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Cooperativity in the two-domain arginine kinase from the sea anemone *Anthopleura japonicus*. II. Evidence from site-directed mutagenesis studies

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ABSTRACT

The arginine kinase (AK) from the sea anemone *Anthopleura japonicus* has an unusual two-domain structure (contiguous dimer; denoted by D1–D2). In a previous report, we suggested cooperativity in the contiguous dimer, which may be a result of domain–domain interactions, using MBP-fused enzymes. To further understand this observation, we inserted six-Lys residues into the linker region of the two-domain AK (D1–K6–D2 mutant) using His-tagged enzyme. The dissociation constants, K_a and K_{ia}, of the mutant were similar to those of the wild-type enzyme but the catalytic constant, k_{cat}, was decreased to 28% that of the wild-type, indicating that some of the domain–domain interactions are lost due to the six-Lys insertion. Y68 plays a major role in arginine binding in the catalytic pocket in *Limulus* AK, and introduction of mutation at the Y68 position virtually abolishes catalytic activity. Thus, the constructed D1(Y68G)–D2 and D1–D2(Y68G) mutants mimic the D1(inactive)–D2(active) and D1(active)–D2(inactive) enzymes, respectively. The k_{cat} values of both Y68 mutants were decreased to 13–18% that of the wild-type enzyme, which is much less than the 50% level of the two-domain enzyme. Thus, it is clear that substrate-binding to both domains is necessary for full expression of activity. In other words, substrate-binding appears to act as the trigger of the functional cooperativity in two-domain AK.

1. Introduction

Arginine kinases (AKs) are enzymes that catalyze the reversible transfer of the gamma phosphoryl group of ATP to arginine, yielding ADP and a phosphorylated arginine. AKs typically function as monomeric enzymes, each with a molecular mass of 40 kDa (single-domain AK). Members of the phosphagen (guanidino) kinase enzyme family, such as AKs and creatine kinases (CKs), play key roles in the ATP buffering systems in animal cells that have high and variable rates of ATP turnover [1–10]. AK is the most widely distributed among invertebrates and genes encoding AK have been identified in protozoa and bacteria [11–13], suggesting its ancient origin [14,15].

In 1997, we isolated and sequenced the gene from the sea anemone *Anthopleura japonicus* that encoded a unique, contiguous two-domain AK for the first time [16]. The gene had a "bridge intron" between the two domains, suggesting gene duplication and subsequent fusion. The same type of AK was later isolated in clams of the genera *Pseudocardium* [17], *Solen*, *Corbicula* [18,19], *Ensis* [20] and *Calyptogena* [21]. A recent DNA database search showed that the two-domain AK is also present in the protozoan *Tetrahymena* [14]. Given the presence of a two-domain phosphagen kinase which clearly originated from the AK gene in *Schistosoma mansoni* [22,23], two-domain enzymes seem to have arisen independently at least four times during evolution, namely in the Phyla Protozoa, Cnidaria, Platyhelminthes and Mollusc [14,24].

Using two-domain clam AKs, several studies have been undertaken to elucidate the functional properties of the two-domain enzyme compared to the normal, single-domain enzyme [19–21]. However, problems of solubility of the recombinant enzymes prevent elucidating the exact nature of the two-domain enzyme and its separated domains. In a previous paper, we reported success in expressing and determining the kinetic constants of the *Anthopleura* two-domain AK and domains 1 and 2 separately as soluble enzymes fused with maltose-binding protein (MBP, 40 kDa) [25]. Based on these parameters, we assumed that the domain–domain interactions (cooperativity) are present in the two-domain AK. However, the large MBP tag appeared to prevent more precise analyses of the cooperativity.

Besides phosphagen kinases, poly-domain enzyme (or protein) is often observed in invertebrate globins. We previously reported that the formation of a poly-domain in myoglobin affects on the function of oxygen storage [26]: the autoxidation rates of 2-, 3-, 4- and 8-domain myoglobins, each constructed artificially, were reduced to 30–50% that of the single-domain myoglobin.

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In the present study, we developed the methods for expressing the recombinant enzymes of *Anthopleura* two-domain AK and domains 1 and 2 separately, and we finally succeeded in obtaining soluble enzymes with the 6× His-tag. The effect of the His-tag on the AK activity is not zero but is minimal. We can use this tag for rapidly isolating enzymes that are highly purified. We determined the kinetic constants for the AK reaction using the His-tagged two-domain AK and separated domains D1 and D2. Consistent with a previous study [25], the catalytic constant, k_{cat} for the two-domain AK, was significantly larger than the sum of the k_{cat} values for D1 and D2. To corroborate the presence of domain–domain interactions in the two-domain AK, we constructed three mutants, D1–K6–D2 (insertion of six Lys in the linker region), D1(Y68G)–D2 (mimicking D1-inactive enzyme) and D1–D2(Y68G) (mimicking D2-inactive enzyme), and determined their kinetic parameters. All the data supported the enhanced activity arising from cooperativity in the two-domain enzyme.

2. Materials and methods

2.1. Cloning of domain 1 (D1) of the two-domain AK into pET30 plasmid

The open reading frame of domain 1 of *Anthopleura* AK (D1) was amplified with the two primers, AnthAK2DHis-tagN: 5′-GGCATATGCACCATCATCATCATCATGCAGATCCAGAGACAGCT (*Nde*I site underlined, 6× His-tag boxed) and AnthAKD1His-tagC: 5′-GGGGATCCTAACGCAATTGCTTCCTTCTC (*Bam*HI site underlined), using the two-domain AK/pMAL plasmid as template [25], and cloned into the *Nde*I/*Bam*HI site of pET30 vector (Novagen, WI, USA). Ex Taq DNA polymerase (Takara, Tokyo, Japan) was used for amplification. The cDNA insert was sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA), using ABI PRISM 3130-Avant Genetic Analyzer.

