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Crystal structure of human adenylate kinase 4 (L171P) suggests the role of hinge region in protein domain motion

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ABSTRACT

It is well known that motion of LID and NMP-binding (NMP_{bind}) domains in adenylate kinase (AK) is important in ligand binding and catalysis. However, the nature of such domain motions is poorly characterized. One of the critical hinge regions is hinge IV, which connects the CORE and LID domains. In addition, the hinge IV contains a strictly conserved residue, L171, in the AK family. To investigate the role of hinge IV, crystal structure of human adenylate kinase 4 (AK4) L171P mutant was determined. This mutation dramatically changes the orientation of the LID domain, which could be described as a novel twisted-and-closed conformation in contrast to the open and closed conformations in other AKs. This mutant provides a new example of domain motions in AK family.

Protein structures are dynamic and undergo considerable conformational changes. Domains can move with respect to others, while their own folds remain essentially unaffected. Hinge region between domains is usually structurally unconstrained, thereby allowing flexible changes in domain orientations. The domain motions can be triggered by substrate binding, changes in ion concentration, and sometimes protein–protein or DNA–protein interactions. Upon substrates binding event, Adenylate kinases are good models for the study of domain motion and protein conformational change [1].

Adenylate kinases (AKs) are ubiquitous enzymes that are involved in energy metabolism and homoeostasis of cellular adenine nucleotide composition [2]. They catalyze reversible transfer of γ-phosphate group from Mg^{2+}ATP (or GTP) to AMP, releasing Mg^{2+}ADP (or GDP) and ADP [3]. AKs belong to the nucleoside monophosphate kinase (NMPK) family, which also includes other members such as guanylate kinases, thymidylate kinases, and UMP/CMP kinases. All of these enzymes share a common typical α/β-fold that consists of a β-sheet CORE surrounded by α-helices, with a P-loop motif at the N-terminus that binds the phosphoryl donor.

Adenylate kinase is a three-domain protein, containing a central CORE domain and two flanking domains, the LID and NMP_{bind} domains. The CORE and NMP_{bind} domains are conserved in all AKs, whereas the LID domain is quite different. Based on the length of LID, AKs are classified into two groups: short form AKs, in which the LID is simply a variable loop; and long form AKs, in which the LID is a four-stranded anti-parallel β-sheet.

To date, two major conformations in long form AKs have been determined. Ligand-free enzyme is characterized by an "open" state in which the LID is distant from the CORE and NMP_{bind} regions [4,5]. A "closed" state is formed upon substrates or bi-substrate analog AP_5A binding, in which both LID and NMP_{bind} domains move closer to the CORE, forming active site and arranging two substrates together [5–13]. Although the amplitudes of opening or closing show more or less variations in different AKs, the direction and orientation of such domain motions are the same [5]. This motion is considered essential for enzymatic activity, without which the substrates cannot be correctly oriented and the phosphate transfer center cannot be shielded from solvent [14,15]. Recent studies showed that substrate-free AK can also form the "close" conformation, at the microsecond to millisecond time scale, along the enzymatic reaction trajectory [16,17]. Such conformational change in AKs is considered an excellent model to study domain movements in proteins [1,18].

In vertebrates, six AK isoforms named AK1–AK6 have been identified. AK1, AK5, and AK6 are short form enzymes while the long forms include AK2, AK3, and AK4. These enzymes are highly

Abbreviations: AK, adenylate kinase; NMPK, monophosphate kinase; NMP, monophosphate; AP_5A, P^1,P^5-bis (adenosine-5′-)pentaphosphate

