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Impact of inter-subunit interactions on the dimeric arginine kinase activity and structural stability

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ABSTRACT

Arginine kinase (AK) is a key enzyme for cellular energy metabolism, catalyzing the reversible phosphoryl transfer from phosphoarginine to ADP in invertebrates. In this study, the inter-subunit hydrogen bonds between the Q53 and D200 and between D57 and D200 were disrupted to explore their roles in the activity and structural stability of *Stichopus japonicus* (*S. japonicus*) AK. Mutating Q53 and/or D57 to alanine (A) can cause pronounced loss of activity and substrate synergism, and cause distinct conformational changes. Spectroscopic experiments indicated that mutations destroying the inter-subunit hydrogen bonds impaired the structure of dimer AK, and resulted in a partially unfolded state. The inability to fold to the functional compact state made the mutants prone to be inactivated and aggregate under environmental stresses. Restoring hydrogen bonds in Q53E and D57E mutants could rescue the loss of activity and substrate synergism, and conformational changes. All those results suggested that the inter-subunit interactions played a key role in keeping the activity, substrate synergism and structural stability of dimer AK. The result herein may provide a clue in understanding the folding and self-assembly processes of oligomeric proteins.

Introduction

Arginine kinase (ATP: L-arginine phosphotransferase EC 2.7.3.3) (AK)[1] catalyzes the reversible phosphorylation of arginine by ATP, yielding the phosphoarginine [1]. As a member of the phosphogen kinase (PK) family and analog of creatine kinase (CK) in vertebrates, AK is widely distributed in invertebrates, and plays a key role in cells, by buffering the ATP concentration according to cellular energy requirements [2–4]. Due to its presumed prominent role in energy metabolism and absence in vertebrates, AK could be chosen as a target to screen effective and harmless pesticide in agriculture [5,6].

In contrast to other PKs which are mostly dimeric or octameric, AKs are typically functional as monomers [6–8]. Unlike the monomeric 40 kDa AKs from molluscs and arthropods [5,6], *Stichopus japonicus* AK is dimeric [7–10], the same as cytoplasmic isoenzymes of the vertebrate CKs. The *S. japonicus* AK has raised interest recently because of its special position in evolution. Sequence analysis indicated that the dimer AK was evolutionarily closer to CK, while the conserved amino acids in its active sites are more like those of AKs though still significantly different from other AKs. Thus, it has been proposed that *S. japonicus* AK evolves at least twice during the evolution of PK: first at an early stage of PK evolution (its descendants are molluscan and arthropod AK), and second, from CK at a later time in metazoan evolution [10,11]. Therefore, studying the amino acid residues critical to catalytic activity of dimer AK may provide clues in understanding the difference of monomer AK and CK.

Just like other PK, the catalytic mechanism of dimer AK belongs to the random-sequential bi mechanism as indicated in Scheme 1 [12,13]. If the binding of the first substrate facilitates the binding of the second substrate, synergism occurs. The parameter K_d/K_m is often used to denote synergism, where K_d and K_m are the dissociation constant in the absence and in the presence of the second substrate, respectively. A higher ratio of K_d/K_m ($K_d/K_m > 1$) indicates stronger synergism [12]. The synergism in substrate binding was suggested to be associated with substrate-induced conformational

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[1] *Abbreviations used:* AK, arginine kinase; CK, creatine kinase; PK, phosphogen kinase; IPTG, isopropyl-D-thiogalactopyranoside; ANS, 1-anilinonaphtalene-8-sulfonate; SEC, size exclusion chromatography; CD, circular dichroism; GdnHCl, guanidine hydrochloride; MG, molten globule intermediate; *E. coli*, *Escherichia coli*; SDS, sodium dodecyl sulfate; WT, wild type; $[\theta]_{MRW}$, mean residue ellipticity; E_{max}, emission maximum wavelength of the intrinsic fluorescence.

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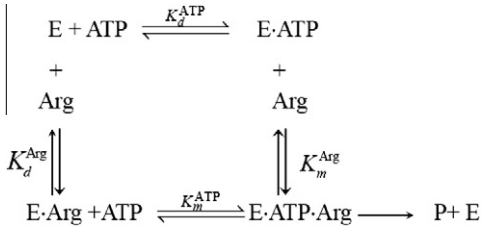

Scheme 1.

changes within the tertiary complex [6,14]. However, little is known about the effect of amino acid residues outside of active sites on the synergism.

The amino acid sequence of a protein determines the three-dimensional conformation of the functional protein [15]. *S. japonicus* AK, as a special dimer protein, is a valuable model for studying the subunit dissociation and inter-subunit interactions of oligomeric proteins. Understanding the folding and self-assembly processes of oligomeric proteins remains a challenge. For oligomeric proteins, folding is generally thought to be hierarchical, the individual domains with different stabilities fold autonomously and independently [16]. However, the folding mechanism of oligomeric proteins may be much more complicated than monomer proteins because it involves both the folding of the individual subunit and the docking of these subunits [16]. To achieve the native tertiary structures, adjacent domains need to recognize each other through the domain-domain and the inter-subunit interface(s) [17–20]. Previous studies presumed that the inter-subunit interactions of *S. japonicus* AK may be crucial in keeping its activity and structural stability [21–23]. Furthermore, the hydrogen bonds between D-200 and Q-53 and between D-200 and D-57 were deduced to play a key role in the dimerization of dimer AK [23]. However, little is known about the roles of inter-subunit hydrogen bonds in dimer AK activity, folding and structural stability. In this research, our study suggested that the mutations Q53A, D57A and Q53A/D57A can cause pronounced loss of AK activity and substrate synergism, and distinct conformational changes. Moreover, those mutations led to the conformational changes by destroying the inter-subunit hydrogen bonds and hindered the dimerization of AK. The partially unfolded state of mutant AKs made them susceptible to environmental stresses and prone to be inactivated and unfolded, form insoluble aggregates. All those results suggested that the inter-subunit interactions played a key role in the dimer AK activity, substrate synergism and structural stability.

