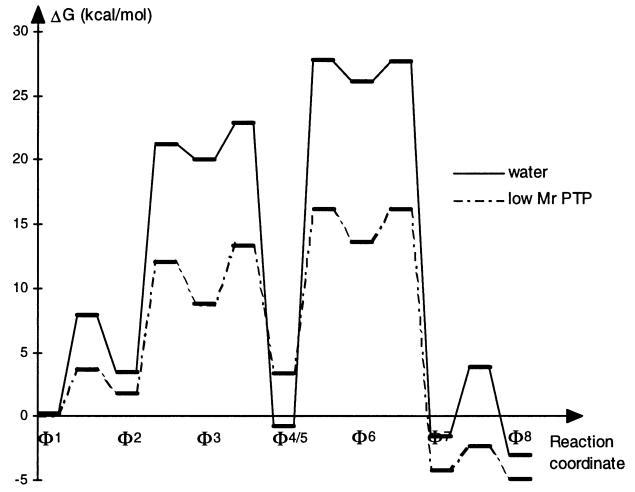

Fig. 2. Free energy profiles for the reaction mechanism in aqueous solution based on experimental data (upper curve) and the same reaction in low M_r PTP obtained from MD/FEP/EVB calculations (lower curve).

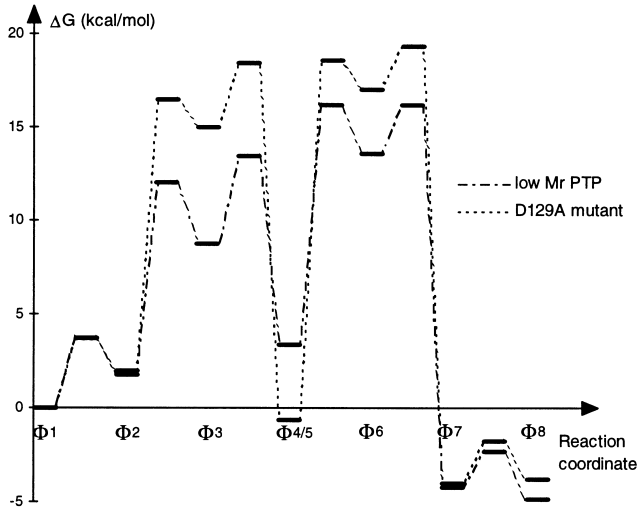

Fig. 3. Free energy profiles for the reaction mechanism catalyzed by the wild-type low M_r PTP and the alternative reaction mechanism (involving Φ'_3–Φ'_6) proposed for the D129A mutant.

[23]. In Fig. 2, the equilibrium constant on the enzyme becomes −5 kcal/mol in favor of the products. Here, the apparent shift of the equilibrium constant includes the difference in binding of the product versus the substrate. Since inorganic phosphate is a competitive inhibitor of low M_r PTP [30], it is not unexpected that the product binds somewhat stronger than the substrate and thus lowers the level of Φ_7 and Φ_8 relative to Φ_1 and Φ_2. The reaction could also involve a free energy change when the leaving phenol group is exchanged for an incoming water molecule ($\Phi_4 \rightarrow \Phi_5$). The difference in binding free energy between a phenol molecule and a water molecule was therefore estimated using the linear interaction energy method [31,32]. The absolute binding affinities of phenol and water to the active site were calculated as in reference [32], giving as a result that the binding free energies were very similar (−2 kcal/mol) for the two ligands. The energetics of ligand exchange do therefore not affect the free energy profiles in Figs. 2 and 3.

The D129A mutant of low M_r PTP has been extensively studied in enzymological experiments. This mutant lacks the catalytically important general acid/base residue. However, the mutant is not entirely inactive, but retains an activity around 3000 times less than that of the wild-type enzyme [15]. We have found that protonation of the leaving group is essential for catalysis of phenyl phosphate hydrolysis since release of negatively charged phenolate species is energetically disfavored. If the leaving group departs as an anion, we predicted an energy barrier that is not compatible with the experimentally observed activity. We therefore proposed that the phosphate group itself may act as an acid in the first reaction step of this mutant and protonate the leaving group concerted with its release. This hypothesis yielded an activation barrier consistent with experiments [20]. It then seems reasonable that the −2-charged phosphocysteine could itself abstract a proton from the attacking water molecule in the second hydrolysis step of the D129A mutant enzyme. This mechanism would then be similar to the substrate-assisted reaction mechanism proposed for the acylphosphatase [33].

The alternative reaction mechanism for D129A was simulated in the same way as the wild-type reaction and our previous results showed that the activation barrier of the first step is 5 kcal/mol higher than the corresponding wild-type reaction step. This corresponds to a decrease in rate by a factor of 4000. Here, we report the complete free energy profile for this reaction mechanism in the D129A mutant. As shown in Fig. 3, the free energy level of the phosphoenzyme intermediate lies somewhat below the initial enzyme-substrate level. From this lowest point of the profile, the rate limiting barrier $\Phi_5 \rightarrow \Phi_6$ is predicted to be 20 kcal/mol which is in accordance with the observed turn-over rate of 0.012 s^{-1}