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conserved in primary sequence except the recently characterized AK6 [19]. Human AK4 was first named as AK3 based on its 58% homology to bovine AK3 [20]. It was renamed as AK4 when it was found in mammalian central nervous system [21]. AK4 is a unique member of the adenylate kinases (AK) family which shows no enzymatic activity in vitro although it shares high sequence homology with other AKs [22]. It remains unclear what physiological function AK4 might play or why it is enzymatically inactive. The crystal structure of human AK4 was available from Protein Data Bank (PDB: 2AR7 and 2BBW) and showed similar "open" and "closed" conformations as other AKs. In human AK4, the AMP-bind domain is connected to the core domain by hinge I (residues 34–35) and hinge II (residues 78–84) while the hinge III (residues 113–124) and hinge IV (residues 158–178) connect the CORE and LID domains. The motion of the AK LID domain upon substrate binding is a good example of a domain motion triggered by substrate. To investigate the role of hinge in the LID domain motion, mutation of AK4 was designed based on structural and sequence alignment information. L171, located in hinge IV, is strictly conserved in AK family and therefore chosen for further study. Interestingly, AK4 L171P mutant revealed a novel conformation in NMPK family, which suggested direct regulation on LID domain motion by such hinge region.

Materials and methods

Gene cloning, expression, and protein purification. Full-length human AK4 cDNA fragment was amplified from human brain cDNA library (Clontech). With MutanBEST Kit (Takara), a pair of primers 5′-TTGCTGCCAGGCCAAGACAGTACA-3′ and 5′-CTGCTTCGGGTTTAT CATCCTCCT-3′ was used to introduce a single mutation into human AK4 gene for L171P. The native and mutant AK4 genes were constructed into vector pET22b (Novagen) and expressed in *Escherichia coli* BL21 (DE3) with six histidines at the C terminus. Highly soluble expression was induced by 0.5 mM IPTG with overnight incubation at 16 °C in LB. Cells were harvested by centrifugation, resuspended in 20 mM Tris–HCl (pH 7.5), 500 mM NaCl, 5 mM imidazole, 1 mM PMSF and sonicated. After insoluble material was removed by centrifugation, the protein was purified by affinity chromatography on a Chelating Sepharose™ Fast Flow column (Amersham Biosciences), followed by gel filtration chromatography with Superdex™ 75 (Amersham Biosciences). AK4(L171P) were concentrated to 10 mg/ml for crystallization. For phase determination, the recombinant plasmids were transferred into Met-auxotrophic strain B834 to obtain the selenomethionyl derivative samples. Protein expression and purification procedures were similar as above except that the M9 medium was used.

Crystal structure determination of AK4 L171P. The initial crystallization conditions for AK4 L171P were screened using Crystal Screen kits I and II from Hampton Research. After optimizing the crystallization conditions, good crystals of L171P were obtained by hanging drop vapor diffusion method at 4 °C under conditions of 1.22–1.28 M $(NH_4)_2SO_4$ and 0.1 M Tris–HCl, pH 8.5. Selenomethionine substituted L171P crystals were obtained under the same condition.

Diffraction data were collected at 100 K at beamline 3W1A, Beijing Synchrotron Radiation Facility, Institute of High Energy Physics, Chinese Academy of Sciences. A MAD (multi-wavelength anomalous dispersion) data set was collected from a Se-Met L171P crystal for phasing. A native data set of higher resolution was also collected for structure refinement. All data was processed with DENZO and SCALEPACK [23].

AK4 L171P crystal belongs to the space group $P4_12_12$ but the Se-Met crystal changes the space group to $I422$ with similar cell parameters. Three selenium positions corresponding to Met53, Met73, and Met74 were found by SOLVE [24] using the anomalous dispersion data set, and the initial phases were calculated to 3.0 Å. RESOLVE [25] was used for density modification and the initial model building. Subsequently, the L171P structure was refined to 2.3 Å resolution using the native data set in CNS [26] and iterative manual adjustments were carried out with the program O. The quality of final model was evaluated by PROCHECK [27]. The statistics of diffraction data analysis and structure refinement are presented in Table 1.

