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Role of amino-acid residue 95 in substrate specificity of phosphagen kinases

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Abstract The purpose of this study is to elucidate the mechanisms of guanidine substrate specificity in phosphagen kinases, including creatine kinase (CK), glycocyamine kinase (GK), lombricine kinase (LK), taurocyamine kinase (TK) and arginine kinase (AK). Among these enzymes, LK is unique in that it shows considerable enzyme activity for taurocyamine in addition to its original target substrate, lombricine. We earlier proposed several candidate amino acids associated with guanidine substrate recognition. Here, we focus on amino-acid residue 95, which is strictly conserved in phosphagen kinases: Arg in CK, Ile in GK, Lys in LK and Tyr in AK. This residue is not directly associated with substrate binding in CK and AK crystal structures, but it is located close to the binding site of the guanidine substrate. We replaced amino acid 95 Lys in LK isolated from earthworm *Eisenia foetida* with two amino acids, Arg or Tyr, expressed the modified enzymes in *Escherichia coli* as a fusion protein with maltose-binding protein, and determined the kinetic parameters. The K95R mutant enzyme showed a stronger affinity for both lombricine ($K_m = 0.74$ mM and $k_{cat}/K_m = 19.34\ s^{-1}\ mM^{-1}$) and taurocyamine ($K_m = 2.67$ and $k_{cat}/K_m = 2.81$), compared with those of the wild-type enzyme ($K_m = 5.33$ and $k_{cat}/K_m = 3.37$ for lombricine, and $K_m = 15.31$ and $k_{cat}/K_m = 0.48$ for taurocyamine). Enzyme activity of the other mutant, K95Y, was dramatically altered. The affinity for taurocyamine ($K_m = 1.93$ and $k_{cat}/K_m = 6.41$) was enhanced remarkably and that for lombricine ($K_m = 14.2$ and $k_{cat}/K_m = 0.72$) was largely decreased, indicating that this mutant functions as a taurocyamine kinase. This mutant also had a lower but significant enzyme activity for the substrate arginine ($K_m = 33.28$ and $k_{cat}/K_m = 0.01$). These results suggest that *Eisenia* LK is an inherently flexible enzyme and that substrate specificity is strongly controlled by the amino-acid residue at position 95.

Keywords: Phosphagen kinase; Lombricine kinase; Creatine kinase; Arginine kinase; Substrate specificity; *Eisenia foetida*

1. Introduction

Members of the phosphagen kinase enzyme family play 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 naturally occurring guanidine compounds, producing phosphorylated high-energy guanidine compounds referred to as phosphagens.

$$Mg^{2+}ATP + \text{natural guanidine} \overset{\text{PhosphagenKinase}}{\Longleftrightarrow} Mg^{2+}ADP + \text{phosphagen}$$

While phosphocreatine and creatine kinase (CK) is the only phosphagen and phosphogen kinase pair found in vertebrates, various phosphagens and corresponding enzymes are found in invertebrates [1,4]: phosphocreatine and CK; phosphoglycocyamine and glycocyamine kinase (GK); phosphotaurocyamine and taurocyamine kinase (TK); phospholombricine and lombricine kinase (LK); and phosphoarginine and arginine kinase (AK). Although the evolutionary processes are not fully understood, enzymes CK, GK, TK and LK appear to have evolved from a common ancestor [5–7] and the cytoplasmic forms of these four enzymes are known to have a conventional dimeric structure consisting of two 40 kDa subunits. The structure and function of CK has been well characterized and the presence of at least three isoforms (cytoplasmic, flagellar and mitochondrial) has been confirmed in the ancestral CK gene [8]. The functional properties of GK, TK and LK are not well known and these enzymes are found only in annelid-like worms. Of these phosphagen kinases, AK is the most widely distributed in invertebrates; its activity has been identified in protozoa [9] and its gene has been detected in the genomic databases of *Paramecium* and *Tetrahymena*, suggesting that AK has an ancient origin.