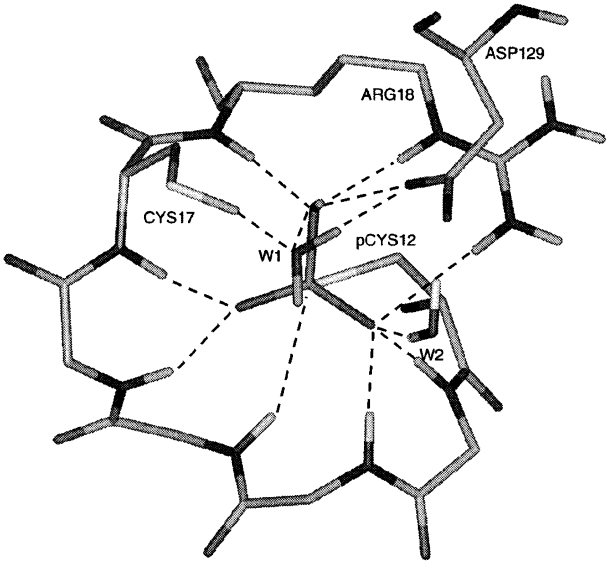

Fig. 4. MD structure of the active site in the phosphoenzyme intermediate state (Φ_5) viewed along the P-S bond. The two water molecules (W1 and W2) interacting with the phosphate group are shown, whereas the side chains of residues 13–16 are omitted for clarity. W1 is in the position for an attack on the phosphorus atom. The network of hydrogen bonds is shown with dashed lines.

[15]. For the wild-type enzyme, the phosphoenzyme intermediate is higher in energy than the initial enzyme-substrate complex, while in the mutant, it is slightly lower. This would imply that more phosphoenzyme intermediate should accumulate in the D129A mutant than in the wild-type, which has also been observed by phosphoenzyme trapping experiments [15]. The good compatibility of this proposed reaction mechanism with available experimental data suggests that this pathway may actually be utilized by mutant low M_r PTP lacking the general acid/base Asp-129.

Due to restricted rotation of the mono-anionic phosphate group when bound to the active site (both in the Michaelis complex and in the covalent intermediate), there are three different oxygens that can be protonated. The entire reaction of low M_r PTP has been simulated with all three possible protonations. In both steps, it is clear that protonation of the O3 oxygen (that accepts a hydrogen bond from N_ε of Arg-18 in the crystal structure) is most favorable. When positioned on this oxygen, the proton can participate in a hydrogen bond to Asp-129 which lowers the energy level of Φ_4 and Φ_5 as well as the flanking barriers compared to the other two possible protonations where the same interaction cannot be formed. The free energy profile presented in Figs. 2 and 3 is calculated with the proton positioned in the most favorable position at O3.

MD trajectories of the wild-type phosphoenzyme intermediate show that two water molecules interact directly with the phosphate group (Fig. 4). One of these water molecules will be in the right position for the hydrolysis reaction to occur. In PTP1B, Gln-262 has been found to be an important residue for coordinating the nucleophilic water molecule. Mutating this residue to alanine resulted in phosphoenzyme trapping which made it possible to crystallize the reaction intermediate [10]. Although very similar in active site structure, there is no corresponding glutamine present in low M_r PTP. However, our simulations of the water attack ($\Phi_5 \rightarrow \Phi_6$) reveal that Cys-17 interacts with the nucleophilic water. It seems that this interaction is responsible for coordinating the water molecule in favor of the reaction. The involvement of Cys-17 in the phosphoenzyme hydrolysis step was proposed by Cirri et al. [7], already in 1993, before the structure was solved. When Cys-17 was mutated to a serine, the enzyme displayed a low activity, but significant amounts of phosphoenzyme intermediate were trapped. This suggests that the larger thiol group orients the water molecule better than the smaller hydroxyl group in position 17. We also calculated the free energy profile (not shown) for the water attack ($\Phi_5 \rightarrow \Phi_6$) in the C17S mutant enzyme and it was found that the free energy barrier increases with 1.6 kcal/mol. The polar and steric interaction between Cys-17 and the water molecule that is responsible for its appropriate positioning can be directly appreciated in Fig. 4. Superimposing the active site residues of PTP1B and low M_r PTP reveals that the proposed water coordinating residues (Gln-262 in PTP1B and Cys-17 in low M_r PTP) are in the same spatial position relative to the active site, although not sequentially related. Whereas Cys-17 is a residue in the phosphate binding loop, Gln-262 is positioned in a flexible loop that can apparently move in and out of the active site [10].

4. Discussion

In summary, we have used the MD/FEP/EVB approach to simulate the rate limiting step (hydrolysis of the phosphoenzyme intermediate) of the reaction catalyzed by the low M_r PTP. The resulting free energy profile was combined with that of the substrate dephosphorylation step reported earlier. The results provide a complete and detailed picture of catalysis in the low M_r PTPs with implications also for other tyrosine specific protein phosphatases. It is clear that the characteristic signature motif corresponding to the phosphate binding loop is the key catalytic factor in these enzymes. It appears to be optimized for stabilizing a double negative charge in the center of the loop (it was earlier shown that the enzyme cannot accommodate a total charge of -3 [21]). This explains both the efficient stabilization of the transition states and why significant accumulation of the reaction intermediate does not occur. In the wild-type enzyme, the intermediate is somewhat destabilized since one of the transition state negative charges is effectively moved to Asp-129. In the D129A mutant, this does not occur and the intermediate remains double negatively charged resulting in its accumulation.

The fact that negative charges only appear to be stabilized in the center of the P-loop is consistent with the raised pK_a value of the general acid/base residue. The high pK_a of Asp-129 clearly facilitates activation of the hydrolytic water molecule in the rate limiting step. We have also investigated an earlier proposed alternative reaction mechanism that might be utilized by mutant enzymes lacking the catalytic acid/base residue. Calculations on the D129A mutant gave a rate decrease that agrees with kinetic data and thus supports the notion that mutation of Asp-129 could enforce a change of mechanism.

The fact that our calculated reaction energetics for both the wild-type and the D129A and C17S mutant enzymes are consistent with available experimental data shows that the present computational modeling approach can successfully describe the catalytic process in low M_r PTPs. The results reported here now also provide a detailed mechanistic framework in which enzymological experiments may be interpreted.

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