An Alternative Role for the Conserved Asp Residue in Phosphoryl Transfer Reactions

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Reversible phosphorylation is the major form of posttranslational modification in proteins and is a key control mechanism in biochemistry for processes such as cellular regulation. In protein kinase and protein tyrosine phosphatase (PTPase) enzymes, which are responsible for phosphorylation and dephosphorylation of protein residues, respectively, there is a conserved aspartate (Asp) residue whose role is considered to be as either a general acid (in the first stage of PTPase[1−3]) or a general base (in the kinase[3,4] and second stage of the PTPase[1−3]). Thus, Asp is proposed to either protonate the leaving group (Figure 1a) or abstract a proton to produce a powerful nucleophile (Figure 1b,c) and hence promote nucleophilic attack. However, recent experimental results have questioned the ability of Asp to act as a general base in the kinase reaction.[5,6] Modeling studies that we have carried out on these enzymes and on some simple model systems have suggested an alternative role for the conserved Asp residue and thus led to alternative mechanisms for the mode of action of these important and widely occurring classes of enzymes based upon the protonation states of the Asp and the phosphate group.

Calculations on the alternative protonation states of the model system, methyl phosphate and acetic acid (Figure 2), located both alternative protonation structures as minima using the PM3 Hamiltonian,[7] where the protonated phosphate (Figure 2a) was favored by 27.7 kcal/mol, but not at the ab initio level (both Hartree−Fock (3-21G*, 6-31G*) and density functional theory (B3LYP/3-21G*) models), where all optimizations led to a structure with a protonated phosphate. At a higher level of theory, MP2/6-31+G*//PM3, an energy difference of 12.8 kcal/mol was found. This is somewhat smaller than the PM3 value, suggesting that PM3 tends to overestimate the energy separation of the two different protonation motifs. The preference for the protonated phosphate was reduced upon solvation (to 20.2 kcal/mol using a SM3-PM3[8] calculation at the PM3 geometries), but even allowing for inaccuracies in the PM3 calculation, it is clear that the preferential solvation of the phosphate dianion is not sufficient to reverse the order of the gas-phase stabilities, in line with the experimental pK_a values for acetic acid (4.75)[9] and methyl phosphate (second pK_a 6.3).[10] Although perturbations of pK_a values of residues in enzyme active sites may occur, the general belief that the effective dielectric constant of an enzyme active site is less than that of the aqueous phase[11] would suggest that the enzyme environment will not lead to preferential protonation of Asp rather than of the phosphate dianion. However, as a number of authors[12] have emphasized, it is the local electrostatic microenvironment that is important in determining the effective pK_a's of the residues. Modeling studies such as those described here have a central role to play in identifying and quantifying these electrostatic effects.

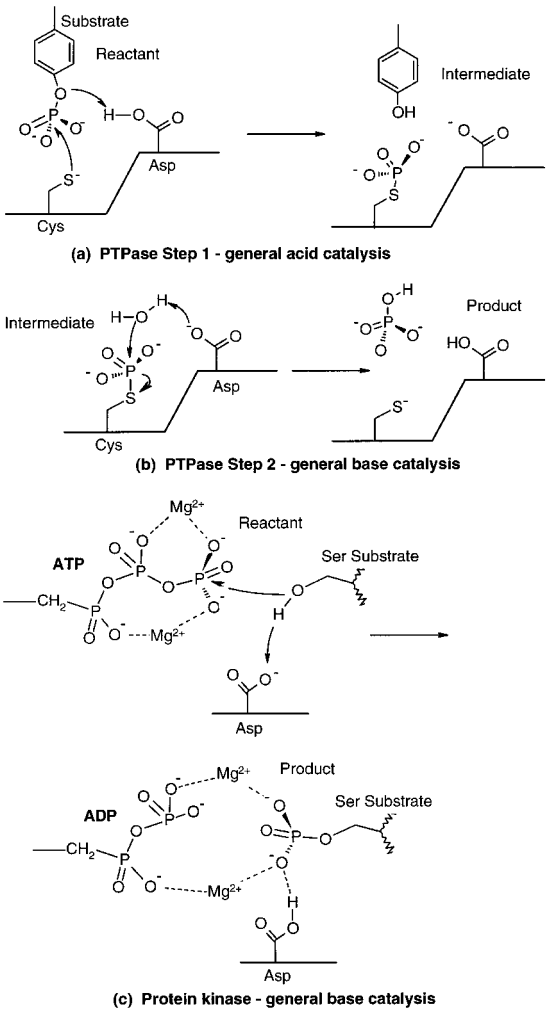

Figure 1. Reaction mechanisms of PTPase and protein kinases with Asp acting as a general acid or base.

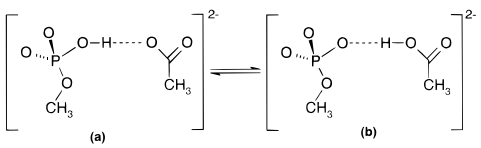

Figure 2. Model systems for phosphate and Asp protonation.

