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REGULAR ARTICLE

Quantum mechanical studies on the existence of a trigonal bipyramidal phosphorane intermediate in enzymatic phosphate ester hydrolysis

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Abstract Phosphate ester hydrolysis is a key step in several enzymatic processes, which follow either a dissociative or an associative mechanism. While in the aqueous phase both pathways are favoured to about the same extent, the associative mechanism is relatively rarely observed. In this paper we report on quantum mechanical calculations for three enzymes HIV integrase, β-phosphoglucomutase and dUTPase, and try to find an explanation for the preference of the associative mechanism in a given enzyme. It is reasonable to suppose that the stabilisation of the pentacovalent, trigonal bipyramidal phosphorane moiety by formation of a covalent bond, one or more hydrogen bonds, or by coordination of a divalent metal cation with the equatorial oxygen atoms is the key factor. In all three enzymes studied one of the equatorial oxygen atoms is co-ordinated to a magnesium dication, while a second one is involved in a covalent bond. While in HIV integrase the third oxygen atom may only form a weak hydrogen bond with a solvent water molecule, in β-phosphoglucomutase this atom is stabilised by two strong hydrogen bonds with adjacent protein side chains and in dUTPase it is involved in a covalent bond.

Keywords dUTPase · Phosphate ester hydrolysis · Quantum mechanical modelling · Reaction path · Penta coordinated intermediate

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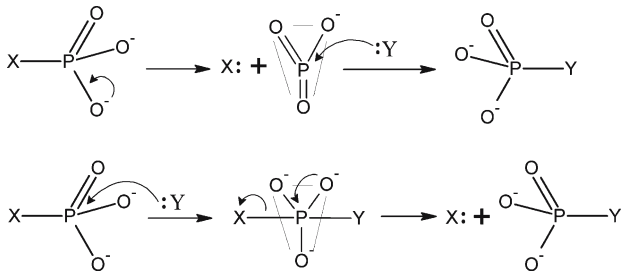
1 Introduction

Most enzymes involved in cell signalling (e.g. staphylococcal nuclease [1], protein kinases [2] or F1 ATPase [3]) catalyse nucleophilic substitutions at phosphorus. Hydrolysis of phosphate ester bonds is a key process in biological systems and many enzymes require one or more metal ions as cofactors to catalyse this reaction. However, the exact roles of metal ions as well as specific features of the enzymatic reaction path are still unclear. Two basic mechanisms are considered; the so-called *dissociative* and *associative* pathways, see Refs. [4,5] and the Scheme. In the dissociative pathway a trigonal metaphosphate intermediate is formed, while the associative route proceeds via a trigonal bipyramidal species. It is stressed that between these extremes there are no intermediates but trigonal bipyramidal transition states [6]. According to Florián and Warshel [7] both mechanisms are similarly favoured in the aqueous phase, while Zalatan and Herschlag [8] stress that in solution, phosphate monoester and diester hydrolysis follow, respectively, dissociative and associative pathways.

Mildvan [4] characterises possible mechanisms by considering the axial distances between the attacking and leaving axialo oxygen atoms to phosphorus in the trigonal bipyramidal intermediate or transition state. This may serve as a measure of the strength of the



Scheme Dissociative (*top*) versus associative (*bottom*) pathways of phosphate ester hydrolysis

P–O bond. In the associative mechanism the P–O bond distance is equal to the single-bond value, while for the dissociative one it is larger than 330 pm, the sum of van der Waals radii of the P and O atoms. The associative character of the reaction is then measured by the fractional bond order, *n,* which is calculated using the following formula given by Pauling [9]

$$n = \exp\,[0.0167(d_1 - d_n)] \qquad (1)$$

where d_n is the bond distance (in pm) for bond order n.

It has to be stressed that the definition in Eq. (1) is incomplete, since the existence of an intermediate or a transition state can be proven exactly only by the presence of a local minimum or a maximum (saddle point) on the reaction path, rather than by bond distances alone. However, their use is justified because they can be determined experimentally, e.g., X-ray diffraction, while at present there is no experimental method available to determine the reaction path of an enzymatic reaction directly. This can be done by quantum mechanical calculations on a suitable model of the enzyme-substrate complex.

