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Mutation of residue arginine 330 of arginine kinase results in the generation of the oxidized form more susceptible

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ABSTRACT

Arginine kinase (AK), a crucial enzyme for the energy metabolism of invertebrates, catalyzes the reversible phosphorylation of arginine by Mg^{2+}ATP to form phosphoarginine and Mg^{2+}ADP. Arginine 330 (R330), not involved in the catalysis of phosphoryl transfer, is a residue highly conserved in the phosphagen kinase family. In order to investigate the role of R330 in AK, it was replaced by lysine (R330K). Non-reduced SDS-PAGE analysis suggested that wild type AK (Wt-AK) and R330K existed in two forms, the reduced form (R-AK or R-R330K) and the oxidized form (O-AK or O-R330K), whereas O-R330K was more susceptible to generate than O-AK. Subsequently, an intramolecular disulfide bond in O-R330K was demonstrated to be formed between Cys201 and Cys271 by site-directed mutagenesis. Biochemical analysis revealed that conformational changes of R330K were concomitant with the sharp decline of catalytic activity. These results were further confirmed by structure modeling of AK and R330K. Therefore, it can be concluded that R330 residue plays an important role in the structural stability and activity of AK.

1. Introduction

Arginine kinase (AK) (ATP: L-arginine phosphotransferase EC 2.7.3.3) is a member of the phosphagen kinase family and a homolog of creatine kinase (CK). It participates in cellular energy metabolism and catalyzes the reversible phosphorylation of arginine by MgATP to form phosphoarginine and MgADP in invertebrates [1,2].

Typically, AKs are found as monomers in solution, unlike CK that adopts either dimeric or octameric physiological forms [3,4]. Although the crystal structures of several invertebrate arginine kinases are currently available, *Limulus* AK, as a monomeric AK model, has been structurally characterized in the first place and extensive studied. It consists of two domains: a small α-helical N-terminal domain (N domain) of residues 1–111 and a large C-terminal domain (C domain) possessing an 8-stranded antiparallel β-sheet which is flanked by 7 α-helices of residues 112–357 [5]. One substrate, ATP or ADP, is accommodated in the C-domain, and another substrate, arginine or arginine phosphate, contacts mainly with the N-domain. Catalytic center, where reversible transfer of the phosphate occurs, is located in the C-domain [6].

Domain–domain interactions might be important to the structure and functions of AK. Asp62 and Arg193 residues are located in the N- and C-domains, respectively. The hydrogen bond and an ion pair formed between Asp62 and Arg193 are thought to link the N- and C-domains, and the interactions between these two residues play a key role in stabilizing the closed substrate-bound structure of AK [4–9]. The hydrogen bond between the Pro272 (conserved) main chain nitrogen and Tyr72 (conserved) hydroxyl oxygen might be one of the constraints linking the segment containing Cys271 of C-domain to the small N-domain [2,10]. Recently, mutations of Ile121 residue, which located in the linker between the N- and C-domains of AK, have been demonstrated to affect the correct positioning of the two domains, and thus disrupt the efficient recognition and interactions between the N- and C-domains [8].

The roles of other amino acid residues have been investigated on AK in terms of structural stability and catalytic functions [5,8–12]. Multiple sequence alignments indicated that nine positively charged Arg residues (R124, R126, R129, R208, R229, R245, R280, R309, R330) are highly conserved in AKs and CKs [13–15]. Accumulating evidence suggests that the five conserved arginines (R124, R126, R229, R280, and R309) are associated with

Abbreviations: AK, arginine kinase; CK, creatine kinase; Wt, wild type; R, reduced form; O, oxidized form; CD, circular dichroism; ANS, 1-anilinonaphtalene-8-sulfonate; SEC, size exclusion chromatography; MMCK, muscle type creatine kinase; BBCK, brain type creatine kinase.

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the catalysis of phosphoryl transfer [5,9,16]. One exception is R330, which is strictly conserved within phosphagen kinase family founded at present [9,13]. It is not clear whether R330 only plays a general role in the structural maintenance of AK.

Changes in the redox state of cysteine residues in proteins play important roles in many physiological and pathological processes [17]. The redox-sensing cysteine residues and the disulfide bonds formed between these cysteine residues serve as redox-sensing molecular switches [18]. Studies on CK have shown that it exists in two forms, the reduced form (R-CK) and the oxidized form (O-CK), and O-CK contains an intrachain disulfide bond in each subunit [19–21]. The generation of O-CK was a negative regulation of R-CK that O-CK can be rapidly ubiquitinated and degraded by ubiquitin-dependent pathway [20]. AK contains five free cysteine thiols in the molecule, and among which Cys271 is essential for the catalytic activity [10,12]. Although oxidized dithiothreitol (DTT) could lead to the conformational change and the activity loss of AK [22], it is unclear yet whether AK has the reversibly oxidized form and O-AK has intramolecular disulfide bonds or not.

In the present study, we used a mutant R330K, in which Arg330 was replaced by Lys to assess the role of Arg330 in the regulation of AK activity and structural stability. We have first confirmed that wild type AK (Wt-AK) and R330K existed in two forms, the reduced form (R-AK or R-R330K) and the oxidized form (O-AK or O-R330K), and found that the R330K mutant was more susceptible to oxidation than Wt-AK. Biochemical analysis revealed that decreased secondary structures, changed microenvironment of Trp residues and increased hydrophobic surface exposure of R-R330K were concomitant with the sharp decline of catalytic activity, and the results were further confirmed by structure modeling of AK and R330K. Our findings provided the insights into the structural and functional roles of R330 residue of AK.

