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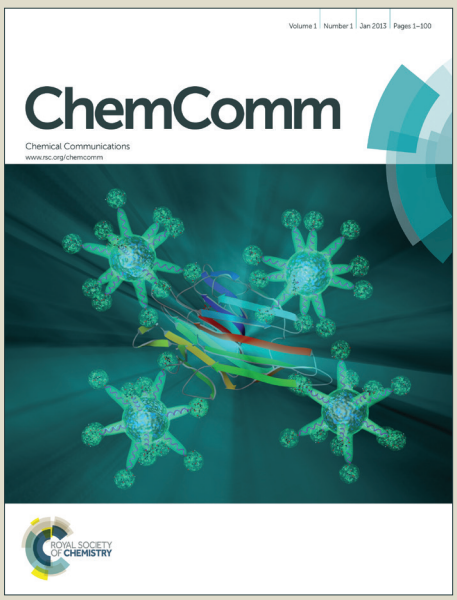

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Phosphatase mechanism of bifunctional kinase/phosphatase AceK†

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We have revealed that bifunctional AceK kinase/phosphatase utilizes a stepwise addition-elimination mechanism in its dephosphorylation reaction. This work explains how AceK enables opposite kinase and phosphatase activities with Asp477 and a single Mg^{2+} ion.

Protein serine/threonine phosphatases comprise three major families.[1] The two major families, PPP (phosphoprotein phosphatase) and PPM (protein phosphatase, Mg^{2+}/Mn^{2+}-dependent), are thought to share the mechanism in which the bimetal-activated water molecule initiates single step dephosphorylation process. The other FCP/SCP family, which was recognized recently, is thought to depend on aspartic acid based hydrolysis in dephosphorylation process. So far, the mechanisms are thought to employ a concerted pathway[2], except the FCP/SCP family the mechanism of which is still not clear.

A unique bifunctional enzyme, *Escherichia coli* (*E. coli*) isocitrate dehydrogenase kinase/phosphatase (AceK) phosphorylates and dephosphorylates isocitrate dehydrogenase (IDH) in response to nutrient availability, resulting in the inactivation or activation of IDH[3] respectively. The structures of AceK and its complex with the substrate IDH have been determined recently,[3b, 4] providing the first structural glimpse of this unusual enzyme. The AceK structure consists of a canonical eukaryotic kinase scaffold domain and a novel regulatory domain with an AMP molecule bound between the two domains.[3b, 4] In addition to possessing two opposing activities, AceK exhibits an unusually strong ATPase activity. As a protein phosphatase, AceK exhibits unique characteristics such as kinase scaffold and ATP/ADP dependence. Interestingly, although a sequence motif "-DYDEIC-" can be readily detected in AceK, which is similar to the FCP with "-DxDx(T/V/I)L-"[5] signature motif, AceK and PPMs share more conserved aspartic acids in the catalytic center. Therefore, it is still elusive whether AceK belongs to PPM or FCP/SCP, or it forms a novel phosphatase family. An additional interesting feature of AceK is the presence of only one Mg^{2+} ion in the active site whereas, in general, protein phosphatases usually contain two or more metal ions. The dephosphorylation mechanism, in which a single Mg^{2+} ion enables AceK phosphatase function, as well as, the specific role of ADP/ATP in the reaction, remains unclear.

AceK represents the first case in which a typical eukaryotic protein kinase scaffold possesses phosphatase activity. Furthermore it has been shown that kinase, phosphatase and ATPase activities all share the same active site, leading to the suggestion that the IDH phosphatase function of AceK is a mere reversal of its kinase mechanism.[3b, 4] A better understanding of the AceK dephosphorylation mechanism may provide insights into the proposed reversible reaction catalyzed by the shared active site, as well as, the regulation of the delicate balance between AceK's kinase and phosphatase activities.

In this work, we carried out extensive characterization of AceK's phosphatase function including crystallography, site-directed mutagenesis, activity measurements and effects of ligands. Overall structure optimization and mechanism investigation were also performed with detailed DFT calculations on various active site models including number of metal ions and ATP in combination of solvation effects. Our findings have, for the first time, shed lights on AceK's elusive phosphatase mechanism and the functional switch between kinase and phosphatase activities. Previous studies have already demonstrated mutation of Asp477 to alanine fully abolishes AceK phosphatase activity while maintaining its kinase function.[1b] Of all the mutations screened, this is the only AceK mutant which affects phosphatase activity specifically. In this work, we determined the crystal structure of an AceK D477A mutant derivative in complex with substrate IDH. The similar overall structure and conformation of the mutated active site in comparison with the wild-type AceK-IDH complex[3b] indicates that the switch from one activity to the other is not accompanied by any appreciable conformational change of the active site. As a result, we proceeded to use the AceK-IDH complex structure for quantum mechanics (QM) calculations to investigate the dephosphorylation mechanism of AceK. We discovered that the phosphatase reaction follows a general acid-base catalysis associative mechanism in a stepwise mode, in which one Mg^{2+} ion cooperates with ADP in the active site to facilitate the phosphotransfer process. Our results have revealed

the structural basis for AceK phosphatase activity based on a kinase fold, the first of its kind. We have also shown that the single Mg^{2+} ion in AceK leads to its unique stepwise mechanism, which is also the key factor in the reversibility of the process.

