A Novel Enzymatic Decarboxylation Proceeds via a Thiol Ester Intermediate

Takayasu Kawasaki, Masakatsu Watanabe, and Hiromichi Ohta*

Department of Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223

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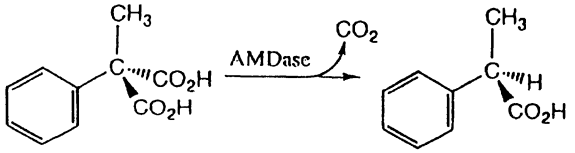
It is proposed that arylmalonate decarboxylase (AMDase)-catalyzed decarboxylation proceeds via a thiol ester intermediate. Kinetics and CD spectra indicated that α-bromophenylacetate is a competitive inhibitor. TOF mass data indicated that the inhibitor bound with the enzyme through a thiol ester bond which was formed between a cysteine residue of the enzyme and the carboxyl group of the inhibitor. This result was also supported by reactivation of the enzyme by the addition of 2-mercaptoethanol, which is expected to cleave the enzyme–inhibitor bond via nucleophilic attack on the thiol ester linkage.

Arylmalonate decarboxylase (AMDase) is a novel enzyme from *Alcaligenes bronchisepticus* KU 1201.[1] It catalyzes an enantioselective decarboxylation of prochiral α-aryl-α-methylmalonates to yield optically active 2-arylpropionates (Eq. 1)[1,2]



(1)

This enzyme has no significant homology with known decarboxylases, and thus studies on the mechanism of this reaction are very fascinating. We have already disclosed some characteristic features of this novel enzyme. This decarboxylation reaction proceeds smoothly without the aid of any co-factors such as ATP, coenzyme A, or biotin, and is inhibited by SH reagents such as iodoacetate (IA), *p*-chloromercuribenzoate (PCMB), mercury(II) chloride, and silver nitrate.[3] In addition, cloning and sequencing of genomic DNA lead to the conclusion that this enzyme consists of 240 amino acids containing four cysteine residues (Cys101, 148, 171, 188).[4] It can be concluded from the above inhibition studies that at least one of these Cys residues plays a crucial role in the activation of the substrate. However, a more detailed mechanism of the enzymatic reaction, including how the cysteine works on the substrate, has not yet been proposed.

In this report, we present the results of the following experiments aiming to disclose the role of the cysteine located in the active site of AMDase.

1) Kinetics of an active site-directed inhibitor,

2) Deactivation of AMDase by the inhibitor and recovery of the activity,

3) Mass spectrometric studies on the AMDase-inhibitor complex.

Results and Discussion

Kinetics of an Active Site-Directed Inhibitor. First, we screened for a potent inhibitor against the AMDase catalyzed-decarboxylation reaction of α-methyl-α-phenylmalonate to give 2-phenylpropionate. Among the compounds (**1**—**6** in Fig. 1) which have structures similar to the substrate, (±)-α-halophenylacetates (**1** and **2**) remarkably inhibited the reaction. Especially, α-bromophenylacetate (**1**) showed a striking inhibitory effect on the AMDase-catalyzed decarboxylation reaction. On the contrary, other substrate analogues (**3**—**6**) showed no inhibitory effects. A Lineweaver-Burk plot (Fig. 2) for inhibitor **1** indicated that this compound was a competitive inhibitor,[5] with a K_i value of 3.6 μM (1 M=1 mol dm^{-3}) at 24 °C.

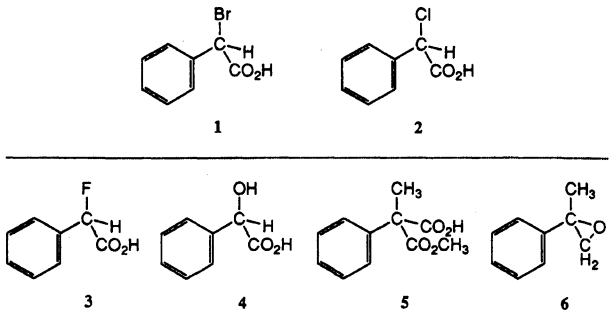


Fig. 1. Compounds tested as active site directed inhibitor.

#Abbreviations: AMDase, arylmalonate decarboxylase; IA, iodoacetate; BPA, α-bromophenylacetate; ME, 2-mercaptoethanol; PCMB, *p*-chloromercuribenzoate.