Results and discussion

Structure of AK4 L171P

To investigate the roles of linkage residues in domain motion of AK4, the strictly conserved residue Leu171 in hinge IV was mutated to a proline, which would make the peptide backbone more rigid. Surprisingly, this mutation generated a new conformation of AK4. The final model of human AK4 L171P mutant has been refined to 2.3 Å resolution with *R*-factor and R_{free} values of 0.226 and 0.287, respectively (Table 1). Two protein molecules, A and B, were identified in the asymmetric unit and most residues could be well located in electron density maps except residues 1–4, 162–174 and the His-tag. In molecule B, residues 115–121 in hinge III and 175–177 in hinge IV are also disordered. The two AK4 L171P molecules in the asymmetric unit are highly similar, with RMSD of 0.67 Å when all of Cα atoms, except those of residues 115–121 and 175–177, were superimposed. Four sulfate ions from the crystallization solution are bound in each protein molecule. Three of them bind at the molecular surface and one binds at the P-loop.

Similar to other AKs, the structure of human AK4 L171P contains the CORE, NMP_{bind}, and LID domains (Figs. 1 and 2). One pair of hinges (hinge I: residues 34–35 and hinge II: residues 78–84) link the CORE and NMP_{bind} domains. Another pair of hinge regions (hinge III: residues 113–124 and hinge IV: residues 158–178) connect the CORE and LID domains. The fold of CORE domain is similar to that of native AK4. One sulfate ion was observed to bind to the P-loop. In bovine AK3 complexed with AMP [4], bovine AK2 [5], porcine AK1 [28], and human AK6 [19], a sulfate ion was found at the same position, which corresponds to the α-PO_4 of ATP bound in *E. coli* AK. The structure of AK4 L171P further confirms that the CORE domain is very structurally conserved in all the known AKs, as well as NMPKs.

The NMP_{bind} domain is part of the molecule where the nucleotide monophosphate binds. Similar to other AKs, AK4 NMP_{bind} domain is formed by three α-helices, α2, α3, and α4. The NMP_{bind} orientation is quite similar to that in native AK4. It is worth noticing that the NMP domain in native apo AK4 (PDB entry: 2AR7) shows the same orientation with NMP domain in AK4 bound with diguanosine pentaphosphate GP5 (PDB entry: 2BBW). Interestingly, the NMP_{bind} domain in bovine AK3 bound with an AMP does not show significant movement compared to the apo form either. The above evidence indicates that substrate binding to NMP_{bind} domain does not trigger the conformational changes of hinge I and II in AK3 and AK4. The orientation of NMP_{bind} domain in AK3 and AK4 adapts a "half-open" form, which is a transit state between the typical "open" and "closed" forms as observed in *E. coli* AK (PDB entries: 1AKE and 4AKE). While in other AKs, the NMP_{bind} domain moves close to the CORE domain when bind with NMP [6–8].

The major structural characteristic of AK4 L171P comes from the LID domain. In most of the known AK structures that are free of phosphor donor, the LID domain is obviously away from the CORE and the NMP_{bind} domains, which makes these AKs in an open conformation. In AK4 L171P, the LID domain itself keeps the same

Table 1
Data collection and refinement statistics.

MAD data collection	Edge	Peak	Remote	Native
Wavelength (Å)	0.9795	0.9793	0.9	0.95
Resolution range (Å)	30–2.9	30–2.8	30–2.8	30–2.3
	2.97–2.90	2.87–2.80	2.87–2.80	2.35–2.30
No. of total reflections	101,740	175,987	172,598	420,436
No. of unique reflections	5136 (325)	5729 (368)	5736 (370)	20,375 (1323)
I/σ	26.9 (3.8)	38.1 (4.4)	36.2 (4.5)	17.2 (4.0)
Completeness (%)	98.7 (100)	99.8 (100)	99.9 (100)	99.6 (99.4)
R_{merge}	0.06 (0.303)	0.06 (0.378)	0.057 (0.368)	0.111 (0.465)

Refinement	
Space group	$P4_12_12$
Unit cell dimensions (Å)	77.79 × 77.79 × 144.64
Resolution (Å)	50.0–2.3 (2.44–2.30)
R_{work} (%)[a]	0.226 (0.272)
R_{free} (%)[b]	0.287 (0.342)
No. of atoms	
Protein atoms	3290
Water molecules	282
Sulfate ions	32
RMSD from ideality	
Bond lengths (Å)	0.007
Bond angles (°)	1.3
Average B factor (Å^2)	
Main chain	22.6
Side chain	24.2
Water	26.5
Sulfate ion	49.4

[a] $R_{work} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$, where F_{obs} and F_{calc} are observed and calculated structure factors.
[b] $R_{free} = \Sigma T||F_{obs}| - |F_{calc}||/\Sigma T|F_{obs}|$, where T is a test data set of 10% of the total reflections randomly chosen and set aside prior to refinement.

