

Cysteine188 Revealed as Being Critical for the Enzyme Activity of Arylmalonate Decarboxylase by Site-Directed Mutagenesis

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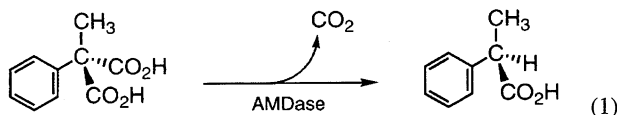
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Arylmalonate decarboxylase (AMDase) catalyzes the asymmetric decarboxylation of α -aryl- α -methylmalonic acid. Since this enzyme is inhibited by SH-reagents, a cysteine residue is supposed to be involved at the catalytic site. Cloning of the gene which codes the enzyme revealed that this enzyme contains four cysteine residues. Titration of free SH groups by *p*-(chloromercurio)benzoate disclosed that all four Cys are in the reduced form. In this study, four mutant enzymes (C101S, C148S, C171S, and C188S) were prepared, in which one of four cysteines was replaced by serine. The CD spectra indicated that the conformational differences of C101S and C188S compared to that of the native enzyme are not so significant. The catalytic activities of the four mutants were measured. Among these mutant enzymes, only C188S showed a drastic decrease in enzyme activity, indicating that cysteine¹⁸⁸ is located at the active center of the enzyme. The catalytic activities of the other mutants are also discussed.

Arylmalonate decarboxylase (AMDase) is a novel enzyme isolated from a kind of bacterium, *Alcaligenes bronchisepticus* KU 1201,¹⁾ and catalyzes the enantioselective decarboxylation of prochiral α -aryl- α -methylmalonic acids to give optically active 2-arylpropanoic acid^{1–3)} (Eq. 1).



This enzyme has no significant homology with other known decarboxylases, providing a unique catalytic mechanism. Unlike other decarboxylases and transcarboxylases, it requires no cofactors, such as biotin, coenzyme A, ATP, or metal ions.⁴⁾ As we presented in previous reports, the reaction is inhibited by SH-reagents, such as *p*-(chloromercurio)benzoate (PCMB), iodoacetate, mercury(II)chloride, and silver nitrate.⁴⁾ Because SH-reagents specifically react with a free cysteine residue, it is apparent that cysteine plays a significant role in decarboxylation reactions. The gene for this enzyme has been cloned and expressed in *E. coli*. Its nucleotide sequence has been elucidated by the dideoxy nucleotide protocol. The deduced amino acid sequence revealed that this enzyme consists of 240 amino acids (calcd MW 24734), including four cysteines at the positions of 101, 148, 171, and 188 from the amino terminal.⁵⁾ It is expected that at least one of four cysteine residues is essential for catalytic activity. The mode of activation was estimated to be the formation of a thiol ester bond with the substrate.^{6,7)} So far, however, no information has been obtained about which cysteine is involved in the active site.

We prepared four AMDase mutants (C101S, C148S,

C171S, and C188S) by site-directed mutagenesis, and measured their catalytic activities. In this paper, we would like to discuss the activities and thermal stabilities of the mutant enzymes, and to propose which one is the catalytic cysteine residue.

Experimental

Materials, *E. coli* Strains and Vectors. TSKgel DEAE-Toyopearl 650M, butyl-Toyopearl 650S, and HPLC column TSKgel ODS-80_{TM}, which were used for purifying the wild and mutant enzymes, are products of Tosoh Corp. Phenylmalonic acid was obtained from Tokyo Chemical Industry Co., Ltd. Mutan-K kit for site-directed mutagenesis, plasmid vector pUC19, and host strain *E. coli* JM109 were purchased from Takara Shuzo Co.

Site-Directed Mutagenesis. The four mutants used in the present study were generated by site-directed mutagenesis according to a method of Kunkel et al.⁸⁾ with a Mutan-K in vitro Mutagenesis kit (Takara Shuzo). The synthetic oligonucleotides used were 5'-CATGGTCGTGCTAGGCAGTCC-3' (21mer) for C101S, 5'-AAGGCTGCGGGATCCGGTGGG-3' (21mer) for C148S, 5'-GGCAGCAGCAGCTCAGGTCGAC-3' (21mer) for C171S, and 5'-CAAGCCGCCGCTAGACAGCAG-3' (21mer) for C188S. The nucleotide sequence of the four mutants around the mutated site were confirmed by the dideoxy chain-termination method with Taq Dye Deoxy Cycle Sequencing kit (Applied Biosystems).