To specifically include the actual enzyme environment in the calculations, we have used hybrid quantum mechanical (QM)/molecular mechanical (MM) methods to model both steps of protein tyrosine phosphatase 1B (PTP1B) as well as the cAMP-dependent kinase (cAPK) reaction, thus studying enzymes that are characteristic of the PTPases and protein kinases. The models for these three systems were built using the available crystal structures. For PTP1B, an enzyme/substrate complex with a Cys-to-Ser mutation was used for step 1,[13] and a crystal structure of the phosphorylated cysteine (pCys) intermediate with a Gln-to-

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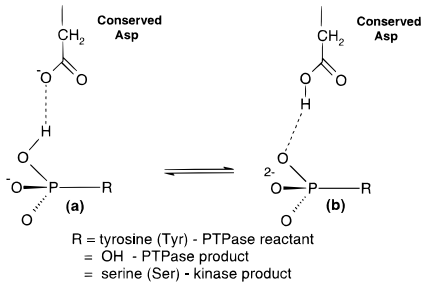


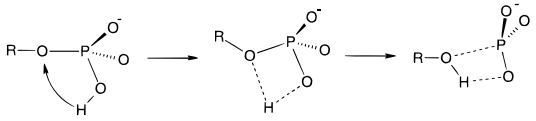
Figure 3. Protonation states involved in PTPase and protein kinase reactions.

Ala mutation was used for step 2.[14] For the kinase reaction, a structure involving the enzyme cAPK complexed with an ATP substrate and a peptide inhibitor[15] was used, where an Ala residue in the inhibitor replaced the Ser in the native substrate. In all cases, the MM models were built by first reversing the mutations to produce models of the native system, followed by energy minimization using standard force fields.[16] The model for PTP1B step 1 contained 17 880 MM atoms (approximately 5000 atoms of the protein and 4300 water molecules), with the phosphorylated tyrosine (pTyr) being replaced with 4-methylphenyl phosphate to allow for substrate mobility. The models for PTP1B step 2 and cAPK were built including crystallographic water molecules and contained approximately 4800 and 6200 MM atoms, respectively.

The QM/MM technique as utilized within this study has been shown to be useful in describing enzyme reactions,[17−21] and the method employed in this study combines Gaussian 94[22] and AMBER[16] and has been described previously.[23] In these systems, the reactive portions of the enzymes and substrates were treated at the QM level using the PM3 Hamiltonian, while the remainder of the enzyme and substrates were considered to be a fixed MM environment. For the PTPase step 1 reaction, the 4-methylphenyl phosphate substrate, along with a crystallographic water, the catalytic Cys (treated as an anion) and Asp residues, and an arginine (Arg) residue involved in phosphate binding gave a total of 59 QM atoms. For the PTPase step 2 reaction, the QM region consisted of the pCys intermediate, the Arg involved in phosphate binding, the catalytic Asp residue, the Gln residue, and two crystallographic waters, to give 59 QM atoms. The QM region of the kinase model contained the Ser substrate, the conserved Asp and Lys, the triphosphate group, and the two Mg^{2+} ions which coordinate the triphosphate, giving 49 QM atoms.

For both the reactants (step 1) and final product (step 2) of PTP1B and the products of cAPK (Figure 1), the most stable structure has the phosphate (Figure 3a) rather than the Asp (Figure 3b) protonated. The calculated QM(PM3)/MM energy differences between these protonation states for the PTP1B reactants and products were 21 and 50 kcal/mol, respectively, and that for the kinase product was 60 kcal/mol. This result is not unexpected when comparing the pK_a values of Asp (around 4)[24] to those for a range of phosphate dianions (from 5.80 for *p*-methylphenyl phosphate[25] to 6.8 for inorganic phosphate,[10] the PTPase step 2 product).

Scheme 1



A further model of the PTP1B reactant system was built with just the Asp residue and methylphenyl phosphate substrate treated at the QM level to allow ab initio optimizations. In this case, optimizations were carried out at the HF/3-21G* and HF/6-31G* levels and using density functional theory at the B3LYP/3-21G* level, starting at the PM3-optimized geometries. As for the model system (Figure 2), the protonated Asp was found to be unstable, and this structure led to the protonation of the phosphate oxygen when optimizations without constraints were carried out. Single-point calculations at the ab initio levels on the PM3 geometries favored protonated phosphate by between 10 and 15 kcal/mol.

Thus, computational studies on the three different enzyme systems and the model system, together with experimental pK_a values for relevant species, suggest that the role of the Asp residue as a general acid or base in phosphoryl transfer reactions should be reconsidered and that the protonation state of the phosphate group in the local environment should be focused on. It is well known that protonated phosphate monoesters hydrolyze much faster than the corresponding unprotonated species,[10,26] which has been attributed to intramolecular protonation of the leaving group oxygen[26] (Scheme 1), and a similar mechanism may be present in the PTPase system. Thus, an alternative role of the Asp residue could be to stabilize the protonated forms of the phosphate species within the active site. Recent modeling studies on a related PTPase system[27] also predicted a protonated phosphate, thus reducing electrostatic repulsion in the active site (though a protonated Asp was also included in these models). This study was criticized on the basis of a number of experimental results[28] including kinetic isotope effects,[29,30] which suggest that the substrate exists as a dianion. These studies, however, use a *p*-nitrophenyl phosphate substrate which has a pK_a value lower than that of the natural substrate (4.96 compared to 5.80 for *p*-methylphenyl phosphate[25]), and the fact that this pK_a value is closer to that of Asp would reduce the energy difference between these protonation states.

Hence, both experimental data based on pK_a values and the modeling results described here support our proposal that, for these systems where Asp and a phosphate group are in close proximity, protonation of the phosphate (to a monoanion) rather than Asp is favored. Thus, the phosphate group rather than the Asp is able to act as a general acid or base in the catalytic mechanisms of PTPase and protein kinases.

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