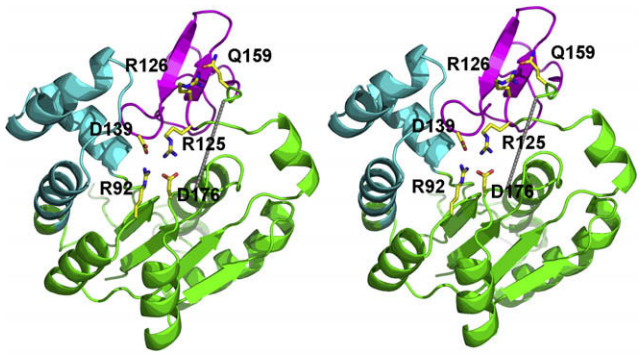

Fig. 1. Stereo view of human AK4 L171P mutant. The CORE, LID, and NMP_{bind} domains are colored with green, cyan and magenta, respectively. Residues 162–174, which are disordered in AK4 L171P mutant, are marked as a gray dotted line. The critical residues involved in stabilization of the conformation are labeled. This figure was generated using Pymol. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

folding as in other long form AKs. The RMSD is only 0.32 Å when the Cα atoms in L171P and native LID domains are superimposed. However, the LID domain of L171P directly contacts the CORE and NMP_{bind} domains, arranging the whole molecule in a compact shape. The structure of this mutant AK4 L171P is in a closed conformation without substrate triggering. More interestingly, this closed conformation is different from other closed forms of AKs, which will be discussed in details below.

Human AK4 L171P is in a twisted-and-closed conformation

As reported in previous crystallographic studies on the long form AKs, most LID domains, whether complexed with single substrate AMP or in the apo form, are in the open state [4]. AK2 is an exception, in which the unliganded Lid domain is somehow closed due to unfavorable crystal packing (PDB entry: 1AK2). Meanwhile, AKs bound with two substrates or their analogs are all in the closed conformation [6,8], where the LID and NMP_{bind} domains move towards each other to cover the active site. It has been suggested that this conformational change is triggered by ATP binding. Here, the human AK4 L171P structure reveals a special conformation never found in other AKs before.

Inter-domain movements are classified into two groups: closure motions and twist motions. Most of the inter-domain movements are observed as closure motions, in which the residues move perpendicularly to the domain interface and the rotation axis is perpendicular to the line connecting the domain centers. Other inter-domain movements could be described as twist motions in which the rotation axis is parallel to the line connecting the domain centers. The domain movements previously observed in AKs belong to the closure motions. The typical example is *E. coli* AK. Upon the substrates binding, the LID rotates by 46° in the mode of closure motion, with the rotation axis passes through both of hinge III and hinge IV. This rotation is also commonly observed in other AKs and NMPKs [29]. In the closed form of *E. coli* AK, the first one-third of the LID, which contains β5 and β6, forms the interface with the CORE domain and is involved in substrate binding. The native structures of AK4 are also similar to those of *E. coli* AK (Fig. 2A, left and middle). However, compared to the open conformation of native AK4, the movement of the LID in AK4 L171P is an 80% twist motion [30,31], with a rotation angle of 108° and the rotation axis only passes hinge III but not hinge IV (Fig. 2A, right). Consequently, β5 and β6 are located outside the molecule. The middle part of the LID (residues 138–146), which is a loop right after β5 and β6, forms the interface to the CORE domain and inserts deeply into the active site. Two pairs of hydrogen bonds, Asp139–Arg92 and Pro142 (carbonyl oxygen)–Arg41, link the LID

