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Inversion of enantioselectivity of arylmalonate decarboxylase via site-directed mutation based on the proposed reaction mechanism

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Abstract

Arylmalonate decarboxylase (AMDase, EC. 4.1.1.76) catalyzes asymmetric decarboxylation of α-arylmalonic acid to give optically pure (*R*)-α-arylpropionic acid. This enzyme has four cysteine residues, one of which, Cys188, is estimated to be located in the active site. It is believed that it delivers a proton from the si-face of the intermediate enolate to give (*R*)-product. Based on database searches, it has been revealed that AMDase has some homology with glutamate racemase and other isomerases. Glutamate racemase has a pair of Cys residues in the active site, at positions 73 and 184, which are believed to abstract and deliver a proton from both sides of the substrate. On the other hand, AMDase has only one Cys at 188, and this is considered to be the reason why this enzyme gives optically pure products. The estimated 3D-structure of AMDase suggests that Gly74 is located in the opposite side of Cys188. Then, it was expected that introduction of one Cys around the region of Gly74, *i.e.*, from 68 to 77 in addition to the replacement of Cys188 with less acidic Ser would invert the enantioselectivity of the enzyme. Thus, 10 mutants were prepared and their activities as well as their enantioselectivities were examined. As expected, two of them, S71C/C188S and G74C/C188S, exhibited decarboxylation activity and gave the opposite enantiomer to that formed by the wild type enzyme.

Keywords: Arylmalonate decarboxylase; Enantioselectivity inversion; Reaction mechanism; Mutagenesis

1. Introduction

Arylmalonate decarboxylase (AMDase, EC. 4.1.1.76) is a unique enzyme, which catalyzes enantioselective decarboxylation of α-aryl-α-methylmalonates to give optically pure α-arylpropionates [1–4] (Scheme 1).

This enzyme was originally isolated from a Gram-negative bacterium, *Alcaligenesis bronchisepticus* KU1201 [5]. The gene coding for the enzyme has already been cloned and overexpressed in *Escherichia coli*, and the resulting enzyme has also been purified [6]. The reaction mechanism of this enzyme has been studied, with mutagenesis studies predicting Cys188 to be located in the active site [7]. AMDase has been revealed to have some homology with some isomerases, such as glutamate racemase [8], aspartate racemase [9], and others [10,11] (Fig. 1).

A common feature of these enzymes is the presence of Cys188 in the active site. On the other hand, the marked difference between AMDase and the other enzymes is that while isomerases have another conserved cysteine residue located on the other side of the substrate bounding in the active site, AMDase has no cysteine residue around the corresponding region.

The reaction mechanism of glutamate racemase has been established by extensive studies as well as X-ray analysis of its tertiary structure. It has been proposed that the key for the racemization activity is the presence of the two cysteine residues. One cysteine residue abstracts the α-proton from the substrate, while the other delivers a proton from the opposite side of the intermediate enolate of the acid. Accordingly, this type of mechanism of racemization is referred to as two-base mechanism [12–14] (Scheme 2).

The likely role of the cysteine residue of AMDase is protonation of the intermediate enolate similar to that of Cys73 in the case of glutamate racemase shown in Scheme 2. However, because there is only one cysteine residue in the active site of AMDase, the proton is delivered from only one enantiomeric face of the enolate resulting in the formation of enantiomerically pure (*R*)-product [15].

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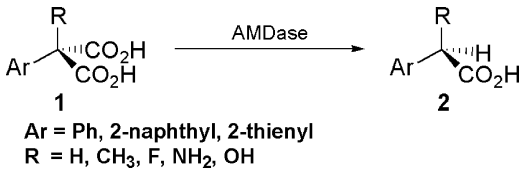


Scheme 1. Arylmalonate decarboxylase (AMDase) catalyzed enantioselective decarboxylation of α-aryl-α-methylmalonate.

