Direct Observation of Thiol Ester Formation between an Enzyme and an Active Site Directed Inhibitor by FT-IR

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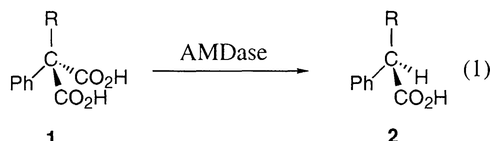
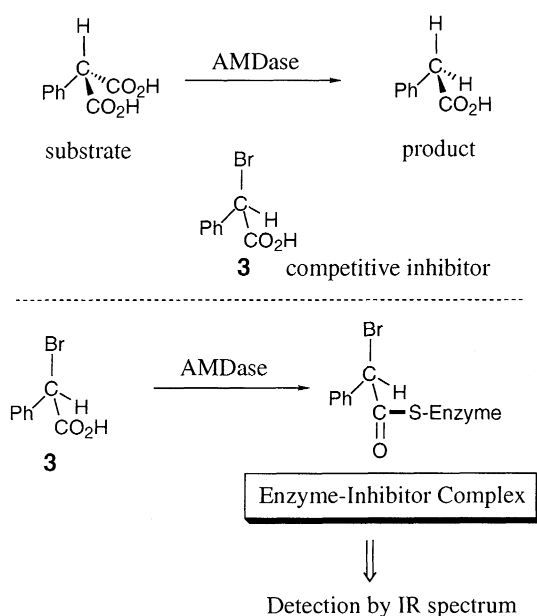
FT-IR measurement disclosed a formation of a thiol ester bond between a cysteine of arylmalonate decarboxylase and an active site directed inhibitor, α-bromophenylacetate, strongly suggesting that the enzyme initially activates the substrates in a similar mechanism.

Arylmalonate decarboxylase (AMDase) isolated from *Alcaligenes bronchisepticus* KU1201 is a novel enzyme which catalyzes a decarboxylation of disubstituted arylmalonates **1** to yield optically pure disubstituted arylacetates **2** (Eq. 1).[1]



This reaction is promoted by the enzyme alone, without the aid of any cofactors such as coenzyme A, ATP and biotin, which are required by usual malonate decarboxylases.[2] Further, this reaction is inhibited by SH specific reagents.[3] These mean that a cysteine residue of the enzyme itself plays an important role in the expression of the decarboxylation activity.



Scheme 1.

Thus, we have been trying to clarify how a cysteine residue interacts with the substrate, and already demonstrated some evidences that suggest a thiol ester formation between a cysteine of the enzyme and one of two carboxyl goups of the substrate.

In our previous paper,[4] we have reported that α-bromophenylacetate (BPA, **3** in Scheme 1) is a potent competitive inhibitor. K_i value is 3.6 μmol dm^{-3} at 24 °C (K_m value of phenylmalonate is 25 mmol dm^{-3}). As shown by Matrix-Assisted Laser Desorption Ionization Time-of-Flight mass spectrometry of the enzyme-inhibitor complex, BPA binds to the enzyme with elimination of one H_2O molecule. Further, the decarboxylation activity of the enzyme recovered by the addition of β-mercaptoethanol to the complex. Although all these facts suggest that BPA forms a thiol ester bond with a cysteine of the enzyme, further evidence is desirable to establish the reaction mechanism because an attack of a thiol group of an enzyme to a free carboxylic acid without the aid of cofactors has not been known so far. Now, we wish to report a direct observation of a thiol ester formation between the enzyme and BPA by FT-IR spectroscopy (Scheme 1).

The enzyme samples for IR measurement were purified homogeneously from *E. coli* transformed by the gene for AMDase.[3] Then, the enzyme-inhibitor complex were prepared as follows according to the previously reported method.[4]