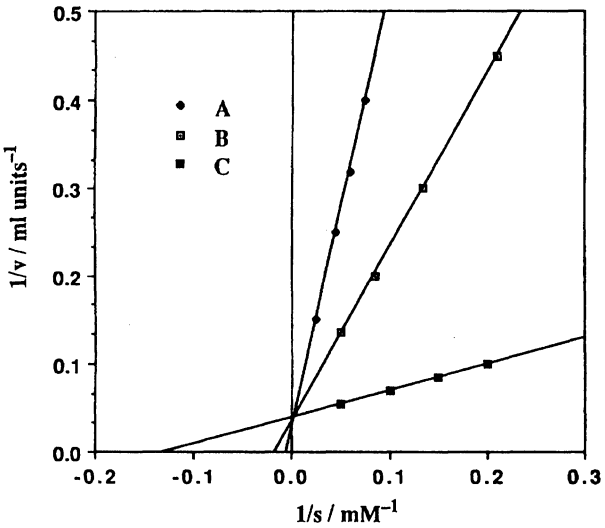


Fig. 2. Inhibition mode of α-bromophenylacetate against AMDase catalyzed decarboxylation. Lineweaver-Burk plot in the presence of α-bromophenylacetate (BPA); A, 100 μM; B, 20 μM; C, 0 μM.

The facts that the mode of inhibition is competitive and the K_i value is extraordinarily small compared with the K_m value of the substrate (25 mM), strongly suggest that this inhibitor blocks the active site and prevents substrate approach to the catalytic site of the enzyme. The high electron-withdrawing effect of the bromine atom could have an important role in the inhibition mechanism. Thus the mode of inhibitor binding to the active site of the enzyme is supposed to closely resemble that of the substrate. Accordingly, to disclose how the inhibitor interacts with the enzyme would give important information on how the enzyme activates the substrate. Then, how does the inhibitor bind to the active site? There are at least three possibilities: (1) formation of a salt between the carboxyl group of the inhibitor and some basic side chain of the enzyme, (2) formation of a sulfide bond with a cysteine residue with elimination of hydrogen bromide, and (3) formation of a thiol ester bond with a cysteine in the active site.

Inactivation by IA and BPA. AMDase has four cysteine groups as is evident from the DNA sequence.[4] In a previous report,[3] it was shown that SH reagents such as PCMB and iodoacetate (IA) irreversibly deactivate AMDase. To elucidate the difference in the mode of inhibition between IA and BPA, we examined the CD spectra of the enzyme–inhibitor complexes (Fig. 3). First, the conformational change of the enzyme due to binding of BPA was studied. The CD spectrum of native enzyme exhibited a curve typical of an α-helix rich structure,[6] and the spectrum of the enzyme–inhibitor complex was essentially the same as that of the native enzyme. This indicates that BPA may bind to the active site pocket of AMDase without causing a gross conformational change of the enzyme itself.[7] On the other hand, the spectrum of the IA–enzyme complex indicates

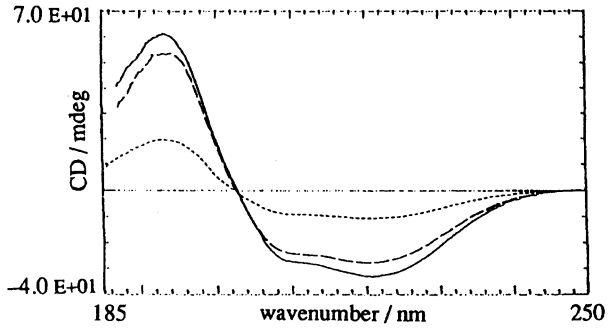


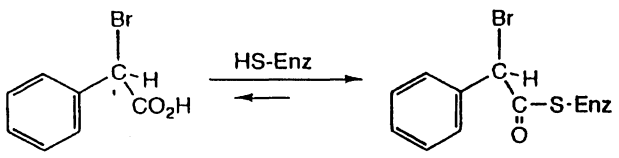
Fig. 3. CD spectra of AMDase (cell length, 1 mm). — AMDase (native) 0.1 mM, --- AMDase (0.09 mM)+PhCH(Br)CO_2H (0.9 mM), ···· AMDase (0.09 mM)+ICH_2CO_2H (9.1 mM).

that binding of IA brings about remarkable conformational changes in AMDase. This CD spectral change shows that IA modifies up to four cysteine residues and causes the deformation of the enzyme into a random coil structure.

