



## Identification of the key amino acid residues in *Sabellastarte* arginine kinase for distinguishing chiral guanidino substrates (D- and L-arginine)

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### ABSTRACT

Arginine kinase (AK, EC 2.7.3.3) is a phosphagen kinase widely distributed in lower and higher invertebrates. *Sabellastarte indica* AKs (AK1 and AK2) are the only AKs that use D-arginine in addition to L-arginine as a substrate. *S. indica* AKs are therefore clearly unrelated to typical AK. The purpose of this work was to identify the amino acid residues responsible for distinguishing the chirality of D- and L-arginine. The three-dimensional structure of *Sabellastarte* AK2 was predicted using the SWISS-MODEL automated modeling server. Two amino acids, L64 in the N-terminal flexible loop and N320 in the C-terminal flexible loop, were found to be closest to the substrate arginine. We introduced several mutations around the predicted binding site of the substrate arginine. In the L64I mutant, the affinity for L-arginine was greatly increased (9.5-fold that of the wild-type), whereas its affinity for D-arginine was increased 2.9-fold, indicating that the L64I mutant enzyme was more specific for L-arginine. On the other hand, the L64V mutant showed a 1.7-fold decrease in affinity for L-arginine, but unchanged affinity for D-arginine. Thus the L64V mutant enzyme was more specific for D-arginine. Since the replacement of amino acid residue L64 by I or V significantly affected the affinity for L-arginine or D-arginine, it can be concluded that amino acid 64 is a key residue for distinguishing D- and L-arginine. Seven mutants at position 320 (N320H, Q, D, E, R, K and A) had considerably reduced enzymatic activity (0.05–52% of the wild-type). The reduction in enzyme activity was more significant when D-arginine was the substrate rather than L-arginine, except for N320D, suggesting that the carbonyl oxygen of the Asn320 side chain forms a hydrogen bond with the  $\alpha$ -amino nitrogen attached to the asymmetric carbon of the D-arginine substrate. In addition, we found that even a residue remote from the guanidino substrate-binding site, such as G54 and Y89 in *Sabellastarte* AK2, significantly affected guanidino substrate specificity.

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### 1. Introduction

Phosphagen (guanidino) kinases catalyze the reversible transfer of the high-energy phosphoryl group of ATP to naturally occurring guanidino compounds. 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 [1–3]. The phosphorylated high-energy guanidines are referred to as phosphagens. In vertebrates, phosphocreatine is the only phosphagen, and the corresponding phosphagen kinase is creatine kinase (CK). In contrast, invertebrates have various phosphagens in addition to phosphocreatine: phosphoglycocyanine (catalyzed by glycocyanine kinase: GK), phosphotaurocyanine (taurocyanine kinase:

TK), phosphohypotaurocyanine (hypotaurocyanine kinase: HTK), phospholombicine (lombicine kinase: LK) and phosphoarginine (arginine kinase: AK). Phosphagen kinases are phylogenetically separated into two distinct groups: the AK group which includes AK and HTK, and the CK group which includes GK, LK and TK [4].

Typical AKs are most widely distributed among organisms such as arthropods, molluscs, nematodes, cnidarians, poriferae, protozoans (ciliates and choanoflagellates) and bacteria, indicating their ancient origin [5–8]. Most AKs are monomers with an average molecular mass of approximately 40 kDa [2]. Unusual dimeric AKs have been isolated from the deuterostome echinoderm, *Stichopus japonicus* [9,10] and the protostome annelid, *Sabellastarte indica* [11], which evolved secondarily and independently from CK-like ancestors [12].

We recently reported that, although typical AKs and the unusual *Stichopus* AK display activity only for L-arginine, two AK isoforms (AK1 and AK2) isolated from *Sabellastarte* showed activity for both L-arginine and D-arginine. The activity for D-arginine was weak in AK1, but stronger in AK2 (50% that of L-arginine). In addition, D-arginine and D-phosphoarginine were isolated directly

Abbreviations: AK, arginine kinase; CK, creatine kinase; GK, glycocyanine kinase; GS region, guanidine specificity region; LK, lombicine kinase; TK, taurocyanine kinase; TSAC, transition-state analog complex.

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from *Sabellastarte* at a high concentration (3–4 mmol/kg). From these results we concluded that, in *Sabellastarte*, D-arginine is the major phosphagen substrate and AK2 catalyzes the synthesis of D-phosphoarginine [11].

