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Structural insights into lethal contractural syndrome type 3 (LCCS3) caused by a missense mutation of PIP5K γ

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Abstract

Signaling molecule phosphatidylinositol 4,5-bisphosphate is produced primarily by phosphatidylinositol 4-phosphate 5-kinase (PIP5K). PIP5K is essential for the development of the human neuronal system, which has been exemplified by a recessive genetic disorder, lethal congenital contractural syndrome type 3, caused by a single aspartate-to-asparagine mutation in the kinase domain of PIP5K γ . So far, the exact role of this aspartate residue has yet to be elucidated. In this work, we conducted structural, functional and computational studies on a zebrafish PIP5K α variant with a mutation at the same site. Compared with the structure of the wild-type (WT) protein in the ATP-bound state, the ATP-associating glycine-rich loop of the mutant protein was severely disordered and the temperature factor of ATP was significantly higher. Both observations suggest a greater degree of disorder of the bound ATP, whereas neither the structure of the catalytic site nor the K_m toward ATP was substantially affected by the mutation. Microsecond molecular dynamics simulation revealed that negative charge elimination caused by the mutation destabilized the involved hydrogen bonds and affected key electrostatic interactions in the close proximity of ATP. Taken together, our data indicated that the disease-related aspartate residue is a key node in the interaction network crucial for effective ATP binding. This work provides a paradigm of how a subtle but critical structural perturbation caused by a single mutation at the

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Author Contribution

J.H. and A.D. formulated the project. X.Z. and D.S. conducted crystallographic and biochemical experiments. A.U. and N.D. conducted computational work. J.H., A.U., A.D. and D.W. wrote the manuscript.

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

Accession numbers

The atomic co-ordinates and structure factors [accession code 6CMW (WT with bound ATP/Ca²⁺), 6CN2 (D236N with bound ATP/Ca²⁺) and 6CN3 (D236A)] have been deposited in the protein data bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

ATP-binding site abolishes the kinase activity, emphasizing that stabilizing substrate in a productive conformational state is crucial for catalysis.

Introduction

Phosphoinositides (PIs) are minor acidic phospholipids in the eukaryotic cell membrane system. Despite low abundance, the PIs are indispensable in many cellular processes, including endo/exocytosis, vesicular trafficking and cytoskeletal dynamics [1]. For instance, phosphatidylinositol 4,5-bisphosphate (PIP₂) is the precursor of signaling molecules [inositol trisphosphate, diacylglycerol and phosphatidylinositol (3,4,5)-trisphosphate] and also recruits an array of cytosolic proteins to the cell membrane [2]. Biosynthesis of PIP₂ in eukaryotic cells is carried out primarily by three isoforms of phosphatidylinositol 4-phosphate 5-kinase (PIP5K) [3–5]. Genetic studies have shown that the three isoforms (α , β and γ) of PIP5K have distinct and non-redundant functions in a variety of biological processes [6–8], and knockout of the γ -isoform in mice led to very early death after birth caused by severe defects in the neurological system [9]. The essential role of PIP5K γ for neural development in human has been exemplified by an autosomal recessive genetic disorder, lethal congenital contractural syndrome type 3 (LCCS3) [10]. LCCS is a group of neonatally lethal genetic disorders characterized by congenital joint contractures. Genetic heterogeneity has been noticed in a total of 11 types of LCCS, which are caused by mutations of genes involved in distinct processes [11]. In LCCS3, a missense mutation occurs in the kinase domain of PIP5K γ , which is expressed at high levels in the brain. A biochemical study has indicated that the substitution of D253 by an asparagine residue largely abolished kinase activity [10]. Because PIP5K γ knockout mice exhibited a very similar phenotype as observed in LCCS3, loss of PIP5K γ activity caused by the mutation and accordingly reduced the level of PIP₂ in the brain account for the severe defects in the neural system.

D253 in human PIP5K γ is located in a 'DLKGSxxxR' motif which is universally conserved in the phosphatidylinositol phosphate kinase (PIPK) family comprising three types of closely related lipid kinases: PIP5K (type I), phosphatidylinositol 5-phosphate 4-kinase (PIP4K, type II) and FYVE finger-containing phosphoinositide kinase (PIKfyve, type III). This motif has been named as the PIP-binding (PIPB) motif, because it has been proposed to associate with the head-group of the lipid substrate [12]. A co-structure of PIP5K with adenylyl-imidodiphosphate (AMP-PNP, a non-hydrolysable ATP analog) has suggested that the aspartate residue mutated in LCCS3 may play dual functions: binding ATP and stabilizing the structure of the catalytically important PIPB motif through a salt-bridge with the arginine residue in the PIPB motif [12]. However, a panel of co-structures of human PIP4K β with bound nucleotides or nucleotide analogs surprisingly showed distinct orientations of the nucleotide in the ATP-binding pocket [13], not supporting a role of the PIPB motif in ATP binding (Supplementary Figure S1), although the importance of the aspartate residue for PIP4K β activity has been demonstrated in a previous mutagenesis study [3]. Because of the conflicting structural information, the exact role of the disease-related aspartate residue and the underlying mechanism has not been clarified.

