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Mechanism of asymmetric decarboxylation of α-aryl-α-methylmalonate catalyzed by arylmalonate decarboxylase originated from *Alcaligenes bronchisepticus*

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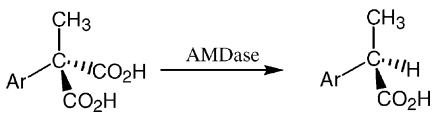
Abstract

Arylmalonate decarboxylase (EC. 4.1.1.76, originated from *Alcaligenes bronchisepticus* KU 1201) is an enzyme which catalyzes asymmetric decarboxylation of arylmalonate. We have once proposed the intermediary formation of a thiol ester between the substrate and the enzyme based on the inhibition studies with α-bromophenylacetate. We misinterpreted the binding mode of this acid as formation of thiol ester and estimated that the substrate also bound to the enzyme in the same manner. However, reinvestigation indicated that the mode of inhibition by this acid is irreversible, different from the previous conclusion. Accordingly the above mechanism became very unlikely. Instead, we would like to propose that Cys 188 is working as a proton donor on the basis of following evidence. The pH-rate of reaction profiles of the native and C188S mutant enzyme greatly differed in alkaline region. This is estimated to come from the difference in pK_a values of Cys and Ser, and suggested that Cys 188 is a proton donor. Homology alignment showed that this enzyme has some homology with glutamete racemase and some other isomerases. The presence of Cys 188 is conserved to all these enzymes as well as to AMDase. The role of this amino acid residue in glutamate racemase has been established to interchange a proton between the substrate. This fact also supports that Cys 188 of AMDase is working as a proton donor to form the asymmetric center of the product.

Keywords: Arylmalonate decarboxylase; Reaction mechanism; Asymmetric protonation; Site-directed mutagenesis; pH profile; Homology alignment

1. Introduction

Some years ago, we have found a novel enzyme which catalyzes asymmetric decarboxylation of α-aryl-α-methylmalonates to give optically active α-arylpropionates (Eq. (1)) [1]. This enzyme was named arylmalonate decarboxylase (AMDase) and registered as EC. 4.1.1.76. It has some characteristic features,



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such as that it requires neither ATP nor coenzyme A unlike other ordinary decarboxylases [2]. Thus we were interested in the application of this novel enzyme in organic syntheses [3,4] as well as the reaction mechanism [5].

From the mechanistic point of view, the characteristics of this enzymatic reaction are summarized as follows.

1. Free SH group is essentially important for this reaction, because representative SH reagents (PCMB, $HgCl_2$, iodoacetate) totally deactivate the enzyme [2].
2. Cys188 is estimated to be located in the active site, as Cys188Ser mutant has extraordinarily low k_{cat} value compared to those of wild-type enzyme and three other mutant enzymes, in which either one of other cysteine residues is replaced by serine [6].
3. Pro-(*R*) carboxyl group is removed as carbon dioxide. Thus the reaction proceeds with inversion of configuration [7].
4. The ρ value of Hammett plot of decarboxylation of *p*- and *m*-substituted phenylmalonate is negative (−1.9). This means that the transition state has some negative charge [2].

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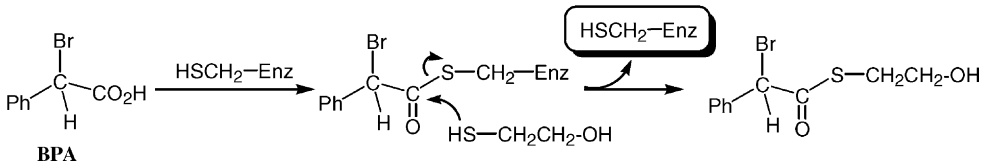
5. The K_m value of cyclic substrate, i.e., indane-1,1-dicarboxylic acid, is smaller than the other substrate by about 10^{-1} [8]. The activation entropy of this compound is also smaller that those of α-methyl-α-phenylmalonate and its *p*-chloro derivative. This fact suggests that the conformation of the transition state will be similar to that of indane-1,1-dicarboxylic acid [9].

In the course of our mechanistic studies, we found that this enzyme is inhibited by α-bromophenylacetic acid (BPA) and the activity was recovered by the addition of β-mercaptoethanol (β-ME) [10]. Thus we supposed that the most possible binding mode of BPA to the enzyme was a thiol ester bond formation with the Cys residue of the active site, because this supposition well explains the effect of β-ME. The thiol can cleave the thiol ester bond via a nucleophilic reaction to give the free Cys-SH and to recover the activity of the enzyme (Eq. (2)). We also speculated that the substrate reacts with the enzyme in the same manner as BPA resulting in the intermediary formation of a thiol ester, which would promote the C–C bond fission by stabilizing the resulting enolate (Eq. (3)). In this case, the role of cysteine residue is similar to that of coenzyme A in the biosynthsis of fatty acids.