In order to investigate whether there is any conformational change accompanying the switch in activity, we sought to determine the crystal structure of the AceK D477A mutant derivative in complex with IDH. The AceK D477A-IDH complex structure was solved to a resolution of 3.3 Å; the overall structure was found to be very close to the complex structure of wild-type AceK with IDH[3b] (PDB ID, 3LCB) (Fig. S1). The side chains of the amino acids in the active site also remain unchanged. Interestingly, despite the presence of 50 mM $MgCl_2$ in the crystallization condition, the structure of the mutant complex contained no Mg^{2+} ion in the active site due to the absence of Asp477, which coordinates the single Mg^{2+} ion in the wild-type structure. The loss of this Mg^{2+} ion in the AceK D477A mutant was also confirmed by inductively-coupled plasma mass spectrometry. In light of the fact that the overall active site conformation of the AceK D477A mutant, which possesses kinase activity only, is the same as the wild-type AceK-IDH complex which has AMP bound and represents phosphatase state,[3b] we hence revealed that AceK employs the same conformation to fulfil its reverse kinase/phosphatase functions. We next proceeded to use the intact active center structure containing Asp477 and Mg^{2+} derived from the wild-type AceK-IDH complex with higher resolution 2.9 Å for further DFT investigations of phosphatase mechanism.

Based on the crystal structure, we optimized the positions of hydrogen atoms and built the cluster model of the active center for DFT investigation. The optimized configuration (Fig. 1) was used as the reactant to explore the potential energy surface (PES) corresponding to dephosphorylation. A stepwise pathway (PathwayI) was identified as the reaction route in the PES (Fig. 2A), which follows a two-step associative pathway: 1) water attacks the phosphorus atom in the substrate (Fig. 3, step 1), and 2) the phosphoryl group is released completely from Ser113 (Fig. 3, step 2). The geometries of the states along the reaction path are shown in Fig. S2. In the first step, a water molecule activates the Mg^{2+} ion, delivers a proton to the β-phosphate of ADP and simultaneously attacks the phosphorus atom of the substrate phosphoryl group (Fig. S2). In the second step, the invariant Asp457 donates its proton through a water molecule (Wat2) to help release the phosphoryl group. The second step is the rate-determining step with the energy barrier of 22.96 kcal mol^{-1}. Two other pathways in the PES were also examined in our calculations. In PathwayII, Asp477 does not interact directly with the substrate phosphoryl group (Fig. S3, PathwayII) and exhibits an energy barrier of 41.10 kcal mol^{-1}. In PathwayIII, the Asp475, instead of ADP, acts as a proton acceptor, and has a high energy barrier of 43.52 kcal mol^{-1}. Both PathwayII and PathwayIII are two-step pathways with significantly higher energy barriers than PathwayI.

Single Mg^{2+} active site is a unique characteristic of AceK. Artificial insertion of a second Mg^{2+} ion into the active site in our calculations changed the reaction from stepwise to concerted mechanism (Fig. S5, PathwayIV). In this process, a water molecule activated by the two Mg^{2+} ions attacks the substrate phosphoryl group to form a new O-P bond and transfer a proton to ADP. Meanwhile on the back-side of the phosphoryl group, another water molecule protonates the O_{Ser113} to help release the phosphoryl group from Ser113. The reaction occurs in one step (Fig. 2B) and exhibits a slightly lower energy barrier than the two-step pathway. The energies of each state of all pathways are given in Table 1.