Expression of the Mutant Genes and Purification of the Mutant AMDase. The site-specific mutants of pAMD101, a plasmid for the expression of AMDase, were transformed into *E. coli* JM109. The transformants were cultivated in 1000 mL of LB-broth (pH 7.0, containing 150 mg dm⁻³ of ampicillin) at 30 °C with shaking (200 rpm). After cultivation for 6 h, starting from 1/100 volume of overnight preculture inoculum, IPTG (isopropyl- β -D-galactopyranoside) was added at 0.1 mM (1 M=1 mol dm⁻³). Cultivation was continued for an additional 12 h. The cell pellet

was then collected by centrifugation at $3000\times g$ for 20 min. The purification procedure was described previously by K. Miyamoto et al.⁴⁾ Mutant enzymes as well as a native one were purified to homogeneity by SDS-PAGE.

Protein Concentration. The protein concentration was determined by supposing that the absorbance at 280 nm of 0.1% (mg mL^{-1}) protein solution to be 1.0.

Titration of Free SH Group with PCMB Solution. *p*-(Chloromercurio)benzoic acid (9 mg) was dissolved in 2 mL of a sodium hydroxide aqueous solution (40 mM) and diluted to 25 mL. A trace amount of insoluble impurities was removed by centrifugation ($800\times g$, 20 min). The concentration of PCMB of this solution was determined by measuring the change in the absorbance at 255 nm. An aqueous solution of cysteine (50 μM , 1 mL) was added to a UV measurement cell. The zero point was adjusted using deionised water as the blank. Then, a 2- to 5- μL aliquot of PCMB solution was added to both cells. The concentration of PCMB was determined to be 714 μM based on the equivalence point.

Titration of the free enzyme: A solution of purified enzyme (0.24 mg mL^{-1} , 200 μM) was diluted with 600 μL of deionised water in a UV cell. To the second cell, was added 800 μL of deionized water, and the zero point was adjusted. Then, a 2- and 5- μL aliquot of PCMB solution was added to both cells, and the absorbance at 255 nm was measured.

Titration of the α -bromophenylacetic acid (BPA)-enzyme complex: To a UV measurement cell were added a solution of enzyme (200 μL), a 100-mM aqueous solution of ($\pm$)- α -bromophenylacetic acid (80 μL) and 520 μL of deionized water. The mixture was allowed to stand at room temperature for 10 min in order to form an inhibitor-enzyme complex. The change in the absorbance with the addition of PCMB was measured in the same way as described for the native enzyme.

Enzyme Assay and Thermal Stability. The enzymatic activity was determined by measuring the rate of formation of phenylacetic acid from α -phenylmalonic acid at pH 8.5 in Tris-HCl buffer at 35 $^{\circ}\text{C}$. A determination of phenylacetic acid was carried out by HPLC, which was performed by using ODS-80_{TM}. The kinetic constants (K_m and k_{cat}) were obtained from Lineweaver-Burk plots. The thermal stability of four mutants and wild-type AMDase were measured by the following two different methods: a) After a heat treatment for 30 min at various temperatures, the remaining activities were measured. b) The activities were measured after incubation at 50 $^{\circ}\text{C}$ for several minutes.

Circular Dichroism Spectra. The CD spectra were measured with a JASCO J-600 spectropolarimeter at room temperature. The light path of the cell was 1 mm for wavelength of between 190–270 nm. The concentration of the enzyme protein was 0.15 mg mL^{-1} in 10 mM Tris-HCl buffer, containing 2-mercaptoethanol (1 mM) and EDTA (0.5 mM).

Results and Discussion

Number of Cysteine Residues in the Active Site of AMDase. As deduced from the DNA sequence of the gene, AMDase contains four cysteines. First, we screened an active site-directed inhibitor to obtain more information about the number and role of cysteines at the active site. Among some compounds structurally related to the substrates, ($\pm$)- α -bromophenylacetic acid was found to be a potent inhibitor. A Lineweaver-Burk plot for this inhibitor, using phenylmalonic acid as the substrate, indicated that this compound was a competitive inhibitor with a K_i value of 3.6 μM at

24 $^{\circ}\text{C}$. The fact that the K_i value of this inhibitor is much smaller than the K_m value (25 mM) of the substrate indicates that this inhibitor blocks the active site of the enzyme.⁶⁾

We tried to clarify how many cysteines are present at the active site by using this inhibitor. It is well-established that when PCMB binds to cysteine, the absorbance at 255 nm increases due to the formation of an aryl-Hg-S bond. Thus, it is possible to estimate the number of free S-H residues of the enzyme by titration using a PCMB solution (Fig. 1). When the native enzyme was reacted with PCMB, the absorbance at 255 nm increased by 0.025. On the other hand, when a PCMB solution was added to the enzyme solution after the enzyme was incubated with α -bromophenylacetic acid, the increase in the absorbance was 0.018, just three fourths of the value for the free enzyme. These results clearly show that one fourth of the cysteine residues was blocked by α -bromophenylacetic acid and could not react with PCMB. As described before, this enzyme has four cysteine residues. Thus, it is concluded from the present titration measurement that all four cysteines are in the free SH form, and that one of them, which reacted with α -bromophenylacetic acid, is located in the catalytic site. Then, which one is at the active site?