Previous amino acid sequence alignments of phosphagen kinases indicated that the guanidino specificity (GS) region can contain significant amino acid deletions, making it a likely candidate for the guanidine-recognition site [13]. For example, LK and AK have a five amino acid deletion in this region and use relatively large guanidine substrates (lombrocin and arginine), CK has a one-amino acid deletion, and GK has no deletion. Additionally, the GS region is overlapped by the flexible loop in the N-terminal domain of *Torpedo* CK [14] and *Limulus* AK [15]. Unlike typical AKs, *Sabellastarte* AK1 and AK2 have three and two amino acid deletions in the GS region, respectively. This is in sharp contrast with the dimeric *Stichopus* AK, which evolved from a cytoplasmic CK gene [16], which has a five amino acid deletion, just like typical AKs. In addition, although the amino acid identity in the GS region for a specific enzyme is generally very high, *Sabellastarte* AK1 and AK2 show low similarity with other AKs [11].

In this work, we predicted the three-dimensional structure of *Sabellastarte* AK2 using SWISS-MODEL, and also estimated the positions of the guanidino substrates, chiral arginines. To identify the key residues responsible for distinguishing the chiral arginines, we introduced several mutations around the arginine binding site. Here we describe the importance of several amino acid residues: L64 and N320 (located closest to the substrate arginine), Y89 (the key residue for guanidino substrate recognition), and G54 (located at the beginning of the GS region).

## 2. Materials and methods

### 2.1. Site-directed mutagenesis and expression of *S. indica* AK2

*S. indica* was collected from the seashore of Tosa city, Kochi, Japan. The internal organs were removed immediately from living *S. indica*, and the body wall musculature was frozen under liquid-nitrogen. mRNA was isolated from the musculature, and the cDNA for *S. indica* AK2 was amplified by PCR [11]. The open reading frame of 1134 base pairs of the AK2 cDNA (GenBank: accession No. AB186406) was cloned into pMAL-c2 [11]. The pMAL-c2 plasmid was used as template of the mutagenesis in this study. Polymerase chain reaction (PCR)-based mutagenesis was done as described previously [16]. The mutations (L64 to I, V, G and A, N320 to Q, D, E, R, K and A, Y89 to Q, and G54 to S, A, V, L and I) were introduced into the template by PCR using mutation-primers shown in sTable 1 (supplementary material). *KOD*<sup>+</sup> DNA polymerase (TOYOBO, Tokyo, Japan) was used as the amplifying enzyme. The PCR products were digested with *DpnI*, and the target DNA fragment (8000 bp) was recovered by EasyTrap Ver. 2 (TaKaRa, Tokyo, Japan). After blunting and ligation, the DNA was self-ligated. The cDNA insert was completely sequenced to confirm that only the intended mutations were introduced.

The maltose-binding protein (MBP)–*Sabellastarte* AK2 fusion protein was expressed in *Escherichia 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] and determined for the forward reaction (phosphagen synthesis). The reaction mixture (total 1.0 ml) for determination of  $K_m^{\text{Arg}}$  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 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 L- or D-arginine made up in 100 mM Tris/HCl (pH 8). The initial velocity values were obtained by varying the concentration of L- or D-arginine under the fixed concentrations of the ATP.

The reaction mixture (total 1.0 ml) for determination of  $K_m^{\text{ATP}}$  contained 0.7 ml of 200 mM L- or D-arginine made up in 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), and 0.05 ml of recombinant enzyme. The reaction was started by adding 0.05 ml of an appropriate concentration of ATP made up in mM imidazole/HCl (pH 7).

Protein concentration was estimated from the absorbance at 280 nm. The extinction coefficient at 280 nm in  $\text{M}^{-1} \text{cm}^{-1}$  (or mg/ml) was obtained using ProtParam (available from the URL <http://ca.expasy.org/tools/protparam.html>). The extinction coefficient of the Y89Q mutant was calculated based on the mutated amino acid sequence. The protein concentration for the AK enzyme moiety was obtained by excluding the portion of the MBP tag. The kinetic parameters were determined by our previous method [18].