Recently, Lahiri et al. [10] reported on a pentacovalent phosphorane intermediate in the phosphoryl transfer reaction catalysed by β-phosphoglucomutase. Their results have been questioned by Blackburn et al. [11], who suggested that this structure is a transition-state analogue with pentacoordinated magnesium bonded to two oxygen and three fluorine ligands. Though the argumentation has been rebutted on the basis of experimental arguments [12], quantum mechanical calculations by Webster [13] indicate again that hydrogen bonding with various residues surrounding the active site may stabilise an MgF_3O_2 transition-state analogue, while the optimisation of a phosphorane intermediate on the reaction path does not converge to a stable minimum, rather to a transition state. A second example for a trapped high-energy intermediate has been published by Barabás et al. [14]. Crystal structures of complexes with a substrate (dUTP and α,β-imino-dUTP, PNP) and the product of the reaction with wild-type and mutant dUTPases were determined. In case of PNP the slow catalytic process allowed following the reaction path by localising snapshot structures, and a relatively stable, high-energy intermediate was trapped, for which the crystal structure is available.

Recently, we did density functional calculations on a model of HIV integrase to follow the reaction path of phosphate hydrolysis and could not locate a high-energy intermediate rather a trigonal bipyramidal transition state [15]. The mechanism of the hydrolysis reaction of guanosine triphosphate by the protein complex Ras-GAP has been modelled by quantum mechanical–molecular mechanical (QM/MM) and ab initio quantum mechanical calculations [16]. It was found that the reaction proceeds via two steps. First, a substantial spatial separation of the γ-phosphate group from the rest of the substrate takes place and this phase of hydrolysis process proceeds through a low-barrier transition state. At the second stage, protons from nearby side chains are abstracted and released within the subsystem through another transition state. Applying the density functional method to a relatively small model of deoxyribonucleotidase, Nordlund and co-workers [17] tried, but failed, to locate a pentacoordinated intermediate for the hydrolysis step in the catalytic reaction.

In the light of the aforementioned experiments and calculations at present we are aware only of two cases, β-phosphoglucomutase and dUTPase, where a high-energy intermediate could be located on the reaction path. In this paper we report on quantum mechanical calculations referring to these two enzymes and compare those results recently obtained for HIV integrase and with experimental findings.

2 Models and methods

We obtained the axial single-bond P–O distance to be used in Eq. (1) by averaging data from the Cambridge Structural Database (CSD) [18]. We found 106 hits in 42 structures with a trigonal bipyramidal PO_5 moiety where the O_{ax}–P–O_{ax} angle varies between 160 and 180°. The average P – O distance is 167 pm, which slightly decreases to 166 pm if ring structures are excluded from the set. Accordingly, in Eq. (1) we use $d_1 = 166$ pm, and thus get $d_n = 184$ for $n = 0.5$ and $d_n > 330$ for $n < 0.02$ ($\approx$0), respectively. In the CSD we found only three structures containing a trigonal bipyramidal PNO_4 moiety with the nitrogen atom in the axial position. The average P–N and P–O distances are 169 and 177 pm, respectively, therefore we use the mean value, 173 pm, for d_1 in Eq. (1). The calculated (ab initio, 6–31G** basis set, GAUSSIAN 03 software package[19])

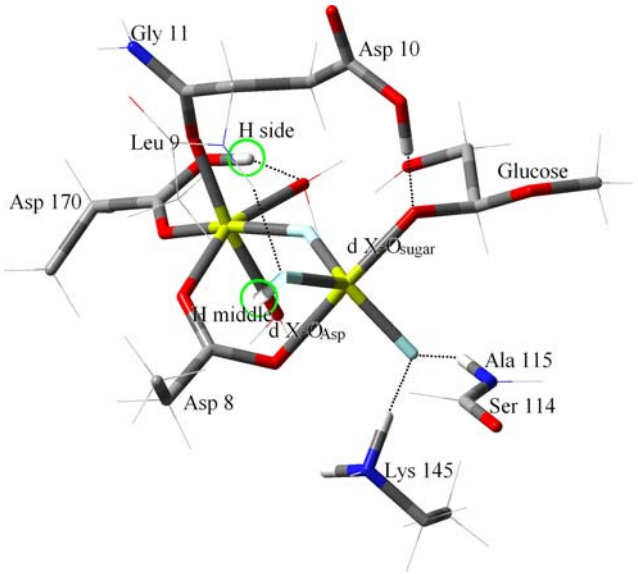


Fig. 1 The β-phosphoglucomutase active site. *Grey*: carbon, *blue*: nitrogen, *red*: oxygen, light *blue*: oxygen (Model A: Asp-170 side chain protonated by Hside, Model B: phosphate equatorial oxygen atom protonated by Hmiddle) or fluorine (Model C), *yellow hexacoordinated*: magnesium, *yellow pentacoordinated*: phosphorus (Models A and B) or magnesium (Model C), *light grey*: hydrogen

