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The role of Arg-96 in *Danio rerio* creatine kinase in substrate recognition and active center configuration

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ABSTRACT

In creatine kinases (CKs), the amino acid residue-96 is a strictly conserved arginine. This residue is not directly associated with substrate binding, but it is located close to the binding site of the substrate creatine. On the other hand, the residue-96 is known to be involved in expression in the substrate specificity of various other phosphagen (guanidino) kinases, since each enzyme has a specific residue at this position: arginine kinase (Tyr), glycocyamine kinase (Ile), taurocyamine kinase (His) and lombricine kinase (Lys). To gain a greater understanding of the role of residue-96 in CKs, we replaced this residue in zebra fish *Danio rerio* cytoplasmic CK with other 19 amino acids, and expressed these constructs in *Escherichia coli*. All the twenty recombinant enzymes, including the wild-type, were obtained as soluble form, and their activities were determined in the forward direction. Compared with the activity of wild-type, the R96K mutant showed significant activity (8.3% to the wild-type), but 10 mutants (R96Y, A, S, E, H, T, F, C, V and N) showed a weak activity (0.056–1.0%). In the remaining mutants (R96Q, G, M, P, L, W, D and I), the activity was less than 0.05%. Our mutagenesis studies indicated that Arg-96 in *Danio* CK can be substituted for partially by Lys, but other replacements caused remarkable loss of activity. From careful inspection of the crystal structures (transition state analog complex (TSAC) and open state) of *Torpedo* cytoplasmic CK, we found that the side chain of R96 forms hydrogen bonds with A339 and D340 only in the TSAC structure. Based on the assumption that CKs consist of four dynamic domains (domains 1–3, and fixed domain), the above hydrogen bonds act to link putative domains 1 and 3 in TSAC structure. We suggest that residue-96 in CK and equivalent residues in other phosphagen kinases, which are structurally similar, have dual roles: (1) one involves in distinguishing guanidino substrates, and (2) the other plays a key role in organizing the hydrogen-bond network around residue-96 which offers an appropriate active center for the high catalytic turnover. The mode of development of the network appears to be unique each phosphagen kinase, reflecting evolution of each enzyme.

1. Introduction

Creatine kinase (CK), a member of phosphagen (guanidino) kinase enzyme family, plays a key role in the regulation of metabolism in energy production and utilization in animals [1–4]. These enzymes catalyze the reversible transfer of high-energy phosphoryl groups of ATP to creatine, producing phosphorylated high-energy phosphocreatine referred to as phosphagen.

Recent transition state analog complex (TSAC) structure analysis of *Limulus* arginine kinase (AK) [5,6] and *Torpedo* cytoplasmic CK [7] has clarified the substrate-binding sites between guanidine compounds (arginine or creatine) and ATP, and large conformational changes accompanying with substrate binding. This structural information is useful for determining the substrate-binding site characteristics of other phosphagen kinases, glycocyamine kinase (GK), taurocyamine kinase (TK) and lombricine kinase (LK), whose structures have not yet been resolved, and for elucidating how the guanidine substrate recognition system developed during phosphagen kinase evolution.

Amino acid residue-96 is one of the key residues in function of phosphagen kinases [8,9]. Multiple alignment of amino acid sequences clearly indicated that the residue is strictly conserved as a specific amino acid in each phosphagen kinase: Arg in CK, Tyr in AK, Ile in GK, His in TK and Lys in LK [10–12]. While this residue does not appear to be directly involved in substrate binding in CK and AK crystal structures, it is located close to the guanidine substrate-binding site.

Edmiston et al. [8] were the first to have pointed out the functional importance of residue-95 in the rabbit CK reaction

Abbreviations: CK, creatine kinase; MiCK, mitochondrial creatine kinase; fCK, flagellar creatine kinase; AK, arginine kinase; GK, glycocyamine kinase; TK, taurocyamine kinase; LK, lombricine kinase; TSAC, transition state analog complex.

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(equivalent to R96 in *Danio* CK). They constructed R95K, R95Y and R95A mutants, and observed that the catalytic efficiencies are 2.7, 0.03 and 0.05%, respectively, compared with that of wild-type. From these results, they concluded that a basic amino acid is required at position 95 for expression of sufficient activity, but R95 is not absolutely essential for creatine binding or CK catalysis [8,13].

On the other hand, we examined the role of residue-95 (equivalent to R96 in *Danio* CK) in terms of substrate specificity of guanidino compounds [14]. Using the wild-type LK with K95, the K95Y mutant was constructed. Surprisingly, it produced a dramatic change in guanidine substrate specificity, resulting in a shift of substrate specificity from lombricine to taurocyamine. The K95Y mutant functions *not* as lombricine kinase *but* as taurocyamine kinase. Thus, it became clear that K95 contributes to the determination of substrate specificity [14].

The purpose of this study is to gain a greater understanding of the role of residue-96. We replaced R96 of *Danio rerio* cytoplasmic CK with other 19 amino acids, expressed them in *Escherichia coli*, and determined their CK activities. Compared with the activity of wild-type, the R96K mutant showed significant activity (8.3% of the wild-type), but other mutants showed a very weak activity. From careful inspection of the crystal structures of *Torpedo* CK, we found that a highly organized hydrogen-bond network exists around residue-96 in TSAC structure. R96 may play a key role in the network for the full expression of CK activity.