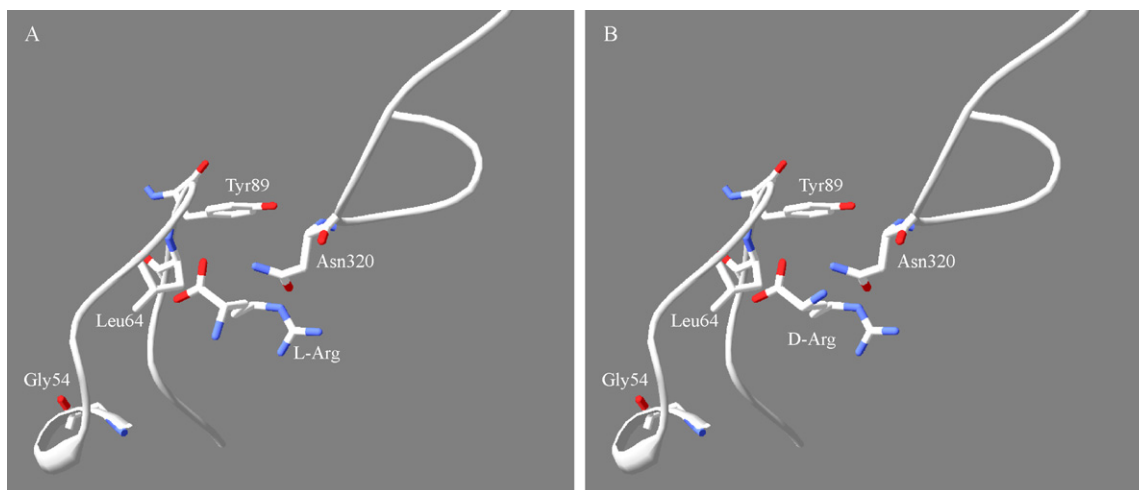
### 2.3. Modeling of three-dimensional structures

The three-dimensional structure of *Sabellastarte* AK2 was generated using the SWISS-MODEL automated modeling server (<http://www.expasy.org/swissmod/SWISS-MODEL.html>; the First Approach Method set at default parameters). Swiss-PdbViewer version 4.0.1 was used to generate a three-dimensional image [19]. In order to predict the L-arginine binding site, the three-dimensional structure of *Sabellastarte* AK2 was superimposed on the transition-state analogue complex (TSAC) structure of *Limulus* AK (PDB number: 1BG0) [15]. The binding site for D-arginine was predicted by fitting it with L-arginine.

## 3. Results and discussion

### 3.1. Prediction of the three-dimensional structure of *Sabellastarte* AK2

The three-dimensional structure of *Sabellastarte* AK2 was generated by SWISS-MODEL based on the TSAC structures of *Limulus* AK and *Torpedo* CK, both of which have a similar substrate-binding site [14,15]. Then we estimated the position of the substrate arginine in *Sabellastarte* AK2 by overlapping the position of L-arginine in the TSAC structure of *Limulus* AK (Fig. 1). The predicted structure indicated that the L- or D-arginine substrate is close to L64 in the N-terminal flexible loop (GS region) and N320 on the C-terminal flexible loop. Y89, which is proposed to be the key residue for guanidino substrate recognition [20,21], is also near the substrate arginines.



**Fig. 1.** Three-dimensional structure of *Sabellastarte* AK2 docked to the substrates L-arginine (A) and D-arginine (B). The structure was generated by SWISS-MODEL and drawn with Swiss-PdbViewer [19].

### 3.2. Expression of *Sabellastarte* AK2 wild-type and mutants, and their kinetic parameters

Each recombinant enzyme was expressed as a soluble protein fused with maltose-binding protein (MBP) and purified to a highly pure state by affinity chromatography. Of the 16 mutants constructed in this study, only five (L64I, L64V, Y89Q G54S and G54A) had sufficient activity to allow estimation of their kinetic parameters. The kinetic parameters ( $K_m$ ,  $k_{cat}$  and  $k_{cat}/K_m$ ) of the wild-type and five mutant enzymes in the presence of either L- or D-arginine were determined for the forward reaction, and are listed in Tables 1 and 3. Seven other mutants at position 320 showed weak enzymatic activity, so only the initial velocities were measured in 4.76 mM ATP and 4.76 mM L- or D-Arginine (Table 2). The remaining five mutants (L64G, L64A, G54V, G54L and G54I) displayed undetectable enzymatic activity under the same conditions.

