Biochimica et Biophysica Acta 1814 (2011) 1219–1225

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Identification of amino acid residues responsible for taurocyamine binding in mitochondrial taurocyamine kinase from *Arenicola brasiliensis*

Kumiko Tanaka, Tamotsu Matsumoto, Tomohiko Suzuki *

Laboratory of Biochemistry, Faculty of Science, Kochi University, Kochi 780-8520, Japan

ARTICLE INFO

Article history:
Received 23 February 2011
Received in revised form 25 May 2011
Accepted 1 June 2011
Available online xxxx

Keywords:
Taurocyamine kinase
Glycocyamine kinase
Substrate inhibition
Arenicola (polychaete)

ABSTRACT

In order to investigate the residues associated with binding of the substrate taurocyamine in *Arenicola* mitochondrial taurocyamine kinase (TK), we performed Ala-scanning of the amino acid sequence HTKTV at positions 67–71 on the GS loop, and determined apparent K_m and V_{max} (appK_m and appV_{max}, respectively) of the mutant forms for the substrates taurocyamine and glycocyamine. The appK_m values for taurocyamine of the K69A, T70A and V71A mutants were significantly increased as compared with wild-type, suggesting that these residues are associated with taurocyamine binding. Of special interest is a property of V71A mutant: its catalytic efficiency for glycocyamine was twice that for taurocyamine, indicating that the V71A mutant acts like a glycocyamine kinase, rather than a TK. The role of the amino acid residue K95 of *Arenicola* MiTK was also examined. K95 was replaced with R, H, Y, I, A and E. K95R, K95H and K95I have a 3-fold higher affinity for taurocyamine, and activity was largely lost in K95E. On the other hand, the K95Y mutant showed a rather unique feature; namely, an increase in substrate concentration caused a decrease in initial velocity of the reaction (substrate inhibition). This is the first report on the key amino acid residues responsible for taurocyamine binding in mitochondrial TK.

1. Introduction

Phosphagen kinases (guanidino kinases), including creatine kinase (CK), arginine kinase (AK), taurocyamine kinase (TK), lombricine kinase (LK), glycocyamine kinase (GK), opheline kinase (OK) and hypotaurocyamine kinase (HTK), are enzymes that catalyze the reversible transfer of the γ-phosphoryl group of ATP to naturally occurring guanidino compounds such as creatine and arginine, yielding ADP and a phosphorylated guanidine typically referred to as a phosphagen (phosphocreatine, phosphoarginine, *etc.*). Members of this enzyme family play a key role in animals as ATP buffering systems in cells that display high and variable rates of ATP turnover [1–4]. Crystal structures have been determined for CK [5,6], AK [7], GK [8] and LK [9]. CK is distributed widely in vertebrates and in vertebrates, and AK is the common phosphagen kinase among invertebrates [10,11]. By contrast, GK, TK, LK, OK and HTK are found only in annelid and annelid-allied worms [10,12,13]. Interestingly, some of the annelid species contain multiple phosphagen kinases, for example both CK and GK are present in *Namalycastis* sp. [14].

TK was first isolated from the body wall muscle of the polychaete lugworm *Arenicola marina* [15,16]. Native, cytoplasmic TK shows considerable activity for hypotaurocyamine (about 50% that of the main target substrate, taurocyamine), and weak activity for lombricine and glycocyamine [17]. The enzyme is dimeric, like CK, LK and GK [16].

Recently, two types of TK cDNAs, cytoplasmic TK and mitochondrial MiTK, were sequenced from the polychaete *Arenicola brasiliensis* [18] and the deep-sea vestimentiferan *Riftia pachyptila* [19]. The amino acid sequence of MiTK has 60–65% identity with invertebrate MiCKs and annelid-specific phosphagen kinases. Analyses of exon–intron organization suggested that annelid-specific enzymes, including MiTK and cytoplasmic GK, LK, TK and *Sabellastate* AK, evolved from a MiCK-like ancestor [20].

MiCK, which is targeted to the intermembrane compartment of mitochondria and exists primarily as a homo-octamer, plays a key role in intracellular energy transport from mitochondria to the cytoplasm [1]. MiCK has an N-terminal extension of 40 residues, a mitochondrial targeting sequence, which is not present in the cytoplasmic CK. This extension is required for translocation of MiCK from the cytoplasm to the mitochondrial intermembrane. It is thought that this system functions in all cells of invertebrates and vertebrates that contain CK [21]. However, the function of other mitochondrial enzymes, such as MiTK and putative MiLK [20], is not well understood.

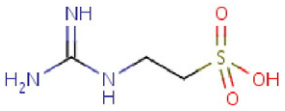
Arenicola MiTK shows considerable enzyme activity for the guanidino substrates, lombricine and glycocyamine, in addition to its original target substrate, taurocyamine [18,19]. Structures for

Abbreviations: TK, taurocyamine kinase; AK, arginine kinase; CK, creatine kinase; GK, glycocyamine kinase; LK, lombricine kinase; HTK, hypotaurocyamine kinase; MiTK, mitochondrial taurocyamine kinase; MiCK, mitochondrial creatine kinase; MiLK, mitochondrial lombricine kinase

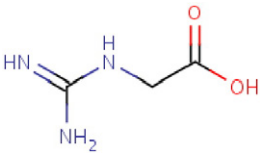
* Corresponding author.
E-mail address: suzuki@kochi-u.ac.jp (T. Suzuki).

1570-9639/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbapap.2011.06.003

taurocyamine and glycocyamine are as follows (cited from ChEBI Home: http://www.ebi.ac.uk/chebi/):



Taurocyamine



Glycocyamine

Thus, the affinity of MiTK for specific guanidino substrates is rather low. This is in sharp contrast to CK and AK, which have high specificity for their substrates [3,10]. We have already shown that the specificity of LK for its substrate is also low [22]. However, the low degree of substrate specificity in annelid enzymes suggests flexibility in substrate recognition, which may act as a driving force in the evolution of phaphagen kinases, which have achieved remarkable diversity in annelids. Indeed, introduction of the mutation K95Y in *Eisenia* LK dramatically altered guanidino substrate specificity, from lombricine to taurocyamine [22].

