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The evolution from asparagine or threonine to cysteine in position 146 contributes to generation of a more efficient and stable form of muscle creatine kinase in higher vertebrates

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Abstract

Creatine kinase, a key enzyme in vertebrate excitable tissues that require large energy fluxes, catalyzes the reversible transfer of phosphate between adenosine triphosphate and creatine. Sequence alignment indicated that the 146th amino acid is cysteine in the muscle creatine kinase of higher vertebrates including Amphibia, Reptilia, Aves and Mammalia. In fishes, it is cysteine in Agnatha and Chondrichthyes, and asparagine or threonine in Osteichthyes, which is the ancestor of Amphibia, Reptilia, Aves and Mammalia. To explore the structural and functional role of this special residue, a series of site-directed mutants of rabbit muscle creatine kinase were constructed, including C146S, C146N, C146T, C146G, C146A, C146D and C146R. A detailed comparison was made between wild-type creatine kinase and the mutants in catalytic activity, physico-chemical properties and structural stability against thermal inactivation and guanidine hydrochloride denaturation. It was found that except for C146S, the mutants had relatively lower catalytic activity and structural stability than Wt-CK. Wt-CK and C146S were the most stable ones, followed by C146N and C146T, and then C146G and C146A, and C146D and C146R were the least stable mutants. These results suggested that the 146th residue plays a crucial role in maintaining the structural stability of creatine kinase, and that the evolution in this amino acid from asparagine or threonine to cysteine contributes to the generation of a more efficient and more stable form of creatine kinase in higher vertebrates.

Keywords: Protein stability; Dimer interface; Molecular evolution; Muscle type creatine kinase; Cysteine

1. Introduction

Phosphagen kinases are a group of highly conserved enzymes that are linked to energy state and ATP hydrolysis by catalyzing the reversible transition from phosphagen and ADP to guanidine receptors and ATP (Ellington, 2001). So far, eight different phosphagen systems have been found distributed along different phylogenetic lines (Ellington, 2001). Of these eight systems, the

Abbreviations: ANS, 1-anilinonaphtalene-8-sulfonate; ATP, adenosine triphosphate; CK, creatine kinase; DTT, dithiothreitol; Gdn-HCl, guanidine hydrochloride; rmCK, rabbit muscle creatine kinase; SDS, sodium dodecyl sulfate; Wt-CK, wild-type CK

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phosphocreatine/creatine kinase (CK, EC 2.7.3.2) system, found in vertebrates and widely distributed throughout the lower chordates and invertebrates, has been the most extensively studied (Ellington, 2001; Wallimann, Wyss, Brdiczka, Nicolay, & Eppenberger, 1992; Watts, 1975). CK functions in temporal ATP buffering, and plays a crucial role in energy homeostasis in tissues with high energy requirement such as muscle fibers, neurons, transport epithelia, photoreceptors and primitive-type spermatozoa (Wallimann & Hemmer, 1994; Wallimann et al., 1992). Since the first purification of rabbit muscle CK (rmCK) (Kuby, Noda, & Lardy, 1954), numerous studies have been carried out on its physico-chemical properties, catalytic mechanism and refolding pathway (Cox et al., 2003; He, Zhang, Zhou, & Yan, 2005; Kenyon & Reed, 1983; Wallimann et al., 1992; Wang, Yang, & Zhou, 1995; Watts, 1973; Zhao et al., 2005).

Four isoforms of CK are known to exist in higher vertebrates: two cytosolic isoforms (muscle (M) and brain (B)), and two mitochondrial isoforms (sarcomeric and ubiquitous). The cytosolic isoforms exist as homodimers (MM, BB) or heterodimers (MB) (Eppenberger, Dawson, & Kaplan, 1967). Muscle type CK (MM-CK) has the unique property of binding with the M-line of sarcomere (Turner, Wallimann, & Eppenberger, 1973), which is not shared by BB-CK or MB-CK (Wallimann, Moser, & Eppenberger, 1983), and the specific interaction is mediated by the NH_2-terminal lysine charge clamps (Hornemann, Stolz, & Wallimann, 2000). Mitochondrial CK exists as octamers and can dissociate into dimers (Wyss, Smeitink, Wevers, & Wallimann, 1992).

It has been proposed that two distinct gene duplication events occurred in the evolution of CK clusters (Mühlebach, Wirz, Brandle, & Perriard, 1996; Qin et al., 1998). One, from an original ancestral gene, gave rise to ancestral mitochondrial and cytosolic CK genes, and the other gave rise to two mitochondrial (ubiquitous and sarcomeric) and two cytosolic (M and B) CK isoforms (Qin et al., 1998). Recent studies suggested that the gene duplication events producing the M and B isoforms seemed to occur at the early radiation of fish (Graber & Ellington, 2001).

According to the crystal structure of rabbit muscle CK (Rao, Bujacz, & Woldawer, 1998), two symmetrical dimer interfaces of rmCK are relatively small and are bridged by polar interactions between E19, D54, Q58, and D62 from one subunit and S147, R152 and D210 from the other subunit. Site-directed mutagenesis indicated that R148 and R152 are crucial for the dimerization of CK (Cox et al., 2003). W211, located near the dimer interface, has also been demonstrated to be important for dimer cohesion (Perraut, Clottes, Leydier, Vial, & Marcillat, 1998). Through chemical modification, some cysteine in rmCK has also been suggested to be crucial for the formation of the dimeric structure of CK (Yang, Park, Yu, & Zhou, 1999). According to the amino acid sequence of rmCK, C146 is near the dimer face, and therefore it is most likely that C146 is involved in the dimer cohesion of CK. Interestingly, the 146th amino acid is cysteine in the CKs of higher vertebrates, but asparagine or threonine in Osteichthyes (Fig. 1). This phenomenon was not observed for other amino acids involved in the dimeric interface and was more like to be specific to the 146th amino acid. So what is the significance of the evolution in the 146th amino acid from asparagine or threonine to cysteine? Another question is why it was cysteine, instead of other amino acids, that was selected in long-term evolution. To elucidate these questions, a series of site-directed mutants of rmCK were constructed in the present study. A detailed comparison between wild-type CK (Wt-CK) and the mutants in their catalytic activity, physico-chemical properties, and structural stability suggested that the evolution from asparagine or threonine to cysteine in the 146th amino acid generated more efficient and stable forms of CK in higher vertebrates.

2. Materials and methods

2.1. Materials

Sodium dodecyl sulfate (SDS), ultra-pure guanidine hydrochloride (Gdn-HCl), Tris, 1-anilinonaphtalene-8-sulfonate (ANS) and ATP were purchased from Sigma. DTT and T_4 DNA ligase were from Promega. All the other chemicals were local products of analytical grade.

2.2. Site-directed mutagenesis

The gene of rmCK has been cloned into the pET21b expression vector (Guo, Wang, Ni, & Wang, 2003). Site-directed mutagenesis was carried out, using 10 ng of double-strand DNA (entire plasmid vector), 15 pmol of each primer, and *Pfu* DNA polymerase. The amplified fragments were cloned into pET21b, and sequenced to verify the mutagenesis. The mutagenic primers (mismatches with the template are underlined) were: C146S (FC146S: 5′-CCCCCGCACT*C*CTCC-CGTGGC-3′; RC146S: 5′-CACGGGAG*G*AGTGC-GGGGGC-3′); C146N (FC146N: 5′-CCGCAC*AA*CT-CCCGT GGCGAG-3′; RC146N: 5′-CACGGGAG*TT*G-