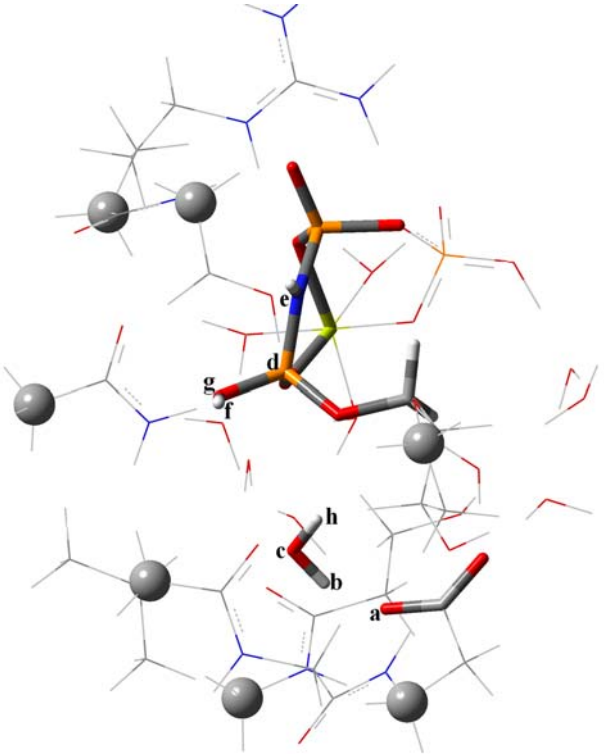


Fig. 2 Atomistic model of dUTPase with the PNP substrate. Colour codes as in Fig. 1. For atoms in bonds represented by tubes we used the 6-31G** basis set, while for other atoms we applied a 3-21G* basis set. Atoms, represented by grey circles, were fixed during geometry optimisation

bond distances, obtained for our enzyme models, were compared with the values obtained for the simplest phosphorane models $(HO)_4PO^-$ and $(HO)_3P(NH)O^-$ with the very same basis set. We obtained 164 pm for the axial P–O distance in $(HO)_4PO^-$, 158 and 165 pm for the axial P–O and P–N distances in $(HO)_3P(NH)O^-$, respectively, the mean is 162 pm.

We modelled the active site of β-phosphoglucomutase as shown in Fig. 1. Atomic co- ordinates were taken from the work of Lahiri et al. [10,20]. We considered two models. In Model A the side chain of Asp-170, while in Model B one of the P–O bonds is protonated. In Model C the PO_3 moiety is replaced by MgF_3 as proposed by Blackburn et al. [11]. We did ab initio Hartree–Fock calculations using the GAUSSIAN 03 software package [19] with a locally dense basis set [21]. This means that we put 6-31G** basis functions on atoms in the central region, while we used a 3-21G basis set elsewhere. Similar dual or multiple level approaches have been proposed by others, too [22–24]. α-carbon atoms of the protein backbone were fixed while performing a full geometry optimisation.

The active site of dUTPase was modelled using the coordinates published by Barabás et al. [14] (cf. Fig. 2). The model is composed of 73 non-hydrogen and 84 hydrogen atoms. We did calculations with the GAUSSIAN 03 software package [19] with a locally dense basis set, as indicated in Fig. 2. We applied our recently developed multi-coordinate driven (MCD) method [25] for the calculation of the reaction path. The rms difference for non-hydrogen atoms between the calculated and experimental structures is 76 pm.

In case of the calculations on HIV integrase our model for the quantum mechanical calculations is similar in size to those in Figs. 1 and 2, it consists of 50 non-hydrogen atoms. For this enzyme we estimated the effect of the distant surroundings by the Poisson–Boltzmann method and found that this is less than 2 kJ/mol [13]. Considering the similar composition of the active site for all three enzymes discussed in the present study, we may suppose that the combined effect of distant protein regions and the biophase on the reaction path can be neglected both for β-phosphoglucomutase and dUTPase.