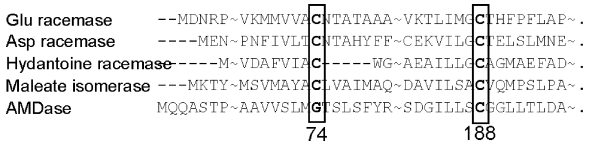


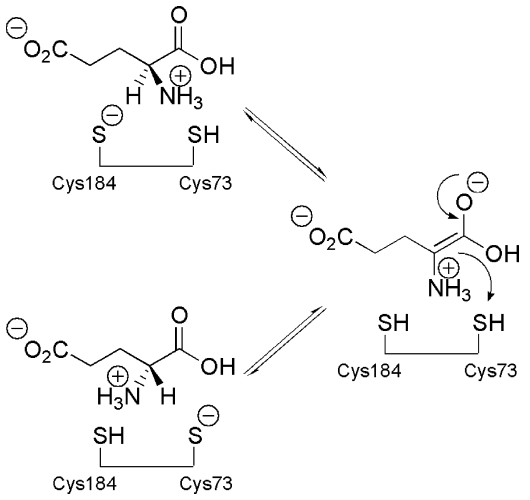
Fig. 1. Amino acid homology between some racemases and AMDase.

These studies on the reaction mechanism and the similarity between AMDase and racemases promoted us to challenge the inversion of enantioselectivity of AMDase by changing the location of the key Cys residue. We supposed that the new proton-donating amino acid residue should be introduced around Gly74 of AMDase for the inversion of the enantioselectivity. Thus, at first, the double mutant AMDase (Gly74Cys/Cys188Ser) was prepared. It exhibited the opposite enantioselectivity, as expected [16]. However, the enzymatic activity was very low (1/1000) compared to that of the native enzyme. It could therefore be presumed that the location of the newly introduced Cys was not the best position for the activity and enantioselectivity of the enzyme. Thus, aiming at increasing the activity of the double mutant, we replaced each of the amino acids in turn between residues 68 and 77, with cysteine.

2. Experimentals

2.1. General procedure

All chemicals were purchased from Aldrich, Kanto Chemical, and Wako Pure Chemical Co., Ltd. Proton NMR spectra were recorded in $CDCl_3$ with a JEOL AE-300 spectrometer



Scheme 2. The reaction mechanism of glutamate racemase.

using tetramethylsilane as the internal standard. Enantiomeric excess (%ee) was determined by HPLC analysis of the reaction mixture with a system consisting of a Jasco pump, a Jasco 875-UV detector, and a Daicel Chiralcel OD or OJ column.

2.2. Preparation of double mutant enzymes of AMDase

A pUC19-based plasmid pAMD101-C188S was used as the template for site-directed mutagenesis. The template plasmid has previously been prepared in our laboratory [16]. To introduce a mutation, partially overlapping PCR products were generated spanning the 5′ and 3′ end of the gene. The synthetic oligonucleotides used for the preparation are summarized in Table 1.

The 5′ fragment was prepared with the forward primer corresponding to the multi-cloning site sequence of the vector (P5ex), and the reverse primer containing the mutation site to change the amino acid residue of 74th glycine to cysteine (G74CR). The 3′ fragment was prepared with the primers (G74CF and P1ex). After preparing of the 5′ and 3′ PCR fragments for mutation, they were combined by mixing and a second PCR amplification with the same primers, P5ex and P1ex. The final PCR product was digested with the restriction enzymes, *Hin*d III and *Pst* I. This mutated gene was ligated with pUC19 which had been digested with the same restriction enzymes. Then *E. coli* JM109 was transformed by the mutant plasmid. The transformant harboring the mutant plasmid was cultivated in 1.5 L of LB-broth (pH 7.0, containing 50 mg/L of ampicillin) at 30 °C with shaking (200 rpm). After cultivation for 2 h, starting from 1/100 volume of over-night preculture inoculum, IPTG (isopropyl-β-D-galactopyranoside) was added to the final concentration of 0.1 mM. Then the cultivation was continued for additional 16 h.

