Towards understanding phosphonoacetaldehyde hydrolase: an alternative mechanism involving proton transfer that triggers P–C bond cleavage†

Borys Szefczyk[a,b]

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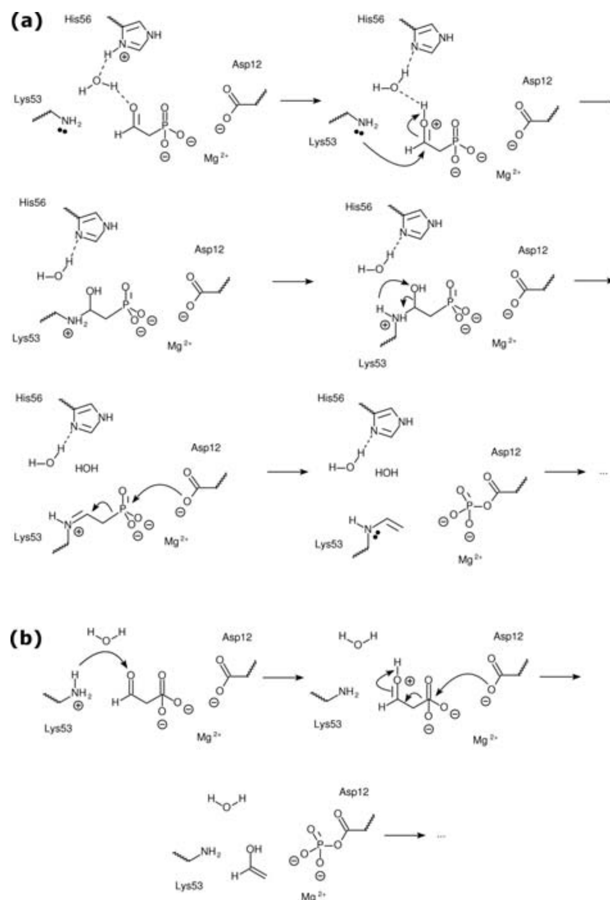
The theoretical QM/MM study of the reaction catalysed by phosphonoacetaldehyde hydrolase indicates a possible alternative mechanism of the P–C bond cleavage: as opposed to the mechanism proposed earlier and that involved formation of a covalently bound intermediate (Schiff-base), in the new mechanism, the bond breaking process is facilitated by proton transfer from catalytic lysine residue to the substrate.

Phosphonoacetaldehyde hydrolase (phosphonatase, EC 3.11.1.1)—enzyme catalysing P–C bond cleavage in phosphonoacetaldehyde (PALD)—has been studied using several experimental methods.[1–4] Based on the results of these experiments and by analogy with the mechanism utilized by decarboxylases and aldolases,[1] a mechanism involving Schiff-base (imine) formation was proposed (called 'Schiff-base mechanism' throughout this work). According to this mechanism, the reaction starts by a nucleophilic attack of a neutral amine (Lys53 residue) on the carbon atom in the carbonyl group of the substrate. Simultaneously a proton is transferred to the oxygen atom of the carbonyl group. After elimination of a hydroxide, the Schiff-base is formed and finally the phosphoryl group is transferred to Asp12. In the present work, QM/MM calculations of the initial phase of this reaction are presented and a new, completely different and much simpler mechanism is proposed. In contrast to the Schiff-base mechanism, no imine formation is needed and P–C bond cleavage starts with the proton transfer from Lys53 to PALD (Scheme 1).

In the experiment performed by Olsen *et al.*,[1] a mixture containing the enzyme and substrate was treated with a reducing agent, $NaBH_4$. After digestion with trypsin, oligopeptides containing *N*-ethyllysine have been found. The *N*-ethyllysine could be a product of the imine reduction. The X-ray crystal structure of the enzyme incubated with the substrate (PALD) and $NaBH_4$ revealed[4] that the *N*-ethyllysine can be found in the active site. Baker *et al.*[2] have shown, that the enzyme loses its catalytic properties after mutation of the Lys53 to arginine. However, the X-ray structure of this mutant,[4] displays a significantly larger angle (*ca.* 20°) between the domains; therefore, the inactivity of the mutant may result from the altered structure of the enzyme, not from the incapability to form the Schiff-base. On the other hand, the quantum-chemical study of the Schiff-base mechanism using a small model of the active site,[5] resulted in barriers that are too high for an enzymatic environment. This can be attributed to the insufficient description of the active site; however, it might also indicate, that the mechanism is in fact different.