In this work, we structurally and functionally characterized a zebrafish PIP5K α (zPIP5K α) variant harboring the same mutation. The mutant enzyme showed no loss of affinity toward either ATP or lipid substrate, but a large decrease in turnover rate. By solving and comparing the crystal structures of the mutant protein and the WT protein, both in the ATP-bound state, we found that the disease-causing mutation did not lead to substantial structural changes at the catalytic site. Instead, the glycine-rich loop, which associates with the phosphate groups of ATP, was severely disordered in the mutant protein structure and the temperature factor of ATP also increased markedly, both suggestive of a higher degree of disorder of ATP. To better understand the molecular basis of ATP disorder, molecular dynamics (MD) simulation was conducted at microsecond scale. The results indicated that the mutation led to structural perturbation near the ATP-binding site and destabilized the key interactions between ATP and the protein. Based on these findings, we propose that the disease-causing mutation abolishes enzymatic activity by reducing the stability of the productive conformational state of ATP.

Materials and methods

Protein preparation

zPIP5K α (GenBank: AAH95318.1) and the mutant proteins were prepared as reported previously [14]. In brief, the plasmid of pET41b harboring the codon-optimized gene of zPIP5K α (kinase domain, residues 49–431) was transformed into Rosetta 2 (DE3) competent cells (Novagen). Overexpression was induced by 0.1 mM IPTG at OD₆₀₀ = 0.4 in LB medium. After culturing for additional 16 h at room temperature, the cells were harvested and suspended in the lysis buffer containing 50 mM sodium phosphate (pH 7.3), 300 mM NaCl, 5% glycerol, 0.5% Triton X-100 (American Bioanalytical) and EDTA-free protease inhibitor cocktail (Roche). The cell suspension was sonicated on ice and applied to centrifugation (10 000×g at 4°C for 30 min). The protein with C-terminal His₆-tag was firstly purified using Co²⁺-resin (Talon, Clontech) and then a size-exclusion column was equilibrated with the gel filtration buffer containing 10 mM HEPES (pH 7.3), 300 mM NaCl, 5% glycerol and 0.03% Triton X-100. The peak was collected for activity assay, and the protein concentration was measured using the Bradford method (Bio-Rad). The site-directed mutations were made using the QuikChange® kit and the mutant proteins were prepared in the same way as the WT protein.

Co-crystallization of zPIP5K α with ATP

Soaking the apo protein crystals, which were prepared in the same way as reported previously [14], with ATP/Mg²⁺ yielded a small block of positive electron density between the N-lobe and C-lobe of the kinase domain, suggestive of ATP binding. However, the electron density map was too poor to model ATP unambiguously. We then tried co-crystallized zPIP5K α with ATP and identified a condition distinct from that for apo protein crystallization. In brief, 1 μ l of protein (10 mg/ml zPIP5K α with 2 mM ATP and 10 mM MgCl₂) was mixed with 1 μ l of well solution containing 14% PEG8000, 100 mM sodium cacodylate, pH 6.5, 160 mM calcium acetate and 20% glycerol. The protein was crystallized at 4°C using the sitting-drop method. The bipyramid-shaped crystals showed up within 1 week and grew to full size in 2 weeks. The crystals were cryo-protected by 25% glycerol

and flash-frozen in liquid nitrogen. The D236N/ATP complex was crystallized under the same condition as the WT protein. D236A was crystallized under the condition for the apo state WT protein [14].

Data collection and structure determination

The diffraction data were collected on the beamline of 21-ID-D at LS-CAT in Advanced Photon Source (APS), and indexed, integrated and scaled by HKL2000 [15]. The structures were solved by the approach of molecular replacement using the structure of zPIP5K α in apo state (PDB access code 4TZ7) as a template in Phenix [16]. Model building was conducted in Coot [17] and refinement was performed by using phenix.refine. It should be noted that the concentration of Ca²⁺ in the well solution (160 mM) is much higher than that of Mg²⁺ in the protein sample (10 mM). Accordingly, calcium ions, instead of magnesium ions, were modeled in the structure. The structure figures were generated using PyMOL v. 1.3 (Schrodinger LLC).

PIP5K kinase activity assay

The activity assay was modified from the approach reported previously [14]. The following components were included in reaction (50 μ l): 100 mM Tris-HCl (pH 7.4), 50 mM EGTA, 100 mM MgCl₂, 100 ng of purified zPIP5K α , 20 μ M ATP with 1 μ Ci [³²P]-gamma ATP (PerkinElmer) and 10 μ M PI(4)P (phosphatidylinositol 4-phosphate; Echelon Biosciences, Inc.). The reaction was initiated by adding 100 ng of purified enzyme. After processing at room temperature for 1 h, it was stopped by the addition of the lipid extraction solution containing chloroform, methanol and HCl with a volume ratio of 3.3 : 3.7 : 0.1, as well as 10 μ g/ml Brain extract from bovine brain (Type I, Folch Fraction I, Sigma). After vortexing for 20 s, the sample was centrifuged at 2000 $\times$ g for 1 min, and the lower organic phase was collected, spotted and separated by thin layer chromatography. The product of the reaction was quantified by a Storm 820 PhosphorImager (GE).