Phosphagen kinases usually show activity only for one guanidino substrate, whereas *Sabellastarte* AKs display activities for at least four guanidino substrates. *Sabellastarte* AK1 is primarily active towards L-arginine, but has weak but significant activity for D-arginine (7.6% compared to L-arginine), D-lombicine (3.0%) and taurocyamine (2.7%). On the other hand, *Sabellastarte* AK2 shows strong activity towards both L-arginine (100% relative activity) and D-arginine (35% relative activity), but shows rather weak activities for D-lombicine (0.2%) and taurocyamine (0.1%) [22]. This large difference in substrate specificity is caused by the difference (30%) in amino acid sequence of the GS region of *Sabellastarte* AK1 and AK2.

The wild-type AK2 enzyme of *Sabellastarte* shows 2.4-fold higher substrate affinity for L-arginine ( $K_m^{L-Arg} = 3.69$  mM) than for D-arginine ( $K_m^{D-Arg} = 9.00$  mM), but it has similar  $k_{cat}$  values for L-arginine ( $k_{cat}^{L-Arg} = 46.0$  s<sup>-1</sup>) and D-arginine ( $k_{cat}^{D-Arg} = 40.8$  s<sup>-1</sup>), as shown in Table 1. It should be emphasized that there were no significant differences between  $k_{cat}^{L-Arg}$  and  $k_{cat}^{D-Arg}$  for all the mutant enzymes whose kinetic parameters were examined in this work, while the  $K_m^{D-Arg}/K_m^{L-Arg}$  ratio for the mutants changed remarkably, ranging from 1.3 to 8.1 (Table 1). This implies that the active center for the phosphoryl transfer mechanism is conserved in the L- and D-arginine substrates.

Five mutants (L64I, L64V, Y89Q G54S and G54A) showed comparable or higher catalytic efficiency for L-arginine ( $k_{cat}^{L-Arg}/K_m^{L-Arg} = 9.65$ – $195$  s<sup>-1</sup> mM<sup>-1</sup>) than the wild-type (12.5) (Table 1); the situation was the same for D-arginine ( $k_{cat}^{D-Arg}/K_m^{D-Arg} = 9.29$ – $29.0$  s<sup>-1</sup> mM<sup>-1</sup> for the mutants, and 4.5 for the wild-type). These results clearly indicate that the native form

of *Sabellastarte* AK2 suppresses the higher catalytic potential for both L-arginine and D-arginine as the wild-type.

### 3.3. Key residues responsible for substrate recognition in *Sabellastarte* AK2

#### 3.3.1. Amino acid 64

Amino acid residue 64 is located in the N-terminal flexible loop (GS region) and is closest to the substrate arginine in the predicted *Sabellastarte* AK2 structure (Fig. 1). The 64th residue is leucine in *Sabellastarte* AK2 but isoleucine in *Sabellastarte* AK1. The corresponding residues in LK, TK, GK, CK and *Limulus* AK are I, I, K, T and G, respectively.

We replaced L64 in *Sabellastarte* AK2 with G, A, I and V. The L64G and L64A recombinant enzymes were expressed as soluble proteins, but no enzymatic activity could be detected. In contrast, the L64I and L64V mutant enzymes showed high activity: 1.4- to 1.6-fold higher  $k_{cat}$  for L-arginine than the wild-type enzyme, and 2.2-fold higher  $k_{cat}$  for D-arginine (Table 1). These results indicate that an amino acid residue with a large hydrophobic side chain, such as L, I or V, is required at position 64 in order to suitably accommodate the substrate arginine and allow full expression of activity.

In the L64I mutant, the affinity for L-arginine ( $K_m^{L-Arg} = 0.389$  mM) was increased greatly (9.5-fold that of the wild-type), and  $k_{cat}^{L-Arg}/K_m^{L-Arg}$  was increased 15.6-fold (Table 1). The affinity for D-arginine ( $K_m^{D-Arg} = 3.14$  mM) was increased 2.9-fold (the  $k_{cat}^{D-Arg}/K_m^{D-Arg}$  increased 6.4-fold); although significant, these increases are smaller than for L-arginine. Comparison of the values of  $(k_{cat}^{D-Arg}/K_m^{D-Arg})/(k_{cat}^{L-Arg}/K_m^{L-Arg})$ , namely, 0.362 for wild-type and 0.149 for L64I (see Table 1 and Fig. 3), indicated that the L64I mutant had enhanced specificity for L-arginine. Finally, it should be noted that the high specificity for L-arginine of the *Sabellastarte* AK2 L64I mutant approaches that of *Sabellastarte* AK1 (wild-type), which has Ile at position 64.

