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The interaction between residues 62 and 193 play a key role in activity and structural stability of arginine kinase

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ABSTRACT

The purpose of this study is to clarify that the amino acid residues (Asp62 and Arg193) are responsible for the activity and stability of arginine kinase (AK). The amino acid residues Asp62 (D62) and Arg193 (R193) are strictly conserved in monomeric AKs and form an ion pair in the transition state analogue complex. In this research, we replaced D62 with glutamate (E) or glycine (G) and R193 with lysine (K) or glycine (G). The mutants of D62E and R193K retained almost 90% of the wild-type activity, whereas D62G and R193G had a pronounced loss in activity. A detailed comparison was made between the physic-chemical properties and conformational changes of wild-type AK and the mutants by means of ultraviolet (UV) difference and fluorescence spectra. The results indicated that the conformation of all of the mutants had been changed and the stability in a urea solution was also reduced. We speculated that the hydrogen bond and electrostatic interactions formed between residues 62 and 193 play a key role in stabilizing the structure and mediating the synergism in substrate binding of arginine kinase from greasyback shrimp (*Metapenaeus ensis*).

1. Introduction

Arginine kinase (AK, EC 2.7.3.3) participates in cellular energy metabolism in invertebrates, which is analogous to the creatine kinase (CK) reaction. As a member of the phosphagen kinase family, it buffers cellular ATP levels by catalyzing the reversible phosphoryl transfer between ATP and arginine, leading to the production of phosphoarginine and Mg^{2+}ADP [1,2]. The latter is considered to be an energy reservoir which is able to supply ATP, the primary energy source in bioenergetics, on demand [3].

Protein folding is the process by which the amino acid sequence of a protein determines the three-dimensional conformation of the functional protein [4]. The proper folding of a protein is important to maintain its function. Various amino acid residues have been widely investigated for their role in protein folding, structural stability and function in horseshoe crab (*Limulus*) AK. The crystal structure for the transition-state analogue complex (TSAC) [5,6] and substrate-free structure [7] of horseshoe crab AK have been determined. Many amino acids located in the active site were proven to play an important role in the activity and conformation stability of AK or CK [8,9], but little is known about the role of amino acid residues outside of the active site. The conserved residue P272 of AK is involved in substrate binding and protein folding which might be because residue P272 is adjacent to the "essential" C271 [10].

The substrate recognition region (GS region) of AK is highly conserved, which is composed of Ser63, Gly64, Val65 and Tyr68 [11]. Supporting evidence came from enzyme kinetic and thermal stability experiments on wild-type and mutated AK which showed that the activity of the enzyme is significantly reduced and the substrate synergism distinctly altered after the conserved sites are mutated [12,13]. Asp62 does not bind to the substrate [5] but may be involved in a substrate-recognition system [14]. The amino acids D62 and R193 are strictly conserved in monomeric AKs and form an ion pair in TSAC of *Limulus* AK [5]. Usually, replacement of D62 or R193 causes a pronounced loss of enzyme activity, which is presumed to indicate that the hydrogen bond formed between them linking the N-terminal and C-terminal domains plays a key role in stabilizing the closed substrate-bound structure [11,12]. However, in *Crassostrea* AK, D62 and R193 are notably replaced by an N and a K, and 50% or more of the wild-type enzyme activity remained after site-specific mutagenesis of these residues, which indicates that the amino acid residues N62 and K193 play a key role in mediating the synergism in substrate binding [15]. In this paper, we show that

Abbreviations: WT-AK, wild-type arginine kinase; CK, creatine kinase; SEC, size exclusion chromatography; TSAC, transition state analogue complex; IPTG, isopropyl-β-D-thiogalactoside; ANS, 1-anilinonaphtalene-8-sulfonate.

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the hydrogen bond and ion pair formed between the amino acid residues 62 and 193 plays a key role in stabilizing the structure and mediating the synergism in substrate binding of arginine kinase from greasyback shrimp (*Metapenaeus ensis*).

2. Materials and methods

2.1. Cloning, and site-directed mutagenesis of the greasyback shrimp AK

ATP, ADP, arginine, ANS, 2-mercaptoethanol and IPTG were Sigma products. All other reagents were local products of analytical grade. The pET-28a plasmid with the insert of greasyback shrimp AK gene (WT-AK) [16] was used as a template for mutagenesis.

The replacement of amino acid Asp62 with glutamate (E) or glycine (G) and Arg193 with lysine (K) or glycine (G) was achieved by PCR using mutation-primers, which were denoted D62E, D62G, R193K and R193G, respectively. KOD-Plus DNA polymerase (TOYOBO, Tokyo, Japan) was used as the amplifying enzyme. The PCR products were digested with DpnI and self-ligated with T4-ligase. The primers used for PCR amplification (mutated codons underlined) were as follows: for D62E, 5′-GGCTCTG-GTGTTGGTATCTATGCTCCCGA-3′ and 5′-CAGGTTCTC-CACACCGGACTGGA-TGACGT-3′; for D62G, 5′-GAGTCTGGTGT-TGGTATCTATGCTCCCGA-3′ and5′-CAGGTTCTCCACACCGGACTGG-ATGACGT-3′, for R193K, 5′-AAGTTCCT-GCAGGCCGCCAACGCTT-3′ and 5′-GTCACCCTCCTTGAAGAGGAAGTGGTC-G-3′; for R193G, 5′-GGATTCCTGCAGGCCGCCAACGCTT-3′ and 5′-GTCACC-CTCCTTGAAGAGGAAGTGGTCG-3′. For all of the mutations, the fidelity of the PCR amplification was confirmed by sequencing to ensure that only the intended mutations were introduced.