Site-Directed Mutagenesis. Site-directed mutagenesis is one of the most powerful techniques for studying the mechanisms of enzyme-catalyzed reactions. Since this technique provides a method for replacing a specific amino acid residue

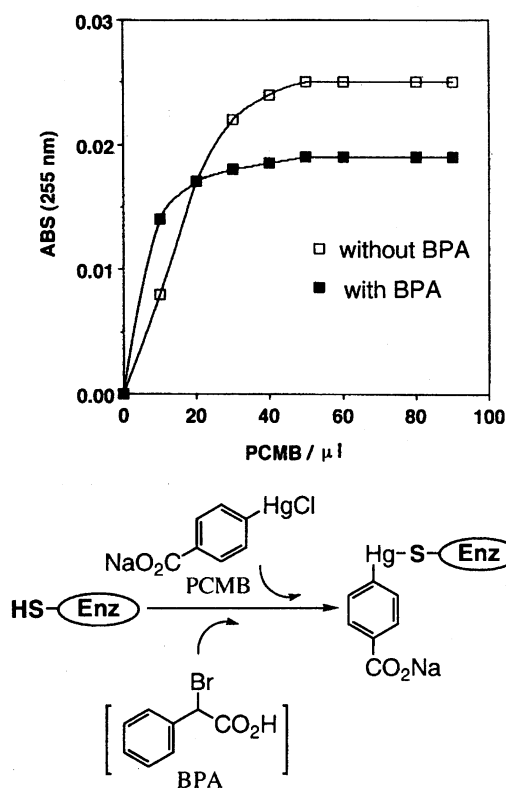
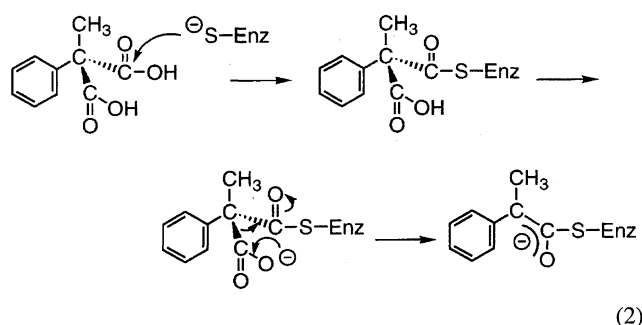


Fig. 1. Titration of the free cysteine residues of the enzyme. PCMB solution was added to a solution of AMDase in the absence or presence of BPA, and the increase of absorbance at 255 nm was measured.

of an enzyme to an arbitrary one, it is especially useful to specify the catalytic amino acid residue(s) of an enzyme.^{9–11)}

In the case of AMDase, one of four cysteine residues was supposed to be involved in the catalytic site, as shown by the titration experiments. Preparation and kinetic studies of four mutant enzymes in which one of cysteines is replaced by some amino acid are expected to be most informative. Which amino acid should be introduced in place of cysteine? To determine the best candidate, the mechanism of the reaction should be considered. To this end, the electronic effects of the substituents of the aromatic ring of phenylmalonic acid on the rate of reaction was examined, indicating that the sign of the Hammett ρ value is positive.⁴⁾ This fact clearly shows that the transition state is negatively charged.

Taking into consideration the fact that in an ordinary enzymatic decarboxylation reaction coenzyme A forms a thiol ester with the substrate, we supposed the mechanism shown in Eq. 2.



Thus, the rate-determining step is supposed to be either a nucleophilic attack of cysteine to one of the two carboxyl groups or C–C bond cleavage to form an enolate-type intermediate, because in both cases the transition state will have a negative charge. This supposition leads to an estimation that in either case, substitution of cysteine at the active site for serine would greatly decrease the reaction rate because of relatively small nucleophilicity and anion-stabilizing effect of a hydroxy group compared to a thiolate functionality.