Recent transition state analog complex structure analysis of *Limulus* AK [10] and *Torpedo* cytoplasmic CK [11] has clarified the substrate-binding sites between guanidine compounds (arginine or creatine) and ATP. This structural information is useful for determining the substrate-binding site characteristics of other phosphagen kinases, GK, TK and LK, whose structures have not yet been resolved, and for elucidating how the guanidine substrate recognition system developed during phosphagen kinase evolution.

Based on previously published amino acid sequence alignments of CK, GK, LK and AK, we proposed that the guanidine specificity (GS) region, which displays remarkable amino acid deletions, is a possible candidate for the guanidine-recognition site [7] (see the boxed region in Fig. 1). Within the GS region, there is a proportional relationship between the size of the amino-acid deletion and the mass of the corresponding guanidine substrate [7]: LK and AK have a five-amino-acid deletion in this region and recognize relatively large guanidine substrates (lombricine and arginine), CK has a one-amino-acid

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Fig. 1. Alignment of amino-acid sequences around GS region of CK, GK, LK and AK. The GS region is enclosed in a box. Amino-acid residue 95 is indicated by an arrow.

deletion, and GK, which uses the smallest guanidine substrate, glycocyamine, has no deletions. The GS region is partly overlapped by a so-called flexible loop in the crystal structures of chicken and *Torpedo* CKs [11,12] and *Limulus* AK [10]. In addition, we earlier proposed that many candidate amino acids play a role in distinguishing guanidine substrates [6]. One of these, amino-acid residue 95, is strictly conserved in various phosphagen kinases: Arg in CK, Ile in GK and Tyr in AK (Fig. 1). While this residue is not directly involved in substrate-binding in CK and AK crystal structures, it is located close to the guanidine substrate-binding site (Fig. 2).

LK is found only in earthworms and the echiuroid worms [13] and the LK amino-acid sequences are known for representative species, *Eisenia foetida* [7] and *Urechis caupo* [14], respectively. In contrast to very high guanidine substrate specificity observed for CK and AK, that for LK is low. LK shows considerable activity for taurocyamine (about 1/3 that of the main target substrate, lombricine) [7,15,16] and in some cases, weak activity for arginine [17]. However, this characteristic appears to be almost physiologically silent, because the species containing LK do not contain taurocyamine [18]. Thus, LK appears to be an excellent model enzyme to elucidate the control mechanisms of guanidine substrate binding.

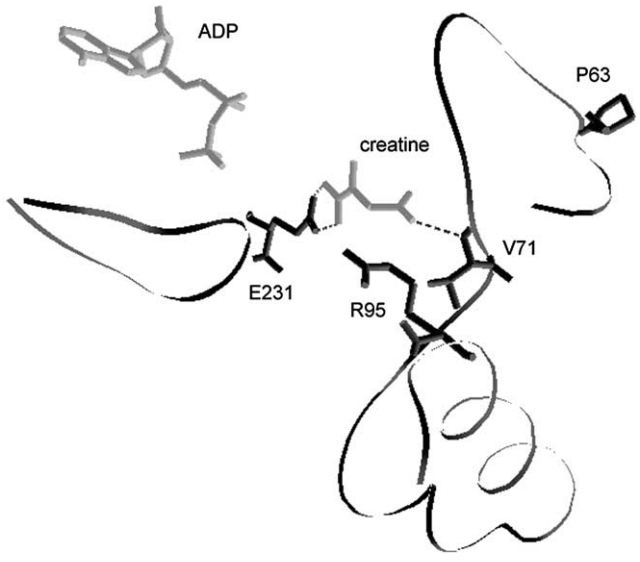

Fig. 2. Crystal structure of the region around the substrate binding site of *Torpedo* CK [11]. The substrate creatine is interacting with V71 and E231, while R95 is located close to creatine. This figure was made with Swiss-Pdb-Viewer (http://kr.expasy.org/spdbv/).

In the present study, we cloned the cDNA of *Eisenia* LK in pMAL plasmid and expressed it in *E. coli* as a fusion protein with maltose-binding protein (MBP). We also constructed two mutant LKs, K95Y and K95R, determined the kinetic parameters of each mutant, and compared these parameters with those of the wild-type enzyme. Of particular interest, the K95Y mutation produced a dramatic change in guanidine substrate specificity, resulting in a shift of substrate specificity from lombricine to taurocyamine.