3 Results and Discussion

In the analysis by Mildvan only two enzyme reactions, those catalysed by Giα1 GTPase [26] and alkaline phosphatase [27], can be considered as at least partly associative, i.e. the associative character, as calculated by

Table 1 Calculated and experimental distances (in pm) for some models of the high-energy intermediate of the β-phosphoglucomutase reaction

Structure (rms deviation)	d(X–O_1)	d(X–O_2)	d(O_1–O_2)	n of Eq. (1)
Model A, this work (89)	168	362	520	0.015
Model A, Webster [13] (54)	170	294	–	0.079
Model B, this work (21)	176	188	363	0.541
Model C, this work (7)	193	223	413	–
Model C, Webster [13] (8)	210	210	–	–
X-ray diffraction [20] (0)	195	211	407	0.241

Models refer to Fig. 1, root mean square deviations (in pm) between co-ordinates of the trigonal bipyramidal moiety and experimental values are given in parentheses. X stands for a P or an Mg atom. d_n in Eq. (1) is calculated as the average of d(X–O_1) and d(X–O_2)

Eq. (1) on the basis of experimentally determined axial P–O distances that exceeds 0.5. In the following, we compare three enzymatic reaction paths, HIV integrase, β-phosphoglucomutase and dUTPase, respectively, and try to derive a conclusion on the factors determining the nature of the mechanism.

Our calculations for HIV integrase [15] support an S_N2-type mechanism with only an associative character of 0.23, as estimated from the calculated bond distances by Eq. (1). This is comparable to the value of 0.16, obtained for the same reaction in the gas phase. We calculated the reaction path carefully and found a very shallow minimum, rather a shoulder on the reactant side. There are two main factors that may influence the shape of the reaction path, one is the electrostatic interaction with the solvent [28] or the protein environment [29], and another is whether any of the equatorial oxygen atoms of the phosphate moiety is involved in a covalent bond [8]. For HIV integrase the electrostatic effect of the close surroundings on the rate enhancement is quite large, the gas-phase activation energy (295 kJ/mol) decreases to 141 kJ/mol in the enzyme, which is mainly due to the hydrogen bonds formed between neighbouring side chains and the trigonal bipyramidal transition state. However, this is not enough to stabilise a phosphorane-like intermediate, probably because in our model, for which we did the calculations, the first equatorial oxygen atom is not involved in any hydrogen bond [15]. Though the second oxygen atom is co-ordinated by a magnesium dication and the third is involved in a covalent bond, these contributions to stabilisation are insufficient.

Our calculations for the β-phosphoglucomutase intermediate are in agreement with the experimental findings if we suppose that the phosphate group is protonated on one of the equatorial oxygen atoms. In Table 1, we display calculated versus. experimental distances between phosphorus (or magnesium) and the axial oxygen atoms. As it is seen, if the phosphate moiety is not protonated, no trigonal bipyramidal intermediate can be formed, the very large X–O_2 distance indicates a dissociation of one of the axial oxygen atoms. A similar result has been obtained by Webster [13], who used a different quantum mechanical method. On the other hand, we obtained results in reasonable agreement with experiment for Model B, where one of the equatorial oxygen atoms is protonated. It is quite interesting to note that the best agreement with experiment is observed for Model C, both by us and Webster [13]. This latter model corresponds to the hypothesis by Blackburn et al [11], who proposed a model where the phosphate group is replaced by a MgF_3^- anion. The associative character of the reaction, as obtained for different models is displayed in Table 1. It is seen that the experimental bond distances indicate only a low associativity in spite of the presence of the trigonal bipyramidal intermediate in the crystal. This may be due to the overestimation of the P–O bond lengths from the electron density map, but may also indicate that the fractional bond order, calculated by Eq. (1), is not an appropriate measure of associativity. At the very end, the associative character can be proven by the location of a high-energy intermediate on the reaction path, which was the case for Model B. This intermediate is stabilised by a covalent bond to the proton, by a strong Mg. . .O interaction and by two hydrogen bonds involving the side chains of Ala-115 and Lys-145.

We calculated the reaction path for a model of dUTPase, where two of the equatorial phosphate oxygen atoms are involved in covalent bonds; one is a proton (atom f in Fig. 2). The third oxygen atom is co-ordinated to the catalytic magnesium ion. The relative energies and inter atomic distances for the axial bonds are given in Table 2. We could locate an unstable high-energy intermediate on the reaction path, which transforms easily to the stable product (see Fig. 3). The activation energy to the first transition state (TS1) is 20.6 kJ/mol, to the second one (TS2) it is much less, 3.7 kJ/mol, which indicates that the lifetime of the intermediate is quite low. As it is seen from Table 2 the geometry of TS1 is reactant-like,

Table 2 Calculated relative energies and axial interatomic distances for critical points on the energy path of the dUTPase catalytic reaction, as calculated for the model on Fig. 2

	Reactant	Transition state 1	Intermediate	Transition state 2	Product
Full model (kJ/mol)	0.0	20.6	−34.8	−31.1	−305.6
Mg dropped (kJ/mol)	0.0	61.5	49.8	64.6	−186.5
P–O (pm)	341	222	179	169	160
P–N (pm)	166	183	177	246	353