We previously proposed that the residues 62–72 (designated the GS region), which have a unique deletion pattern in an alignment of phosphagen kinases sequences, are involved in distinguishing the size of a guanidino substrate (see Fig. 1) [23]. This region partially overlaps with the N-terminal flexible loop in the crystal structures of AK and CK, and some of the amino acid residues in the GS region have been shown to interact with a guanidino substrate in *Limulus* AK and *Torpedo* CK [6,7]. The amino acid sequence of GS region of MiTK is quite different from that of cytoplasmic TK (Fig. 1), reflecting their evolutionary processes [20]. Consequently, the responses to the guanidino substrates, taurocyamine, lombricine, glycocyamine and arginine, differ significantly in the two enzymes [18,19].

Additional studies have revealed the functional relevance of another residue. Namely, the importance of the amino acid residue 95 was demonstrated by Edmiston et al. [24] and by our group [25,22]. The residue is identical or conserved in various phosphagen kinases: Arg in CK, Ile in GK, Lys in LK and Tyr in AK (see Fig. 1). Based on the CK and AK crystal structures, residue 95 does not appear to be directly associated with substrate binding; however, it is located close to the binding guanidino substrate site [6,7,26]. Cytoplasmic TKs and mitochondrial MiTKs have His and Lys, respectively, at residue 95 (Fig. 1).

In this study, we performed Ala scanning mutagenesis in the GS region of *Arenicola* MiTK, and also replaced K95 with R, H, I, and Y residues found in native enzymes, and with A or E. The K69A, T70A and V71A mutant proteins have a lower affinity for taurocyamine, and conversely, K95 mutants have an increased affinity for this substrate. Surprisingly, the V71A mutant protein shows a higher catalytic efficiency for the substrate glycocyamine as compared with the original substrate, taurocyamine. Thus, K67, T70, V71 and K95 appear to act as key residues that help determine affinity and specificity for guanidino substrates. Throughout this report, we use sequence numbering based on rabbit muscle CK [27].

2. Materials and methods

2.1. Site directed mutagenesis and expression of Arenicola brasiliensis MiTK

The pMAL-c2 plasmid with the insertion of *Arenicola* MiTK [18] was used as a template of mutagenesis. Polymerase chain reaction (PCR)-based mutagenesis was done as described previously [28]. Primers used in PCR are shown in Table 1. KOD^{+} DNA polymerase (TOYOBO, Tokyo, Japan) was used as the amplifying enzyme. The PCR products were digested with *Dpn*I and the target DNA fragments (7000 bp) were 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.

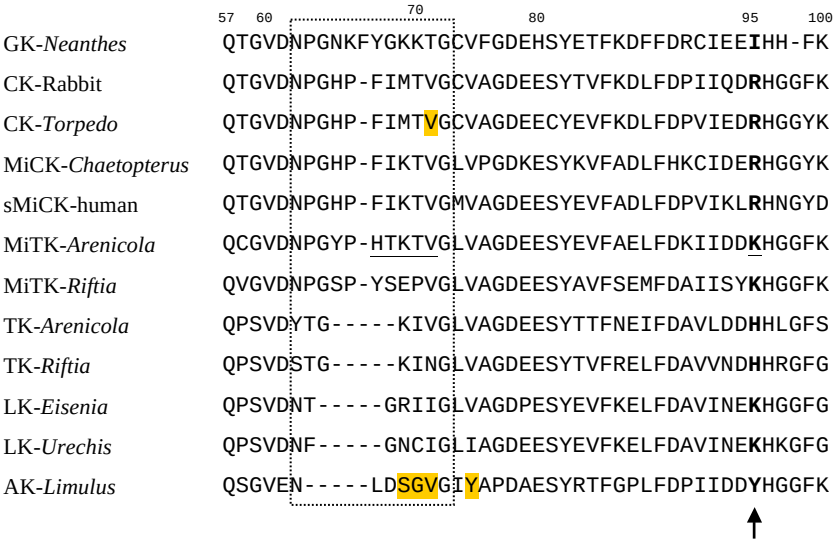


Fig. 1. Alignment of amino acid sequences around the GS region and amino acid residue 95 of GK, CK, MiCK, MiTK, TK, LK and AK. The GS region is boxed. Amino acid residue 95 is indicated by an arrow. The mutated residues in *Arenicola* MiTK are underlined. Shaded, residues interacting with substrates in the *Torpedo* CK and *Limulus* AK crystal structures. Accession numbers: GK-*Neanthes* (AB017257), CK-Rabbit (K02831), CK-*Torpedo* (M36427), MiCK-*Chaetopterus* (AF139589), sMiCK-human (BC029140), MiTK-*Arenicola* (AB186412), MiTK-*Riftia* (AB18409), LK-*Eisenia* (AB017257), LK-*Urechis* (AF421182), TK-*Arenicola* (AB186411), TK-*Riftia* (AB186410) and AK-*Limulus* (U09809).

Table 1
Primers used for the site-directed mutagenesis of Arenicola MiTK.

Mutant	Primer name	Sequence
K95Y	AreMiTKm01F	ATCATGGTGGCTTCAAGCCGAC
	AreMiTKm02R	AGTCGTCAATGATCTTGTCAAACAG
K95R	AreMiTKm03F	GACATGGTGGCTTCAAGCCGAC
	AreMiTKm03R	TGTCGTCAATGATCTTGTCAAACAG
K95H	AreMiTKm01F	ATCATGGTGGCTTCAAGCCGAC
	AreMiTKm01R	GGTCGTCAATGATCTTGTCAAACAG
K95I	AreMiTKm04F	TACATGGTGGCTTCAAGCCGAC
	AreMiTKm03R	TGTCGTCAATGATCTTGTCAAACAG
K95E	AreMiTKm05F	AACATGGTGGCTTCAAGCCGAC
	AreMiTKm05R	CGTCGTCAATGATCTTGTCAAACAG
K95A	AreMiTKm11F	CACATGGTGGCTTCAAGCCGAC
	AreMiTKm05R	CGTCGTCAATGATCTTGTCAAACAG
H67A	AreMiTKm10F2	CAGGGCTCGTCGCCGGAGACG
	AreMiTKm10R	CTGTCTTTGTGTGGGGGTATCCGGG
T68A	AreMiTKm08F	CAAAGACAGTGGGGCTCGTCGCCG
	AreMiTKm08R	CGTGGGGGTATCCGGGGTTGTCCAC
K69A	AreMiTKm06F	CGACAGTGGGGCTCGTCGCCGG
	AreMiTKm06R	CTGTGTGGGGGTATCCGGGGTTGTC
K69R	AreMiTKm13F	GGACAGTGGGGCTCGTCGCCG
	AreMiTKm13R	TTGTGTGGGGGTATCCGGGGTTGTC
T70A	AreMiTKm07F	CAGTGGGGCTCGTCGCCGGAG
	AreMiTKm07R	CCTTTGTGTGGGGGTATCCGGGGTT
V71A	AreMiTKm09F	CCACAAAGACAGTGGGGCTCGTCG
	AreMiTKm09R	CGGGGTATCCGGGGTTGTCCACTCC