2.3. Purification of AMDase [17]

A potassium phosphate buffer of various concentrations containing 0.5 mM EDTA and 5 mM β-mercaptoethanol was used throughout the purification. All procedures for the purification of the enzyme were performed below 4 °C. The *E. coli* cells containing AMDase were collected by centrifugation at 6000 × *g* for 20 min. The cells were suspended in 200 mL of 100 mM buffer (pH 7.0) and homogenized by French press (1500 kg/cm^2). The suspension was centrifuged (12,000 × *g*, 20 min) to remove the insoluble materials. To the resulting solution was added 1% volume of aqueous solution of protamine sulfate (concentration 2%), and the mixture was stirred for 30 min. The resulting precipitate was removed by centrifugation (12,000 × *g*, 20 min). To the obtained enzyme solution, ammonium sulfate was applied to the concentration of 60% saturation and the solution was stirred for 1 h. The precipitated protein was isolated by centrifugation (12,000 × *g*, 20 min), dissolved in 10 mM Tris–HCl buffer (pH 8.0) and dialyzed against the same buffer. The enzyme solution was charged to a DEAE-Toyopearl column, which had been equilibrated with 10 mM Tris–HCl buffer (pH 8.0) and eluted by an aqueous solution of NaCl with a linear gradient of 10–50 mM. The active fractions were collected and concentrated to 30 mL by ultrafiltration. Ammonium sulfate was added to this solution to 25% saturation and this mixture was applied

Table 1
PCR primers used in this study

Primer name	Primer sequence (5′ → 3′)
P5ex	CACAGGAAACAGCTATGACCATGATTACGCCAAGC
G74CR	GCTGAGCGAGGTGCACATCAGCGAAACCAC
P1ex	GTAAAACGACGGCCAGTGAATTCGAGCTCGGTA
G74CF	GTTTCGCTGATGTGCACCTCGCTCAGCTTC
A68CR	CCATCAGCGAAACCACGCACGCGCCCTGCTTTTGC
A68CF	GCAAAAGCAGGGCGCGTGCGTGGTTTCGCTGATGG
V69CR	GGCCATCAGCGAAACGCACGCCGCGCCCTGC
V69CF	GCAGGGCGCGGCGTGCGTTTCGCTGGATGGGC
V70CR	GGTGCCCATCAGCGAGCACACCGCCGCGCCC
V70CF	GGGCGCGGCGGTGTGCTCGCTGATGGGCACC
S71CR	AGCGAGGTGCCCATCAGACAAACCACCGCCGC
S71CF	GGCGCGGCGGTGGTTTGTCTGATGGGCACCTCG
L72CR	GCTGAGCGAGGTGCCCATACACGAAACCAC
L72CF	GGCGCGGCGGTGGTTTCGTGTATGGGCACCTCG
M73CR	CGGTAGAAGCTGAGCGAGGTGCCGCACAGCGAAACCACCGC
M73CF	GCGGCGGTGGGTTTCGCTGTGCGGCACCTCGCTCAGCTTCTAC
T75CR	CGGTAGAAGCTGAGCGAGCAGCCCATCAGCGAAACCACCGCC
T75CF	GCGGCGGTGGGTTTCGCTGATGGGCTGCTCGCTCAGCTTCTAC
S76CR	CCCCGGTAGAAGCTGAGGCAGGTGCCCATCAG
S76CF	TCGCTGATGGGCACCTGCCTCAGCTTCTACCGG
L77CR	CCCGGTAGAAGCTGCACGAGGTGCCCATCAGCG
L77CF	CGCTGATGGGCACCTCGTGCAGCTTCTACCGGG

Note: The underlined sequences are the mutation sites in the primers.

to a Butyl-Toyopearl column which had been equilibrated with a 25% ammonium sulfate solution in 10 mM Tris–HCl buffer (pH 8.0). The enzyme was eluted with an aqueous solution of ammonium sulfate with a linear gradient of 25–15%. The active fractions were combined and dialyzed against 10 mM Tris–HCl buffer.

The concentration of protein was determined by Bio-Rad Protein Assay kit using BSA as the protein standard or by the measurement of the absorbance at 280 nm. For the mutant enzymes, the protein which was identical to AMDase on SDS-PAGE was recovered after purification by column chromatography.

2.4. Reactivity of mutant enzyme

The purified AMDase was used for the measurement of reactivity and enantioselectivity. An aqueous solution of α-aryl-α-methylmalonic acid (200 mM, pH 7.5, 100 μL), 100 μL of 1 M Tris-HCl buffer (pH 8.5), 300 μL of water, and 500 μL of purified AMDase solution was mixed and the mixture was stirred at 35 °C for 12 h. The reaction was quenched by adding 1 mL of 2 M HCl and the mixture was filtered through a pad of cerite. The filtrate was extracted with diethyl ether. The ether layer was washed with brine, and dried over anhydrous $MgSO_4$. After filtration and removal of the solvent, the residue was purified by preparative TLC to give arylpropionic acid. After esterification with TMS-diazomethane, the enantiomeric excess of the product was determined by HPLC.