Throughout this paper, the sequence numbering of rabbit muscle CK [19] is used.

2. Materials and methods

2.1. Purification of lombricine and other guanidine substrates

Lombricine was purified as a natural product from the body wall muscle (about 1000 g) of *Pheretima sieboldi* as described by Hoffmann [17] with some modifications. The lombricine was identified according to the method of Voges-Proskauer [20] and the concentration was determined on the basis of standard curve calibrated on various concentrations of L-arginine.

Other guanidine compounds, taurocyamine, creatine, glycocyamine and arginine, were purchased from Wako (Tokyo, Japan).

2.2. Cloning, site-directed mutagenesis and expression of Eisenia foetida LK

The open reading frame of 1116 base pairs (bp) of *Eisenia* LK [7] was amplified with the two primers (TTGGATCCATGCCGAAGTTCACCGCTCG and TTGGATCCCTAGCCCTTGAGGCTCGCAGG: *Bam*HI site underlined) and cloned into the *Bam*HI site of pMAL-c2 (pMAL/*Eisenia* LK-wild).

Polymerase chain reaction (PCR)-based mutagenesis was done as described previously [21,22]. The mutations (deletion of T62, T62G63 to TGG, G63D, G63R, K95Y and K95R) were introduced into the

template of pMAL/*Eisenia* LK-wild by PCR using mutation-primers (for deletion of T62, GGTCGCATCATCGGATTAGTC and GTTGTCGACAGAGGGCTGAATG; for T62G63 to TGG, GCGGTCGCATCATCGGATTAG and CGGTGTTGTCGACAGAGGG; for G63D, ATCGCATCATCGGATTAGTC and CGGTGTTGTCGACAGAGGG; for G63R, CGTCGCATCATCGGATTAGTC and GGTGTTGTCGACAGAGGG; for K95Y, TCACGGAGGGTTCGGACC and TACTCGTTGATGACGGCATC; for K95R, GACACGGAGGGTTCGGACC and TCTCGTTGATGACGGCATC: mutated sequence underlined). KOD$^+$ DNA polymerase (TOYOBO, Tokyo, Japan) was used as the amplifying enzyme. The PCR products were digested with *Dpn*I 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-*Eisenia* LK fusion protein was expressed in *E. coli* TB1 cells by induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 25, 22 °C (for T62G63 to TGG mutant) or 20 °C (for deletion of T62 mutant) for 24 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.

2.3. Enzyme assay

Enzyme activity was measured with an NADH-linked assay at 25 °C [1,29,23] 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 guanidine substrate made up in 100 mM imidazole/HCl (pH 7). The initial velocity values were typically obtained by varying the concentration of one substrate (guanidine) versus five fixed concentrations of the second substrate (ATP), resulting in a 7×5 matrix. Protein concentration was estimated from the absorbance at 280 nm (0.77 AU at 280 nm in a 1-cm cuvette corresponds to 1 mg protein/ml). To estimate kinetic constants, a Lineweaver–Burk plot was made and fitted by the leastsquare method using Microsoft Excel. The kinetics of phosphagen kinase can be explained as a random-order, rapid-equilibrium kinetic mechanism [29], and the K_d, the dissociation constant in the absence of one substrate, is obtained graphically [24] or by fitting data directly according to the method of Cleland [30], using the software written by Dr. R. Viola (Enzyme kinetics Programs, ver. 2.0).

3. Results and discussion

3.1. Expression of Eisenia foetida recombinant LKs and their kinetic parameters

All of the recombinant enzymes, with the exception of the T62 deletion mutant, were expressed as soluble proteins, successfully purified by affinity choromatography, and confirmed to be highly purified by SDS–PAGE. An amount of recombinant enzyme sufficient for analysis was not obtained for the T62 deletion mutant due to its instability.