The maltose binding protein (MBP)-*Arenicola* MiTK fusion protein was expressed in *Escherichia coli* TB1 cells by induction with 1 mM isopropyo-1-thio-β-D-galactopyranoside at 25 °C for 24 h. The cells were resuspended in 50 mM Tris/EDTA buffer, sonicated and the soluble proteins were extracted. All of recombinant enzymes are purified by affinity chromatography using amylose resin (New England BioLads, Ma, USA). Purity was confirmed 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

Enzyme activity was measured with an NADH-linked assay at 25 °C [29,30] using Ultrospec 4300 UV/Visible Spectrophotometers (Amersham Biosciences) 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 100 mM 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 100 mM 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 guanidine substrate made up in 100 mM Tris–HCl (pH 8). The values for initial velocity were obtained by varying the concentration of guanidine substrate in 4.76 mM ATP, and the apparent K_m and V_{max} (appK_m and appV_{max}, respectively) were calculated using the SigmaPlot 12 (Systec Software, Inc.). Protein concentration was estimated from the absorbance at 280 nm with ProtParam (http://ca.expasy.org/tools/protparam.html).

3. Results and Discussion

3.1. Expression and kinetic properties of Arenicola recombinant MiTK (wild-type)

All of the recombinant enzymes were expressed as MBP-tagged soluble proteins, successfully purified by affinity chromatography, and confirmed to be highly purified by SDS-PAGE.

The phosphagen kinase reactions, which utilize two substrates, guanidino substrate and ATP for the forward reaction and phosphagen and ADP for the reverse reaction, can be explained as a random-order, rapid-equilibrium kinetic mechanism [29], and in many cases there is a synergy in the binding of the two substrates. The enzyme reaction of *Arenicola* MiTK was determined for the forward reaction and the kinetic parameters, appK_m and appV_{max}, of the recombinant enzymes were obtained for two guanidino substrates (taurocyamine and glycocyamine) in the presence of 4.46 mM ATP. Assuming from the K_m^{ATP} values for MiTK wild-type (1.26 ± 0.15 mM) and K69A/K95Y mutant (0.91 ± 0.11 mM) (Matsumoto and Suzuki, unpublished data), ATP is not fully saturated at the binding site of the enzyme in this condition (at most 80% saturation). However the parameters, appK_m^{tau}, appK_m^{gly}, appV_{max}^{tau} and appV_{max}^{gly}, may be used to elucidate the binding properties for the guanidino substrates of MiTK.

The parameters of the wild-type and mutants were compared, considering the values of standard error and 95% confidence interval calculated by the SigmaPlot.

The appK_m^{tau} and appV_{max}^{tau} for wild-type *Arenicola* MiTK were determined to be 0.91 mM and 43.1 μmoles Pi•min^{-1} mg protein^{-1}, respectively, for the substrate taurocyamine. The corresponding catalytic efficiency, appV_{max}/appK_m, was calculated to be 47.2 μmoles Pi•min^{-1} mg protein^{-1} mM^{-1} (Table 2). On the other hand, the appK_m^{gly} and appV_{max}^{gly} were determined to be 10.6 and 20.4, respectively, for the substrate glycocyamine. The corresponding catalytic efficiency was 1.9 (Table 2). Based on appK_m values, the affinity of wild-type *Arenicola* MiTK for taurocyamine, its main target substrate, is 12-fold higher than that for glycocyamine. Recombinant wild-type *Arenicola* MiTK has weak activity for arginine and creatine (data not shown).

3.2. Ala-scanning of the amino acid sequence H-T-K-T-V at positions 67–71 on the GS loop of Arenicola MiTK

In 1997, we proposed that residues 62–72, which have a unique deletion pattern in the sequence alignment of CK, GK, AK and LK (Fig. 1), are associated with distinguishing the size of a guanidino substrate and thus, was designated the "GS region" [23]. The GS region partially overlaps with the N-terminal flexible loop in the crystal structures of CK and AK. Indeed, S69, G70 and V71 in *Limulus* AK have been shown to interact with the guanidino substrate arginine [7], and I69 and V71 in CK [6,31] are the key residues in the active center pocket. In order to determine which residues are involved in taurocyamine binding in *Arenicola* MiTK, we performed Ala scanning mutagenesis, altering the amino acid sequence H-T-K-T-V at positions 67–71 on the GS loop, and then measured the enzyme activities of the mutant proteins for the substrates taurocyamine and glycocyamine.

3.2.1. Arenicola MiTK H67A and T68A mutants

The H67A and T68A mutants have a property similar to wild-type; that is, they have affinities for taurocyamine (appK_m^{tau} = 0.72–0.81 mM) and for glycocyamine (appK_m^{gly} = 12.6–14.2 mM) that are similar to those of wild-type (0.91 and 10.6 mM, respectively) (Table 2 and Fig. 2). The appV_{max}^{tau} (38.7–39.2 μmoles Pi•min^{-1} mg protein^{-1}) and appV_{max}^{gly} (18.2–22.5) for these two mutant forms of *Arenicola* MiTK were also comparable to those of wild-type (43.1 and 20.4, respectively). Moreover, their catalytic efficiencies, as calculated by appV_{max}^{tau}/appK_m^{tau} and appV_{max}^{gly}/appK_m^{gly}, which were 48.1–53.9 and 1.5–1.6 μmoles Pi•min^{-1} mg protein^{-1} mM^{-1}, respectively, were also similar to those of wild-type (47.2 and 1.9, respectively). Taken together, these results indicate that residues H67 and T68 are not significantly associated with taurocyamine binding or the phosphoryl transfer reaction.