2.5. Enzymatic decarboxylation by the mutant enzymes

The substrates, **1a** and **1b**, were prepared as described in Ref. [17].

2.5.1. Decarboxylation of α-methyl-α-(2-thienyl)malonic acid (*1a*)

α-Methyl-α-(2-thienyl)malonic acid (**1a**, 400 mg, 2 mmol) was dissolved in water (8 mL) and the pH of the mixture was adjusted to 8.0 with 2 M NaOH. Final volume was adjusted to 10 mL by adding water. To a 20-mL round-bottomed flask was added 1 mL of 10 mM Tris-HCl buffer (pH 8.5), 2 mL of the substrate solution (80.0 mg, 0.4 mmol) and 1 mL of AMDase solution (*ca.* 1 mg protein). The mixture was stirred at 30 °C for 3–72 h, and quenched with 1 mL of 2 M HCl, followed by filtration with a pad of cerite. The filtrate was extracted with diethyl ether. The ether layer was washed with brine, and dried over anhydrous $MgSO_4$. After filtration and removal of the solvent, the residue was purified by preparative TLC (hexane/ethyl acetate/acetic acid = 33/66/1) to give **2a** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.61 (3H, d, J = 7.3 Hz), 4.04 (1H, q, J = 7.3 Hz), 6.95–7.23 (3H, m); IR ν_{max}: 2982, 1715, 1457, 1417, 1236, 699 cm^{-1}

After esterification with TMS-diazomethane, the enantiomeric excess of the product was determined by HPLC: Chiralcel OJ (Daicel Chemical Industries Ltd.); eluent: hexane/2-propanol = 50/1; flow rate: 0.5 mL/min.

Retention time: (*R*)-form 19 min, (*S*)-form 32 min.

2.5.2. Enzymatic decarboxylation of α-methyl-α-(2-naphthyl)malonic acid (*1b*)

α-Methyl-α-(2-naphthyl)malonic acid (**1b**, 488 mg, 2 mmol) was dissolved in water (8 mL) and the pH of the mixture was adjusted to 8.0 with 2 M NaOH. Final volume was adjusted to 10 mL by adding water. To a 20-mL round-bottomed flask was added 1 mL of 10 mM Tris-HCl buffer (pH 8.5), 2 mL of the substrate solution (97.7 mg, 0.4 mmol) and 1 mL of AMDase solution (*ca.* 1 mg protein). The mixture was stirred at 30 °C

for 3–72 h and quenched with 1 mL of 2 M HCl followed by filtration with a pad of cerite. The filtrate was extracted with diethyl ether. The ether layer was washed with brine, and dried over anhydrous $MgSO_4$. After filtration and removal of the solvent, the residue was purified by preparative TLC (hexane/ethyl acetate/acetic acid = 50/50/1) to give **2b** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.61 (3H, d, J = 7.1 Hz), 3.93 (1H, q, J = 6.9 Hz), 7.44–7.83 (7H, m); IR ν_{max}: 2980, 1698, 1419, 1273, 1225, 963, 747 cm^{-1}

After esterification with TMS-diazomethane, the enantiomeric excess of the product was determined by HPLC: Chiralcel OD (Daicel Chemical Industries Ltd.); eluent: hexane/2-propanol = 100/1; flow rate: 0.5 mL/min.

Retention time: (*R*)-form 20 min, (*S*)-form 22 min.

2.6. Measurement of the kinetic parameters

The kinetic parameters of the mutant AMDase were determined as follows. To a 1.5-mL microtube were added water (350 μL), 1 M Tris–HCl buffer (pH 8.5, 50 μL), an aqueous solution of purified AMDase (50 μL, 0.05 mg), and an aqueous solution of α-aryl-α-methylmalonic acid (**1a**, **1b**: pH 8.0, 50 μL). Several such solutions were prepared with different concentrations of the substrates and incubated at 37 °C for 10 min. The reaction was quenched by the addition of 2 M HCl (125 μL). The activity of the enzyme was evaluated by determining the amount of the resulting products by HPLC. The kinetic parameters, K_m and k_{cat} were obtained from the Lineweaver–Burk plot.