In this way, if the mutant enzyme partially retains its catalytic activity, even when the essential cysteine in the active site is replaced by serine, the k_{cat} value would greatly decrease, while K_{m} value would not be seriously affected. On the other hand, if the cysteine residue other than the catalytic one is replaced by serine, the effect on the reactivity will be moderate, because the steric bulkiness of serine resembles that of cysteine, and it would more or less keep the hydrogen bonding(s) of the wild enzyme. Thus, we prepared four mutant genes in which one of four codons corresponding to cysteine was replaced by that of serine, via site-directed mutagenesis. Four AMDase mutants expressed in a mutant of *E. coli* were purified to homogeneity and analyzed as follows.

First, the enzyme activity was measured and the kinetic parameters were determined by Lineweaver–Burk plots (Table 1). Among four mutants, C188S showed a drastic decrease in activity ($k_{\text{cat}}/K_{\text{m}}$). This low activity was due to a decrease in the catalytic turnover number (k_{cat}) rather than the affinity to the substrate (K_{m}).

Table 1. Relative Activities and Kinetic Parameters of the Wild Type and Four Mutant Enzymes

	Relative activity U/mg	K_{m} mM	k_{cat} s^{-1}	$k_{\text{cat}}/K_{\text{m}}$
Wild type	406.4	13.3	365.9	27.5
C101S	547.6	4.3	247.6	57.6
C148S	166.5	11.5	100.1	8.7
C171S	118.0	9.1	62.3	6.8
C188S	1.3	4.9	0.62	0.13

The CD spectrum (Fig. 2) of the C188S mutant revealed that the tertiary structure of this mutant changed little compared to that of the wild-type enzyme. The calculation results of the content of the secondary structure of the mutant enzymes based on the J-600S Secondary Structure Estimation system (JASCO) are given in Table 2. These data also show that there is no significant change in the tertiary structure of the C188S mutant. The fact that the k_{cat} value of this mutant is extremely small, despite a slight change in conformation, clearly indicates that Cys¹⁸⁸ is located in the active site.

Another mutant which showed only a small change in conformation was C101S, which exhibited higher activity than the wild enzyme. The higher activity is attributed to a smaller K_{m} value. A concrete reason for the higher activity is not presently clear, but it is estimated that Cys¹⁰¹ is located near to the catalytic site, or mutation brought about some increase in flexibility which made the induced fit of the enzyme to the substrate more tight.

On the other hand, the catalytic activity of C148S and C171S decreased in spite of the smaller K_{m} values than that of the wild enzyme. It can be assumed that a decrease in α -helix structure caused a decrease in the k_{cat} value. The distance between the catalytic amino acid and the binding substrate would become longer because of a change in the conformation. It was thus concluded that cysteine¹⁸⁸ is located at the catalytic site of the enzyme.

We previously reported that AMDase forced the substrate to take a specific conformation at the active site to bind to the enzyme.^{12,13)} It is thus supposed that the retained or smaller K_{m} values of the mutant enzymes may be due to an increased flexibility of the enzymes, which allows the activation entropy of the reaction to be smaller.

Thermal Stability of Mutant Enzymes. The thermal stabilities of the mutant enzymes were examined. The data (Fig. 3) show that all of these mutants were thermally more

Table 2. Content of Secondary Structure of the Wild Type and Four Mutant Enzymes

	α -Helix %	β -Sheet %	Turn %
Wild type	39.6	4.8	11.1
C101S	38.0	3.2	5.0
C148S	29.6	5.7	5.6
C171S	21.5	4.2	5.0
C188S	32.9	4.1	9.1