In this study, the first step of the reaction in the active site of phosphonatase was investigated using hybrid QM/MM approach. The initial model was built from the crystal structure of the *Bacillus cereus* enzyme complexed with an inhibitor.[3] Reaction paths were explored using the Conjugate Peak



Scheme 1 (a) P–C bond cleavage and Schiff-base formation in the original mechanism as proposed by Lahiri *et al.* See ref. 4 for full description of the mechanism. (b) The alternative mechanism of P–C bond cleavage in phosphonatase. Concerted proton transfer from Lys53 and nucleophilic attack from Asp12 results in the P–C bond breaking.

[a] *Institute of Physical and Theoretical Chemistry, Wroclaw University of Technology, Wybrzeze Wyspianskiego 27, 50-370 Wroclaw, Poland. Fax: +48 71 320 33 64; Tel: +48 71 320 32 00*
[b] *REQUIMTE, Department of Chemistry, Faculty of Science, University of Porto Portugal. E-mail: borys.szefczyk@pwr.wroc.pl*

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Refinement (CPR)[6] method implemented in CHARMM.[7] The calculations of the QM region, at the Density Functional Theory (DFT) level, applying the B3LYP functional and the 6-31G(d) basis set were performed using the GAMESS package.[8] The MM part of the system was treated with the CHARMM22 force field.[9] The procedure of preparation of the structures for calculations was similar to our earlier work on chorismate mutase[10] and is described in the ESI.† The Density Functional Theory has been successfully applied to model other enzymatic reactions involving a Schiff-base intermediate.[11,12]

According to the mechanism proposed in the literature,[3] the Schiff-base formation is accompanied by the proton transfer to the oxygen atom in the carbonyl group of PALD. If the Lys53 residue is protonated, there is a possibility of direct proton transfer from lysine to PALD or indirect proton transfer from lysine to the water molecule (HOH120 in the crystal structure) and then, from the water molecule to PALD. When the proton is transferred from Lys53 to the oxygen atom of PALD in the initial model of the R1 or R2 complex, and the resulting geometry is optimized—it does not yield the structure expected for the Schiff-base mechanism. Instead, the P–C bond is broken, the phosphoryl group is transferred to Asp12 and vinyl alcohol is formed (which could later isomerize to acetaldehyde). In other words, the proton transfer itself induces the P–C bond cleavage. A similar event was observed for both proton transfer routes—direct, from Lys53 to PALD and mediated by HOH120. In these two routes, two conformations (Pd, Pm) of the product are formed, differing in the orientation of the vinyl alcohol bound in the active site, whereas the phosphonyl group is bound to Asp12. Earlier calculations at the QM/MM HF/3-21G(d) level (unpublished results), resulted in similar observations: the putative intermediate of the Schiff-base mechanism could not be found and the proton transfer led to a synchronous P–C bond cleavage. In order to estimate the energetic cost of this proton transfer, the reaction paths were calculated using the TREK approach. Assuming the two initial conformations (R1 and R2) and two routes of the proton transfer, four pathways are possible: (i) R1 → TS1d → Pd, (ii) R1 → TS1m → Pm, (iii) R2 → TS2d → Pd and (iv) R2 → TS2m → Pm. Numbers 1 and 2 denote initial conformation of the reactant complex, 'd' means direct proton transfer, 'm' means proton transfer mediated by HOH120. Fig. 1 shows the geometry of the optimized species and Table 1 displays the energetics of the paths. All paths exhibit low barriers (from 5 to 8 kcal mol^{-1}) and the direct proton transfer is the energetically preferred route; however, the predicted difference is within the accuracy of the method used. Based on these results, the reaction is concerted and both events—the proton transfer and the P–C bond cleavage happen at the same time. The barriers calculated here (5–8 kcal mol^{-1}, Table 1) are significantly lower, compared to the earlier gas-phase calculations of the Schiff-base mechanism[5] where the barrier for the nucleophilic attack of Lys53 on PALD, assisted by a water molecule is *ca.* 12 kcal mol^{-1} and the barrier for the water molecule abstraction is around 31 kcal mol^{-1}. These results show, that the QM/MM approach is necessary for the correct description of the reaction in phosphonatase. To estimate the influence of the choice of the QM region, arginine Arg160 has been included into the QM region and the barriers have been recalculated (using geometries optimized with the smaller QM region). As shown in Table 1 (column TS[b]), in three paths the barrier is underestimated by 0.7–2 kcal mol^{-1}, probably because of too strong stabilization of the transition state by the force field charges on arginine. Since some negatively charged systems sometimes require diffuse basis functions to account for the further penetration of the electron density, the barriers have also been recalculated using the 6-31+G(d) basis set (with the geometry optimized at the B3LYP/6-31G(d)/CHARMM level). In three paths, the barriers increase by 2–2.5 kcal mol^{-1} if diffuse functions are added (Table 1, column TS[a]).

