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Protein Phosphatase 1 Catalyses the Direct Hydrolytic Cleavage of Phosphate Monoester in a Ternary Complex Mechanism

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Abstract—The catalytic subunit of the Ser/Thr protein phosphatase 1 ($PP1_{cat}$) hydrolyses *N*-acetyl Arg-Arg-Ala-phosphoThr-Val-Ala ($K_M = 3.7$ mM) in a reaction that is inhibited competitively by inorganic phosphate (P_i, $K_i = 1.6$ mM) but unaffected by the product peptide alcohol at concentrations up to 3 mM. The enzyme does not catalyse the incorporation of ^{18}O-label from ^{18}O-labelled water into P_i whether, or not, the product alcohol is present. The dephosphorylated product alcohol of phosphorylated histone, an alternative substrate for the enzyme, serves as a competitive inhibitor for phosphopeptide hydrolysis ($K_i = 60\,\mu M$) and co-mediates ^{18}O-label exchange into P_i in a concentration-dependent manner ($K_M = 64\,\mu M$). These results indicate that hydrolysis occurs through the direct attack of an activated water molecule on the phosphate ester moiety of the substrate in a ternary complex mechanism.

The balanced transfer of phosphoryl groups from one entity to another as catalysed by the phosphatases and kinases is the basal mechanism by which cellular function is controlled[1] and some 4% of the eukaryotic genome codes for kinases and phosphatases.[2] In the cytoplasm of eukaryotic cells most, if not all, of the phosphoseryl/phosphothreonyl phosphatases can be accounted for by four different catalytic subunits, types 1, 2A, 2B and 2C[3] where such subunits are targeted towards a specific substrate by a regulatory protein which controls location and specificity.[4] The natural substrates for these regulated enzymes are themselves proteins, but the activity of the protein phosphatases can be examined using small synthetic phosphorylated peptides as substrates.[5–7] The active site structure of $PP1_{cat}$ has been solved for complexes that contain both essential divalent metal ions and either tungstate[8] or the inhibitor microcystin.[9] PP2A, which is also inhibited by microcystin, shows complete homology to $PP1_{cat}$ for amino acid residues involved in binding to the catalytic metal ions and to microcystin and ca. 50% overall homology.[10] While this high degree of structural homology at the active sites explains why PP1 and PP2A display overlapping substrate specificities, an important objective in understanding the control of protein dephosphorylation is to determine how active each regulated enzyme is with a given common substrate. Such questions might be most reasonably addressed using highly selective inhibitors for each enzyme.

To aid in the design of selective inhibitors for PP1 and PP2A, we set out to determine the catalytic mechanism of phosphate ester hydrolysis. In particular we wished to learn in what order the products are released and whether the phosphoryl moiety of the substrate was transferred directly to water in one step, or, in two or more steps through the intermediacy of an enzyme bound nucleophile as for alkaline and acid phosphatases. We chose to use the method of ^{18}O-label exchange from ^{18}O-labeled water into inorganic phosphate (P_i) as this method has proved to give reliable mechanistic information for a wide range of phosphatases that employ each of the common substituted-enzyme (ping-pong)[11,12] and ternary complex mechanisms[13,14] (Scheme 1). A substituted enzyme mechanism is expected to form a phosphoryl enzyme intermediate from P_i through reverse steps in the absence of the product alcohol and to allow the incorporation of ^{18}O-label from water through the hydrolysis of the phosphoryl enzyme in the forward reaction direction. A ternary complex mechanism is not expected to mediate ^{18}O-exchange into P_i in the absence of the product alcohol, unless water can serve as a surrogate for the product alcohol[13] but is expected to display facile label exchange at saturating concentration of the product alcohol.[15]