The above results support that BPA attacks the active site pocket of AMDase, as suggested by the Lineweaver-Burk plot, while IA attacks several cysteines and deactivates the enzyme. In other words, the spatial arrangement as well as the binding of BPA in the active site could be also very close to those of the substrate.

Mass Measurement of the Enzyme–Inhibitor Complex. BPA was established to be an active site-directed inhibitor. However, the bonding between the inhibitor and amino acids in the active site has not been clarified. The molecular weight of the enzyme–inhibitor complex was then measured with matrix-assisted laser desorption ionization (MALDI)–time of flight (TOF) mass spectrometry in order to investigate the mode of binding of BPA to AMDase.[8]

Figure 4 shows the results of the mass measurements. The mass number of the native enzyme was measured to be 24722. This value was consistent with the calculated value, 24734, within the range of error (resolution is ±100) of TOF mass measuring. Next, the molecular weight of the AMDase–BPA (MW=215) complex was measured to be 24971. This mass value is consistent with that calculated for a complex which is formed via a thiol ester bond between the carboxyl group of BPA and an SH group of the enzyme (calcd: 24734+215−18=24931) or formation of a carboxylate salt (calcd: 24949). However, the formation of a 1:1 complex via a sulfide bond with elimination of hydrogen bromide (calcd: 24734+215−81=24868) is not likely (Eq. 2).



(2)

Recovery of AMDase Activity. From the kinetics and mass measurement results, BPA was found to bind with AMDase in a reversible manner, and the resulting bond was estimated to be a thiol ester or a simple salt. If the former is true, some thiol compounds would attack the carbonyl group to liberate the free enzyme resulting in the recovery of AMDase activity, while these thiol compounds would have no effect on the dissociation of the carboxylate-enzyme complex. In this way, two possibilities are expected to be distinguished, and this prediction turned out to be true as indicated in Table 1.

The activity of the enzyme gradually increased when a large excess of 2-mercaptoethanol (ME) was added to the enzyme–inhibitor complex, until the activity finally recovered to 100%. This result clearly shows that BPA was released from the active site of AMDase when ME was added to the AMDase–BPA complex. If the bonding between the inhibitor and the active site of the enzyme is the result of the formation of a carboxylate salt, it is unlikely to be broken by the addition of a neutral compound, such as ME. On the contrary, if BPA is bound to AMDase via a thiol ester bond, it could be cleaved via a nucleophilic attack of excess free thiol, which was actually observed.[9]

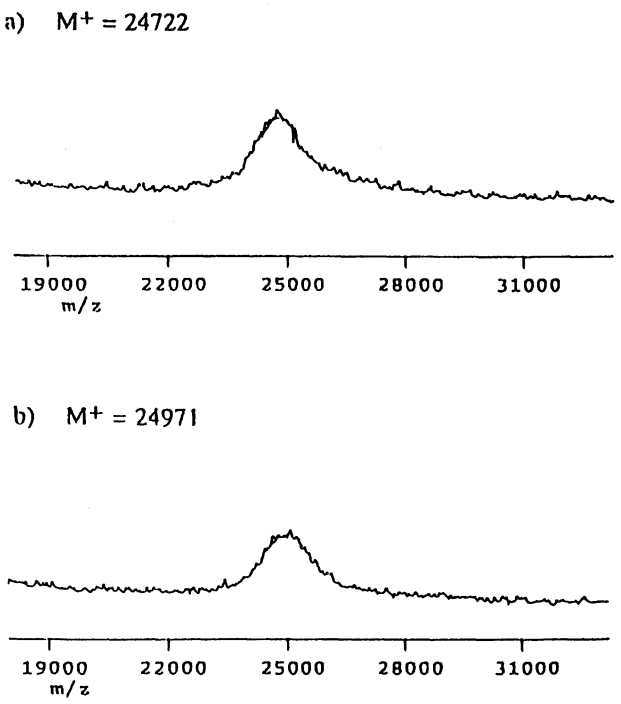


Fig. 4. Time of Flight mass spectra of AMDase and its complex with BPA. Positive ion was detected in each spectrum. a) Mass spectrum of native AMDase. Calculated value is 24734. b) Mass spectrum of AMDase after incubation with BPA.

Table 1. Conversion of α-Phenylmalonate in the Presence of 2-Mercaptoethanol by the Inhibited Enzyme and Native Enzyme

Reaction time	Yield of phenylacetate / %	
h	Native enzyme	Inhibited enzyme[a)]
10	70	53
23	100	100

a) The enzyme was once treated with BPA. Then, the reaction was performed in the presence of excess 2-mercaptoethanol.

