The Mode of Substrate-Recognition Mechanism of Arylmalonate Decarboxylase

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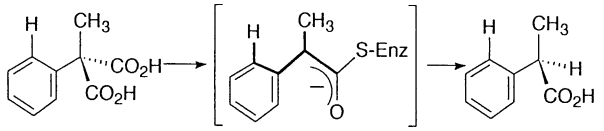
A mode of substrate-recognition mechanism by an enzyme, arylmalonate decarboxylase, is proposed. ^{19}F- and ^{31}P-NMR spectra of a complex consisting of the enzyme and an active site-directed inhibitor demonstrated that a hydrophobic interaction between the enzyme and its substrate via their aromatic rings was especially important for the enzyme function.

We have already demonstrated that arylmalonate decarboxylase (AMDase) catalyzes asymmetric decarboxylation of disubstituted malonic acids to give the corresponding monocarboxylic acids.[1] One of the characteristic points of the substrate specificity is that the substrate should have an aryl group directly bound to the α-carbon.[2] It has been revealed that aromatic ring have to occupy a periplanar conformation with the α-substituent in the active site of the enzyme (Scheme 1).[3] Moreover, when the benzene ring has a substituent at one of its ortho positions, the reaction proceeds only when the substituent is allowed to occupy *syn*-position with α- substituent.[4] How does the enzyme control the conformation of the aromatic ring?



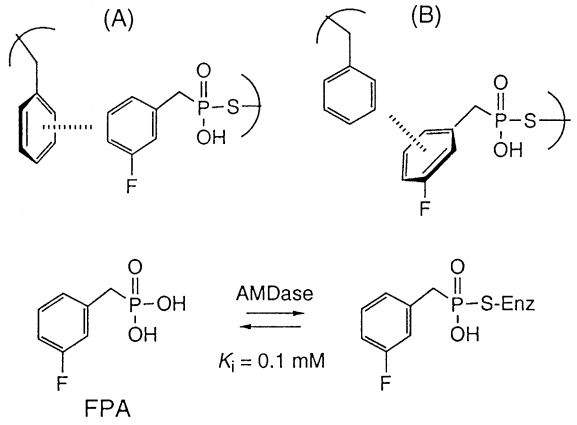
Synperiplanar conformation

Scheme 1.

One of the most probable interpretations is a hydrophobic CH-π interaction between the substrate and a hydrophobic amino acid residue(s), such as phenylalanine and tyrosine in the active site makes some specific conformation favorable.[5] Then, in what relative conformation are the two aromatic rings of the enzyme and the substrate arranged? Two binding modes can be proposed: A) The face of the aromatic ring of the enzyme is crossed by the edge of the benzene ring of the substrate. B) The edge of the aromatic ring of the enzyme crosses to the face of the benzene ring of the substrate. Thus, we have designed a novel inhibitor, 3-fluorophenylmethanephosphonic acid (FPA)[6] to distinguish the two possibilities (Scheme 2).

Because a phosphorus atom has a strong affinity to a thiol group, it was anticipated that phosphonic acid analogue would be a potent inhibitor against AMDase. FPA has a fluorine atom at *meta* -position, and then, we expected to detect the conformation of the benzene ring of FPA bound with the enzyme by the use of ^{19}F-NMR.

If case A is true (the benzene ring of the inhibitor acts as π-acceptor), anisotropic effect would cause a upfield-shift of the fluorine resonance.[7] On the contrary, if case B is operating (inhibitor acts as π-donor), binding of the inhibitor with the enzyme would cause a downfield-shift of ^{19}F signal. Herein, we would like to demonstrate the results of NMR studies on the enzyme-inhibitor complex, and a mode of substrate-recognition mechanism by the enzyme. FPA exhibited the time-dependent inhibition against AMDase-catalyzed decarboxylation of phenylmalonate (Figure 1), K_i value being 0.1 mM (cf. K_m of the substrate: 12 mM).



Scheme 2.

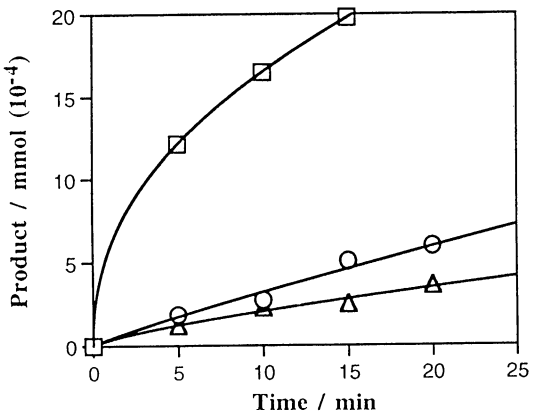


Figure 1. Effect of concentration of FPA on AMDase-catalyzed decarboxylation. Substrate; α-Phenylmalonate (20 mmol dm^{-3}), Product; Phenylacetate. □ I = 0 mmol dm^{-3}, ○ I = 5 mmol dm^{-3}, △ I = 7 mmol dm^{-3}.