3.2.2. Arenicola MiTK K69A mutant

The K69A mutant form of *Arenicola* MiTK has a slightly decreased affinity for taurocyamine (appK_m^{tau} = 1.23 mM) and for glycocyamine (appK_m^{gly} = 13.2 mM) as compared with wild-type, and also decreased

Table 2
Comparison of apparent kinetic parameters of *Arenicola* MiTK and mutants for the forward reaction.

	Taurocyamine				Glycocyamine		
	appK_m^{tau} [mM]	K_i [mM]	appV_{max}^{tau} [μmoles Pi•min^{-1} mg protein^{-1}]	appV_{max}/appK_m^{tau} [μmoles Pi•min^{-1} mg protein^{-1} mM^{-1}]	appK_m^{gly} [mM]	appV_{max}^{tau} [μmoles Pi•min^{-1} mg protein^{-1}]	appV_{max}/appK_m^{gly} [μmoles Pi•min^{-1} mg protein^{-1} mM^{-1}]
WT	0.913 ± 0.116		43.06 ± 2.145	47.2	10.58 ± 0.702	20.41 ± 0.793	1.93
H67A	0.813 ± 0.037		39.15 ± 0.709	48.1	14.18 ± 1.012	22.52 ± 0.970	1.59
T68A	0.718 ± 0.042		38.74 ± 0.881	53.9	12.56 ± 0.694	18.24 ± 0.565	1.45
K69A	1.228 ± 0.059		35.43 ± 0.781	28.8	13.16 ± 1.045	16.75 ± 0.784	1.27
K69R	0.742 ± 0.096		30.06 ± 1.433	40.5	6.27 ± 1.185	11.76 ± 0.948	1.88
T70A	1.868 ± 0.077		31.07 ± 0.594	16.6	24.22 ± 4.117	9.07 ± 1.027	0.37
V71A	2.091 ± 0.109		23.89 ± 0.561	11.4	0.89 ± 0.047	20.31 ± 0.400	22.78
K95Y	0.082 ± 0.029	4.61 ± 0.314	10.33 ± 0.358	125.7	1.68 ± 0.061	7.16 ± 0.103	4.25
K95R	0.287 ± 0.015		29.62 ± 0.475	103.3	5.89 ± 0.285	15.45 ± 0.343	2.62
K95H	0.313 ± 0.036		33.67 ± 1.245	107.6	9.20 ± 1.341	13.89 ± 1.115	1.51
K95I	0.278 ± 0.038		22.17 ± 0.901	79.9	7.37 ± 0.924	12.79 ± 0.814	1.74
K95E	13.218 ± 3.403		16.41 ± 2.401	1.2	–	–	–
K95A	0.205 ± 0.021		15.89 ± 0.361	77.7	1.02 ± 0.055	14.55 ± 0.367	14.31
H67A/K95Y	0.184 ± 0.108	5.10 ± 1.130	13.29 ± 1.352	72.2	2.14 ± 0.059	9.74 ± 0.100	4.55
T68A/K95Y	0.140 ± 0.074	5.62 ± 0.917	12.01 ± 0.931	86.1	1.69 ± 0.415	7.49 ± 0.801	4.43
K69A/K95Y	0.153 ± 0.011		6.72 ± 0.102	44.0	1.49 ± 0.073	5.56 ± 0.088	3.73
K69R/K95Y	0.217 ± 0.049	7.52 ± 0.868	13.07 ± 0.606	60.2	5.10 ± 0.381	8.62 ± 0.289	1.69
T70A/K95Y	0.332 ± 0.037	6.38 ± 0.417	17.14 ± 0.528	51.7	6.93 ± 0.337	8.37 ± 0.201	1.21
V71A/K95Y	0.603 ± 0.081		6.47 ± 0.330	10.7	0.49 ± 0.021	5.35 ± 0.083	10.89

All kinetic parameters were obtained using SigmaPlot 12.

values for appV_{max}^{tau} (35.4 μmoles Pi•min^{-1} mg protein^{-1}) and appV_{max}^{gly} (16.8) as compared with wild-type (Table 2 and Fig. 2). The catalytic efficiencies, calculated by appV_{max}^{tau}/appK_m^{tau} and appV_{max}^{gly}/appK_m^{gly}, for the K69A mutant (28.8 and 1.3 μmoles Pi•min^{-1} mg protein^{-1} mM^{-1}, respectively), were decreased to 61–68% those of wild-type (Fig. 3), suggesting a weak relationship between K69 and taurocyamine binding. To test this, we constructed an additional mutant form, K69R. The affinity of the K69R form for taurocyamine (appK_m^{tau} = 0.74 mM) was comparable to that of wild-type (appK_m^{tau} = 0.91 mM) (Fig. 2), suggesting the importance of a positively charged side chain at residue 69.

In CK, residue 69 (I69) has been shown to play a key role in substrate binding by forming a part of a specificity pocket for creatine [31]. On the other hand, our data suggest a weak relationship between residue 69 (K69) and taurocyamine binding in MiTK. It is likely that the above difference is derived from the difference in their active pocket structures. In fact, CK has strict substrate specificity relatively [1,10,31,32], but MiTK has relaxed substrate specificity [10,17–19].

3.2.3. *Arenicola* MiTK T70A mutant

The T70A mutant has 2-fold decreased affinity for taurocyamine (appK_m^{tau} = 1.87 mM) and for glycocyamine (appK_m^{gly} = 24.2 mM) as compared with wild-type, and relatively lower values for appV_{max}^{tau} (31.1 μmoles Pi•min^{-1} mg protein^{-1}, 72% of the wild-type) or appV_{max}^{gly} (9.1, 45% of the wild-type) (Table 2 and Fig. 2). Consequently, the catalytic efficiencies of the T70A mutant protein were remarkably decreased in comparison with wild-type (*i.e.* appV_{max}^{tau}/appK_m^{tau} = 16.6 μmoles Pi•min^{-1} mg protein^{-1} mM^{-1}, or 35% that of wild-type, and appV_{max}^{gly}/appK_m^{gly} = 0.4, or 21% that of wild-type) (Fig. 3). These data indicate a stronger relationship of T70 with taurocyamine binding and phosphoryl transfer reaction.