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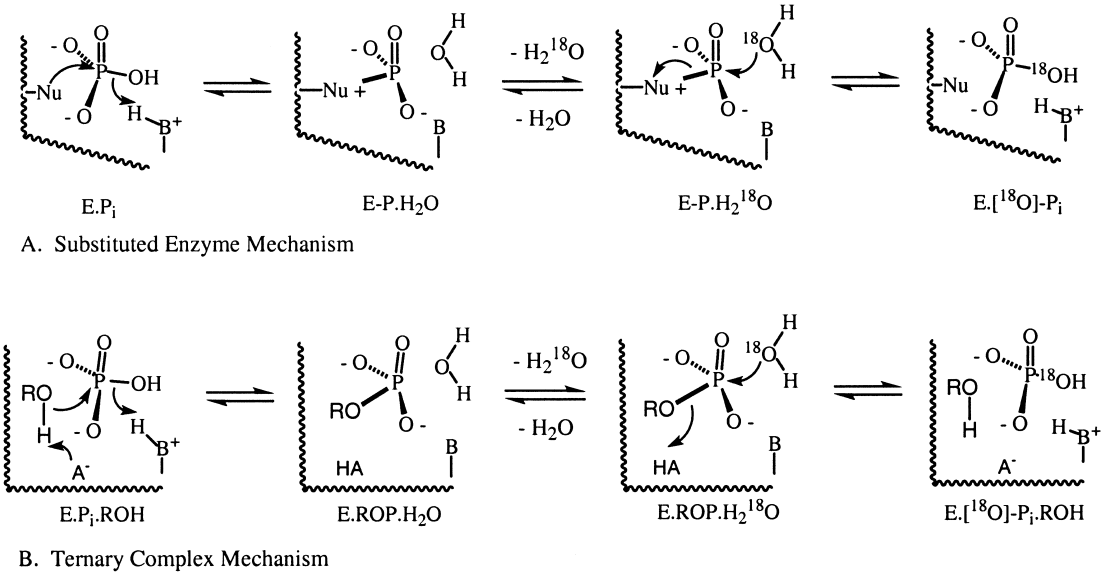

Scheme 1.

In order to define the conditions for ^{18}O-label exchange it was first necessary to determine whether the two products of the hydrolysis reaction, the peptide alcohol and P_i, could be recognised by the enzyme at accessible concentrations. Accordingly the inhibitory properties for the products were investigated. The phosphorylated peptide Arg-Arg-Ala-phosphoThr-Val-Ala is a known PP1 substrate and recently the use of [^{14}C-acetyl]-Arg-Arg-Ala-phosphoThr-Val-Ala (K_M value = 3.7±0.9 mM) was developed in a generic protein phosphatase stopped assay system[7] suitable for use under a wide range of conditions including those in which P_i is present. Activity assays performed with recombinant $PP1_{cat}$ at pH 7.5 in the presence of Mn^{2+} ions at various concentrations of the substrate and P_i gave a K_i value for P_i of 1.6± 0.9 mM for competitive inhibition. Similar experiments performed with the product peptide alcohol, Arg-Arg-Ala-Thr-Val-Ala failed to retard the rate of substrate hydrolysis even at concentrations of up to 3 mM. A 10% systematic reduction in rate at any substrate concentration would have been detected under the conditions of the assay indicating that the K_i value for the peptide alcohol is >30 mM. Thus, it was apparent that the enzyme recognised P_i at an accessible concentration but not the peptide alcohol.

In order to test for a substituted enzyme mechanism, $PP1_{cat}$ was incubated with 2 mM P_i in buffer containing 49% [^{18}O]-water in the presence of Mn^{2+} ions in duplicate experiments and aliquots of the solution were removed periodically over 36 h. Each aliquot was frozen in liquid nitrogen and then lyophilised. The residue was suspended in dry ethyl acetate saturated with $HCl_{(g)}$ and the suspension was centrifuged to precipitate the protein. The supernatent solution was added to a large excess of ethereal diazomethane solution and after removal of the excess diazomethane in a stream of argon, the solvents were removed under reduced pressure to give trimethyl phosphate as an oil. Analysis by GC–MS was performed for each sample using authentic trimethyl phosphate as a reference standard. No increase in the ratio of the mass of the parent ion at 142 compared to 140 Da was detected indicating that, under the conditions of the experiment, no phosphorylated enzyme intermediate is formed.

Given that the peptide product alcohol had failed to serve as product inhibitor in an accessible concentration range, it was not expected that the compound would co-mediate the exchange of ^{18}O-label from water into P_i. Indeed, repetition of the ^{18}O-exchange experiment in the presence of 3 mM Arg-Arg-Ala-Thr-Val-Ala did not lead to the incorporation of ^{18}O-label into P_i as judged by GC–MS analysis of the trimethyl phosphate derivative. Since it was apparent that the phosphate moiety of the phosphopeptide provided most if not all of the binding affinity for the enzyme, the product alcohol of a phosphorylated protein substrate (which was expected to display a higher affinity for the enzyme than peptide alcohols) was sought and tested.