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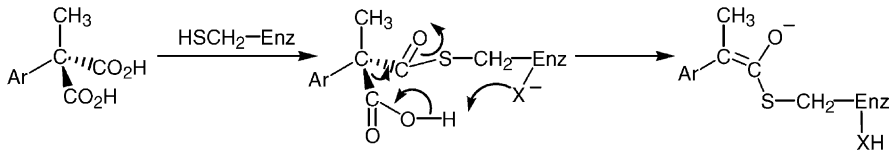

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Based on this assumption, the reaction mechanism was further investigated in detail. In due course, we have noticed that we had overestimated the effect of β-ME in the inhibition–recovery studies. In conclusion, we would like to propose that Cys 188 in the active site works as a proton donor rather than a nucleophile as hitherto estimated.

2. Materials and methods

2.1. Materials

Phenylmalonic acid, methyl phenylacetate, β-mercaptoethanol, and α-bromophenylacetic acid were purchased from Tokyo Kasei Co. ^{18}O containing water is 99% grade of Kanto Chemical Co.

The LB medium consisting of trypton (1%), yeast extract (0.5%), and NaCl (0.5%) was used. For the preparation of plates, 1.5% of agar was added.

AMDase has been originally isolated from *Alcaligenes bronchisepticus* (KU 1201, registered to International Patent Organism Depositary, 1-1 Higashi, Tsukuba 305-8566, Japan as IPOD 11670). In this study, this enzyme was purified from *Escherichia coli* DH5α-MCR/pAMD 101 (IPOD 12968) [2,12]. The sequence data of the gene coding AMDase is available from DNA Data Bank of Japan (DDBJ) with the accession number D13116.

The biomaterials were the products of Takara Shuzo Co.

2.2. Preparation of C188S mutant enzyme of AMDase

The mutant gene for C188S mutant enzyme was generated by site-directed mutagenesis according to the method of Kunkel et al. [11] as described in our previous paper [6], with a Mutan-K in vitro Mutagenesis kit (Takara Shuzo Co.). The template plasmid used was pAMD 101 [12]. The synthetic oligonucleotide used was 5′-CAAGCCGCCGCTAGACAGCAG-3′ (21mer). The nucleotide sequence of the mutant around the mutated site was confirmed by the dideoxy chain-termination method with Taq Dye Dideoxy Cycle Sequencing kit (Applied Biosystems). The *E. coli* JM 109 was transformed by the mutant plasmid of pAMD 101. The transformant harboring the mutant plasmid was cultivated in 1500 ml of LB-broth (pH 7.0, containing 150 mg/l of ampicillin) at 30 °C with shaking (200 rpm). After cultivation for 3 h, starting from 1/100 volume of overnight preculture inoculum, IPTG (isopropyl-β-D-galactopyranoside) was added at 0.1 mM. The cultivation was continued for additional 15 h.

2.3. Purification of AMDase

The potassium phosphate buffer of various concentrations containing 0.5 mM EDTA and 5 mM β-mercaptoethanol was used throughout purification. All the 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). This suspension was centrifuged (12,000 g, 20 min) to remove the insoluble precipitates. A solution of 1% volume of protamine sulfate (2% aqueous solution) was added to the resulting solution 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,

was added ammonium sulfate to the concentration of 60% saturation and stirred for 1 h. The precipitated protein was isolated by centrifugation (12,000 g, 20 min) and 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 with aqueous solution of NaCl with a linear gradient of 10–50 mM. The active fractions were collected and concentrated to 40 ml by ultrafiltration. Ammonium sulfate was added to this solution to 25% saturation and applied to a butyl-toyopearl column which had been equilibrated with 10 mM Tris–HCl buffer (pH 8.0), and 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 or from the absorbance at 280 nm. As to the C188S mutant enzyme, the protein which was identical with AMDase on SDS-PAGE was recovered after the purification by column chromatography.

2.4. Measurement of the activity of AMDase

To a mixture of 50 μl of aqueous solution of phenylmalonate (200 mM, pH 7.0) and 50 μl of Tris–HCl buffer (pH 8.5) was added an appropriate amount of enzyme solution and made up to 500 μl with deionized water. The reaction was performed for 5 min at 35 °C and quenched by adding 125 μl of 1 M hydrochloric acid. The conversion of the substrate was less than 20% under these conditions. The resulting phenylacetic acid was determined by HPLC. One unit of activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of phenylacetate from phenylmalonate per minute.