To determine the kinetic parameters, the measured initial reaction rates were plotted against different concentrations of the substrate (0.2–10 times of the corresponding K_m). The K_m and V_{max} were calculated by curve fitting to Michaelis–Menton equation in OriginPro® 8.0.

MD simulation

We performed five independent CHARMM [18,19] MD simulations for each of the WT and the mutant (D236N) zPIP5K α structures bound to ATP using the CHARMM36 force-field [20] (in total 1.3 μ s for the WT and 1.2 μ s for the mutant). Missing residues (residue ID: 1–56, 311–356 and 385–401) were omitted from the simulations. Each structure is solvated in a cubic box with at least 12 Å of distance between the protein and the cell boundaries. The two calcium ions in the binding site were preserved, and chloride ions (six for the WT and seven for the mutant) were added to neutralize the system. The systems were heated from 50 to 300 K over 100 ps and equilibrated for 0.5 ns at 300 K. Then, Langevin dynamics was performed for the final structures at 300 K using OpenMM version 7.0 [21]. The first 124 ns of each simulation was taken as equilibration and not included in further analysis. Visual Molecular Dynamics (VMD) [22] was used to generate Figure 4A and Supplementary

Figure S6, and MDTraj [23] software was used to calculate distances in Figure 4 and Supplementary Figure S6.

Results

Functional characterization of the zPIP5K α variants

Because zPIP5K α bares high homology (~70%) with the kinase domain of human PIP5K γ , the structural and functional features of PIP5K γ should be preserved in zPIP5K α . Indeed, D236 in zPIP5K α , which is equivalent to D253 in human PIP5K γ , is essential for its kinase activity (Table 1). The substitution of D236 by alanine, asparagine or glutamate similarly abolished activity, indicating that both negative charge and proper length of the side chain are required for activity. Kinetics study showed that the D236N mutation did not affect the K_m s toward ATP or the lipid substrate PI(4)P, whereas we observed a 100-fold reduction in k_{cat} (Table 2). D236A mutation reduced k_{cat} to a similar extent, and a moderately reduced affinity toward ATP by three-fold suggested that D236 plays either a direct or indirect role in ATP binding.

Structural characterization of the zPIP5K α /ATP complex

The co-structure of zPIP5K α with AMP-PNP (PDB access code 5E3U) and the co-structure of human PIP4K β with AMP-PNP (PDB access code 3X03) have been reported recently [12,13]. However, the orientations of the ATP analog are not consistent in these structures (Supplementary Figure S1). Considering the conserved ATP-coordinating residues and the highly superimposable ATP-binding pockets of these two closely related PIPKs, it is unlikely that such a discrepancy in ATP orientation can be attributed to the different catalytic mechanisms. We also noticed that these co-structures were obtained by soaking the apo protein crystals with externally added ATP analog. To avoid the potential issues caused by crystal soaking, we co-crystallized the WT zPIP5K α with ATP at 4°C and solved the structure at a resolution of 3.15 Å by molecular replacement (Table 3 and Figure 1A).

The structure of ATP-bound zPIP5K α can be well superimposed on the apo state structure (PDB access code 4TZ7) with a root-mean-squared deviation (RMSD) of 0.56 Å for C α (Figure 1B) [14], and structural comparison with the other protein kinase structures suggested that the ATP molecule adopted a productive or productive-like conformation. Structural overlapping of the N-lobe (residues 57–223, RMSD = 0.42 Å for C α) revealed a small rotation (~5°) of the C-lobe (residues 224–426), narrowing the cleft between the two lobes for better ATP binding. This conformational change led to an ~2 Å displacement of the PIPB motif toward the N-lobe, making the catalytic residue K238 1.5 Å closer to the γ -phosphate (PG) of ATP. Because the crystallization buffer contains 160 mM calcium acetate, we modeled two Ca²⁺ ions in the structure. Although Mg²⁺ is preferred, Ca²⁺ ion also supports the kinase activity of zPIP5K α (Supplementary Figure S2). The bound ATP adopted an orientation very similar to AMP-PNP in 5E3U, and the small structural differences in the β -phosphate and PGs are likely due to the phosphorus–nitrogen bonds in AMP-PNP. Notably, both the ATP-bound structure and the AMP-PNP-bound structure support that the side chain of D236 forms a hydrogen bond with the ribose of ATP (Figure 1A and Supplementary Figure S1).

One remarkable feature of the ATP-bound structure is that the glycine-rich loop, which has not been solved in the previous PIPK structures, formed extensive interactions with ATP (Figure 1C). Sequence analysis revealed that there is a conserved ‘¹⁵⁴GxSGS¹⁵⁸’ motif in the glycine-rich loops of the PIP5Ks from distinct species (Supplementary Figure S3), which is reminiscent of the ‘GxGxxG’ motif in protein kinases [24], including cAMP-dependent protein kinase A (PKA) [25]. When zPIP5K α was co-crystallized with ATP, the glycine-rich loop folded down and directly interacted with the phosphates of ATP, but in a way distinct from that in PKA: the PG formed two hydrogen bonds with the backbone nitrogen atoms of A155 and S156, respectively, and the β -phosphate unexpectedly formed a hydrogen bond with the hydroxyl group of S158. The unusual interaction between S158 and the β -phosphate appears to account for the extrusion of G157 from the plane where the glycine-rich loop resides, leaving the glycine-rich loop of zPIP5K α less compact and more rippled than its counterparts in PKA and many other kinases.