Inhibition constant was determined by Lineweaver-Burk plots.[8] This kinetic analysis revealed that FPA binds into the active site competitively with the substrate. (1 M = 1 mol dm^{-3})

Purified enzyme[9] and the inhibitor solution[10] were mixed in an NMR tube at room temp. with a molar ratio of the enzyme vs FPA being 1:1.6 (1.5 mM:2.4 mM). The fluorine resonance bound with the enzyme was observed at −112.95 ppm (Figure 2). This signal was broader compared to the one due to the free compound, which was observed at −112.72 ppm. While $J_{F\text{-}P}$

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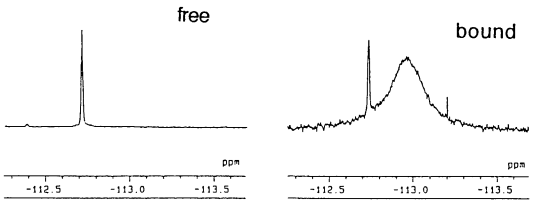

Figure 2. ^{19}F-NMR spectra of FPA and FPA-AMDase complex.

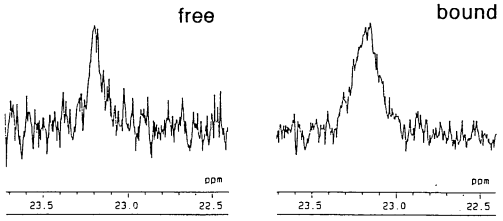

Figure 3. ^{31}P-NMR spectra of FPA and FPA-AMDase complex.

value of the signal of the free inhibitor was assigned to be 2.29 Hz, that of the signal due to the complex could be hardly assigned. An increase of the half-band width of the signal can be interpreted to come from the decrease of the freedom of FPA bound with the enzyme. In order to confirm that FPA was binding with the enzyme, ^{31}P-NMR spectra of the free and the complexed inhibitor were measured (Figure 3). The signal of the enzyme-inhibitor complex was broader compared to the free one in analogy with the spectrum of ^{19}F-NMR. Both spectra show that the inhibitor was binding into the active site of the enzyme accompanied by *freezing* of its conformation. The most emphatic result is that the fluorine resonance of the complexed inhibitor shifted 0.23 ppm upfield from where that of free inhibitor was observed. This result evidently discloses that the fluorine atom of the inhibitor is present above the face of the aromatic ring of the enzyme, i.e., FPA-binding mechanism is not B but A in Scheme 2. Based on the above NMR study, substrate-recognition mechanism of AMDase is proposed in Figure 4. Aromatic side chains of the enzyme would be arranged to be near the benzene ring of the substrate in the active site. It can be estimated that the face of aromatic ring of the enzyme would be crossing to the edge of the benzene ring of the substrate. The enzyme would control the benzene ring of the substrate to occupy synperiplanar conformation with α-substituent by a hydrophobic interaction, that is a CH-π interaction.

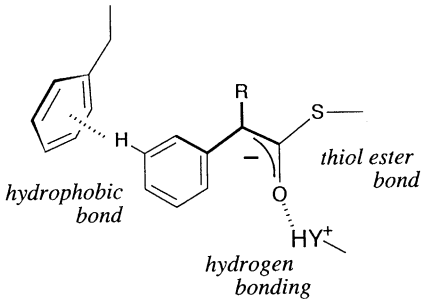

Figure 4. Proposed binding mode of the substrate with the enzyme.

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References and Notes

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6 FPA was synthesized from 3-fluorobenzyl bromide and triethyl phosphite according to the known methods (Arbuzov reaction). A. K. Bhattacharya and G. Thyagarajan, *Chem. Rev.*, **81**, 415 (1981). ^{1}H-NMR (400 MHz, D_2O) δ 2.98-3.03 ppm (2H, d, J = 20.8 Hz), 6.93-7.07 (3H, m), 7.25-7.31 (1H, m); mp 158-158.5 °C.
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8 The substrate, phenylmalonate (2 - 20 mmol dm^{-3}), was incubated with the enzyme solution (0.06 mg ml^{-1}, 22 μmol min^{-1}ml^{-1}) in the presence of FPA (0, 5, 7 mmol dm^{-3}) at 35 °C for 5 min. The assay method was the same as the reported one (Ref. 4).
9 Enzyme samples for NMR study were purified by the reported methods in Ref. 4.
10 FPA was dissolved in Tris buffer (100 mmol dm^{-3}, pH 8.0) to result a solution of 10 mmol dm^{-3}.