2.5. Lyophilization and $^{18}OH_2$ exchange of the enzyme

A solution of AMDase (100 μl, 600 U/ml) and 100 μl of 10 mM Tris–HCl buffer (pH 8.5) was mixed and lyophilized. The resulting powder was dissolved in 100 μl of $^{18}OH_2$ and allowed to stand at 4 °C for 1 h. This solution was lyophilized again and dissolved in 100 μl of $^{18}OH_2$. To this solution, 11 mg (50 μmol) of disodium phenylmalonate was added and the mixture was incubated at 35 °C for 5 min. Non-aqueous ion exchange resin (Amberlist 15, 20 mg) was added to the reaction mixture to make the pH of the solution to 5 and the mixture was extracted with ether after the addition of NaCl. The obtained crude phenylacetic acid (6.0 mg, 90%) was methylated by diazomethane and purified by column chromatography on silica gel (eluent; hexane/ethyl acetate 10/1). MS: 150 (Calcd for $C_9H_{10}{}^{16}O_2$, 150).

2.6. Inhibition of the enzymatic reaction with α-bromophenylacetic acid (BPA)

To a solution of AMDase (100 μl, 600 U/ml) was added a 5 mM aqueous solution of BPA (5 μl) and the mixture was incubated for 10 min at 35 °C. After incubation for 16 h at 4 °C, the activity of the enzyme was measured using phenylmalonic acid as the substrate [Ex-C].

For the evaluation of the effect of β-mercaptoethanol, the experiment was performed as follows. To 20 μl of a solution of the mixture of the enzyme (12 U) and the inhibitor was added 980 μl of 10 mM Tris–HCl buffer (pH 8.5) containing 50 mM β-mercaptoethanol. This mixture was allowed to stand for 16 h at 4 °C. The enzyme activity was determined for a 50 μl aliquot of this solution [Ex-B].

For the control experiments, the same procedure was carried out using deionized water instead of BPA solution and 980 μl of 10 mM Tris–HCl buffer (pH 8.5) containing β-mercaptoethanol [Ex-A].

2.7. Measurement of pH profile of the enzymatic decarboxylation

The following buffer solutions were used to obtain the pH profile of the reaction: AcOH–AcONa buffer for pH 4.0–5.5; MES–NaOH buffer for pH 6.0–6.5; Tris–HCl buffer for pH 7.1–8.9; glycine–NaOH buffer for pH 9.0–10.5.

A mixture of 50 μl of a solution of phenylmalonic acid (200 mM, pH 7.0), 50 μl of buffer solution (1 M), and a solution of AMDase was diluted to 500 μl with deionized water. The enzymatic reaction was performed for 5 min at 35 °C. The amount of the enzyme was controlled to the level that the conversion reached 10–20% under the reaction conditions mentioned above. The reaction was quenched by the addition of 125 μl of 1 M HCl and the resulting phenylacetic acid was determined by HPLC.

The pH profile of the reaction of C188S mutant enzyme was also obtained in the same manner as that of native enzyme except for the reaction time. Because the activity of the mutant enzyme was far lower than that of the native one, the conversion of the substrate was determined after 30 min under the same reaction conditions for the native enzyme. The conversion was controlled to be around 10%.

2.8. Measurement of the pH stability of the enzyme

A mixture of a solution of the enzyme and 50 μl of each buffer solution (1 M) was diluted to 500 μl with deionized water, and the solution was allowed to stand at 35 °C for 30 min. To 50 μl of this enzyme solution was added 50 μl of 1 M Tris–HCl buffer (pH 8.5) and the mixture was made up to 500 μl with deionized water. Then, phenylmalonic acid was added and the activity of the enzyme was measured as described above.

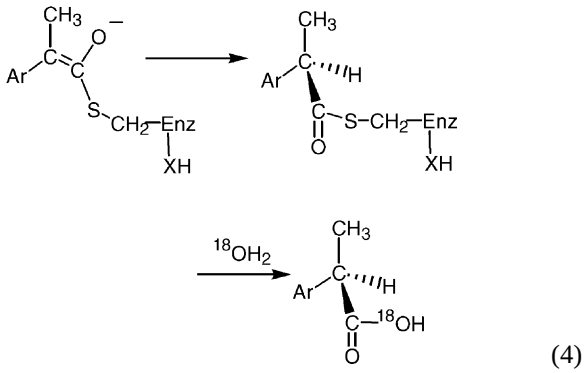
2.9. Instrumentation

NMR spectra were recorded on a JEOL JNM GX-400 (^{1}H NMR, 400 MHz) spectrometer. IR spectra were measured with a JASCO FT/IR-410 spectrometer. For the measurement of absorbance of proteins Shimadzu UV-2100S UV-Vis Recording Spectrophotometer and Hitachi U2000 Spectrophotometer were employed. EI MS spectra were recorded on a HITACHI M-80 instrument and ESI MS spectra were recorded on a JEOL JMS-700 instrument equipped with HP-1100 HPLC (Agilent Technologies Co.). ESI MS experiments were carried out at +5 kV acceleration voltage using 50% aqueous MeOH containing 0.2% trifluoroacetic acid as a solvent.HPLC was performed using Shimadzu LC-5A: column, TSK-GEL ODS-80TM of Tosoh Co.