Structural characterization of the D236N mutant/ATP complex

We co-crystallized the mutant protein with ATP under the same crystallization condition as the WT protein and solved the structure at a similar resolution (3.10 Å). The D236N/ATP complex structure showed no apparent difference from the WT structure with an RMSD of 0.25 Å for C α . Such a small structural difference can be attributed to variations among crystals. However, the glycine-rich loop of the mutant was severely disordered, even though the mutant still bound ATP (Figure 2). The residues 152–157, which are involved in association with the β -phosphate and PGs of ATP, could not be modeled due to lack of electron density. To examine the importance of the glycine-rich loop, we mutated the conserved S156 and S158 into alanine, respectively, and found that the mutations had little effects on kinase activity. We then deleted the whole ‘GxSGS’ motif, and the mutant showed reduced but still robust activity (Tables 1 and 2). The moderately reduced $V_{\max}$ (~70% reduction) and increased K_m toward ATP (by 4–5-fold) indicate that — although the glycine-rich loop of zPIP5K α is contributive to ATP binding and probably helps to orientate the PG of ATP for efficiency phosphoryl transfer, which has been proposed for the glycine-rich loop of PKA [25] — it does not play a vital role in catalysis and therefore the disorder of the glycine-rich loop in the mutant structure cannot itself account for the 100-fold decrease in activity.

Similar to D236, N236 formed two hydrogen bonds with the ribose of ATP and the hydroxyl group of S301, respectively (Figure 2 and Supplementary Figure S4). Its side chain still associated with R244, but through a hydrogen bond, rather than a salt-bridge. Because the D236–R244 salt-bridge was thought to be important to keep the PIPB motif in a compact and active conformation, it has been proposed that loss of this salt-bridge would result in structural changes of the PIPB motif, which thereby accounts for the abolished activity [12]. However, the structure of D236N mutant showed that the conformation of the PIPB motif was well maintained. This is unexpected, but not completely surprising because R244 forms additional interactions with the backbone carbonyl oxygen of N236 and the side chain of D260, respectively. Indeed, when we crystallized and solved the structure of the D236A mutant at 3.35 Å, we found that side chain elimination of D236 did not result in any observable conformational change of the PIPB motif, suggesting that the interactions of

R244 with the backbone oxygen of A236 and the side chain of D260 play more important roles in maintaining the conformation of the PIPB motif (Supplementary Figure S5).

Although the mutant structure is quite similar to the WT protein structure, we found that the temperature factor (*B*-factor) of ATP in the mutant structure ($95 \text{ \AA}^2$) was markedly higher than that in the WT protein structure ($68 \text{ \AA}^2$) (Figure 3 and Table 3). Because the two structures were solved at very similar resolutions, refined with the same protocol and had similar average *B*-factors (WT $81 \text{ \AA}^2$ vs. D236N mutant $91 \text{ \AA}^2$), the significant difference in ATP *B*-factors cannot be explained by a random variation between the two datasets. The *B*-factor of ATP in the WT protein structure was comparable to the ATP-interacting residues within the ATP-binding pocket, indicating that the bound ATP molecule has the same degree of thermal motion as the surrounding environment (Table 4). In contrast, the degree of motion of ATP in the mutant was significantly higher than that of the interacting residues. A detailed analysis indicated that the PG and the adenosine are the most disordered moieties in the ATP molecule (Table 4). Because *B*-factor is coupled with occupancy, a higher *B*-factor of a ligand could also be interpreted as a low occupancy. In this case, the ATP occupancy could be refined to be as low as 0.85 in the mutant protein structure. However, we do not attempt to explain the discrepancy in this way because the protein was co-crystallized with 2 mM ATP, which is 5000 times higher than the $K_{m,ATP}$ ($0.4 \text{ \mu M}$) for the mutant protein. Low temperature (4°C) crystallization may further strengthen ATP binding. In fact, as indicated by the $K_{m,ATP}$ (Table 2), the mutant protein did not show any difference in ATP-binding affinity. Admittedly, K_m , which is measured in solution, is not a good predictor for occupancy in crystal. However, since the WT protein and the mutant were crystallized under the same condition with the same space group, if there is any factor in the crystal affecting ATP binding, it would affect both the WT protein and the mutant equally. Therefore, there is no reason to believe that the occupancy of ATP in the mutant structure should be lower than that in the WT protein structure, and we instead interpret that the higher *B*-factor is a sign of higher degree of thermal motion of the bound ATP. Consistently, the severe disorder of the ATP-associating glycine-rich loop in the mutant protein can be explained by an increased mobility of ATP, whereas it cannot be explained merely by a slightly lowered ATP occupancy or a random variation among different crystals.