3.2.4. *Arenicola* MiTK V71A mutant with strong GK activity

The V71A mutant form of *Arenicola* MiTK has a 2-fold decreased affinity for taurocyamine (appK_m^{tau} = 2.1 mM) and a decreased appV_{max}^{tau} value (23.9 μmoles Pi•min^{-1} mg protein^{-1}) (Table 2 and Fig. 2). Surprisingly, it also showed remarkably strong affinity for glycocyamine (appK_m^{gly} = 0.89 mM, a 2-fold higher affinity than taurocyamine) with an appV_{max}^{gly} value of 20.3, resulting in 2-fold higher catalytic efficiency for glycocyamine (appV_{max}^{gly}/appK_m^{gly} = 22.8 μmoles Pi•min^{-1} mg protein^{-1} mM^{-1}) as compared with taurocyamine (appV_{max}^{tau}/appK_m^{tau} = 11.4) (Fig. 3). Thus, V71 is significantly associated with taurocyamine binding and recognition of guanidino substrates. Interestingly, in making a single amino acid change (*i.e.* V71A), *Arenicola* MiTK has been altered to behave as a GK. It is perhaps surprising that at least for the annelid enzymes MiTK (this work) and LK [22], the main target of guanidino substrates can easily be changed.

The residue V71 has been conserved in all CKs (Fig. 1) and CK has a slight activity for glycocyamine. The fact that the *Arenicola* MiTK V71A mutant form displayed a very strong activity for glycocyamine suggests that the V71 in CK might have a role in minimizing its GK activity. In agreement with this idea, Jourden et al. [32] reported that a 6-fold larger catalytic efficiency of CK for glycocyamine as compared with creatine was obtained by introducing eight amino acid mutations on the GS region, including V71T replacement.

4. Amino acid replacement of K95 in *Arenicola* MiTK with R, H, I, A, E or Y, and kinetic properties of the mutant proteins

Amino acid residue 95 is strictly conserved in various phosphagen kinases: Arg in CK, Ile in GK, Lys in LK, and Tyr in AK (Fig. 1). This residue is not directly associated with substrate binding as revealed by the CK and AK crystal structures. However, it is located close to the guanidino substrate-binding site [6,7,26], and it is known to be a determinant of guanidino substrate specificity [22,24,25]. Residue 95 is occupied by H and K in cytoplasmic and mitochondrial TKs, respectively.

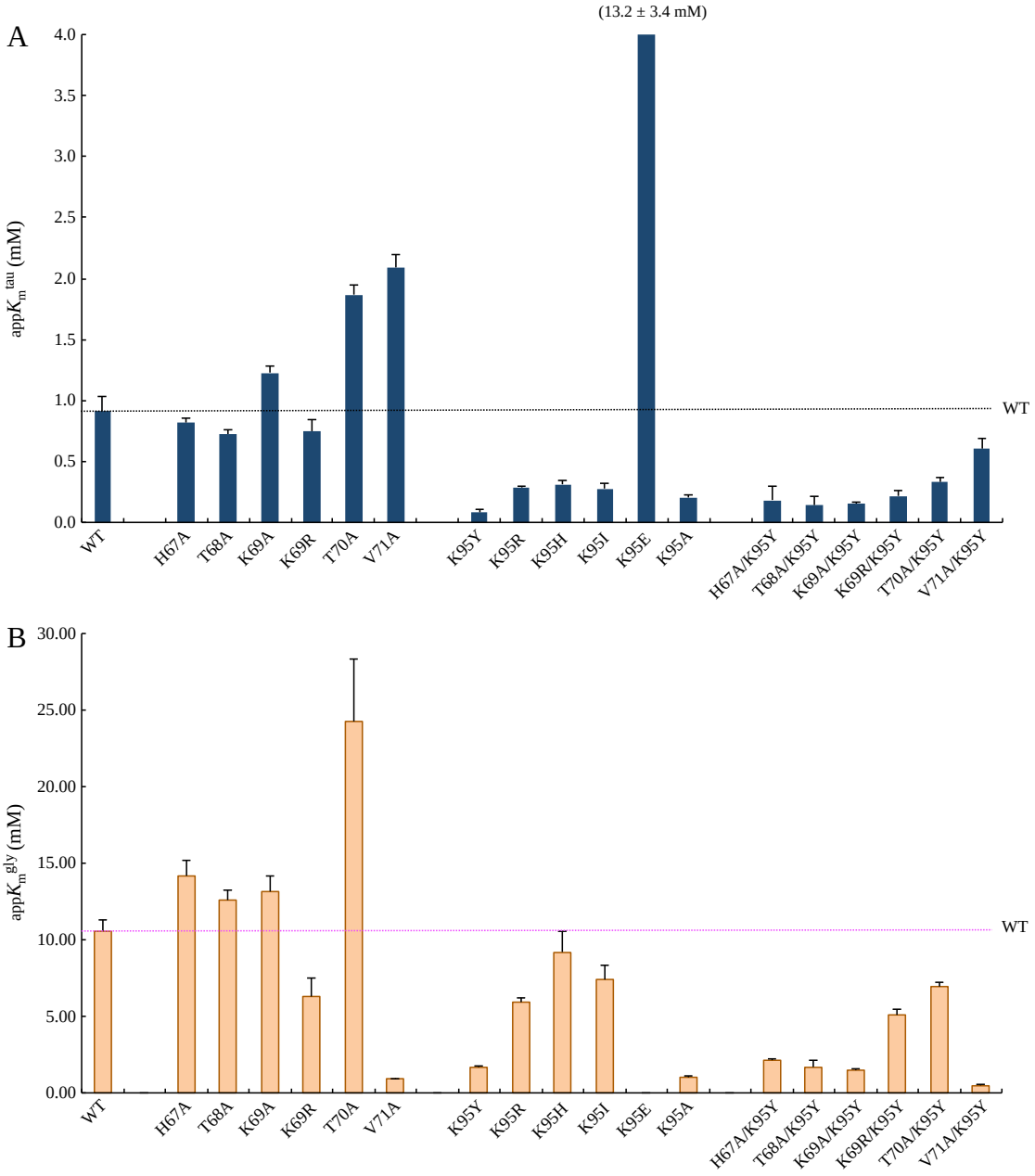


Fig. 2. Comparison of appK_m^{tau} and appK_m^{gly} in wild-type and mutant forms of *Arenicola* MiTK. (A) appK_m^{tau}, and (B) appK_m^{gly}.