On the other hand, the L64V mutant showed a 1.7-fold decrease in affinity for L-arginine ( $K_m^{L-Arg} = 6.45$  mM), but unchanged affinity for D-arginine ( $K_m^{D-Arg} = 9.55$  mM) (Table 1). Thus the L64V mutation makes the enzyme more specific for D-arginine ( $(k_{cat}^{D-Arg}/K_m^{D-Arg})/(k_{cat}^{L-Arg}/K_m^{L-Arg}) = 0.986$ ) (Fig. 3).

As shown above, since the replacement of L64 by I or V significantly affected affinity for L-arginine or D-arginine, it can be

**Table 1**  
Kinetic parameters for the forward reaction of the wild-type and mutants of *Sabellastarte* AK2.

	L-Arg			D-Arg			$K_m^{D-Arg}/K_m^{L-Arg}$	$k_{cat}/K_m$ for D-Arg/ $k_{cat}/K_m$ for L-Arg
	$k_{cat}^{L-Arg}$ [1/s]	$K_m^{L-Arg}$ [mM]	$k_{cat}^{L-Arg}/K_m^{L-Arg}$	$k_{cat}^{D-Arg}$ [1/s]	$K_m^{D-Arg}$ [mM]	$k_{cat}^{D-Arg}/K_m^{D-Arg}$		
Wild-type	46.0 ± 3.4	3.69 ± 0.20	12.5 ± 1.0	40.8 ± 5.2	9.00 ± 0.39	4.52 ± 0.40	2.44	0.362
L64I	75.4 ± 4.4	0.389 ± 0.027	195 ± 11	90.7 ± 4.3	3.14 ± 0.13	29.0 ± 1.8	8.07	0.149
L64V	62.3 ± 4.4	6.45 ± 0.12	9.65 ± 0.61	90.7 ± 6.0	9.55 ± 0.79	9.52 ± 0.40	1.48	0.986
L64G	N.D.			N.D.				
L64A	N.D.			N.D.				
Y89Q	77.8 ± 4.1	5.07 ± 0.35	15.4 ± 1.3	61.5 ± 4.3	6.45 ± 0.12	9.54 ± 0.60	1.27	0.619
G54S	81.2 ± 3.7	2.05 ± 0.09	39.5 ± 0.1	86.7 ± 1.8	9.34 ± 0.41	9.29 ± 0.25	4.55	0.235
G54A	121 ± 8	2.72 ± 0.12	44.7 ± 2.8	121 ± 2	4.40 ± 0.16	27.5 ± 1.4	1.62	0.615
G54V	N.D.			N.D.				
G54L	N.D.			N.D.				
G54I	N.D.			N.D.				

concluded that the 64th residue plays a critical role in distinguishing D- and L-arginine.

### 3.3.2. Amino acid 320

As shown in Fig. 1, it was predicted that amino acid N320 in the C-terminal loop, as well as L64, is close to the substrate arginine in *Sabellastarte* AK2. Residue 320 is strictly conserved in CK as D and in typical AKs as H. We constructed seven mutants differing at position 320 (N320H, Q, D, E, R, K and A). All the mutants displayed weak enzymatic activity, so the exact kinetic parameters were not obtained. However, the initial velocities of these mutants were measured at the same substrate concentrations (Table 2). All the mutants showed a considerable reduction in enzymatic activity (0.05–52% of the wild-type), but the decrease was significantly larger with D-arginine than with L-arginine, except for the N320D mutant. In N320D, enzymatic activity towards L-arginine was decreased 3-fold and towards D-arginine 2-fold, making this the only enzyme with higher specificity for D-arginine among the seven mutants (see the value for D-Arg activity/L-Arg activity in Table 2 and Fig. 3). It is also noted that among the mutants, N320D maintain relatively high activity for both of L-arginine (32%) and D-arginine (48%), and N320E only for D-arginine (48%). These results suggest that the carbonyl oxygen of the N320 side chain is involved in recognizing the D-arginine substrate via a hydrogen bond with the  $\alpha$ -amino nitrogen attached to the asymmetric carbon of D-arginine. Thus, D320 and E320 could partly substitute for the role played by N320.