2. Materials and methods

2.1. Site-directed mutagenesis and expression of Danio rerio CK

The cDNA (open reading frame of 1143 base pairs) of *D. rerio* cytoplasmic CK, cloned into pMAL-c2 (pMAL/*Danio* CK-wild), was used as template [15].

Polymerase chain reaction (PCR)-based mutagenesis was done as described previously [16]. The twenty mutations (R96K, A, S, E, G, H, F, T, Y, C, V, N, Q, M, P, L, W, D, I and deletion) were introduced into the template by PCR using mutation-primers shown in Table S1 in the supplementary section. KOD$^+$ DNA polymerase (TOYOBO, Tokyo, Japan) was used as the amplifying enzyme. The PCR products were digested with DpnI, and the target DNA fragment (7000 bp) was recovered by EasyTrap Ver.2 (TaKaRa, Tokyo, Japan). After blunting and kination, the DNA was self-ligated. The cDNA insert was completely sequenced to confirm that only the intended mutations were introduced.

The MBP (maltose-binding protein)-*Danio* CK fusion protein was expressed in *E. coli* TB1 cells by induction with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 25 °C for 20 h. The soluble protein was extracted with the Bugbuster (Novagen, WI, USA), and purified by affinity chromatography using amylose resin (New England BioLabs, MA, USA). Purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The enzymes were placed on ice until use, and enzymatic activity was determined within 12 h.

2.2. Enzyme assay and determination of kinetic constants

Enzyme activity was measured with an NADH-linked assay at 25 °C [17,18] and determined for the forward reaction (phosphagen synthesis). The reaction mixture (total 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 creatine made up in 100 mM imidazole/HCl (pH 7). The initial velocity values were typically obtained by varying the concentration of one substrate (creatine) versus five fixed concentrations of the second substrate (ATP), resulting in a 8×5 matrix.

Protein concentration was estimated from the absorbance at 280 nm. The extinction coefficient at 280 nm in $M^{-1}\,cm^{-1}$ (or mg/ml) was obtained using ProtParam (available from the URL http://ca.expasy.org/tools/protparam.html). The protein concentration for the CK enzyme moiety was obtained by excluding the portion of the MBP-tag.

Since the kinetics of phosphagen kinase can be explained as a random-order, rapid-equilibrium kinetic mechanism [17], the reaction velocity (v) is given by the following equation.

$$v = \frac{V_{max}}{(K_a^{ATP} \cdot K_{ia}^{Cr})/[Creatine][ATP] + K_a^{ATP}/[ATP] + K_a^{Cr}/[Creatine] + 1} \quad (1)$$

$$K_a^{Cr} \cdot K_{ia}^{ATP} = K_a^{ATP} \cdot K_{ia}^{Cr} = K_T = [E][Cr][ATP]/[E{-}Cr{-}ATP] \quad (2)$$

and

$$V_{max} = k_{cat}[E_0] = k_{cat}([E] + [E{-}Cr] + [E{-}ATP] + [E{-}Cr{-}ATP]) \quad (3)$$

Here, K_a is the ternary dissociation constant in the presence of the second substrate, K_{ia} is the binary dissociation constant in the absence of the second substrate, and K_T corresponds to the dissociation constant of the [E–Cr–ATP] complex (see Eq. (2)). To determine the kinetic parameters, data were fitted directly to Eq. (1) according to the method of Cleland [19], using the software written by Dr. R. Viola (Enzyme kinetics Programs, ver. 2.0).

3. Results and discussion

3.1. Enzymatic properties of residue-96 mutants of Danio CK

All the recombinant enzymes with MBP-tag, with the exception of the R96-deletion mutant, were expressed as soluble proteins, successfully purified by affinity chromatography, and confirmed to be highly purified by SDS-PAGE. An amount of recombinant enzyme sufficient for activity measurement was not expressed for the R96-deletion enzyme.

Expression of recombinant proteins with the MBP-tag gave, in most cases, a soluble enzyme that could be easily purified by affinity chromatography. This is advantageous when dealing with many mutants generated by site-directed mutagenesis. We used the MBP-fusion AKs directly for determination of their kinetic parameters because in our previous studies, it was shown that the tag gave no significant effect on the activity and catalytic constants [26].

All the MBP-tag enzymes were bound tightly to the column of amylose resin under the same conditions, suggesting that at least MBP moiety was refolded properly. We believe that AK moiety would also be refolded properly.

Among 19 mutants examined, only one mutant, R96K, gave a sufficient activity for the calculation of kinetic constants with a random-order, rapid-equilibrium kinetic mechanism (Eq. (1)). Table 1 compares the kinetic constants of wild-type and R96K mutant of *Danio* CK, with those of rabbit CK [8]. The catalytic efficiency of the two-substrate reaction is given by $k_{cat}/K_a^{Cr}K_{ia}^{ATP}$ [8], because the Eq. (1) can be approximated to $v = (k_{cat}/K_a^{Cr}K_{ia}^{ATP})$ [E][Cr][ATP] in concentration of creatine and ATP close to zero.

In *Danio* R96K mutant, the ternary dissociation constant for creatine (K_a^{Cr}) was 4.7-fold (39.4/8.36) decreased compared with the wild-type, while that for ATP (K_a^{ATP}) was 2.2-fold (2.31/1.03)