MD simulation of the WT and the D236N zPIP5K α –ATP complex

To better understand the underlying mechanism of the higher degree of ATP thermal mobility caused by the D236N mutation, we performed five independent CHARMM [18,19] MD simulations each for the WT and the D236N mutant zPIP5K α structures bound to ATP (in total $1.3 \text{ \mu s}$ for the WT and $1.2 \text{ \mu s}$ for the mutant). Compared with the WT protein, the D236N mutant exhibited similar overall conformational dynamics throughout the whole polypeptide chain (Supplementary Figure S6A). During simulation, ATP was constantly in the bound state for both WT protein and the mutant protein, as seen by the hydrogen bonding of the adenosine with the hinge region connecting the N-lobe and the C-lobe (Supplementary Figure S6B).

We then examined the key interactions mediated by D236 (or N236 in the mutant protein) shown in Figures 2 and 4A by calculating probability distributions of atomic distances

averaged from five independent simulations for each form. Consistent with the crystallographic data, the mutation did not affect the interactions of the side chain of R244 with the side chain of D260 or the backbone of N236 (Figure 4B), confirming that the conformation of R244 was not affected by the mutation. In contrast, although the interaction of the side chain of D236 with the guanidine of R244 was well preserved in the WT protein, the distance was significantly greater with a broader range distribution in the mutant, indicative of loss of interaction (Figure 4C). Similarly, the hydrogen bonding between D236 and S301 was not observed in the mutant, but partially preserved in the WT protein (Figure 4D). As the S301A mutation caused a loss of activity by 20 times (Table 1) and the hydroxyl group of S301 interacts only with D236, the D236–S301 interaction must be important for activity. In contrast, the hydrogen bond between D236 and the hydroxyl hydrogen of the ribose of ATP were equally unstable for both WT and the mutant protein, suggestive of a weak association and likely a relatively small contribution to ATP binding (Figure 4E). Because an electrostatic association of K238 with the PG of ATP is crucial for catalysis (see the next section), we further examined this key interaction in the simulations and found significant differences between the WT and the mutant protein (Figure 4F). In the WT, the K238–PG interaction (~ 5.6 Å) was well preserved and a close association with a distance of ~ 4 Å, which has been seen in other kinases (such as PKA [26]), was also observed with a small probability. However, the K238–PG association was largely diminished in the mutant protein and the close association was negligible. We postulate that the negative charge elimination by the D236N mutation disrupted the balance of the local electrostatic network, leading to an increased repulsion among the positively charged entities, including R244, K238 and the two Ca^{2+} ions, which is likely responsible for the disrupted K238–PG interaction. If both the interactions of D236–S301 and K238–PG are necessary for maintaining a productive conformational state of the kinase–ATP complex, this occurs with a small but substantial probability ($\sim 17\%$) for the WT protein and with an essentially zero probability for the mutant (Figure 4G).

The roles of the positively charged residues at the catalytic site

Since our data did not support a role of the D236–R244 salt-bridge for the PIPB motif structure, we examined the role of the conserved R244 with the mutants R244K and R244E. Interestingly, although both our study and the recent report indicated that the R244A mutation diminished activity (Table 1) [12], R244K and R244E had no effect on $K_{m,\text{PI}(4)\text{P}}$ or V_{max} (Table 2). This result indicated that the D236–R244 salt-bridge indeed is not essential for catalysis, and also suggested that R244 may not even play a role in binding the phosphate group of PI(4)P because R244E would otherwise reduce the affinity toward the negatively charged lipid substrate. Besides, R244, K238 and K259 are the other two positively charged residues in the proximity of the catalytic site. We then conducted mutagenesis on these two residues to examine their functions. Because the $K_{m,\text{PI}(4)\text{P}}$ values of K259E and K259R markedly increased by ~ 10 times and the catalytic efficiency (V_{max}/K_m) of K259R was ~ 6 times higher than that of K259E, it is likely that K259 directly associates with 4'-phosphate of PI(4)P. For K238, K238R and K238E mutations completely abrogated the activity, suggesting that K238 plays a fundamental role in the reaction. Structural comparison with the members of the IPK family, which has been suggested to be a close relative of the PIPK family in kinase evolution [27], provides additional hints about

the functionality of this residue (Supplementary Figure S7). In the structure of IP₃-3K, a representative member of the IPK family, the lysine residue from the 'DxK' motif, which is topologically equivalent to the PIPB motif in PIPKs, is the only residue forming a direct interaction with the target hydroxyl group [27]. Because IP₃-3K lacks a catalytic aspartate residue corresponding to D166 in PKA (equivalent to D299 in zPIP5K α), it has been proposed that the conserved lysine residue may function as a catalytic residue. Accordingly, it is conceivable that K238 in zPIP5K α has a dual function in catalysis (Figure 5): (1) by electrostatically associating with the PG of ATP, K238 neutralizes the developed negative charge during the nucleophilic reaction, which is analogous to the well-established function of K168 in PKA [28]; (2) K238, together with D299, orients the target hydroxyl group of the substrate through a hydrogen bond for an efficient phosphoryl transfer reaction. A similar scenario has been observed in the structure of another IPK, IP₅-2K [29]. One unresolved issue with this mechanism is the substrate recognition by the activation loop, which is folded only when the lipid kinase is associated at the membrane surface and therefore disordered in all the PIPK crystal structures [30].