The kinetic parameters, K_m, V_{max} or V, and k_{cat} of the forward reaction (phosphagen formation) were determined for the recombinant wild-type and mutant enzymes for each of the five different guanidine substrates (lombricine, taurocyamine, arginine, glycocyamine and creatine). Detailed parameters were obtained for the substrate taurocyamine (Table 1) due to a limited amount of lombricine available. K_d values were obtained only for wild-type LK and K95R mutant with the substrate taurocyamine. To compare the relative substrate specificity among recombinant enzymes, a catalytic efficiency (k_{cat}/K_m) was also calculated (Table 1).

3.2. Characteristics of Eisenia recombinant LK (wild-type)

In addition to its original target substrate lombricine, native LK also recognizes taurocyamine [7,15,16] and in some cases, arginine [17], with the activity for taurocyamine being about 1/3 that for lombricine [7]. K_m and V_{max} for the recombinant *Eisenia* wild-type LK with substrate lombricine were determined to be 5.33 ± 0.67 mM and 26.6 ± 0.35 μmol Pi/min mg of protein ($k_{cat}/K_m = 3.37\ s^{-1}\ mM^{-1}$), respectively, and those with substrate taurocyamine were 15.31 ± 0.75 mM and 10.92 ± 0.67 μmol Pi/min mg of protein ($k_{cat}/K_m = 0.48$), respectively (Table 1). Thus, recombinant *Eisenia* wild-type LK shows strong activity for both lombricine and taurocyamine, with the $K_m^{Lombricine}/K_m^{Taurocyamine}$ ratio of 0.35 being comparable to 0.29 for native *Eisenia* LK [7]. In this study, recombinant *Eisenia* LK did not show any activity for substrates such as creatine, glycocyamine and arginine. By contrast, native *Tubifex* LK is reported to show a low level of activity for arginine [17].

CK and AK undergo a large conformational change upon substrate binding (*open* to *closed* structure) [25,26]. We assume that this conformational change is reflected in the apparent kinetic parameters K_m (the value comparable to the dissociation constant of a guanidine substrate in the presence of ATP) and K_d (the dissociation constant of a guanidine substrate in the absence of ATP) [24]. We determined the K_m and K_d for recombinant *Eisenia* LK with the substrate taurocyamine to be 15.31 ± 0.75 and 81.79 ± 7.97 mM, respectively (Table 1). The K_d/K_m ratio of 5.3 is comparable to those (3–7) observed for CK and AK [27], suggesting that a synergism or a large conformational change also occurs in LK upon substrate binding, provided *Eisenia* LK has a homologous three-dimensional structure with CK and AK.

3.3. Importance of GS region amino acid residues on substrate recognition in Eisenia LK

In previous papers [21,22,27], we proposed that the GS region, which displays remarkable amino acid deletions, is a possible candidate for the guanidine-recognition site (Fig. 1) [7]. Therefore, as a first step, we introduced several amino-acid mutations in the GS region: deletion of T62, insertion of Gly (T62G63 to TGG), G63D and G63R.

The T62-deletion mutant appeared to be structurally unstable and statistically useful kinetic parameters could not be obtained. Nonetheless, this mutant appears to have a very low V_{max} compared to the wild-type enzyme (Table 1). The TGG mutant showed a 2-fold reduction in substrate affinity for taurocyamine ($K_m = 32.75$ mM) accompanied by decreased V_{max} (2.46 μmol Pi/min/mg of protein) and k_{cat}/K_m (0.05) (Table 1), indicating that the length of the GS region and the size of the guanidine substrate are directly related. In addition, the TGG mutant showed lower enzyme activities for arginine and glycocyamine (Table 1).

We replaced Gly at position 64 in the GS region with charged amino acids, Asp or Arg to produce G63D and G63R mutants, respectively. The kinetic parameters for these two mutants were comparable, those of the wild-type enzyme (Table 1). Thus, a single amino acid replacement in the GS region of *Eisenia* LK does not appear to have a significant affect on guanidine substrate affinity. It must be noted that

Table 1
Comparison of kinetic parameters of *Eisenia* wild-type and mutant LKs with those of *Tubifex* LK for the forward reaction