We suggested recently that residue 95 in *Danio* CK and equivalent residues in other phosphagen kinases have dual roles [33]: (1) distinguishing guanidino substrates, and (2) 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 in each phosphagen kinase, reflecting evolution of each enzyme.

We next examined the role of amino acid residue K95 of *Arenicola brasiliensis* MiTK. K95 was replaced by R (CK type), H (cytoplasmic TK type), Y (AK type), I (GK type), A or E, and the activities of the resultant mutant proteins were determined for the substrates taurocyamine and glycocyamine (Table 2).

4.1. Arenicola MiTK K95R, K95H and K95I mutants

Three mutant forms of the MiTK protein, K95R, K95H and K95I, shared similar properties, *i.e.* 3-fold higher affinity for taurocyamine (appK_m^{tau} = 0.28–0.31 mM) as compared wild-type and a higher affinity for glycocyamine (appK_m^{gly} = 5.9–9.2 mM) than wild-type (Table 2 and Fig. 2). However, the appV_{max}^{tau} and appV_{max}^{gly} for the three mutants was decreased to 51–78% those of wild-type.

The catalytic efficiencies for the three mutants, as calculated by appV_{max}^{tau}/appK_m^{tau} (79.9–108 μmoles Pi•min^{-1} mg protein^{-1} mM^{-1}), were 2-fold higher than that of wild-type (47.2), and the appV_{max}^{gly}/appK_m^{gly} for these mutants (1.5–2.6) were comparable to that of wild-type (1.9).

Interestingly, replacement of K95 with R or H had a significant effect on the affinity for taurocyamine (3-fold higher than wild-type), an effect that was shared by the K95I mutation (Fig. 2). Thus, even another positively charged amino acid residue, *i.e.* R or H, cannot substitute for K95. This idea is further supported by the fact that residue 95 is strictly conserved among phosphagen kinases (see Fig. 1).

Finally, it is interesting to note that *Arenicola* MiTK and cytoplasmic TK have distinct residues (K or H, respectively) at residue

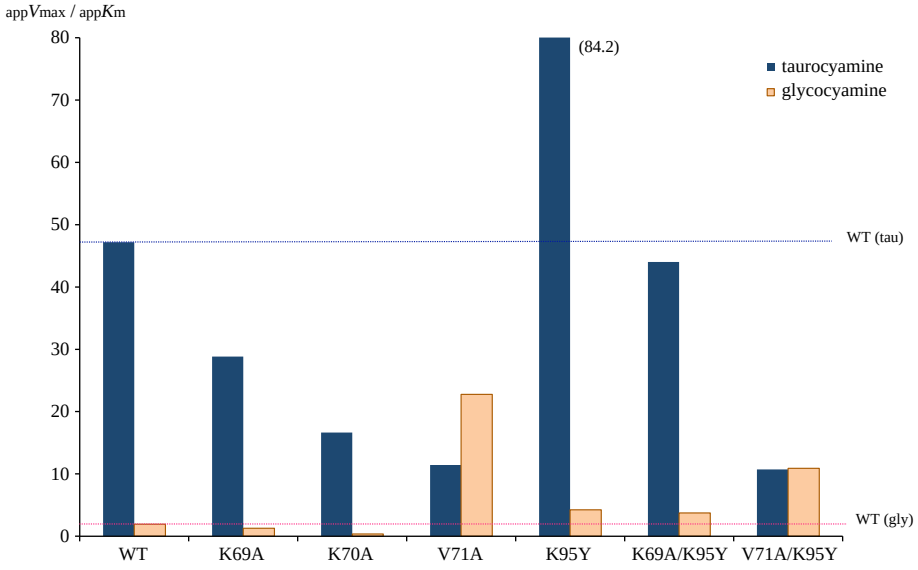


Fig. 3. Comparison of apparent catalytic efficiencies ($appV_{max}/appK_m$) in wild-type and mutant forms of *Arenicola* MiTK.

95, in addition to the unique sequences of their GS regions (see Fig. 1). This appears to be sufficient to explain the significant difference in responses of the two enzymes to guanidino substrates [34].

4.2. Arenicola MiTK K95A mutant

The K95A mutant protein showed a similar affinity for taurocyamine ($appK_m^{tau} = 0.21$ mM) as those observed for K95R, K95H and K95I, with a slightly lower $appV_{max}^{tau}$ value (15.9 μmoles Pi•min^{-1} mg protein^{-1}). However, K95A also showed a remarkable increase in affinity for glycocyamine ($appK_m^{gly} = 1.0$ mM, or 10-fold higher than the wild-type) and had an $appV_{max}^{gly}$ value of 14.6, resulting in 7.5-fold higher catalytic efficiency for glycocyamine than the wild-type (Table 2 and Fig. 2). We do not know the reason why the mutated Ala facilitates the binding of glycocyamine.

4.3. Arenicola MiTK K95E mutant

The K95E mutant protein showed an extraordinarily low affinity for taurocyamine ($appK_m^{tau} = 13.2$ mM, a 15-fold value that of wild-type) and its $appV_{max}$ (16.4 μmoles Pi•min^{-1} mg protein^{-1}) was lower than those observed for other mutant forms as described above (Table 2 and Fig. 2). This mutant form has almost completely lost activity for glycocyamine. Clearly, amino acid residue E, which has a negatively charged side chain, is unfavorable for binding of taurocyamine. This suggests that the SO_3^- group of taurocyamine is normally oriented towards residue 95, which is repulsed by the negatively charged side chain present at that position in the K95E form. Consistent with this, none of the phosphagen kinases has the negatively charged amino acids E or D at residue 95.