3. Results and discussion

3.1. Reaction in ^{18}O-water

If the cysteine residue at the active site works as a nucleophile and forms a thiol ester bond with the substrate as described in the introductory part, the C–O bond of the substrate is once cleaved and formed again in the final step of the hydrolysis of the intermediate thiol ester. Thus when the reaction is carried out in $^{18}OH_2$, half of the oxygen atoms of the carboxyl group of the product should be ^{18}O (Eq. (4)).



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However, there is one important problem. Since the active site of the enzyme is generally hydrophobic and located inside of the polypeptide chain, the bulk solvent water may not enter there by simple diffusion. Thus, when the enzyme is purified in ^{16}O- and ^{18}O-water is used only for the performance of the reaction, $^{16}OH_2$ (inside the tertiary structure) and $^{18}OH_2$ (bulk) may not reach the diffusion equilibrium within the reaction time. In this case, ^{18}O will not be incorporated in the product even when the supposed mechanism is really operating.

Thus we first carried out the reaction of phenylmalonic acid in deuterium oxide (D_2O). If the water molecules inside and outside the enzyme are thoroughly equilibrated by proper pre-treatment, a deuterium atom should be introduced to the α-position of the resulting monobasic acid. In due course, before carrying out the reaction, the enzyme solution was lyophilized and treated with D_2O. After repetition of this treatment, the reaction was carried out in D_2O. The resulting phenylacetic acid was proved to have one deuterium atom on the α-position of the carboxyl group by mass spectroscopy and NMR [4]. Thus it can be concluded that lyophilization of the enzyme solution and treatment of the resulting dry enzyme with water is enough to exchange the water molecule in the active site. With this evidence in hand, we carried out the reaction of phenylmalonic acid in $^{18}OH_2$ at 35 °C after the same pre-treatment. The resulting phenylacetic acid was extracted and methylated with diazomethane. The methyl phenylacetate obtained was purified by column chromatography on silica gel. The isolated sample was identified by the comparison of the retention time of HPLC and NMR spectrum with those of commercially available authentic specimen. The mass spectrum of the methyl ester was entirely identical with that of the authentic sample (M^+ 150). This fact clearly shows that the product obtained from the reaction in ^{18}O-water contains no ^{18}O. Although we cannot totally exclude a little possibility that the ^{16}OH eliminated from the substrate at the step of thiol ester formation is retained in the active site and incorporated again in the final product at the hydrolysis step of the thiol ester, it is unlikely that the reaction proceeds via a C–O bond fission. Accordingly, formation of a thiol ester bond between the cysteine residue of the enzyme and the substrate became doubtful.

3.2. Reinvestigation of the mode of inhibition by BPA

One of the bases on which we previously supposed the formation of the thiol ester was the observation that the inhibition of the enzyme activity by BPA was recovered by the addition of β-mercaptoethanol (β-ME). We supposed that the effect of β-ME to recover the enzyme activity indicated that the inhibitor is bound to the enzyme not via a sulfide linkage but a thiol ester bond with the Cys residue at the active site. We considered that if Cys 188 is able to form a C–S bond with a free carboxylic acid, such as BPA, it will also give a thiol ester with the substrate. However, because these suppositions are in conflict with the results obtained by the reaction in $^{18}OH_2$, we reinvestigated the inhibition experiments. The results are summarized in Fig. 1.

First, as the control experiment, the effect of the addition of β-ME was examined. The enzyme kept its full activity after treatment with β-ME for 16 h at 4 °C [Ex-A]. On the other hand, when BPA was added to the solution of the enzyme first, and then treated with β-ME, only negligibly small activity was observed, indicating that β-ME had only little effect on the recovery of the enzyme activity [Ex-B]. The effect of β-ME to recover the activity in a small extent might be accounted for by supposing that this thiol was working in place of Cys residue which was blocked by BPA. However, we have not examined in further detail. In the previous experiment [10], we overestimated the amount