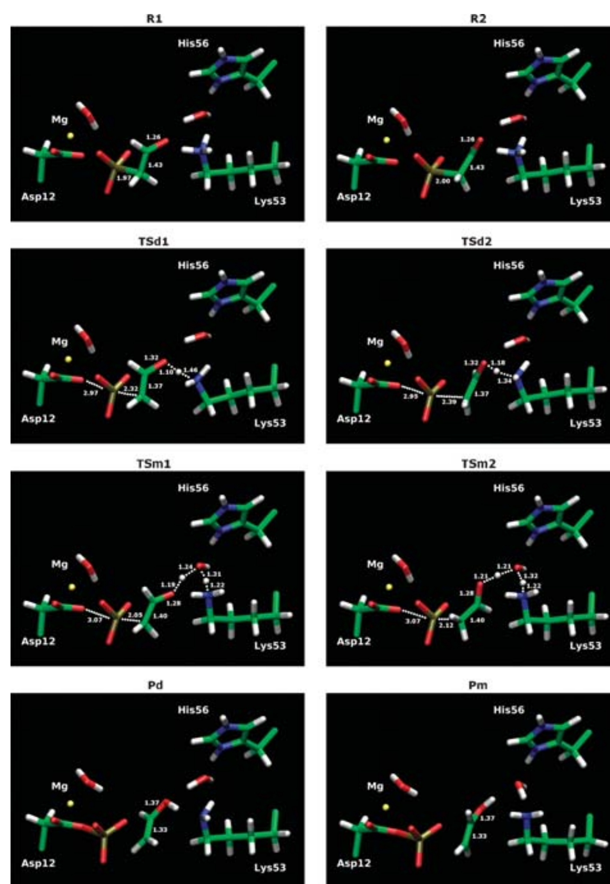

Fig. 1 Reactants (R1, R2), transition states (TSd1, TSm1, TSd2, TSm2) and products (Pd, Pm) of the P–C bond cleavage reaction in phosphonatase, calculated at the QM/MM level. Selected bond lengths are given in Å (see also ESI†).