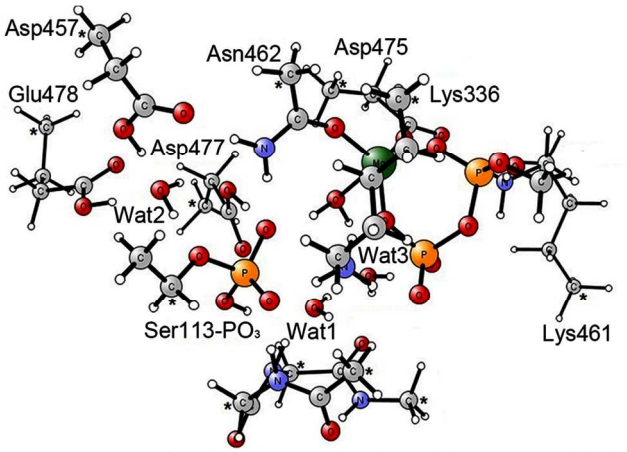

Fig. 1 Quantum-chemical computational model. Energy-minimized structure of the active site of AceK in complex with the substrate fragment. Main-chain carbons are identified with asterisks.

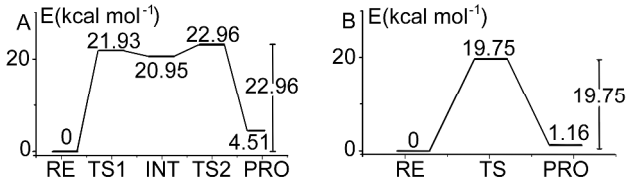

Fig. 2 Potential energy surface (PES) of the dephosphorylation reaction. (A) PathwayI-stepwise pathway of AceK; (B) PathwayIV-concerted pathway of AceK with double Mg^{2+} ions.

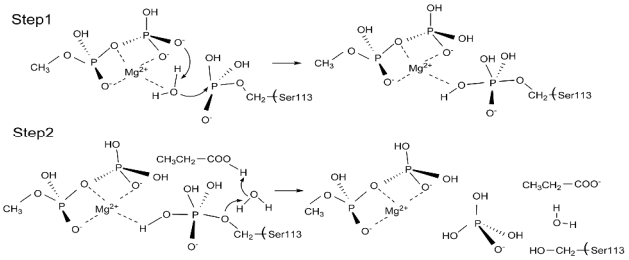

Fig. 3 Reaction scheme for the dephosphorylation steps. It is an associative acid/base-catalyzed reaction through addition-elimination mechanism. The panel above is the first step and the panel below is the second step.

Table 1 Summary of the calculated energetics for the various models (kcal mol^{-1})

Single Mg^{2+} model: stepwise				Double Mg^{2+} model: concerted	
	PathwayI	PathwayII	PathwayIII		PathwayIV
RE	0.0	0.0	0.0	RE	0.0
TS1	21.93	41.10	43.52		
INT	20.95	38.65	26.84	TS	19.75
TS2	22.96	40.43	35.41	PRO	1.16
PRO	4.51	-3.38	25.06		
Energy barrier	22.96	41.10	43.52	Energy barrier	19.75

The calculations identified several key residues such as Asp457, Asp477 and Glu478 coupled with Mg^{2+} ion and ADP that participate in AceK phosphatase catalysis. To validate the phosphatase mechanism proposed from QM calculation, the AceK mutants of these catalytic residues were generated and the phosphatase activities, in the presence of ADP, ATP and

AMPPCP respectively, were measured to compare with that of the wild-type AceK. As seen in Fig. 4A, AceK with ADP shows higher phosphatase activity than ATP, followed by AMPPCP which is a non-hydrolyzable ATP analogue. The D457A, D457S, D457T mutant derivatives, in which the proton donor Asp457 was removed, caused the complete loss of phosphatase activity (Fig. 4B). Though the mutation of Glu478 (E478Q) also caused a decrease of phosphatase activity, its activity is slightly higher than that of the Asp457 mutants (Fig. 4B, D457A and E478A, D457A, D457S, D457T).

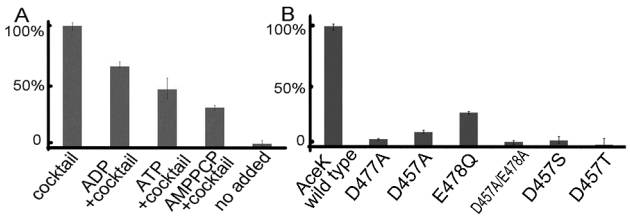

Fig. 4 Results of phosphatase activity assay. (A) Relative phosphatase activity of AceK with different nucleotides; kinase inhibitor/phosphatase activator cocktail contain 5 mM AMP and 5 mM pyruvate. (B) Relative phosphatase activity of AceK Asp457 and Glu478 mutants; error bars indicate the standard deviations.

Compared with other protein phosphatases, the lack of the second Mg^{2+} ion in AceK prevents the reaction from occurring in a one-step concerted mechanism. As such, AceK might be the first protein phosphatase which utilizes two-step associative mechanism. In this scenario, ADP in the active site acts to receive the proton and a single Mg^{2+} ion with the assistance of Asp477 underlies the reversibility, thus providing the rationale for two opposing activities within the same active site and utilizing the same overall conformation.