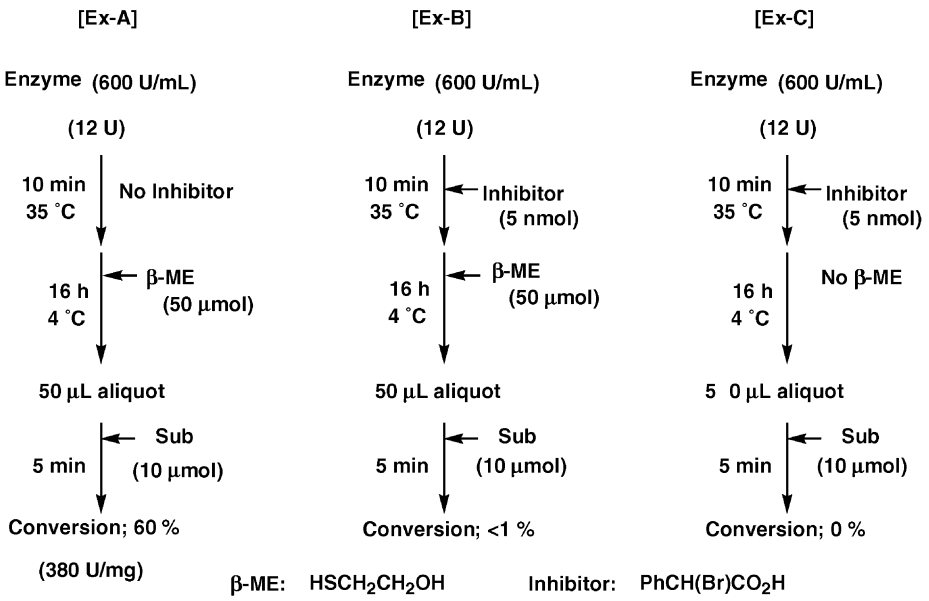


Fig. 1. The effect of addition of α-bromophenylacetic acid (BPA, inhibitor) and β-mercaptoethanol (β-ME). [Ex-A]; The addition of mercatoethanol had no effect on the enzyme activity. [Ex-B]; The effect of β-mercaptoethanol was negligible when the inhibitor was added. [Ex-C]; The enzyme activity was totally lost by the addition of the inhibitor.

of recovery of the enzyme activity because (1) too large an amount of enzyme was used and (2) lack of the positive control experiment [Ex-A]. No activity was observed when the enzyme solution was treated only with BPA [Ex-C]. There was no recovery of activity even when this solution was dialyzed against Tris–HCl buffer. If the binding between BPA and the enzyme is reversible, dialysis would remove BPA at least to some extent and therefore some recovery of the enzyme activity should be observed. These experiments lead to the conclusion that the binding of BPA and the enzyme is irreversible, i.e., via a covalent sulfide bond (Eq. (6)). In fact this was confirmed by ESI mass spectroscopy.

3.3. Mass spectrum of the enzyme–inhibitor complex

To examine the mode of binding between BPA and the enzyme, ESI mass spectra of the complex and inhibitor-free enzyme were measured. To obtain clean spectra, the enzyme solution in buffer was dialyzed against deionized water to remove the inorganic salts. The concentration of the enzyme was made up to 0.2 mg/ml and 20% volume of methanol containing 1% acetic acid was added prior to the measurement. The ESI mass spectrum of the native enzyme gave several multiply-charged ions. The deconvolution process gave molecular weight (MW) of the enzyme at 24,744 (Table 1). When BPA and the enzyme bind with the formation of a sulfide bond (Eq. (6)), the molecular weight of the complex should be 134 units larger than that of the native enzyme. On the other hand, if the inhibitor and enzyme bind via a thiol ester, the increase of mass number should be 197 (Eq. (5)). There is one more possibility to bind the two components, i.e., the formation of a salt between the carboxylate group of BPA and some basic amino acid residue of the enzyme (Eq. (7)). In this case, the mass number of the salt should be 214 units larger than that of enzyme itself.

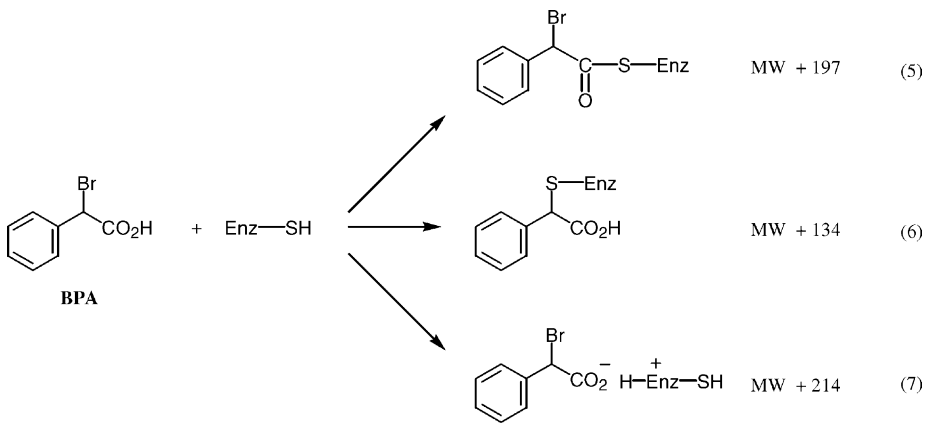


Table 1
The ESI mass spectra and molecular weights of native and BPA-binding enzymes

Enzyme	*m/z* on ESI mass spectrum (charge number)	MW (obsd)	MW (calcd)
Native enzyme	1905 (+13), 1767 (+14), 1650 (+15) 1547 (+16), 1456 (+17), 1375 (+18) 1303 (+19)	24744	24734
Enzyme–BPA complex	1925 (+13), 1787 (+14), 1668 (+15) 1564 (+16), 1472 (+17), 1391 (+18) 1317 (+19)	25011	25001

Actually, when the enzyme was treated with BPA, the mass number of the enzyme increased by 267 (Table 1). Although this number is different from any of the predicted values, it is just twice of the number calculated for the case of formation of a sulfide bond (Eq. (6)). Thus it is probable that two moles of the bromo acid bound to the enzyme irreversibly with elimination of hydrogen bromide, although it is not clear which amino acid residues reacted with BPA.