3. Results and discussion

Ten different mutants were prepared via site-directed mutagenesis utilizing the overlap PCR method. The sequences of the mutated region of the gene were confirmed by sequence analysis. The site-directed mutation points were two amino acid residues, *i.e.*, at the 188th position in addition to either one of 68th to 77th. The cysteine residue at position 188 in the active site was changed to serine, which is a weaker proton-donor compared to cysteine. Accordingly, this mutation is expected to decrease the efficiency of the protonation from the original side of the enolate intermediate. The newly introduced cysteine residue in place of either one amino acid residues between 68 and 77 is expected to be located on the opposite side of the enantiomeric face of the intermediate and thus will deliver a proton from the opposite direction compared to that to the native enzyme. The constructed mutant genes were ligated with the vector pUC19 and transferred into *E. coli*. The recombinant *E. coli* harboring the mutant AMDase genes were cultivated in LB medium to overexpress the mutant enzymes, which were purified as described in Section 2. The steric structures of all the purified mutant enzymes were little different from that of native one as judged by the measurement of the CD-spectra (data not shown). Specific activities of the mutant enzymes were measured by using phenylmalonic acid as the substrate. As shown in Table 2, the wild type AMDase exhibited 467 unit/mg (1 unit is defined as the amount of protein to catalyze the decarboxylation of 1 μmol of phenylmalonic acid in 1 min). On the other hand, the specific activity of two double mutant enzymes, S71C/C188S and G74C/C188S, were 0.07 and 0.05, respectively. The eight other double mutant enzymes showed no activity within the error of measurement. As mentioned above, CD-spectra demonstrated that the introduction of two mutations does not cause a serious distortion to the 3D-structure of the proteins, and it can thus be presumed that these mutant enzymes lost their activity due to the incorrect positioning of a proton-donating cysteine residue relative to the substrate.

Table 2
Reactivity of mutant enzymes

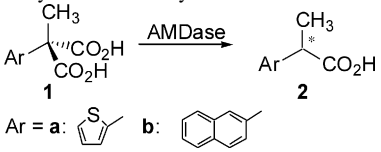
Entry	Mutant	Concentration (mg/mL)	Specific activity (Unit/mg)
1	wild type	1.74	467
2	A68C/C188S	1.05	–
3	V69C/C188S	1.11	–
4	V70C/C188S	1.08	–
5	S71C/C188S	3.33	0.07
6	L72C/C188S	1.73	–
7	M73C/C188S	0.80	–
8	G74C/C188S	1.04	0.05
9	T75C/C188S	0.78	–
10	S76C/C188S	1.57	–
11	L77C/C188S	1.23	–
12	C188S	0.77	1.30

Next, the reactivity and the enantioselectivities of the double mutant enzymes were measured using other two substrates which exhibit higher activities compared to α-methyl-α-phenylmalonic acid with wild type AMDase (1.16 s^{-1} mM^{-1}), *i.e.*, α-methyl-α-(2-thienyl)malonic acid (**1a**; 16.0 s^{-1} mM^{-1}) and α-methyl-α-(2-naphthyl)malonic acid (**1b**; 71.8 s^{-1} mM^{-1}). Two compounds were synthesized via four steps from commercially available compounds. The enantioselectivities of the mutant enzymes for these two substrates are summarized in Table 3. Unfortunately, only two mutants that showed activity to phenylmalonic acid promoted the decarboxylation reactions of **1a** and **1b**, and no activity was observed for the other eight mutant enzymes even with these relatively active compounds.

Wild type AMDase gave enantiomerically pure products, *i.e.*, **2a** (entry 1, *Y*: 99%, 99% ee, *S*) and **2b** (entry 13, *Y*: 96%, 99% ee, *R*). In contrast, the two double mutant enzymes gave opposite enantiomers compared to those of formed by the wild type. The S71C/C188S mutant enzyme gave **2a** (*Y*: 97%, 58% ee) of (*R*)-form (entry 5) and **2b** (*Y*: 22%, 80% ee) of (*S*)-form (entry 17). Likewise, the G74C/C188S mutant enzyme gave (*R*)-**2a** in 60% yield with 94% ee (entry 8), and (*S*)-**2b** in 17% yield with 96% ee (entry 20). It has been shown that the enantioselectivity of G74C/C188S mutant is higher than that of S71C/C188S mutant, although the reactivity of the former is lower compared to that of the latter. To confirm the activity of the mutants, the kinetic parameters were obtained from Lineweaver–Burk plot as shown in Table 4.