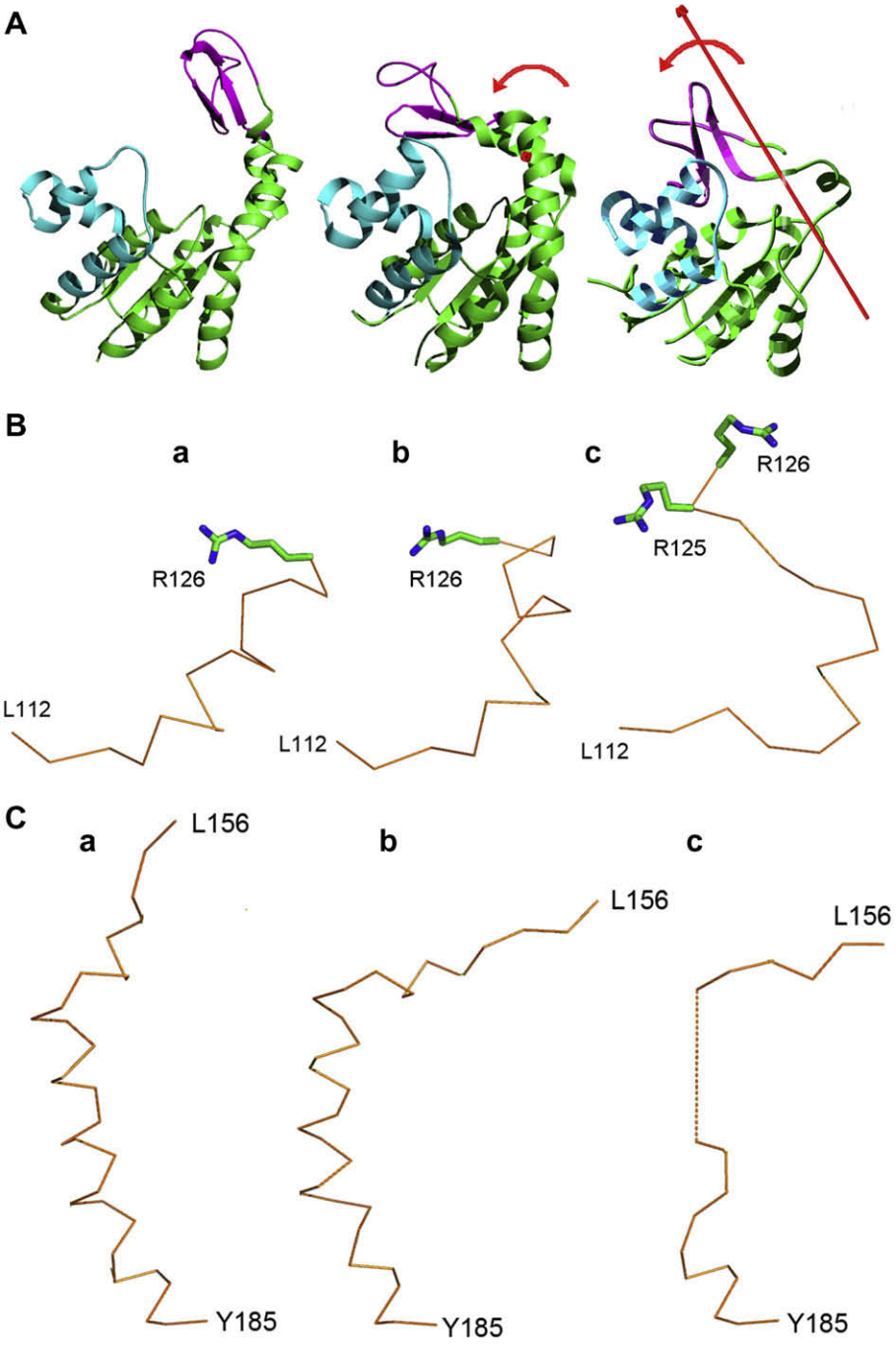

Fig. 2. Structural comparison of human AK4 L171P mutant with the native AK4 in open and closed form (left is native AK4 open form, middle is native AK4 bound with GP5 in close form and right is the AK4 L171P.). (A) Overall structural comparison. (B) Hinge III comparison. (C) Hinge IV comparison.