The most important basis on which we supposed the formation of thiol ester between the enzyme and substrates was the estimation that BPA bound with the enzyme via a thiol ester bond. Now this prerequisite is proved to be incorrect. Thus we have to reexamine other possible mechanisms.

3.4. Effect of pH on the activity of AMDase

The pH-rate profiles of the native and C188S mutant enzymes using phenylmalonate as the substrate are illustrated in Fig. 2. The figure shows the relative activity of each enzyme taking the maximum activity of each enzyme as 100%, although the net activity of the mutant enzyme is far lower than that of wild-type enzyme (The relative activity of the mutant enzyme to that of native enzyme at pH 8.5 is 4.8×10^{-3}. WT enzyme: K_m, 13.9 mM; k_{cat}, 364 s^{-1} C188S mutant enzyme: K_m, 4.9 mM; k_{cat}, 0.62 s^{-1}) [6].

The activity of the wild-type enzyme gradually increased with increasing pH of the medium until it showed the maximum at pH 8.0. In the region of pH above 8.0, a drastic decrease of activity was observed. It was confirmed that this decrease in activity was not due to the irreversible denaturation of the enzyme. When the enzyme was treated in alkaline medium under the same conditions as those employed in the reaction, and then it was subjected to the reaction at pH 7.5, it showed its full activity. Thus it is clear that the decrease of activity only depends on the change of the pH of the medium. Then we carried out kinetic studies at pH 9.5 as the representative basic region to clarify either which of the change in K_m or k_{cat} is responsible for the lowering of the enzyme activity. According to the changing of the pH of the medium from 8.5 to 9.5, while the value of k_{cat} decreased from 364 to 251 s^{-1} (69%), the change in the affinity between the substrate and the enzyme was found to be relatively small (89%, K_m value; from 13.9 to 15.6 mM). Thus it is clear that the major reason of the lower enzyme activity in the basic region is the lowering of k_{cat} rather than the change in K_m.

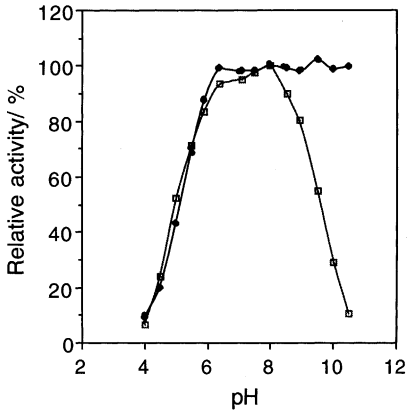
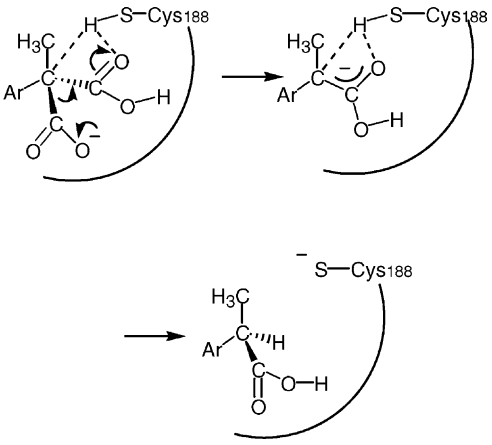


Fig. 2. pH-Rate profiles for WT and C188S mutant of AMDase at 35 °C: (□) activity of the wild-type enzyme; (●) activity of the C188S mutant enzyme.

In contrast to the bell-shaped pH profile of the activity of the wild-type enzyme, the activity of the C188S mutant retained its full activity until the pH of the medium exceeds 10. There may be some ambiguities on the mechanism how the pH of the medium affect the reactivity of two enzymes, such as change in dissociation of the carboxyl groups of the substrate, change in conformation of the enzyme, and etc. However, as all the reaction conditions are the same for WT and mutant enzymes, the most promising key to explain this marked difference will be the difference between some property of cysteine and serine. However, because the activity of the C188S mutant is extremely lower compared to that of WT enzyme, it is apprehended that not only Ser188 but also some other amino acid residues are working together to compensate for the lack of Cys188. In any event, there will be no possibility that the candidates are other Cys residues, because changing them to Ser had no serious effect on the k_{cat} of the reaction (minimum: 1/6 for C171S mutant [6]). Thus the conclusion obtained from these experiments will be more or less the same regardless of the participation of other amino acid residue other than Ser188.