2.2. Expression and purification

To facilitate the expression of the mutagenic DNA, the recombinant plasmids were transformed into the *Escherichia coli* strain Rosetta. Cultures for mutant AKs were grown at 37 °C by inoculating 6 mL overnight culture into 300 mL of LB medium containing 50 mg L^{-1} kanamycin. Incubation was continued until the culture reached an A_{600} between 0.6 and 0.8, and the mutant proteins were induced at 22 °C for approximately 16 h by the addition of 0.8 mM isopropyl-β-D-thiogalactoside (IPTG). The WT-AK was expressed as described previously [16]. All of the enzymes were purified in three steps as follows: (a) The cells were collected by centrifugation (5000 × *g*, 10 min) and then resuspended in lysis buffer (50 mM Tris/HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0). (b) The cells were lysed by ultrasonication and the debris was removed by centrifugation (12,000 × *g*, 20 min, 4 °C). (c) The supernatant was collected and purified by metal affinity chromatography using a Ni^{2+} matrix. The Ni^{2+} column was equilibrated first with binding buffer (50 mM Tris/HCl, 500 mM NaCl, pH 8.0) before the proteins were applied to it, and then it was washed gradually with the same buffer containing a gradient of imidazole. Afterwards, the recombinant proteins were dialyzed against binding buffer for later use. The purified enzymes were proven to be homogeneous by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Enzyme concentration and activity

The activity of the wild-type and mutant AK in the forward reaction (arginine phosphate synthesis) was assayed using direct continuous pH-spectrophotometric assay [17]. The enzyme concentration was estimated from the absorbance at 280 nm (absorbance 0.67 at 280 nm in a 1 cm cuvette corresponds to 1 mg protein/mL) [18]. The enzymatic activity and concentration of the proteins were measured with an Ultrospec 4300 pro UV/visible spectrophotometer.

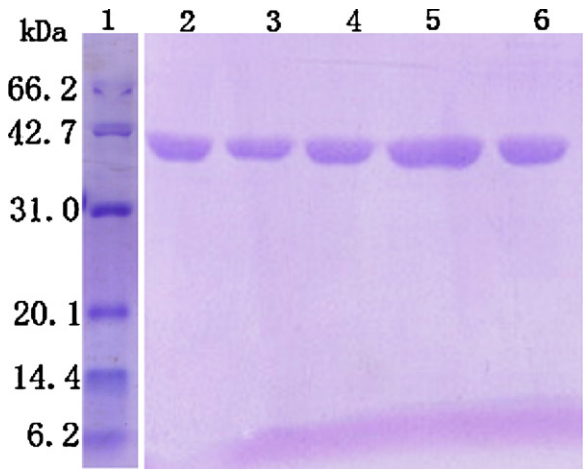

Fig. 1. Analysis of the purified WT-AK and mutated AK on 12% SDS-PAGE: 1, the molecular mass standard; 2, D62E; 3, D62G; 4, R193K; 5, R193G; 6, AK.

2.4. Spectroscopic measurements

Intrinsic fluorescence emission spectra were recorded on an F-4500 fluorescence spectrophotometer (Hitachi) with 1 cm path-length cuvettes, excitation at 295 nm and emission wavelength in the range of 300–400 nm. ANS was used as an extrinsic probe [19], and a 20-fold molar excess of ANS was added to the samples for 30 min in the dark. An excitation wavelength of 380 nm was used to determine the ANS fluorescence intensity of mutant enzymes in the range of 400–600 nm.

2.5. Stability experiments

In the unfolding studies, the mutants were denatured by dilution into the standard buffer (50 mM Tris/HCl, 500 mM NaCl, 1 mM 2-mercaptoethanol at pH 8.0) containing urea solutions of various concentrations ranging from 1 M to 5 M for 1 h. The intrinsic and ANS fluorescence spectra of the unfolding mutants were collected on an F-4500 fluorescence spectrophotometer (Hitachi) with 1 cm path-length curettes as described earlier.

3. Results

3.1. Purification and activity measurement of the site-directed mutants

All of the recombinant enzymes with 6×His-tag were obtained from the *E. coli* strain Rosetta transformed with plasmids containing the genes for the wild-type and mutated AK, successfully purified by affinity chromatography, and confirmed to be highly purified by SDS-PAGE (Fig. 1). In addition, the enzymatic activity was measured in the same condition as described above and the results are shown in Fig. 2. The mutants, D62E and R193K, retained almost 90% of the wild-type activity, whereas D62G and R193G had a pronounced loss in activity, which reflected that the ion pair formed between residues 62 and 193 were mediating the synergism in substrate binding of arginine kinase.

3.2. Effects of mutations on AK structure

Concerning the tertiary structure, the emission maximum of the intrinsic fluorescence (Fig. 3A and B). was found to have a slight red