Based on our calculations, the role of Mg^{2+} is to activate the attacking water with its positive charge. This implies that the replacement of Mg^{2+} by another positive divalent ion would not lead to the loss of AceK phosphatase activity. The fact that the activity of AceK phosphatase in the presence of Mn^{2+} is even higher than that of Mg^{2+} supports this hypothesis.[3b, 6]

Interestingly, the number of Mg^{2+} ions present in the active site has a large impact on the catalytic mechanism. Our theoretical analysis reasonably explains how AceK catalyzes dephosphorylation through a specific stepwise pathway featuring only one Mg^{2+} ion. In the double Mg^{2+} ions model (Fig. S6, $2Mg^{2+}$), the critical Asp477 residue coordinates with the Mg^{2+} ion, significantly weakening the interaction with the substrate resulting in destabilization of the metastable intermediate. Consequently, the reaction occurs in a single step. In addition, since two metal ions anchor the phosphoryl group of the substrate Ser113-PO_3, it is easier for water to attack the P_{sub} forming a new bond and breaking the old P-O bond simultaneously in a relatively small space.

The calculated results of the double Mg^{2+} ions model indicate that a concerted reaction is the most energetically favorable pathway for a dephosphorylation reaction involving two Mg^{2+} ions (Table 1, energy barrier). The presence of a single Mg^{2+} ion leads to higher energy barrier pathway which would suggest the lower phosphatase activity than other bimetal protein phosphatase. Previous calculations provided insights into the phosphotransfer reaction catalyzed by AceK kinase activity.[7] It revealed that AceK preferred a single Mg^{2+} in the kinase activity and that a second Mg^{2+} ion would unfavorably increase the reaction energy barrier. Moreover, the invariant Asp477 also acts as an inhibitor during the kinase reaction. This observation, together with the fact that the Mg^{2+} ion and Asp477 have opposing effects on kinase and phosphatase activities, has led to the further confirmation that the single Mg^{2+} ion is a key factor in the AceK's reversible catalysis. The single Mg^{2+} ion and Asp477 inhibit kinase activity but activate the phosphatase activity thus balancing the two opposing reactions. The higher energy barrier in phosphatase caused by a single Mg^{2+} ion is in accordance with the physiological function of AceK, a bifunctional enzyme that phosphorylates or dephosphorylates IDH in response to environmental changes. AceK phosphatase activity is highly regulated only when *E. coli* is under the nutrient-limiting conditions, in which the Krebs cycle is shut down by the phosphorylated IDH and channels the isocitrate to the glyoxylate bypass.[3a, 8] Under such inactive conditions, the phosphorylated IDH are usually maintained at a physiologically low level. Therefore compared with the other specific phosphatases, the catalytic activity of AceK phosphatase may not need to be very high.

The role of ADP in AceK is distinct as no similar cofactors are implicated in any other phosphatases. In the favorable reaction pathway we calculated, ADP not only coordinates with Mg^{2+} ion to stabilize the entire reaction system, but also accepts the proton of the attacking water molecule. It is a phenomenon that rarely occurs in phosphotransfer reactions.

We are able to rationalize the role of ADP based on our calculation results. In the pathway in which Asp475, instead of ADP, acts as the proton acceptor (PathwayIII), the energy barrier increases to 43.52 kcal mol^{-1}. The lower electronegativity of the oxygen atom in the carboxyl group (-0.61) makes Asp475 less capable to act as a proton acceptor than the oxygen atom (-0.67) in the β-phosphoryl group of ADP. Our experimental results also support a role of ADP as a proton acceptor. AceK exhibits higher phosphatase activity in the presence of ADP, with the presence of ATP and AMPPCP resulting in successively lower activities, respectively (Fig. 4A). The higher phosphatase activity in the presence of ADP supports the vital role of ADP in the dephosphorylation reaction. Furthermore, our calculations and phosphatase activity assays confirm the speculation that AceK's ATPase activity is an integral part of its phosphatase activity. The steric hindrance of ATP blocks the effect of the Mg^{2+} ion on the substrate, and prevents the γ-phosphoryl group from accepting the proton. Thus ADP, instead of ATP, acts as the proton acceptor in this mechanism. AMPPCP, an ATP analogue which cannot be hydrolyzed, exhibits the lowest activity. In contrast, ATP hydrolysis generates ADP. Consequently, while ATP cannot directly catalyze the dephosphorylation reaction, its hydrolysis product, ADP, can. In our calculation model, the γ-phosphoryl group in both ATP and AMPPCP would occupy the space between the substrate phosphoryl group and the Mg^{2+} ion, preventing the substrate Ser113-PO_3 from moving close to the catalytic center, resulting in the inability of ATP and AMPPCP to catalyze the reaction. Our experimental results verify the ADP's role in the first step of the reaction.