Species	Origin	Lombricine				Taurocyamine						Arginine			Glyco-cyamine	Creatine
		K_m^{Lomb}	k_{cat}	V_{max}	k_{cat}/K_m^{Lomb}	K_m^{Tauro}	K_d^{Tauro}	K_m^{ATP}	K_d^{ATP}	k_{cat}	V_{max} or V^*	k_{cat}/K_m^{tauro}	K_m^{Arg}	k_{cat}	V_{max} or V^*	k_{cat}/K_m^{Arg}	V	V
Tubifex	Native [19]	4.3		112									5.1		22			
Eisenia	Native [8]	13.16				44.44									N.A.			
	Recombinant WT	5.33 ± 0.67	17.73 ± 0.24	26.60 ± 0.35	3.37 ± 0.38	15.31 ± 0.75	81.79 ± 7.97	0.29 ± 0.056	1.67 ± 0.33	7.28 ± 0.45	10.92 ± 0.67	0.48 ± 0.02			N.A.		N.A.	N.A.
	Deletion of T63	–	–	–	–	–	–	–	–	–	0.014*	–	–	–	–	–	–	–
	T63G68 to TGG	16.25	12.09	18.14	0.74	32.75 ± 4.11	–	–	–	1.64 ± 0.14	2.46 ± 0.21	0.051 ± 0.006	–	–	0.011*	–	0.067	N.A.
	G68D	13.3	22.06	33.1	1.66	23.98 ± 0.77	–	–	–	4.64 ± 0.06	6.95 ± 0.10	0.19 ± 0.008	–	–	0.008*	–	0.004	0.003
	G68R	–	–	–	–	17.31 ± 0.5	–	–	–	5.53 ± 0.26	8.29 ± 0.39	0.32 ± 0.008	–	–	0.012*	–	N.A.	0.006
	K95Y	14.15 ± 2.12	9.95 ± 0.68	14.92 ± 1.02	0.72 ± 0.15	1.93 ± 0.07	–	–	–	12.35 ± 0.51	18.52 ± 0.77	6.41 ± 0.06	33.28 ± 0.90	0.37 ± 0.002	0.56 ± 0.003	0.01 ± 0.0003	0.095	N.A.
	K95R	0.74 ± 0.10	14.00 ± 0.64	21.00 ± 0.96	19.34 ± 2.59	2.67 ± 0.08	21.00 ± 1.60	0.29 ± 0.04	2.20 ± 0.32	7.47 ± 0.61	11.21 ± 0.92	2.81 ± 0.31	–	–	0.070*	–	0.078	N.A.

K_m and K_d in mM, V_{max} and V in mmol Pi/min mg protein, k_{cat} in s^{-1}, and k_{cat}/K_m in s^{-1} mM^{-1}. N.A., no activity; –, not determined.
*Activity was measured in a final concentration of 13.3 mM for glycocyamine, 133 mM for arginine, and 66.7 mM for creatine.

these mutants also show weak activity for arginine, creatine and glycocyamine (only for G63D) (Table 1).

3.4. Amino acid 95 is a key residue for distinguishing guanidino substrates

Amino-acid residue 95 is strictly conserved in various phosphagen kinases: Arg in CK, Ile in GK, Lys in LK and Tyr in AK. We previously suggested that this residue is one of the many candidates involved in the recognition of specific guanidino substrates [6]. We replaced 95-Lys in *Eisenia* LK with Tyr, a residue characteristic of AK. In the K95Y mutant, the affinity for lombricine ($K_m = 14.15 \pm 2.12$ mM) decreased to 1/3 that of the wild-type (the $k_{cat}/K_m^{Lombricine}$ decreased 1/5 that of the wild type), and the affinity for taurocyamine ($K_m = 1.93 \pm 0.07$ mM) increased 8-fold (the $k_{cat}/K_m^{Taurocyamine}$ increased 13-fold) (Table 1), indicating that this mutant shows higher substrate affinity for taurocyamine than for lombricine. Thus, this mutant functions as a TK. This dramatic, functional conversion of the mutant and wild-type enzymes can be clearly demonstrated by comparing their catalytic efficiency k_{cat}/K_m values. As seen in Fig. 3, the K95Y mutant shows an inverse character to that of wild-type LK with the catalytic efficiencies for the substrates lombricine (black) and taurocyamine (gray). In addition, the mutant form shows weak, but significant activity for arginine ($K_m = 33.28$, $V_{max} = 0.56$, $k_{cat}/K_m = 0.01$) and for glycocyamine ($V = 0.095$). It is clear that considerable activity for arginine cannot be achieved simply by replacing residue 95 in *Einenia* LK with the residue typical for AK (Tyr).