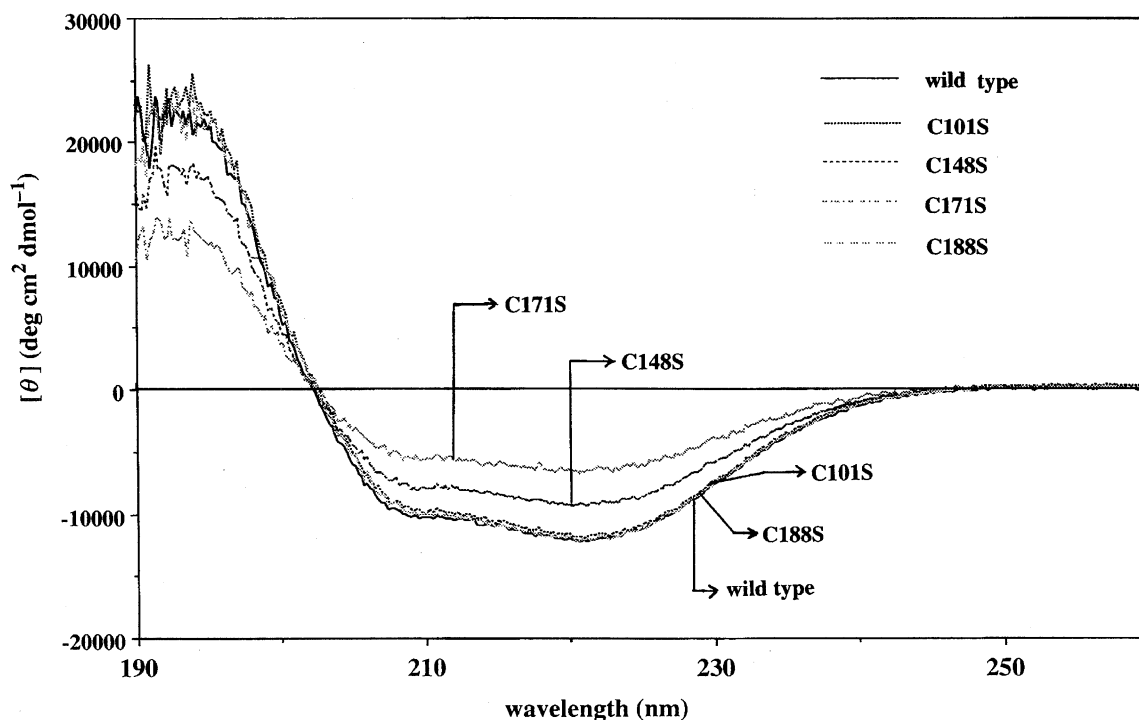


Fig. 2. CD spectra of the wild type and four mutant enzymes.

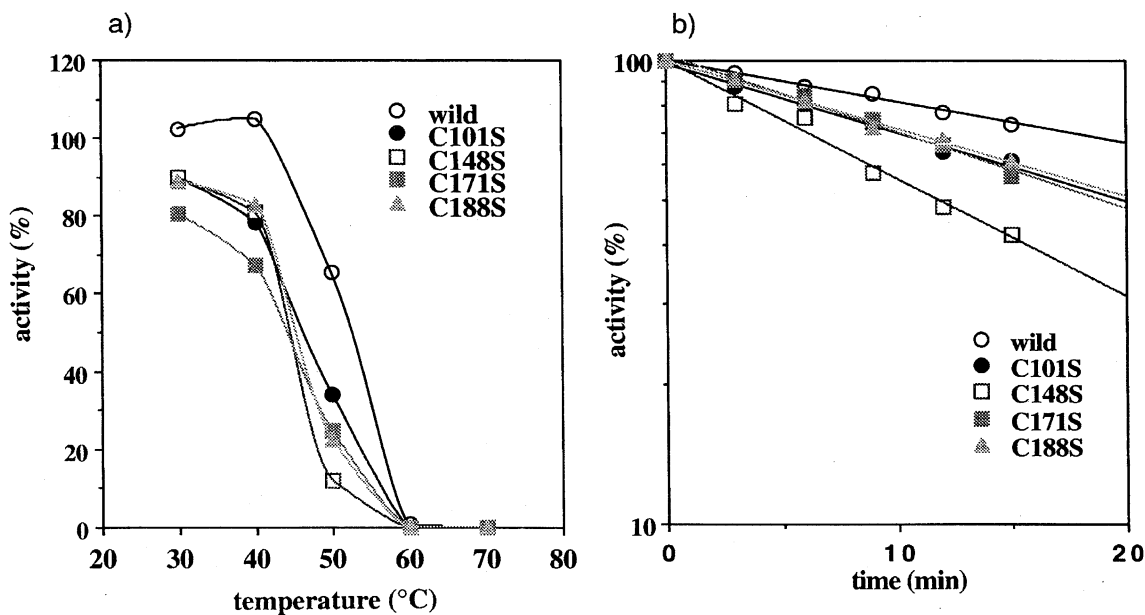


Fig. 3. Thermostability of the wild type and four mutant enzymes. a) The remaining activities were measured after heat treatment for 30 min at various temperatures. b) Activities were measured after incubation at 50 °C for periods depicted in the Figure.

labile than the wild-type enzyme. While the half-life of the wild enzyme at 50 °C is about 32 min, that of the most unstable mutant (C148S) is less than 12 min, and those of other mutants are around 18 to 19 min (Fig. 3b). It may be possible that cysteines are present in the hydrophobic region of the tertiary structure of the enzyme. In this case, the replacement of Cys to a more hydrophilic Ser would bring about some decrease in the thermal stability. We, therefore, have the view that all of the four cysteine residues contribute to the stability of the enzyme, two of which (cysteine 148 and 171) are more important for maintaining the overall tertiary

structure. More detailed investigations by X-ray and NMR structure analyses are now under way.

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