The most remarkable difference between Cys and Ser is considered to be that of their pK_a values. Because cysteine is more acidic than serine, there will be a pH region in which the SH of Cys residue will dissociate to thiolate anion, while serine still retains the free OH form. Such a pH region should be a slightly basic region, which is between pH 8 and 10 in this case. It can be said that the free SH or OH is essential for the enzyme activity and when SH group loses its proton, the enzyme became inactive. This suggests that the Cys residue of AMDase works as the proton donor to the substrate. It is estimated that Cys188 will partially protonate the α-carbon and/or the carbonyl group, and assists the formation of enolate type transition state triggered by

the deprotonation of one carboxylic group. Because it has been already revealed that the Hammett's ρ value is negative for the reactions of substituted phenylmalonic acid [2], the transition state should have some negative charge. However, the present experiment suggested that the partial assistance of the protonating amino acid residue is inevitable to lower the activation energy to promote the C–C bond fission. In other words, the present decarboxylation reaction can be said to proceed similarly to two-base mechanism (Eq. (8)).



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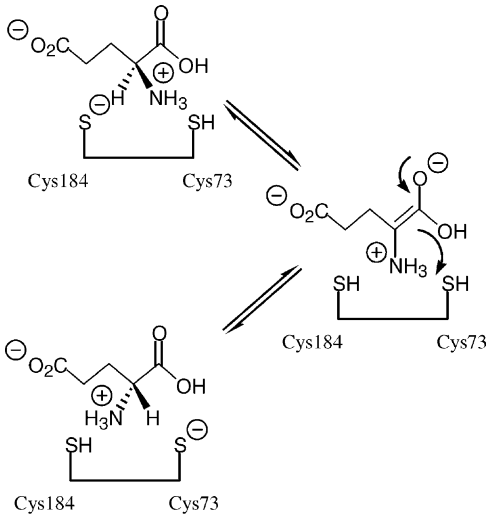
3.5. Homology of AMDase with glutamate racemase and other isomerases

At this stage, we searched other enzymes which have some similarity to AMDase in both function and amino acid sequences, which will be useful to make clear the reaction mechanism of AMDase.

Unfortunately, no known enzyme which had high (over 50%) homology with AMDase was found by the search of database obtained via internet according to the PSI-BLAST algorithm. However, there were some enzymes that had about 30% homology and some common points in their functions (Fig. 3). They were glutamate racemase from *Lactobacillus fermenti* [13], aspartate racemase from *Streptococcus thermophilus* [11], hydantoin racemase from *Pseudomonas* sp. Strain NS671 [15], and maleate isomerase from *A. faecalis* [16].

The important point that is conserved for all these enzymes is the presence of Cys188. On the other hand, the critical difference between AMDase and isomerases is the fact that while all of the isomerases have another cysteine residue around 73, AMDase has no corresponding Cys around this region.

The reaction mechanism is extensively studied for glutamate racemase [17–19]. It has been proposed that the key for the racemization activity is that two cysteine residues of the enzyme are located in both sides of the substrate bound to the active site. Thus 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 amino acid. In this way the racemase catalyzes the racemization of glutamic acid via the two-base mechanism (Eq. (9)). The tertiary structure of glutamate racemase has been dissolved, and it has been also clarified that a substrate analogue glutamine binds between the two cysteine residues [20]. This fact is a strong support to the proposed mechanism.



(9)

In contrast to racemases, AMDase has only one cysteine residue in the active site. The marked difference between racemization and decarboxylation reactions is that the substrate of racemization is neutral, while that of decarboxylation is anionic. Thus it is estimated that the decarboxylation reaction is promoted only by the presence of a proton donor. The cysteine residue is presumed to deliver a proton from just one side of the enolate intermediate resulting in the formation of single enantiomer. It is estimated that partial

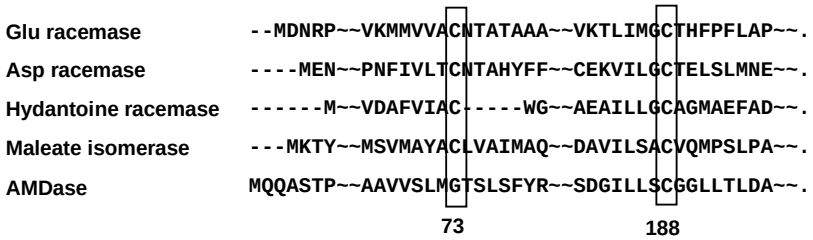


Fig. 3. Amino acid homology between some racemases and AMDase. Origins of the listed isomerases: glutamate racemase, *Lactobacilllus fermenti* [13]; aspartate racemase, *S. thermophilus* [14]; hydantoin racemase, *Pseudomonas* sp. strain NS671 [15]; maleate racemase, *A. faecalis* [16]; AMDase, *A. bronchiseptica* (former name; *A. bronchisepticus*) [5].

protonation from the cysteine residue would assist the C–C bond fission, although the complete C–H bond is formed after the formation of enolate type transition state because the Hammett's ρ value was negative as mentioned earlier.