In the dephosphorylation reaction, the Asp477 residue makes important contributions to the hydrogen bond network in the active site. Asp477 forms two vital hydrogen bonds with the substrate phosphoryl group to stabilize the **TS**; these strong hydrogen bonding interactions persist throughout the dephosphorylation process (Fig. S7). The simultaneous hydrogen bonding interactions maximize the effect of Asp477 on the phosphoryl group by reducing the electronegativity of the adjacent oxygen atom. Collectively, these structural features

favor the metal-activated water interaction with the substrate phosphorous atom (P_{sub}) and assist the release of the phosphoryl group. Through the two hydrogen bonds, the electronic distribution on the phosphoryl group is polarized by Asp477, which therefore influences the nucleophilic attack on the P_{sub}.

Once the hydrogen bonds between Asp477 and the substrate phosphoryl group are broken, even if one of them is disrupted by a water molecule, as observed in PathwayII, the energy barrier would rise drastically (41.10 kcal mol^{-1}). When Asp477 is mutated to alanine, the hydrogen bond interaction is abolished so that **TS** for the model cannot be located. The other vital role of Asp477 is to anchor the Mg^{2+} ion, which is confirmed by the complex structure of AceK D477A-IDH where the Mg^{2+} ion is missing in the active site.

Structured-based sequence alignment of the AceK active site with eukaryotic protein kinases shows that Asp477 in AceK is replaced by a conserved glycine residue in other kinases. Mechanism studies have shown that Asp477 has significant inhibitory influences on kinase activity[7] as well as activating effects on phosphatase activity. The opposing effects on kinase and phosphatase activities imply that Asp477 is involved in the regulation of AceK's reversible processes. As such, Asp477 is a key residue that enables AceK's phosphatase activity in the absence of double Mg^{2+} ion in active center. Moreover, it inhibits the kinase activity to help balance the two opposing functions.

In conclusion, through theoretical calculations and experiments we have obtained evidence to support our proposed novel stepwise mechanism that distinguishes it from PPMs or PPPs. Our mutation experiments have confirmed the proposed phosphatase mechanism of AceK and the roles of individual residues in its active site. DFT calculations suggest the critical roles of the single Mg^{2+} and Asp477 in the dephosphorylation function. The elucidation of AceK dephosphorylation mechanism for the first time gives insights into its unusual phosphatase function which is ADP/ATP dependent. The establishment of the working model of AceK phosphatase provides crucial foundation for further understanding its essential role in helping microorganisms cope with environmental stress. It is expected that more research will be done to gain more insights into the kinase fold-enabled phosphatase function of AceK.

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Notes and references

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† Electronic Supplementary Information (ESI) available: details of methods, as well as supplementary results. See DOI: 10.1039/b000000x/

1 Y. Shi, *Cell*, 2009, **139**, 468-484.
2 G. Hou and Q. Cui, *Journal of the American Chemical Society*, 2013, **135**, 10457-10469.
3 (a) D. C. LaPorte and D. E. Koshland, Jr., *Nature*, 1982, **300**, 458-460; (b) J. Zheng and Z. Jia, *Nature*, 2010, **465**, 961-966.
4 R. A. Bradshaw and E. A. Dennis, in *Handbook of Cell Signaling*, ed. N. Tonks, Beijing, 2nd ed., 2011, pp. 881.
5 (a) S. P. Yates, T. E. Edwards, C. M. Bryan, A. J. Stein, W. C. Van Voorhis, P. J. Myler, L. J. Stewart, J. Zheng and Z. Jia, *Biochemistry*, 2011, **50**, 8103-8106; (b) J. Zheng, S. P. Yates and Z. Jia, *Philosophical Transactions of the Royal Society B-Biological Sciences*, 2012, **367**, 2656-2668.
6 C. S. Stueland, K. Gorden and D. C. LaPorte, *The Journal of biological chemistry*, 1988, **263**, 19475-19479.
7 Q. Li, J. Zheng, H. Tan, X. Li, G. Chen and Z. Jia, *PloS one*, 2013, **8**, e72048.
8 (a) M. Garnak and H. C. Reeves, *The Journal of biological chemistry*, 1979, **254**, 7915-7920; (b) J. C. Hoyt and H. C. Reeves, *Biochemical and biophysical research communications*, 1988, **153**, 875-880.