and CORE domains (Fig. 1). The hydrophobic interactions between Phe140 and the hydrophobic patch formed by Ile58, Leu40, and Ile44 strengthen the interactions between the LID and NMP_{bind} domains. Other interactions are bridged by water molecules forming hydrogen bonds among the three domains. Distinguished from the open and closed conformations found in other AKs, the AK4 L171P structure may be named as a twisted-and-closed conformation.

The novel orientation of the LID in AK4 L171P could result from the special conformations of hinge III and hinge IV. In other AKs, hinge III and hinge IV are two α-helices proximately parallel to each other. The bending of the two hinge α-helices in the same direction causes the movements of the LID (Fig. 2A and B). While in AK4 L171P, hinge III and hinge IV are twisted so that they are almost perpendicular to each other. The connecting region between hinge III and LID (residues 122–126) stretches as a β-strand and forms a sharp turn at Arg125, instead of forming the third turn of helix 6 (Fig. 2B). The conformational changes in hinge IV are more dramatic although part of it (Asp162–Tyr174) is disordered. Compared to the open form of AKs, the conformational changes of hinge IV mainly result from the following aspects: first, the peptide fragment from Asp162 to Val177 rotates more than 90° around the line connecting the Cα atoms of Asp161 and Ala178; second,

residues Gln159–Asp161 form a β-strand instead of a 3_{10} helix in other AKs (Fig. 2C).

This special large conformational change in structure of the AK4 L171P reveals that the completeness of hinge IV helix is important to keep the apo-AK4 in an open conformation. Furthermore, the two hinges connecting the LID and CORE domains are dependent to each other. The destruction of Hinge IV consequently influences the structure of Hinge III (Fig 2B). However, in contrast to the large motion of LID domain, the orientation of NMP domain was not affected, which may suggest that the domain motions of LID and NMP_{bind} are independent events in adenylate kinase 4.

We measured AK activities of human AK4 and AK4 (L171P) by a coupled pyruvate kinase (PK)/lactate dehydrogenase (LDH) assay [32] with several different phosphor donors and acceptors, however, neither of human native AK4 or AK4 (L171P) mutant shows any AK enzymatic activity in vitro. It remains unclear why AK4 is enzymatically inactive, and its physiological function is largely unknown; however, recent large-scale genomic studies suggested that it could respond to various stress conditions [33,34]. The level and regulation of the expression of AK4 indicate an important role, which may be of regulatory rather than enzymatic nature. The structure of AK4 L171P provides an example of domain motion directly regulated by hinge region, which exhibit a novel conformation different from native AK4. The domain motion and large conformational changes suggest the structural flexibility of AK4, which may facilitate AK4 to function in vivo as a regulatory protein.

Conclusion

To investigate the role of hinge region in domain motion of AK4, the conserved residue leu171 in hinge IV, which is important for the helix formation, was mutated to a proline. The crystal structure of AK4 (L171P) reveals that the helix in the hinge was destroyed as expected. However, the large conformational change of AK4 (L171P) was very surprising. The LID domain was closed to the CORE domain with a manner never reported in AK family before. The structure analysis shows that the movement of the LID in AK4 L171P is an 80% twist motion with a rotation angle of 108°. Distinguished from the open and closed conformations found in other AKs, the AK4 L171P structure may be named as a twisted-and-closed conformation. The structure of AK4 L171P provides an example of domain motion directly regulated by hinge region without the effect from substrate binding.

Note added in proof

We recently published that the enzymatically inactive AK4 can protect cells from oxidative stress and the protective function is likely to be mediated by the interaction between AK4 and mitochondrial ADR/ATP translocase [35].

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