In the *Torpedo* CK TSAC structure, D320 forms a hydrogen bond with H66 on the N-terminal flexible loop and stabilizes the substrate-bound structure [14]. Moreover, in human muscle CK, it was reported that the disruption of the hydrogen bond (ion pair) between D320 and H66 caused a remarkable loss of activity (0.1% that of wild-type activity) [23]. If N320 in *Sabellastarte*

AK2 is also hydrogen bonded with a given residue on the N-terminal loop, as in CK, the remarkable loss of activity in the N320 mutants might be attributed partly to destabilization of the catalytic pocket formed by the N-terminal and C-terminal flexible loops.

### 3.3.3. Amino acid 89

Amino acid residue-89 is proposed to be one of the key residues for recognition of guanidino substrates in phosphagen kinases [20,21]. Multiple alignments of amino acid sequences clearly indicated that the residue is strictly conserved as a specific amino acid in each phosphagen kinase: R in CK, Y in typical AK, I in GK, H in TK, and K in LK [24] (see Fig. 2). While this residue does not appear to be directly involved in substrate binding in the TSAC structures of CK and AK, it is located close to the guanidine substrate-binding site [14,15]. Site-directed mutagenesis studies of this residue using rabbit CK and *Einenia* LK have shown that it has a significant effect on enzymatic activity and guanidino substrate specificity [20,21]. A recent report investigating *Danio* CK and *Nautilus* AK also showed that the 89th residue plays a key role in organizing the hydrogen bond network between the four dynamic domains [25] of AK and CK, thereby helping provide an appropriate active center for high catalytic turnover [26].

We replaced Y89 in *Sabellastarte* AK2 with Q, the amino acid at this position in AK1 (see Fig. 2). The catalytic constants of the Y89Q mutant were increased 1.7-fold for L-arginine ( $k_{cat}^{L-Arg} = 77.8 \text{ s}^{-1}$ ) and 1.5-fold for D-arginine ( $k_{cat}^{D-Arg} = 61.5 \text{ s}^{-1}$ ), compared with those of the wild-type (46 and  $40.8 \text{ s}^{-1}$ , respectively, see Table 1). On the other hand, the affinity for L-arginine was decreased 1.4-fold ( $K_m^{L-Arg} = 5.07 \text{ mM}$ ) compared with that of the wild-type (3.69 mM), but that for D-arginine was increased 1.4-fold ( $K_m^{D-Arg} = 6.45 \text{ mM}$ ). Clearly, Y89Q has more specificity for D-arginine. These results are consistent with the idea that the 89th amino acid is the key residue for guanidino substrate recognition [20,21].

**Table 2**  
Enzyme activity of the wild-type and Asn320 mutants of *Sabellastarte* AK2.

	Enzyme activity [ $\mu\text{moles min}^{-1} \text{ mg protein}^{-1}$ ]		D-Arg activity/L-Arg activity
	L-Arg	D-Arg	
Wild-type	30.8 ± 1.2 (100)	14.9 ± 0.3 (100)	0.483
N320H	9.08 ± 0.26 (29.5)	1.51 ± 0.15 (10.2)	0.166
N320Q	2.77 ± 0.05 (8.97)	0.386 ± 0.030 (2.60)	0.139
N320D	10.0 ± 0.2 (32.4)	7.20 ± 0.50 (48.4)	0.719
N320E	16.1 ± 0.4 (52.3)	1.50 ± 0.42 (10.1)	0.093
N320R	1.27 ± 0.18 (4.11)	0.216 ± 0.029 (1.46)	0.171
N320K	0.189 ± 0.011 (0.612)	0.0726 ± 0.0066 (0.489)	0.385
N320A	5.27 ± 0.34 (17.1)	0.771 ± 0.081 (5.19)	0.146

The reaction was measured in the concentration of 4.76 mM each of creatine and ATP at 25 °C. Values in parentheses are the relative value (%) to its wild-type.



**Table 3**  
 $K_m^{ATP}$  and  $k_{cat}$  of the wild-type and mutants of *Sabellastarte* AK2. Kinetic parameters were obtained in 133 mM L- or D-arginine.