4. Conclusions

The asymmetric decarboxylation of disubstituted malonate is a unique reaction characteristic of biocatalysis. We once proposed the formation of a thiol ester intermediate between the substrate and the enzyme. However, careful reinvestigation of inhibition reaction revealed that we had overestimated the effect of β-mercaptoethanol for the recovery of the activity of the enzyme that is once deactivated by BPA. In addition, pH profiles of the reactions of native enzyme and the C188S mutant, as well as the homology alignment of amino acid sequence lead us to conclude that the cysteine residue in the active site will be working as a proton donor to create the asymmetric center of the product. Apparently, enzymatic reactions are the events in water, which is of course a proton donor. Nontheless, enzymes are able to control the direction of approaching protons. There are known not a few examples, such as isomerization of citric acid to isocitric acid in TCA cycle, formation of malic acid from fumalic acid (TCA cycle), and conversion of isobutyric acid to β-hydroxyisobutyric acid as a representative example of biotransformation of synthetic substrates [21,22]. The present decarboxylation reaction is possibly one example of this type of enzymatic reactions. The main reason that the asymmetric protonation in aqueous medium becomes possible when enzyme is used as the catalyst will be the hydrophobicity of the active site of enzymes, which prevents bulk water molecules to join the events inside of the enzymes.

If the proposed role of Cys188 is true, introduction of a cysteine residue around the region that is supposed to be located at the opposite side of the Cys188 might result in the lowering or inversion of enantioselectivity. The experiments in due course are now underway, and the preliminary results obtained so far support the present proposal.[1]

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References

[1] K. Miyamoto, H. Ohta, J. Am. Chem. Soc. 112 (1990) 4077.
[2] K. Miyamoto, H. Ohta, Eur. J. Biochem. 210 (1992) 475.
[3] Y. Fukuyama, K. Matoishi, M. Iwasaki, E. Takizawa, M. Miyazaki, H. Ohta, S. Hanzawa, T. Sugai, Biosci. Biotechnol. Biochem. 63 (1999) 1664.
[4] K. Matoishi, S. Hanzawa, H. Kakidani, M. Suzuki, T. Sugai, H. Ohta, J. Chem. Soc. Chem. Commun. (2000) 1519.
[5] H. Ohta, Bull. Chem. Soc. Jpn. 70 (1997) 2895, and the references cited therein.
[6] M. Miyazaki, S. Hanzawa, H. Kakidani, H. Ohta, Bull. Chem. Soc. Jpn. 70 (1997) 2765.
[7] K. Miyamoto, S. Tsuchiya, H. Ohta, J. Am. Chem. Soc. 114 (1992) 6256.
[8] K. Miyamoto, H. Ohta, Y. Osamura, Bioorg. Med. Chem. 2 (1994) 469.
[9] T. Kawasaki, E. Horimae, H. Ohta, Bull. Chem. Soc. Jpn. 69 (1996) 3591.
[10] T. Kawasaki, M. Watanabe, H. Ohta, Bull. Chem. Soc. Jpn. 68 (1995) 2017.
[11] T.A. Kunkel, J.D. Robents, R.A. Zakour, Methods Enzymol. 154 (1987) 367.
[12] K. Miyamoto, H. Ohta, Appl. Microbiol. Biotechnol. 38 (1992) 234.
[13] K.A. Gallo, J.R. Knowles, Biochemistry 32 (1993) 3981.
[14] M. Tohda, H. Okada, H. Kumagai, Biochim. Biophys. Acta 1089 (1991) 234.
[15] K. Watanabe, T. Ishikawa, Y. Mukohara, H. Nakamura, J. Bacteriol. 174 (1992) 3461.
[16] K. Hatakeyama, Y. Asai, Y. Uchida, M. Kobayashi, M. Terasawa, H. Yukawa, Biochem. Biophys. Res. Commun. 239 (1997) 74.
[17] S. Glavas, M.E. Tanner, Biochemistry 38 (1999) 4106.
[18] K.A. Gallo, M.E. Tanner, J.R. Knowles, Biochemistry 32 (1993) 3991.
[19] M.E. Tanner, K.A. Gallo, J.R. Knowles, Biochemistry 32 (1993) 3998.
[20] K.Y. Hwang, C.-S. Cho, S.S. Kim, H.-C. Sung, Y.G. Yu, Y. Cho, Nat. Struct. Biol. 6 (1999) 422.
[21] C.T. Goodhue, J.R. Schaeffer, Biotech. Bioeng. 13 (1971) 203.
[22] J. hasegawa, M. Ogawa, S. Hamaguchi, M. Shimazaki, H. kawaharada, K. Watanabe, J. Ferment. Tech. 59 (1981) 203.

[1] In the reaction of α-methyl-α-(β-naphthyl)malonate, the G74C mutant gave nearly the racemic form of the corresponding monobasic acid in contrast to the fact that the wild-type enzyme gave the (*R*)-enantiomer.