Discussion

PIP₂ generated by PIP5K γ is required for the development of the neurological system. A missense mutation in the signature PIPB motif of human PIP5K γ diminishes kinase activity and causes LCCS3. To understand how the aspartate-to-asparagine substitution results in a 100-fold decrease in activity, we conducted structural, functional and computational studies on a zPIP5K α variant harboring the same mutation. Our results suggest that the aspartate residue plays a key structural role in stabilizing the productive conformational state of ATP.

The previously solved PIP5K/AMP-PNP complex structure has implied multiple roles of the aspartate residue mutated in LCCS3 [12]: (1) it binds ATP through a hydrogen bond with the ribose ring of ATP; (2) it forms a hydrogen bond with S301 and (3) it also forms a salt-bridge with the invariable R244 and therefore may play a structural role in maintaining the structure of the PIPB motif in an active conformation. Substitution of the aspartate by an asparagine likely preserves the hydrogen bonds, but abolishes the salt-bridge with R244. Accordingly, it was postulated that the loss of the D236–R244 salt-bridge may be primarily responsible for the diminished activity [29]. However, the current data do not support such a hypothesis. Firstly, the conservative mutation D236E has little activity (Table 1), indicating that preserving a negative charge on the side chain is not sufficient to support activity. Secondly, the D236N/ATP complex is structurally superimposable with the WT complex. No meaningful structural changes have been observed in the proximity of the PIPB motif (Figure 2), which is further supported by the MD simulation results (Figure 4). Thirdly, the D236A mutant structure also showed no structural changes in the PIPB motif (Supplementary Figure S5). Therefore, it seems that the D236–R244 salt-bridge does not play a key role in maintaining the conformation of the PIPB motif. Lastly, the R244E mutation did not affect kinase activity (Table 2), confirming that the salt-bridge is dispensable.

Although the D236N mutation did not affect hydrogen bond formation (Figure 2), it would likely weaken the hydrogen bonds because a carboxamide is a weaker hydrogen bond

acceptor than a carboxylic acid. Indeed, in MD simulation, we found that the hydrogen bonds of D236 with R244 and S301 were largely destabilized. Although the former appears to be very unstable in MD simulation and possibly a weak interaction (Figure 4E), the latter must be important because S301A mutation reduced activity by 20 times (Table 1). It is likely that the D236–S301 association orients the hydroxyl group of S301 in such a way that S301 facilitates binding and orienting the divalent metal bound with ATP (3.3 Å between the hydroxyl oxygen and Ca^{2+}). Although we did not observe an alternative conformation of ATP in the mutant protein structure, probably due to the dynamic feature of ATP within the binding pocket (fast exchange among distinct conformational states), a close inspection did allow us to find that the *B*-factor of the ATP molecule in the mutant protein structure was markedly higher than that in the WT protein structure (Figure 3 and Table 4), indicative of a higher degree of thermal motion and therefore an instability of the productive conformational state. Consistently, the glycine-rich loop of the mutant protein was severely disordered even though the protein was still in an ATP-bound state (Figure 2). In contrast with its counterpart in PKA, the glycine-rich loop of zPIP5K α plays a minor role in binding and orienting ATP (Table 1), and its conformational stability appears to be a sensitive indicator of ATP thermal mobility. Interestingly, the MD simulation study revealed that the key interaction between the catalytic residue K238 and the PG of ATP was lost in the mutant protein (Figure 4F), providing another piece of evidence explaining the instability of ATP and the loss of activity (Figure 4G).

Conclusion

The lethal genetic disorder LCCS3 is caused by a single missense mutation in PIP5K γ . In this work, we conducted extensive structural, functional and computational studies to unravel the underlying mechanism how an aspartate-to-asparagine substitution at the signature PIPB motif drastically affects enzymatic activity. Our results demonstrated that a subtle but critical structural perturbation caused by a mutation at the ATP-binding site is sufficient to destabilize the productive conformational state of the kinase/ATP complex, which likely accounts for the largely diminished activity. This work reinforces the notion that effective substrate binding is pivotal for catalysis, highlighting the importance of substrate dynamics in enzymatic reaction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AMP-PNP	adenylyl-imidodiphosphate
LCCS3	lethal congenital contractural syndrome type 3
MD	molecular dynamics
PG	gamma phosphate
PI(4)P	phosphatidylinositol 4-phosphate
PIP₂	phosphatidylinositol 4,5-bisphosphate
PIP5K	phosphatidylinositol 4-phosphate 5-kinase
PIPB	PIP-binding
PIPK	phosphatidylinositol phosphate kinase
PIs	phosphoinositides
PKA	protein kinase A
RMSD	root-mean-squared deviation
WT	wild type
zPIP5Kα	zebrafish PIP5K α

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Figure 1. Crystal structure of zPIP5K α with bound ATP and Ca²⁺.