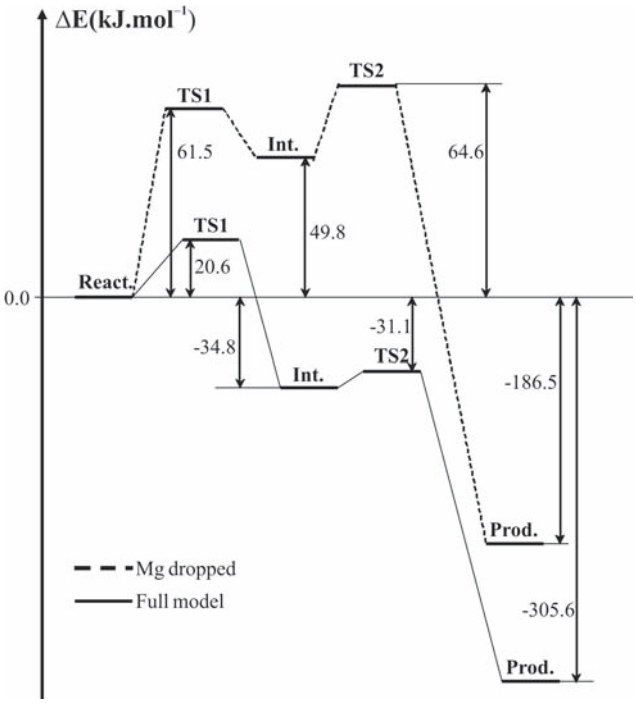


Fig. 3 Schematic reaction path for the dUTPase catalytic reaction. TS1 and TS2 denote the transition states

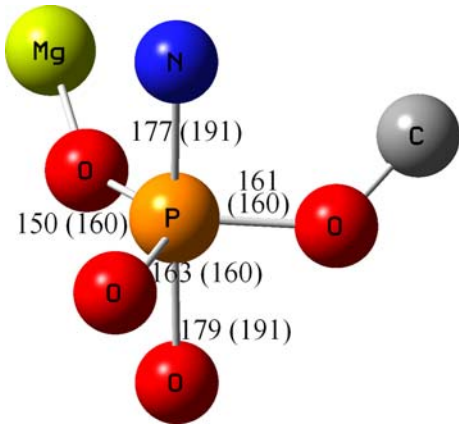


Fig. 4 Bond lengths (in pm, experimental in parentheses) as calculated for the phosphorus fragment of dUTPase

with a P–O distance indicating the formation of a weak single bond, while the P–N bond does not loosen very much. On the other hand, TS2 is product-like with an almost regular P–O single bond.

It is interesting to consider the effect of the catalytic magnesium ion. If we drop it from our model, the activation energy strongly increases (61.5 kJ/mol), indicating that this metal ion has a crucial effect in reducing the barrier on the reaction path. On the other hand, the energy of the intermediate becomes higher and this structure becomes more stable, the activation energy to TS2 increases to 14.8 kJ/mol.

We located a local minimum on the reaction path, which represents a high-energy intermediate with a trigonal bipyramidal structure around phosphorus (cf. Fig. 4). The calculated axial P–O and P–N distances agree reasonably well with the experimental ones obtained for the dUTPase-PNP complex (191 pm for both [14]). If we consider the averaged values, as extracted from the CSD (see the Models and Methods section), which are 169 and 177 pm for the P–N and P–O bonds, respectively, the agreement is much better. Equatorial P–O distances are very close to the experimental values, all of which have been set equal during structure determination because the resolution was not high enough to allow making differences. Our calculations indicate that the oxygen atom, which is co-ordinated to the catalytic magnesium ion, shares a partial double bond with phosphorus, its length is 150 pm. The other two equatorial P–O distances indicate the formation of single-bonds. The associativity of the reaction, as obtained from Eq. (1) with reference to experimental bond distances is 0.520, which is quite close to the value, 0.541, obtained using quantum mechanically calculated bond lengths. These figures are relatively low, so it has to be stressed again that Eq. (1) alone does not necessarily predict the presence of a locally stable intermediate.

4 Conclusions

On the basis of quantum mechanical calculations we provided evidence that the main factor contributing to the stabilisation of a trigonal bipyramidal phosphorane-like intermediate during enzymatic phosphate hydrolysis is the involvement of equatorial oxygen atoms

in covalent, hydrogen or ionic bonds. If all three oxygen atoms are co-ordinated, the formation of a relatively short-lived, high-energy intermediate can be anticipated.

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