2. Materials and methods

2.1. Site-directed mutagenesis

The KOD-Plus-Mutagenesis Kit (TOYOBO) was used. Single amino acid mutation was made by using the template pET28a-AK plasmid, which carries the greasyback shrimp AK gene inserted in pET28a vector [23]. The sequences of the entire coding region of the mutants were confirmed by DNA sequencing.

Six mutations (R330K, R330KC23S, R330KC127S, R330KC139S, R330KC201S, R330KC271S) were made by reverse-PCR-based site-directed mutagenesis [11]. The PCR primers were as follows:

R330K (FR330K, 5′-CAACAAGCGCAAGATGGGTCTGACTGA-3′; RR330K, 5′-GAGATATCGTAGATGCCGCCCTCAGCC-3′), **R330KC23S** (FR330KC23S, 5′-CCAAGTCTCTCCTTAAGAAGTATCTTAC-3′; RR330-KC23S, 5′-AGTCGGTGGCGGCTTCAAGTTTCTTGAAA-3′), **R330KC-127S** (FR330KC127S, 5′-CCGGTCGCTCAATGCAAGGTTACCCCTT-3′; RR330KC127S, 5′-AGCGGACTCGGGTGGAGATAACGAACTT-3′), **R330KC139S** (FR330KC139S, 5′-CTCACTGAGTCTCAGTACAAAGAG-ATGGA-3′; RR330KC139S, 5′-GGAGGGGTTGAAGGGGTAACCCTGC-3′), **R330KC201S** (FR330KC201S, 5′-CCCGCTACTGGCCCGCCG-GACGTGGAAT-3′; RR330KC201S, 5′-AAGCGTTGGCTGCTTGCAGG-AAGCGGT-3′), **R330KC271S** (FR330KC271S, 5′-AGTCCCACCAA-CCTTGGCACCACTGTGC-3′; RR330KC271S, 5′-GAAAGTGAGGAAG-CCCAGGCGGTCGTG-3′).

2.2. Protein expression and purification

The enzymes were overexpressed in the *Escherichia coli* strain Rossetta. Wt-AK and mutants were expressed and purified using the same procedures as described previously [10,11,23]. R-AK or R-R330K was prepared with the buffer containing 2 mM DTT. We failed to prepare fully oxidized form of R330K or AK. R-AK or R-R330K was used immediately after purification, and it was confirmed to show only one band on non-reduced SDS-PAGE.

2.3. Enzyme concentration and activity assay

The activities of AK and mutant in the forward reaction (arginine phosphate synthesis) were assayed using a direct continuous pH-spectrophotometric assay [24]. The enzyme concentration was estimated from the absorbance at 280 nm (absorbance 0.67 at 280 nm in 1 cm cuvette corresponds to 1 mg protein/ml) [25], and confirmed by Coomassie Brilliant Blue method. The enzymatic activity and concentration of the proteins were measured with an Ultrospec 4300 pro UV/visible spectrophotometer.

2.4. Physico-chemical analysis

Far-UV circular dichroism (CD) spectra were recorded on a Jasco J-810 spectrophotometer, using a cell with a path length of 0.1 cm over a wavelength range of 200–250 nm. Fluorescence spectra were recorded on an F-4500 fluorescence spectrophotometer (Hitachi) using a 1-ml cuvette with excitation and emission slit widths of 5 nm, scan speed 1200 nm/min, PMT voltage 400 V. Intrinsic fluorescence emission spectra were measured using an excitation at 295 nm and emission wavelength in the range of 300–400 nm. ANS was used as an extrinsic fluorescence probe [26], and a 20-fold molar excess of ANS was incubated with the samples for 30 min in the dark. ANS fluorescence intensity was measured using an excitation at 380 nm and emission in the range of 400–600 nm. All resultant spectra were collected at 25 °C. The final concentrations of the proteins were 18.3 μM.

2.5. Size-exclusion chromatography (SEC)

To determine the molecular size and profile, SEC experiments were performed with a Superdex 200 10/300 GL column (GE Healthcare) on a Pharmacia AKTA purifier apparatus at 25 °C. All prepared solutions were passed through a filter and degassed. A 500 μl of each sample in binding buffer (0.02 M Tris and 0.5 M NaCl, pH 8.0) with 2 mM DTT was analyzed. The flow rate was set at 0.5 ml min^{-1}, and the results were monitored at 280 nm.

2.6. Modeling the structure of R330K

In order to analyze the effect of the mutation on the AK structure, both the SWISS MODEL WORKSPACE (http://swissmodel.expasy.org) [27–29] and SPDBV software (http://swissmodel.expasy.org/) were used to model the structure of AK and R330K based on PDB files (PDB ID: 1BG0) for *Limulus* AK from the Protein Data Bank.

3. Results

3.1. R330K was more easily oxidized than Wt-AK

CK is known to exist in two forms, R-CK and O-CK [19,20]. To investigate whether a similar oxidized form could form in AK, protein samples were prepared using buffer without 2 mM DTT in the purification process. Non-reduced and reduced SDS-PAGE of Wt-AK and R330K were shown (Fig. 1). The results clearly indicated that the oxidized form of AK (O-AK or O-R330K) generated, and O-R330K was more obvious than O-AK, which meant that R330K was more easily oxidized than Wt-AK (Fig. 1A). Therefore, R330K was selected for subsequent oxidation studies.

Since DTT is a strong reducing agent, and it can stabilize free sulfhydryls (cysteines) and reduce disulfide bonds in peptides and