Phosphorylated histone was known to display a relatively low K_M value of 4 μM (56 μg mL^{-1})[16] as a substrate and when the product alcohol was tested as an inhibitor, competitive inhibition was observed (K_i = 60 μM, 850 μg mL^{-1}). The result verifies that protein product alcohols can serve as product inhibitors in an accessible concentration range and demonstrates that the two products, P_i and alcohol, dissociate from the enzyme in a random fashion. When the ^{18}O-exchange experiment was repeated in the presence of dephosphorylated histone, incorporation of ^{18}O-label into P_i occurred in a time-dependent manner (2.3% at t = 9 h), as judged by GC–MS analysis of the trimethyl phosphate derivatives. Repetition of the ^{18}O-exchange experiment at a range of dephosphorylated histone concentrations gave a K_M value for exchange of 64 μM (900 μg mL^{-1}). Thus, the enzyme appears to operate through ternary complex mechanism in which water or hydroxide directly attacks the phosphate monoester.

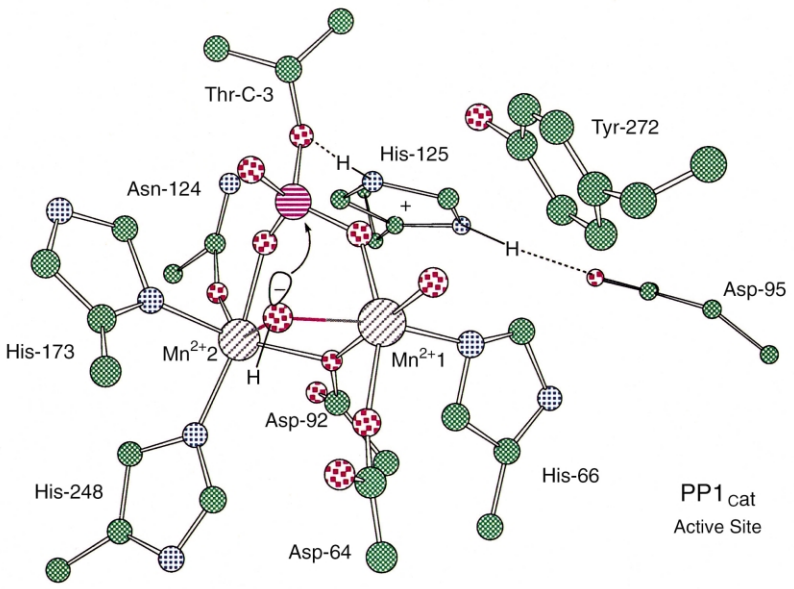

Figure 1. Mechanistic scheme for phosphate ester hydrolysis by $PP1_{cat}$ showing direct nucleophilic attack on the phosphorous atom by hydroxide ion and protonation of the leaving group by the imidazolium side chain of His-125.

The two divalent metal ion binding site of PP2B (calcineurin) is structurally homologous to that for PP1.[10,17] The enzyme is able to process a wide range of low molecular weight phosphate esters including those derived from tyrosine. The enzyme does not catalyse ^{18}O-exchange from ^{18}O-labelled P_i into water in the absence of a product alcohol, in accord with the results reported here.[18] However, PP2B also failed to catalyse ^{18}O-exchange in the presence of 0.5 mM tyrosine, the product of a known substrate for the enzyme. In the light of the findings reported here we would suggest that PP2B also catalyses the direct attack of hydroxide on the phosphate ester. We would expect that the concentration of the product alcohol used in these experiments[18] was too low to allow formation of the ternary complex through reverse steps (Scheme 1B) such that ^{18}O-exchange could not be detected. This explanation is entirely consistent with the failure of the peptide alcohol to co-mediate exchange in the PP1 system as reported here, above.

Other phosphates known to operate via a ternary complex mechanism include fructose 1,6-bisphosphatase[14] and inositol monophosphatase.[15] In both of these systems the nucleophile is derived from a water molecule that is associated with a divalent metal ion and inline displacement of the leaving group occurs with inversion of stereochemistry at phosphorus.[15,19]

No crystal data is yet available for PP1 complexes containing bound substrate-like molecules. However, if direct attack by a metal ion bound water molecule, activated as hydroxide, occurs via inline displacement of a threonine or serine derived alkoxide leaving group, the oxyanion could be stabilised by pre-association with the acidic imidazolium side chain of His-125 (Fig. 1). The His residue forms an H-bonding interaction with Asp-95 and a water molecule exists in approximately the correct position in the crystal structure of tungstate-bound $PP1_{cat}$.[8]

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