Lahiri *et al.*[4] considered a mechanism starting from protonated lysine Lys53, and deprotonated histidine His56, followed by a proton transfer from Lys53 to His56, mediated by a water molecule. As a result, the histidine becomes protonated and an exposed lone electron pair on nitrogen of Lys53 is ready for the nucleophilic attack that leads to the Schiff-base formation. This state—after the proton transfer from Lys53 to His56—is shown in Scheme 1, on the left-hand side of the diagram (a). Earlier, a mechanism starting from protonated histidine His56 was also considered.[3] Here, only the selected variant of the protonation state of Lys53 and His56 that is chemically the most intuitive (both residues, Lys53 and His56, are protonated) is investigated. It has been found[2] that after the Lys53Arg mutation, the catalytic activity of the enzyme is suppressed. This result seems to support the Schiff-base mechanism and

Table 1 Energetics of the reaction in phosphonatase, calculated at the QM/MM level (values are in kcal mol^{-1}). Compare Fig. 1 for geometry of reactants (R1, R2), transition states (TS1d, TS1m, TS2d, TS2m) and products (Pd, Pm)

Path	R	TS	TS^a	TS^b	P
R1 → TS1d → Pd	0.0	4.9	7.5	7.1	−5.5
R1 → TS1m → Pm	0.0	7.7	9.9	8.4	−5.2
R2 → TS2d → Pd	0.0	6.0	8.0	8.2	−8.7
R2 → TS2m → Pm	0.0	6.4	5.9	6.1	−8.3

[a] Single-point barrier height recalculated using a basis set with diffuse functions [6-31 + G(d)] for geometries optimized using the 6-31G(d) basis set. [b] Single-point barrier height recalculated with arginine Arg160 included in the QM region.

contradict the mechanism presented here, because arginine could serve as a proton donor in the same way as lysine does and should not affect the activity if the mechanism presented here is valid. However, it must be kept in mind, that the pK_a of arginine is higher than that of lysine by almost two units, therefore the reaction rate could be expected to drop by about two orders of magnitude. Moreover, Lys53 is located in a loop, that works like a lid, closing after the binding of the substrate. The angle of rotation of the loop is about 22°.[4] According to Lahiri *et al.*,[4] the angle of the loop opening increases in the Lys53Arg mutant to *ca.* 43°. The arginine residue is slightly larger than the lysine residue, therefore it is possible that after binding of the PALD molecule, the lid is unable to close and the contacts required for the reaction are not formed. Although the reduction of the lysine adduct to *N*-ethyllysine[1,2] could be interpreted in favour of the Schiff-base mechanism, this argument can be also questioned. The enzyme was incubated with PALD in the presence of $NaBH_4$, which reduces the covalently bound intermediate to *N*-ethyllysine. The *N*-ethyllysine adduct was also observed in the crystal structure of phosphonatase,[4] however, it may be also produced after the P–C bond cleavage from the acetaldehyde reacting with lysine Lys53. A similar experiment was performed using a mixture of phosphonatase, acetaldehyde (the product of this particular enzymatic reaction) and $NaBH_4$.[1] The inactivation was observed only when a higher concentration of acetaldehyde was used. This seems to eliminate the possibility of inactivation by the product. However one can speculate that when using PALD, the acetaldehyde is produced directly in the active site, where it can react with lysine Lys53 even in low concentration. This is due to the fact, that the PALD molecule is bound specifically in the active site, whereas in the second experiment, when acetaldehyde is used instead of PALD, the acetaldehyde molecule, being smaller, has better access to other lysines in the structure as well. Olsen *et al.*[1] observed a second fraction of digested tripeptides and admitted that it may result from reduction of a lysine other than Lys53. This issue could be resolved experimentally by determination of the crystal structure of phosphonatase incubated with acetaldehyde and $NaBH_4$. However, to the best of our knowledge, the only crystal structure available, was prepared by incubation with PALD and $NaBH_4$.[4] Another method of experimental verification of the mechanism could be through the measurements of kinetic isotope effect (KIE)—*e.g.* deuterium or carbon KIE's. This method has been successfully used in evaluation of mechanisms exploited by enzymes, including those that perform phosphoryl transfer reactions.[13]

The results presented here indicate the possible existence of an alternative mechanism of the reaction catalyzed by phosphonatase that does not involve formation of a covalent imine adduct (Schiff-base). The applied QM/MM methodology involving DFT predicts a new low-energy mechanism of the P–C bond cleavage in PALD that proceeds with a synchronous hydrogen transfer from lysine Lys53, which may be assisted by a water molecule.

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