	L-Phosphoarginine synthesis		D-Phosphoarginine synthesis	
	$k_{cat}^{ATP}$ [1/s]	$K_m^{ATP}$ [mM]	$k_{cat}^{ATP}$ [1/s]	$K_m^{ATP}$ [mM]
Wild-type	39.5 ± 1.9	1.13 ± 0.14	36.6 ± 2.6	2.99 ± 0.40
L64I	50.1 ± 0.7	0.400 ± 0.021	68.3 ± 3.3	0.837 ± 0.077
L64V	43.3 ± 1.3	0.774 ± 0.038	61.8 ± 5.6	3.72 ± 0.52
Y89Q	33.4 ± 2.2	0.930 ± 0.080	41.9 ± 4.7	1.59 ± 0.34
G54S	43.2 ± 0.6	0.926 ± 0.033	60.7 ± 3.9	3.49 ± 0.36
G54A	41.0 ± 1.3	0.661 ± 0.077	65.8 ± 1.3	1.12 ± 0.13

of the N-terminal loop. In contrast, the G54S and G54A mutants showed 1.8- and 2.6-fold increases in  $k_{cat}$  for L-arginine, and 2.1- and 3.0-fold increases in  $k_{cat}$  for D-arginine compared with those of wild-type (Table 1). The G54A mutant showed higher affinities for both L-arginine (1.4-fold that of the wild-type) and D-arginine (2.0-fold). In the G54S mutant, the affinity for L-arginine was increased 1.8-fold, but the affinity for D-arginine was unchanged. Comparison of the values of  $(k_{cat}^{D-Arg}/K_m^{D-Arg})/(k_{cat}^{L-Arg}/K_m^{L-Arg})$  for wild-type (0.362), G54S (0.235) and G54A (0.615) (see Table 1 and Fig. 3) shows that the G54A mutant enzyme was more specific towards D-arginine. These results suggest that even a residue remote from the guanidino substrate-binding site, such as G54 in *Sabellastarte* AK2 (Fig. 1), can significantly affect guanidino substrate specificity.

### 3.4. Comparison of $K_m^{ATP}$ values of wild-type and mutants of *Sabellastarte* AK

The wild-type enzyme of *Sabellastarte* AK2 showed the  $K_m^{ATP}$  value of 1.13 mM in 133 mM L-arginine, while it showed the value of 2.99 mM in the same concentration of D-arginine (Table 3). Thus the enzyme shows 2.6-fold higher affinity for ATP in L-arginine than in D-arginine. In this condition, L- or D-arginine is nearly saturated in its substrate-binding site of the enzyme. On the other hand, the  $k_{cat}$  values are comparable (39.5 and 36.6 s<sup>-1</sup>, respectively) (Table 3).

Five mutants, G54S, G54A, L64I, L64V and Y89Q, showed higher affinity for ATP ( $K_m^{ATP}$  = 0.400–0.930 mM) than that (1.13 mM) of wild-type in the L-arginine saturated condition (Table 3). In the D-arginine saturated condition, the affinities were also higher ( $K_m^{ATP}$  = 0.837–1.59 mM) in the G54A, L64I and Y89Q mutants than that (2.99 mM) of the wild-type, but lower ( $K_m^{ATP}$  = 3.49–3.72 mM) in G54S and L64V. The  $k_{cat}$  values of the mutants were estimated to be 30% lower in the L-arginine saturated conditions.

Thus it is suggested that slightly, but significant difference in the arrangement of the active center caused by the binding of L- or D-arginine, affected on the affinity of the other substrate ATP in *Sabellastarte* AK2 reaction.

## 4. Conclusions

*Sabellastarte* AK is the only phosphagen kinase using D-arginine as substrate. It is clearly unrelated to typical AK, and apparently evolved from a mitochondrial CK-like gene. In this study, we found

several amino acid residues responsible for distinguishing the chiral substrates, D- and L-arginine. The 64th residue appears to be the most important determinant for recognizing the chirality of arginine, because the L64I and L64V mutations caused remarkable changes in substrate specificity for D- and L-arginine. Other residues, such as N320, Y89 and G54, also affect the guanidino substrate specificity of *Sabellastarte* AK2, but these residues might be involved simultaneously in other structural interactions that affect the stability of AK.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2010.02.005.

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