4.4. Arenicola MiTK K95Y mutant

For the substrate taurocyamine, the K95Y mutant showed a unique feature compared with the other mutants. Namely, we observed that in the taurocyamine concentration of more than 1 mM, an increase in substrate concentration caused a decrease in initial velocity of the reaction (substrate inhibition). This phenomenon was tentatively analyzed using the model of single substrate/substrate inhibition (uncompetitive) in the SigmaPlot software ($v = V_{max}/(1 + K_m/[S] + [S]/K_i)$), and the reaction curve was successfully fitted with the value of $R^2 = 0.99$. Although the mechanism for the substrate inhibition is unknown, identification of amino acid residues responsible for substrate inhibition in the K95Y mutant form will help to uncover the relative importance or roles of residues around the active center of *Arenicola* MiTK.

Tentative analyses of the K95Y mutant revealed that its affinity for taurocyamine is 10-fold higher ($appK_m^{tau} = 0.082$ mM) as compared with wild-type (Table 2 and Fig. 2). Further, K_i value, which might correspond to the dissociation constant for the second taurocyamine-binding site, was calculated to be 4.6 mM. The mutant showed 6-fold higher affinity for glycocyamine ($appK_m^{gly} = 1.68$ mM) with a lower $appV_{max}^{gly}$ (7.2 μmoles Pi•min^{-1} mg protein^{-1}) value, and substrate inhibition was not observed for glycocyamine.

To explore the unique feature of K95Y mutant, we used Ala scanning mutagenesis to alter the amino acid sequence H-T-K-K-T-V. In total, we constructed five double mutant forms of *Arenicola* MiTK*; i.e.* H67A/K95Y, T68A/K95Y, K69A/K95Y, T70A/K95Y and V71A/K95Y. The enzyme activities were then measured using taurocyamine and glycocyamine as substrates.

The three mutants H67A/K95Y, T68A/K95Y and T70A/K95Y retained substrate inhibition for taurocyamine, with K_i values of 5.1–6.4 mM (Table 2), a value comparable to that observed for the K95Y mutant (4.6 mM). Thus, the residues H67, T68 and T70 are not likely to be associated with substrate inhibition.

In contrast, substrate inhibition is abolished in the mutants K69A/K95Y and V71A/K95Y (Table 2). Thus, K69 and V71 are likely to be involved in the unique feature of K95Y mutant. To make clear the role of a positively charged side chain at K69, we constructed another double mutant, K69R/K95Y. This mutant still showed substrate inhibition, with a K_i value of 7.5 mM (Table 2). These data strongly suggested that the positive charge of K69 is responsible for substrate inhibition.

Among the double mutants described above, the V71A/K95Y mutant was particularly interesting, as it showed a 20-fold higher affinity for glycocyamine as compared with wild-type, and as for the V71A mutant its catalytic efficiency $appV_{max}^{gly}/appK_m^{gly}$ (10.9 μmoles Pi•min^{-1} mg protein^{-1} mM^{-1}), is very close to its $appV_{max}^{tau}/appK_m^{tau}$ (10.7) (Fig. 3).

5. Conclusion

Transition-state analog complex structures of *Limulus* AK and *Torpedo* CK have revealed the residues interacting with guanidino

substrates: S69, G70, V71 and Y74 in AK and V71 in CK, which are located on the GS region (see Fig. 1). In this work, we showed through Ala scanning mutagenesis that in *Arenicola* MiTK, K67, T70 and V71 are associated with binding of taurocyamine. It should be noted that V71 is relevant in the above-mentioned enzymes, AK, CK and MiTK, suggesting a common role in binding guanidino substrates among various phosphagen kinases. In *Arenicola* MiTK, residue 71 also appears to be associated with the ability to distinguish among various guanidino substrates, as the replacement of V71 by A71 caused a dramatic change in enzyme activity, shifting the protein from a TK to behaving like a GK.

Acknowledgement

This work was supported by Grants-in-Aid for Scientific Research in Japan to TS (17570062 and 20570072). We thank Dr. W. Ross Ellington of Florida State University for his helpful suggestions.