The K_m values of the two double mutant enzymes for **1a** were better compared to that of wild type enzyme, but

Table 3
Enzymatic decarboxylation



Entry	substrate	Mutant	Yield (%)	ee (%)
1	**1a**	Wild type	99[a]	99(*S*)
2		A68C/C188S	0	–
3		V69C/C188S	0	–
4		V70C/C188S	0	–
5		S71C/C188S	97[b]	58(*R*)
6		L72C/C188S	0	–
7		M73C/C188S	0	–
8		G74C/C188S	60[b]	94(*R*)
9		T75C/C188S	0	–
10		S76C/C188S	0	–
11		L77C/C188S	0	–
12		C188S	17[a]	50(*R*)
13	**1b**	Wild type	96[a]	99(*R*)
14		A68C/C188S	0	–
15		V69C/C188S	0	–
16		V70C/C188S	0	–
17		S71C/C188S	22[b]	80(*S*)
18		L72C/C188S	0	–
19		M73C/C188S	0	–
20		G74C/C188S	17[b]	96(*S*)
21		T75C/C188S	0	–
22		S76C/C188S	0	–
23		L77C/C188S	0	–
24		C188S	6[a]	70(*S*)

[a] Reaction time was 3 h.
[b] 72 h.

those for **1b** were a little worse. The k_{cat} values of the mutant enzymes were much smaller than that of the wild type for both substrates. In total, the enzymatic efficiencies (k_{cat}/K_m) of the two double mutant enzymes were one to about several hundredths of that of the wild type for each substrate.

The low activities of the mutant enzymes are mainly due to their low k_{cat} values. Considering the similarities of CD-spectra and K_m values, the mode of interaction between the substrates and both the wild type and mutant enzymes is estimated to be little different. Thus, the low activities of the mutants are suggested to arise from the greater distance between the newly introduced SH residue and the α-carbon of the substrate.

Table 4
Kinetic parameters of mutant enzmyes

Entry	Substrate	Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
1	**1a**	Wild type	200	12.5	16.0
2		S71C/C188S	0.08	1.01	0.08
3		G74C/C188S	0.34	5.03	0.07
4	**1b**	Wild type	30.9	0.43	71.8
5		S71C/C188S	0.05	0.51	0.10
6		G74C/C188S	0.12	1.25	0.09

4. Conclusions

In summary, based on supposition of Matoishi et al. that the role of the cysteine 188 in the active site was protonation of the intermediate enolate [15], the mutant enzymes were prepared, in which a new proton-donating cysteine was introduced at the estimated opposite side of the substrate, compared to the deactivated essential cysteine of the native enzyme. As expected, two of the mutant enzymes exhibited decarboxylation activity, and they were demonstrated to have the opposite enantioselectivity compared to that of wild type enzyme. We believe that the present success is a strong support for the estimation that the key step for determination of enantioselectivity of AMDase-catalyzed decarboxylation is the enantioface differentiating protonation to the intermediate enolate from Cys188. This reaction mechanism is quite similar to the case of glutamate racemase, in that the key step is the formation of enolate of carboxylic acid followed by the protonation. Thus, AMDase is indicated to also be a member of the enolate superfamily.

The enantioselectivity of G74C/C188S was better than S71C/C188S, while the enzymatic efficiency (k_{cat}/K_m) was almost equal. Thus, the amino acid residue at the position 74 is revealed to be the best point to introduce a new active site. As the amino acid residue in the α-helix is known to rotate by 3.6 amino acid residues, amino acid residue position 71 is supposed to face to the intermediary enolate in the active site as well as 74th amino acid residue. However, judging from the fact that the k_{cat} of the S71C/C188S double mutant enzyme was lower than that of G74C/C188S for both substrates, the distance from the α-carbon of the substrate to 71C is considered to be slightly greater than that of 74C, and hence the protonating ability would be a little weaker.

To increase the activity of the resulting G74C/C188S double mutant, random mutagenesis of the corresponding gene was performed and a triple mutant of which activity was increased by 10-fold higher was obtained [17].

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