(A) Identification of ATP/Ca²⁺ complex. The $F_o - F_c$ omit map ($\sigma = 5$) for ATP and calcium ions (black spheres) is shown as green mesh. The key residues in the PIPB motif (in green) and ATP are shown in stick mode. The hydrogen bond is indicated by the red dashed line. (B) Structural comparison of zPIP5K α in apo state (gray, PDB access code 4TZ7) with that in the ATP-bound state (blue). The PIPB motif is highlighted in magentas (apo) and green (ATP), respectively. ATP binding induces a 5° rotation of the C-lobe toward the N-lobe, causing a 1.5 Å displacement of the catalytic residue K238 toward the PG of ATP. (C) Structure of the glycine-rich loop. *Top*: the glycine-rich loop in apo state (gray) and in ATP-bound state (blue). The blue mesh indicates a $2F_o - F_c$ electron density map ($\sigma = 1$) in ATP-bound state. *Bottom*: the interactions of the glycine-rich loop with ATP. The residues in the 'GxSGS' motif are shown in stick mode and the hydrogen bonds are indicated by red dashed lines. The disordered side chain of S156 was not modeled.

Figure 2. Structural comparison of the catalytic site of WT α PIP5K (left, blue) with that of the D236N mutant (right, green).

The residues of the PIPB motif are shown in stick mode and colored in green and magentas, respectively. The hydrogen bonds are shown as red dashed lines. The black spheres are calcium ions. The black dotted line represents the disordered glycine-rich loop in the mutant structure.

Figure 3. Comparison of the *B*-factor of ATP in the WT zPIP5K α (left) with that in the D236N mutant.

Protein surface is colored by *B*-factor in a Blue-White-Red style (blue means low *B*-factor and red means high *B*-factor). ATP molecules (stick mode) and calcium ions (sphere) are colored in the same way. Note that ATP in the WT protein has similar color as the surrounding residues, whereas ATP in the mutant protein is redder than its environment, indicative of higher *B*-factor. For clarity, the glycine-rich loop is removed from the structures.

Figure 4. MD simulation of the WT zPIP5K α /ATP/Ca²⁺ and the D236N mutant/ATP/Ca²⁺ complexes.

(A) The catalytic site of the WT zPIP5K α . Catalytically important residues are shown as sticks and labeled with different colors. ATP is colored by atom type and shown in stick representation. Specific atoms that are used in the distance calculations are shown as spheres. When a residue has more than one interaction of interest, the atoms are distinguished by different sphere sizes. Calcium ions in the catalytic site are shown as cyan spheres. Gly-rich loop is not shown for clarity. (B–F) Probability distributions of distances between sets of atoms in the catalytic site of zPIP5K α . In (B–F), the WT protein is black and the mutant is red. When equivalent atoms are present, both distances are computed and the minimum is used. Atom pairs used in the distance calculations are as follows (B) left: the backbone oxygen atom of D236 (small green sphere) and the NH2 of R244 (small blue sphere), right: the oxygen of carboxylic acid of D260 and NE of R244 (small blue sphere), (C) the oxygen of carboxylic acid of D236 (large green spheres) and HH1 of R244 (large blue spheres), (D) the carbonyl oxygen of D236 and the hydroxyl group of S301, (E) the carbonyl oxygen of D236 and ribose of ATP, (F) NZ of the catalytic residue K238 and the γ -phosphate of ATP and (G) the correlation of the distances between D236–S301 and K238–PG of ATP are shown as a 2D histogram for the WT protein (left) and the mutant (right). The area encircled by the blue rectangle represents the productive conformational state of the zPIP5K α –ATP complex.

Figure 5. Structural model of zPIP5K α /ATP/PI(4)P ternary complex.

The head-group of PI(4)P is modeled in the structure of ATP-bound zPIP5K, where the 5'-OH is 3.5 Å away from the amine group of K238 [comparable with the corresponding distances of 3.3 Å in PKA (PDB access code 1ATP), 2.7 Å in IP₃-3K (PDB access code 1W2C) and 3.6 Å in IP₅-2K (PDB access code 2XAN)], 4.4 Å away from the PG of ATP (two-headed arrow, 5.2 Å in PKA, 4.6 Å in IP₃-3K and 3.2 Å in IP₅-2K) and 2.7 Å away from the carboxylic acid of D299 (3.3 Å in PKA and 2.6 Å in IP₅-2K). D299 is modeled with an alternative rotamer to point toward the 5'-OH. The amine group of K259 is 2.9 Å away from 4'-phosphate of PI(4)P, allowing an electrostatic interaction. The red dashed lines represent the proposed interactions. The activation loop, which is disordered in the PIPK structures, is supposed to bind PI(4)P from the direction indicated by the arrows.

Table 1Activity assay of the zPIP5K α variants

Mutation	Activity (%) ^{*,†}	Mutation	Activity (%) [*]
S156A	80	K238R	<1
S158A	109	R244A	3
154–158	30	R244E	102
D236A	<1	R244K	98
D236E	<1	K259A	3
D236N	<1	K259E	15
K238A	<1	K259R	74
K238E	<1	S301A	4

^{*} Expressed as the percentage of the activity of the WT enzyme.