The open reading frames of *Anthopleura* two-domain AK (D1–D2 AK) and its separated domain 2 (D2) had been cloned into the *Nde*I/*Bam*HI site of pET30 [25].

2.2. Site-directed mutagenesis of Anthopleura two-domain AK (D1–D2)

2.2.1. Construction of D1–K6–D2 mutant

Three Lys residues were first introduced into wild-type two-domain AK (denoted by D1–D2) in pET30 by PCR, using the following mutation-primers, KKKinsert-F: 5′-AAGAAG AAACGTTCAGTGTTTCCTGAA (mutated sequence underlined), and KKKinsert-R: 5′-TTTCTTCGCAATTGCTTCCTTCTC (mutated sequence underlined). KOD-plus-DNA polymerase (Toyobo, Osaka, Japan) was used as the amplifying enzyme. The PCR products were digested with *Dpn*I, and the target DNA fragment (7500 bp) was recovered by EasyTrap Ver.2 (TaKaRa, Tokyo, Japan). After blunting and phosphorylation, the DNA was self-ligated (D1–K3–D2 mutant).

Three Lys residues were further introduced into the D1–K3–D2 in pET30 by PCR, using the following mutation-primers, Kinsert6-F: 5′-AAGAAGAAGAAGAAACGTTCAGTGTTTCCT (mutated sequence underlined), and Kinsert6-R: 5′-CTTTTTCTTCGCAATTGCTTC. The cDNA insert was sequenced completely to confirm that only the intended mutations were introduced (D1–K6–D2 mutant).

2.2.2. Construction of D1(Y68G)–D2 mutant

The mutation was introduced into wild-type D1–D2 in pET30 by PCR, using the following mutation-primers, D1/Y68G-F: 5′-AGGTGCAGGTGATGAAGAGTCATAC (mutated sequence underlined), and D1/Y68G-R: 5′-ACACCGACTCCAGAATCAAGATT.

2.2.3. Construction of D1–D2(Y68G) mutant

The mutation was introduced into wild-type D1–D2 in pET30 by PCR, using the following mutation-primers, D2/Y68G-F: 5′-AGTGGGTGCCGGTGATGAAGAATGCTAC (mutated sequence underlined), and D2/Y68G-R: 5′-CCACAGGAGGAGTCCAGGTTTTC.

2.3. Expression and purification of His-tagged Anthopleura AKs

2.3.1. Wild-type two-domain AK and its mutants

Anthopleura two-domain AK (D1–D2) and its mutants (D1–K6–D2, D1(Y68G)–D2 and D1–D2(Y68G)) with a hexameric His at the N-terminal end, were expressed in *Escherichia coli* BL21 cells by induction with 1 mM IPTG at 18 °C and at shaking speed of 100 rpm for 93 h, using BioShaker BR-21FP (TAITEC, Koshigaya, Japan). The cells were resuspended in 5× TE buffer, sonicated, and purified by affinity chromatography using Ni-NTA Superflow (QIAGEN, CA, USA). The purity of the expressed enzymes was verified by SDS-PAGE. The enzymes were placed on ice until use, and enzymatic activity was determined within 10 h.

2.3.2. D1 and D2 (separated domains)

Anthopleura D1 and D2 with a hexameric His at the N-terminal end, were expressed in *E. coli* BL21 cells by induction with 1 mM IPTG at 20 °C and at shaking speed of 135 rpm for 24 h. The cells were resuspended in 5× TE buffer, sonicated, and purified by affinity chromatography using Ni-NTA Superflow.

2.4. Determination of enzyme concentration

The concentration of His-tagged *Anthopleura* AK was estimated based on absorbance at 280 nm. The extinction coefficient at 280 nm in $M^{-1}\,cm^{-1}$ (or mg/ml) was obtained using the computer program ProtParam (available from the URL http://ca.expasy.org/tools/protparam.html).

2.5. Estimation of molecular weights of His-tagged Anthopleura AKs on gel filtration

The molecular masses for the His-tagged enzymes of *Anthopleura* AK were estimated on a Superdex 75 gel filtration column (1 cm × 30 cm: Amersham Pharmacia Biotech, NJ, USA), which had been equilibrated with 10 mM Tris/HCl buffer, pH 8. The column was eluted with the same buffer at a flow rate of 0.5 ml/min and calibrated with ovalbumin (43 kDa), conalbumin (75 kDa) and aldolase (158 kDa).

2.6. Enzyme assays

Enzyme activity was measured using the NADH-linked spectrophotometric assay at 25 °C [27] and determined for the forward reaction (phosphagen synthesis). The reaction mixture (total volume of 1.0 ml) contained 0.65 ml of 100 mM Tris/HCl (pH 8), 0.05 ml of 750 mM KCl, 0.05 ml of 250 mM Mg-acetate, 0.05 ml of 25 mM phosphoenolpyruvate made up in 100 mM imidazole/HCl (pH 7), 0.05 ml of 5 mM NADH made up in Tris/HCl (pH 8), 0.05 ml of pyruvate kinase/lactate dehydrogenase mixture made up in 100 mM imidazole/HCl (pH 7), 0.05 ml of an appropriate concentration of ATP made up in 100 mM imidazole/HCl (pH 7) and 0.05 ml of recombinant enzyme. The reaction was started by adding 0.05 ml of an appropriate concentration of arginine made up in 100 mM imidazole/HCl (pH 7). The pH of the final reaction mixture was about 7.9. The above reactions were done at least five different concentrations of ATP.

Since the kinetics of phosphagen kinase can be explained as a random-order, rapid-equilibrium kinetic mechanism [28], the reac-