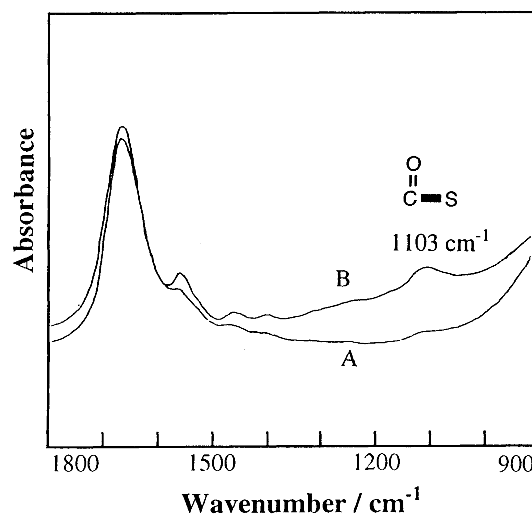
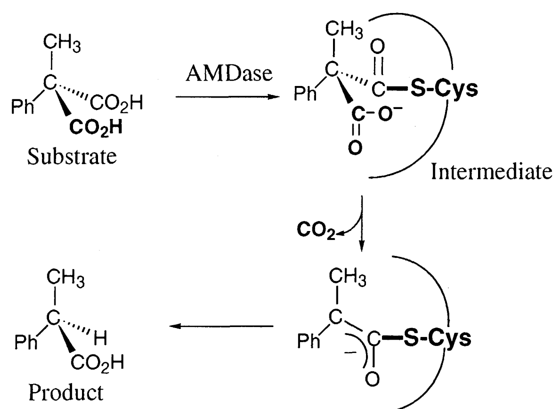


Figure 1. FT-IR spectra in the region of amide band (1500 cm^{-1} – 1800 cm^{-1}) of the enzyme and low wavenumbers containing deformation vibration of a C-S bond (900 cm^{-1} – 1200 cm^{-1}): (A), native AMDase; (B), AMDase inhibited by BPA.

The concentration of the purified enzyme was 1.7 mmol dm^{-3} in Tris-HCl buffer (pH 8.0). A solution of BPA in the same buffer (10 mmol dm^{-3}) was added to the enzyme solution to result enzyme-inhibitor complex.[5] Then, FT-IR spectra were recorded with a JIR-5500 (JEOL LTD) under the following conditions: window, KRS-5; scan, 200; resolution, 2 cm^{-1}; temperature, 25 °C.

The difference spectrum of native enzyme (A) and that of the enzyme-BPA complex (B) are shown in Figure 1. Each spectrum was obtained by differentiation of the spectrum of H_2O. In spectrum (B), a new band is observed at 1103 cm^{-1}. When the concentration of the enzyme was less than 1.0 mmol dm^{-3}, this band was not observed. This absorption can be atributed to deformation vibration of C-S bond of a thiol ester.[6] Other C-S bond, that is, S-alkyl bond, is observed in the region of low wavenumbers (less than 900 cm^{-1}). Thus, a thiol ester formation between a cysteine of the enzyme and the carboxyl group of BPA was confirmed. As BPA is a competitive inhibitor, it will interact with the enzyme in a similar manner with arylmalonates. Thus, it can be concluded that the first event which occurs between the substrate and the enzyme is a formation of a thiol ester. Under the reaction condition (pH 8.5), the carboxyl group of BPA or substrate is a carboxylate salt, but it will be considered to be protonated by some basic amino acid in the active site of the enzyme.

In usual decarboxylases, ATP works as the activator of a carboxyl group of malonate by forming a mixed anhydride. Then, coenzyme A attacks the activated carbonyl group of the mixed anhydride to form a thiol ester.[7] On the other hand, AMDase is a monomeric enzyme which requires no cofactors. Thus, the activation of the carboxyl group of a substrate in a usual manner can be hardly expected.



Scheme 2.

Accordingly, in the reaction of AMDase-catalyzed decarboxylation, the enzyme itself should work as the activator of the acid. The proposed mechanism of the reaction is shown in Scheme 2. We suppose that the active site pocket is very hydrophobic, and some basic amino acid assists a cysteine to form a thiolate which attacks the pro-(*S*) carboxyl group of a substrate.[8] Then, the electron-withdrawing effect of the thiol ester group lowers the potential energy of the negatively charged transition state and facilitate the cleavage of the C-C bond of another carboxyl group.

In this way, the enzyme itself activate the substrate in place of coenzyme A without the aid of ATP. Present decarboxylase, which has both of the role of usual coenzyme A and catalytic activity, is supposed to be a primitive enzyme.

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References and Notes

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5 The formation of an enzyme-inhibitor complex was assigned by TOF mass spectroscopy. Matrix: 2,5-dihydroxybenzoic acid, laser wavenumber: 337 nm, sample concentration: 20 nmol ml^{-1} ($l = dm^3$). The observed mass number of native enzyme was 24766 (calcd: 24734) and that of the enzyme-inhibitor complex was 24967 (calcd: 24931 in the case of thiol ester formation). Resolution is ± 6. The detailed method was shown in Ref. 4.
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