References

[1] M. Wyss, J. Smeitink, R.A. Wevers, T. Wallimann, Mitochondrial creatine kinase: a key enzyme of aerobic energy metabolism, Biochim. Biophys. Acta 1102 (1992) 119–166.
[2] T. Wallimann, M. Wyss, D. Brdiczka, K. Nicolay, H.M. Eppenberger, Intracellular compartmentation, structure and function of creatine kinase isoenzymes: the "phosphocreatine circuit" for cellular energy homeostasis, Biochem. J. 281 (1992) 21–40.
[3] W.R. Ellington, Evolution and physiological roles of phosphagen systems, Ann Rev Physiol 63 (2001) 289–325.
[4] U. Schlattner, M. Tokarska-Schlattner, T. Wallimann, Mitochondrial creatine kinase in human health and disease, Biochim. Biophys. Acta 1762 (2006) 164–180.
[5] K. Fritz-Wolf, T. Schnyder, T. Wallimann, W. Kabsch, Structure of mitochondrial creatine kinase, Nature 381 (1996) 341–345.
[6] S.D. Lahiri, P.F. Wang, P.C. Babbitt, M.J. McLeish, G.L. Kenyon, K.N. Allen, The 2.1 Å structure of torpedo californica creatine kinase complexed with the ADP–Mg^{2+}-NO3–Creatine transition-state analogue complex, Biochemistry 41 (2002) 13861–13867.
[7] G. Zhou, T. Somasundaram, E. Blanc, G. Parthasarathy, W.R. Ellington, M. Chapman, Transition state structure of arginine kinase : implications for catalysis of bimolecular reactions, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 8449–8454.
[8] K. Lim, S. Pullalarevu, K.T. Surabian, A. Howard, T. Suzuki, J. Moult, O. Herzberg, Structural basis for the mechanism and substrate specificity of glycocyamine kinase, a phosphagen kinase family member, Biochemistry 49 (2010) 2031–2041.
[9] D.J. Bush, O. Kirillova, S.A. Clark, O. Davulcu, F. Fabiola, Q. Xie, T. Somasundaram, W.R. Ellington, M.S. Chapman, The Structure of Lombricine Kinase: implications for phosphagen kinase conformational changes, J. Biol. Chem. 286 (2011) 9338–9350.
[10] J.F. Morrison, Arginine kinase and other invertebrate guanidino kinases, in: P.C. Boyer (Ed.), The Enzymes, Academic Press, New York, 1973, pp. 457–486.
[11] W.R. Ellington, T. Suzuki, Evolution and divergence of creatine kinases, in: C. Vial (Ed.), Molecular Anatomy and Physiology of Proteins — Creatine Kinase, NovaScience, New York, 2006, pp. 1–26.
[12] N. van Thoai, Homologous phosphagen phosphokinases, in: N.V. Thoai, J. Roche (Eds.), Homologous Enzymes and Biochemical Evolution, Gordon and Breach, New York, 1968, pp. 199–229.
[13] D.C. Watts, The origin and evolution of phosphagen phosphotransferases, in: N. van Thoai, J. Roche (Eds.), Homologous Enzymes and Biochemical Evolution, Gordon and Breach, New York, 1968, pp. 279–296.
[14] C. Mizuta, K. Tanaka, T. Suzuki, Isolation, characterization, and cDNA-derived amino acid sequence of glycocyamine kinase from the tropical marine worm Namalycastis sp, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 140 (2005) 387–393.
[15] N. van Thoai, S. Zappacosta, Y. robin, Biogenesis of 2 sulfurated guanidines: taurocyamine and hypotaurocyamine, Comp. Biochem. Physiol. 10 (1963) 209–225.
[16] R. Kassab, L.A. Pradel, L.A. Nguyen Van Thoai, ATP:taurocyamine and ATP: lombricine phosphotransferases Purification and study of SH groups. Biochim Biophys Acta. 99 (1965) 397–405.
[17] B. Surholt, Taurocyamine kinase from body-wall musculature of the lugworm *Arenicola marina*, Eur. J. Biochem. 93 (1979) 279–285.
[18] K. Uda, N. Saishoji, S. Ichinari, W.R. Ellington, T. Suzuki, Origin and properties of cytoplasmic and mitochondrial isoforms of taurocyamine kinase, FEBS J. 272 (2005) 3521–3530.
[19] K. Uda, K. Tanaka, X. Bailly, F. Zal, T. Suzuki, Phosphagen kinase of the giant tubeworm *Riftia pachyptila*. Cloning and expression of cytoplasmic and mitochondrial isoforms of taurocyamine kinase, Int. J. Biol. Macromol. 37 (2005) 54–60.
[20] K. Tanaka, K. Uda, M. Shimada, K. Takahashi, S. Gamou, W.R. Ellington, T. Suzuki, Evolution of the cytoplasmic and mitochondrial phosphagen kinases unique to annelid groups, J. Mol. Evol. 65 (2007) 616–625.
[21] W.R. Ellington, K. Roux, A.O. Pineda, Origin of octameric creatine kinases, FEBS Lett. 425 (1998) 75–78.
[22] K. Tanaka, T. Suzuki, Role of amino-acid residue 95 in substrate specificity of phosphagen kinases, FEBS Lett. 573 (2004) 78–82.
[23] T. Suzuki, Y. Kawasaki, T. Furukohri, W.R. Ellington, Evolution of phosphagen kinase. VI. Isolation, characterization and cDNA-derived amino acid sepuence of lombricine kinase from earthworm *Eisenia foetida*, and identification of possible candate for guanidine substrate recognition site, Biochim. Biophys. Acta 1348 (1997) 152–159.
[24] P.L. Edmiston, K.L. Schavolt, E.A. Kersteen, N.R. Moore, C.L. Borders, Creatine kinase: a role for arginine-95 in creatine binding and active site organization, Biochim. Biophys. Acta 1546 (2001) 291–298.
[25] K. Uda, T. Suzuki, Role of amino acid residues on the GS region of *Stichopus* Arginine Kinase and *Danio* Creatine Kinase, Protein J. 23 (2004) 53–64.
[26] M.S. Yousef, S.A. Clark, P.K. Pruett, T. Somasundaram, W.R. Ellington, M.S. Chapman, Induced fit in guanidino kinases—comparison of substrate-free and transition state analog structures of arginine kinase, Protein Sci. 12 (2003) 103–111.
[27] S. Putney, W. Herlihy, N. Royal, H. Pang, H.V. Aposhian, L. Pickering, R. Belagaje, K. Biemann, D. Page, S. Kuby, Rabbit muscle creatine phosphokinase. cDNA cloning, primary structure and detection of human homologues, J. Biol. Chem. 259 (1984) 14317–14320.
[28] T. Suzuk, H. Fukuta, H. Nagato, M. Umekawa, Arginine kinase from *Nautilus pompilius*, a living fossil. Site-directed mutagenesis studies on the role of amino acid residues in the Guanidino specificity region, J. Biol. Chem. 275 (2000) 23884–23890.
[29] J.F. Morrison, E. James, The mechanism of the reaction catalyzed by adenosine triphosphate-creatine phosphotransferase, Biochem. J. 97 (1965) 37–52.
[30] W.R. Ellington, Phosphocreatine represents a thermodynamic and functional improvement over other muscle phosphagens, J. Exp. Biol. 143 (1989) 177–194.
[31] W.R. Novak, P.F. Wang, M.J. McLeish, G.L. Kenyon, P.C. Babbitt, Isoleucine 69 and valine 325 form a specificity pocket in human muscle creatine kinase, Biochemistry 43 (2004) 13766–71374.
[32] M.J. Jourden, C.N. Clarke, A.K. Palmer, E.J. Barth, R.C. Prada, R.N. Hale, D. Fraga, M.J. Snider, P.L. Edmiston, Changing the substrate specificity of creatine kinase from creatine to glycocyamine: evidence for a highly evolved active site, Biochim. Biophys. Acta 1774 (2007) 1519–1527.
[33] K. Uda, A. Kuwasaki, K. Shima, T. Matsumoto, T. Suzuki, The role of Arg-96 in *Danio rerio* creatine kinase in substrate recognition and active center configuration, Int. J. Biol. Macromol. 44 (2009) 413–418.
[34] T. Suzuki, K. Uda, M. Adachi, H. Sanada, K. Tanaka, C. Mizuta, K. Ishida, W.R. Ellington, Evolution of the diverse array of phosphagen systems present in annelids, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 152 (2009) 60–66.