[†] Results from single experiments.

Table 2Kinetic parameters of zPIP5K α and the variants

Protein	$K_{m,ATP}$ (μ M) [*]	$K_{m,PI(4)P}$ (μ M) [*]	Relative k_{cat} (%) [*]
WT	0.45 $\pm$ 0.06	1.5 $\pm$ 0.3	100 $\pm$ 3
D236N	0.40 $\pm$ 0.06	2.1 $\pm$ 0.4	1.2 $\pm$ 0.1
D236A	1.5 $\pm$ 0.3	1.6 $\pm$ 0.4	1.1 $\pm$ 0.1
154–158	1.9 $\pm$ 0.1	—	34 $\pm$ 1
R244E	—	1.7 $\pm$ 0.2	99 $\pm$ 6
R244K	—	1.8 $\pm$ 0.5	110 $\pm$ 9
K259E	—	14 $\pm$ 6	39 $\pm$ 2
K259R	—	17 $\pm$ 5	310 $\pm$ 40

—: Not determined.

^{*} Results are expressed as the means $\pm$ SD of three independent assays.

Table 3

Crystallographic statistics

	WT	D236N	D236A
Data collection			
Wavelength (Å)	1.078	0.979	0.979
Space group	P4 ₃ 2 ₁ 2	P4 ₃ 2 ₁ 2	P4 ₃ 2 ₁ 2
Cell dimensions (Å)	$a = b = 88.6, c = 157.5$	$a = b = 89.0, c = 157.8$	$a = b = 89.3, c = 156.7$
Resolution (Å) *	40 – 3.15 (3.26 – 3.15)	40 – 3.10 (3.21 – 3.10)	40 – 3.35 (3.47 – 3.35)
Redundancy *	28.2 (29.0)	28.3 (29.4)	14.0 (14.3)
Completeness * (%)	99.9 (100.0)	99.9 (100)	100 (100)
$\ \sigma I \ $ *	39.9 (5.3)	31.2 (3.0)	24.6 (3.0)
$R_{\text{merge}}^{\dagger}$ *	0.103 (0.893)	0.118 (>1)	0.114 (0.848)
$R_{\text{pim}}^{\ddagger}$ *	0.02 (0.168)	0.025 (0.274)	0.032 (0.231)
$CC_{1/2}$ of the highest resolution shell ^{\$}	0.944	0.958	0.898
Refinement			
Unique reflections	11 412	12 055	9645
Number of atoms	2394	2360	2305
Protein	2361	2326	2305
ATP	31	31	—
Ca ²⁺	2	2	—
Water	—	1	—
$R_{\text{work}}/R_{\text{free}}^{\parallel}$	0.21/0.26	0.22/0.26	0.23/0.27
Wilson B -factor (Å ²)	91	97	101
Average B -factors (Å ²)	81	91	95
Protein	81	91	95
ATP	68	95	—
Ca ²⁺	72	89	—
Water	—	57	—
R.m.s. deviations			
Bond lengths (Å)	0.010	0.010	0.010
Bond angles (°)	1.40	1.25	1.23
Ramachandran plot (%)			
Flavored	95.7	95.2	92.8
Allowed	3.6	4.5	5.8
Outliers	0.7	0.3	1.4

* Highest resolution shell is shown in parentheses.

$R_{\text{merge}}^{\dagger} = \frac{\sum_{hkl} \sum_j |I_j(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_j I_j(hkl)}$, where I is the intensity of reflection.

$R_{\text{pim}}^{\ddagger} = \frac{\sum_{hkl} [1/(N-1)]^{1/2} \sum_j |I_j(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_j I_j(hkl)}$, where N is the redundancy of the dataset.

${}^sCC_{1/2}$ is the correlation coefficient of the half datasets.

${}^{\parallel}R_{\text{work}} = \frac{\sum |F_{\text{obs}}| - |F_{\text{calc}}|}{\sum |F_{\text{obs}}|}$, where F_{obs} and F_{calc} are the observed and the calculated structure factors, respectively. R_{free} is the cross-validation R factor for the test set of reflections (10% of the total) omitted in model refinement.

Table 4Comparison of the *B*-factors ($\text{\AA}^2$) between the WT and the D236N mutant structures

	WT	D236N	(D236N-WT)/WT $\times 100\%$ *
Overall structure	81	91	12
ATP-coordinating residues	66 ± 10 [†]	72 ± 11 [†]	9
ATP	68	95	40
PA	60	88	47
PB	68	97	43
PG	75	119	59
Ribose	63	83	32
Adenosine	67	101	51

* The percentage reflects how much the *B*-factor of the mutant protein is higher than that of the counterpart of the WT protein.

[†] The *B*-factors of nine ATP-coordinating residues, including F160, I169, L224, L225, D236 (N236 in the mutant), S301, L303, I377 and D378, are analyzed statistically. The results are expressed as the means $\pm$ SD.