Materials and methods

Cloning, site-directed mutagenesis and expression of the mutant S. japonicus AKs

Total RNAs were isolated from the muscle of *S. japonicus* by using the TRIzol reagent (Invitrogen). First strand cDNAs were prepared by reverse transcription of total RNAs with random primers. For amplification of AK cDNA, the primers 5′-GCTGGATCCCCGGTGTTAATCATGGCAAA-3′ and 5′-GCACTCGAGGTCCCCAAGTAAACGGCT-3′ were used, in which the restriction sites introduced were underlined. Then the PCR product was purified and inserted into the pET-28a vector.

Five mutations (Q53A, D57A, Q53A/D57D, Q53E and D57E) were introduced into the template of the pET-28a-WT AK by overlap PCR using mutation primers. The sequences of the mutation primers were as follows: for Q53A, 5′-CTGGACAGAGCCATAGCTAACGGTGTCGAT-3′ and 5′-ATCGACACCGTTAGCTATGGCTCTGTCCAG-3′; for D53E, 5′-CTG GACAGAGCCATAGAAAACGGTGTCGAT-3′ and 5′-ATCGAC ACCGTTTTCTATGGCT CTGTCCAG-3′; for D57A, 5′-CAGAACG GTGTCGCTAATCCCGATTTC-3′ and 5′-GAA ATCGGGATTAGCGACACC GTTCTG-3′; for D57E, 5′-CAGAACGGTGTCGAAAATCCCGATTTC-3′ and 5′-GAAATCGGGATTTTCGACACCGTTCTG-3′ mutated sequences are underlined. As for the double mutant, the Q53A mutant was used as template and the primers for D57A were used to introduce the second mutation. Then the cDNA of the mutants was cloned into expression vector pET-28a, sequenced and transformed into the *Escherichia coli* (*E. coli*) BL21 (DE3) codon plus.

The WT and the mutant AKs fusion protein was expressed in *E. coli* BL21 and purified as described previously [24]. The purity was checked by SDS–PAGE. Protein concentration was determined using Bradford's method [25]. The size exclusion chromatography (SEC) analysis was carried out using a FPLC system (General Electric Company) with a Superdex 200HR column at 25 °C. Each time, a total of 120 μl (0.4 mg/ml) sample was injected into the column pre-incubated with the standard buffer (10 mM glycine-NaOH, 1 mM DTT at pH 8.1).

Enzyme assay and determination of kinetic parameters

AK activity (phosphoarginine synthesis) was assayed as previously described with some modification [26,27]. The assay mixture for AK determination consisted of 100 mM Tris, pH 8.0, 10 mM L-arginine, 8 mM ATP-Na, 10 mM mercapto-ethanol and 10 μl of 0.01 mM enzyme solution. The absorbance at 660 nm was measured at 30 °C using an Ultrospec4300 pro UV–vis Spectrophotometer.

The two-substrate graphical method was used to obtain the kinetic parameters [6]. The activity assays were carried out at the optimum pH (pH 8.1) and temperature (30 °C) with different concentrations of ATP and arginine. All the experiments were repeated at least four times.

Thermal stability of AK

The thermal stability of WT and mutant AKs was determined by activity assay after being incubated at different temperatures. The enzyme solutions were incubated at given temperatures varying from 25 to 65 °C for 10 min, then cooled on ice and the activity was measured at 30 °C. The data were normalized to the activity measured at 25 °C. The aggregation of AKs at a given temperature was monitored by measuring the turbidity at 400 nm. The final protein concentrations of WT and mutant AKs were all adjusted to 2.3 μM.

Unfolding and refolding experiments

For the unfolding experiment, the WT and mutant AKs were added to the standard buffer (pH 8.1) with different concentrations of GdnHCl dissolved for 24 h at equilibrium state. The refolding experiment was initiated by diluting the denatured AKs into the standard buffer (pH 8.1) with final GdnHCl concentrations ranging from 0.1 to 3 M. The intrinsic Trp fluorescence spectra of unfolding and refolding AKs were collected on an F-4500 spectrofluorometer using a 1-cm path-length cuvette. For ANS-fluorescence measurements, 10-fold molar excess of ANS was added to the samples. The samples were equilibrated for 30 min in the dark, and then the extrinsic fluorescence was measured on an F-4500 spectrofluorometer using a 1-cm path-length cuvette. Far-UV circular dichroism (CD) spectra were recorded on a Jasco 715 spectrophotometer with a 1 mm path-length cell. The final concentration of the enzyme for spectroscopic experiments was 2.3 μM and all experiments were carried out at 25 °C.