In conclusion, the potent inhibitory effect of α-bromophenylacetate is very likely to come from the formation of a thiol ester with a cysteine residue which is present in the active site of the enzyme (Eq. 2). The remarkably small K_i value can be accounted for by a strong electron-withdrawing effect from the bromine atom which causes the high reactivity of the carbonyl group in the nucleophilic attack on a thiol. Further, as this inhibitor is competitive with the substrate, the first event which would occur between the substrate and the active site of the enzyme would be an interaction similar to that between the inhibitor and the enzyme. Thus the first step of the activation of the substrate by the enzyme is considered to be attack of cysteine on the pro-(S) carboxyl group of the substrate.[10] The electron-withdrawing effect of the thiol group would lower the potential energy of the negatively charged transition state and facilitate the cleavage of the C–C bond to release carbon dioxide from the free carboxyl group. In this way, the enzyme itself plays the role of coenzyme A, which is required in ordinary decarboxylation reactions of malonate.

Experimental

Screening of Inhibitors. α-Methyl-α-phenylmalonate (20 mM, 1 M=1 mol dm^{-3}) in 40 mM aqueous NaOH (400 μl) was incubated with an enzyme solution (6.1 units[3)]) in 100 μl of Tris-HCl buffer (pH 8.5) in the presence of a substrate analogue (20 mM) at 35 °C. The formation of 2-phenylpropionate was checked by TLC (eluent solvent; ethyl acetate : hexane : acetic acid=6 : 3 : 1). The R_f value of the product was 0.8, and that of the substrate was 0.3.

Kinetic Studies Using α-Bromophenylacetate (BPA). The mode of inhibition by added BPA was established by a Lineweaver-Burk plot measuring the rate constant at various concentrations of the substrate. α-Phenylmalonate (10—40 mM) was incubated with an enzyme solution (14.6 unit ml^{-1} in 50 μl of Tris-HCl buffer, pH 8.5) in the presence of BPA (100, 20, and 0 μM) for 3 min at 35 °C. The reaction was quenched by the addition of aqueous HCl (2 M, 100 μl). The resulting phenylacetate was extracted with diisopropyl ether (500 μl) containing methyl phenoxyacetate as an internal standard (20 mM). After treatment with excess trimethylsilyldiazomethane, methyl phenylacetate was determined by gas chromatographic analysis (column: BDS, carrier gas: N_2, temperature: 100—200 °C, 10 °C min^{-1}, retention time: methyl phenylacetate, 3.0 min; methyl phenoxyacetate, 5.7 min).

CD Spectra Measurements of the Enzyme–Inhibitor Complexes. A solution of IA in water (100 mM, 100 μl) was added to an enzyme solution (100 μM, 1 ml) and the mixture was incubated at 24 °C. The same

procedure was done for a solution of BPA in water (10 mM, 100 μl). After complete inactivation of the enzyme (for IA, 24 h; for BPA, 10 min), the CD spectra of the complexes were recorded on a JASCO C dicrograph.

TOF Mass Measurements of the Enzyme–Inhibitor Complex. A solution of BPA in water (37 μmol ml^{-1}, 1.6 μl, 0.06 μmol) was added to an enzyme solution (0.08 μmol ml^{-1}) in Tris-HCl buffer (pH 8.5, 160 μl). 2-Mercaptoethanol (15 μmol ml^{-1}, 4 μl, 0.06 μmol) was then added to the solution. The resulting solution was incubated at 24 °C for 24 h. The molecular weight of the enzyme-inhibitor complex was then measured by matrix-assisted laser desorption ionization (MALDI)–time of flight (TOF) mass spectrometry on a MALT–TOF JMS-LDI 1700 instrument.[11]

Recovery of AMDase Activity. AMDase (209 unit ml^{-1} in 50 μl of Tris-HCl buffer, pH 8.5) was reacted with BPA (100 μM in 50 μl of water) for 10 min. The enzyme was completely deactivated. ME (50 mM in 50 μl of water) and α-phenylmalonate (20 mM in 50 μl of 40 mM aqueous NaOH) were then added to the solution. The activity of the enzyme began to recover. The conversion of the substrate was determined by gas chromatography as described previously.

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11) Matrix: sinapinic acid, Laser wave number: 337 nm.