Next, we replaced the 95 Lys by Arg, a residue typical of CK. The K95R mutant showed higher affinities for both lombricine (7-fold of the wild-type) and taurocyamine (5-fold) (Table 1). The $k_{cat}/K_m^{Lombricine}$ and $k_{cat}/K_m^{Taurocyamine}$ values also increased about 6-fold, compared with those of wild-type (Table 1 and Fig. 3). The mutant also showed weak activity for arginine ($V = 0.07$) and glycocyamine ($V = 0.078$). These results clearly indicate that *Eisenia* wild-type LK enzyme does not have the highest potential affinity for its natural substrate, lombricine. The amino-acid sequence of *Eisenia* LK is apparently designed to diminish the affinity for lombricine, in order to adapt to the physiological conditions of a worm and to suppress the other activities of other phosphagen kinases, such as AK, GK and CK. In the K95R mutant, CK activity was not detected. This may be due to the unique structure of creatine, which is the only guanidine substrate with a methyl group. The presence of the methyl group has a large effect on the thermodynamic properties of phosphocreatine and distinguishes the CK/creatine system from other phosphagen systems from a physiological point of view [23]. Further refinement of the amino acid groups surrounding creatine and the methyl group will be necessary to generate sufficient CK activity in mutant enzymes.

Similar site-directed mutagenesis studies on the role of residue 95 using CK and AK have been conducted. Edmiston et al. [28] reported that R95Y, R95A and R95K mutants of rabbit muscle CK produced remarkably reduced CK activity. On the other hand, Uda and Suzuki [27] demonstrated a 70% decrease in V_{max} for the Y95R mutant of *Stichopus* AK. These reports, together with our results, indicate that amino acid 95 is a key residue in the guanidine substrate recognition system. Finally, guanidine substrate specificity in phosphagen kinases is likely to be controlled by several regions and amino acids, including the GS region and amino acid residue 95.

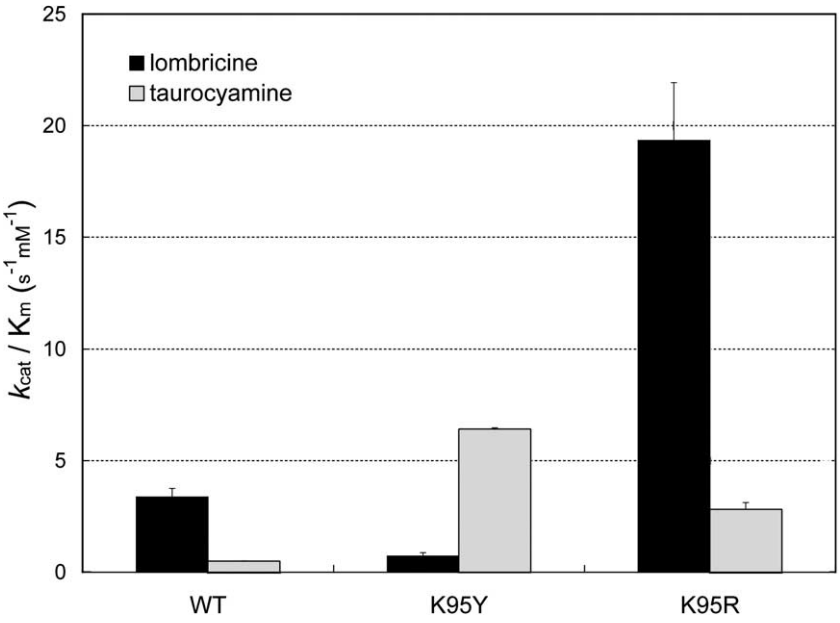

Fig. 3. Comparison of the k_{cat}/K_m values in *Eisenia* wild-type LK, K95Y and K95R mutants for the substrate lombricine (black) and for taurocyamine (gray).

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