On the Conformation of the Substrate Binding to the Active Site during the Course of Enzymatic Decarboxylation

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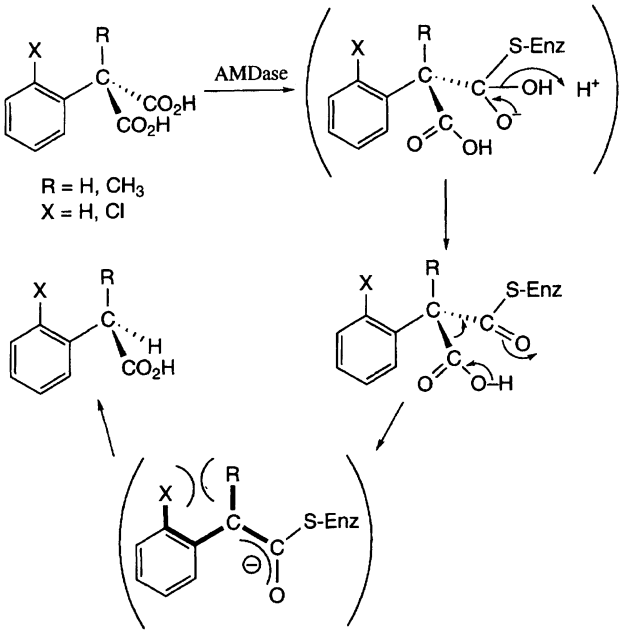
(Received July 1, 1996)

The activation parameters of the enzymatic decarboxylation of malonic acid derivatives catalyzed by arylmalonate decarboxylase (AMDase) were obtained by means of kinetics. In order to understand the conformation of the substrate at the transition state, the activation entropy ($\Delta S^{\ddagger}$) for indane-1,1-dicarboxylic acid, a representative molecule that is conformationally restricted, was compared with those of phenylmalonic acid and *ortho*-chlorophenylmalonic acid, of which the aromatic ring can freely rotate. The obtained value for the former molecule was about 10 cal mol^{-1} K^{-1} smaller than those of the latter compounds, clearly indicating that a conformationally restricted substrate, such as the *indane* derivative, is entropically advantageous for enzymatic decarboxylation. These kinetic studies evidently disclose that the conformation of the substrate at the transition state in the course of AMDase-catalyzed decarboxylation should be synperiplanar with regard to *ortho*- and α-substituents.

Arylmalonate decarboxylase (AMDase) isolated from *Alcaligenes bronchisepticus* KU1201 is a novel enzyme which contains no cofactors, such as coenzyme A, ATP, and biotin, which are required for the usual decarboxylases.[1] Thus, the mechanism of the catalytic activity of this enzyme is very interesting. During the course of our mechanistic studies on AMDase, we have proposed that the enzymatic decarboxylation proceeds via the thiol ester intermediate, based on the results of a Hammett plot[1] and biochemical experiments using an active site-directed inhibitor.[2] It was estimated that the electron-withdrawing effects of the substituents on the phenyl ring and the thiol ester group lower the potential energy of the negatively charged transition state in Scheme 1.[3] Thus, what conformation is required for the substrate in the active site of the enzyme to undergo a smooth reaction? Based on theoretical and kinetic studies,[4] it was suggested that the phenyl ring of the substrate at the transition state would be coplanar to the substituent at the α-position. If this supposition is true, a conformationally restricted substrate, such as indane-1,1-dicarboxylic acid (IDA, **1** in Fig. 1), would fit better than freely rotating ones (**2** and **3**),[5] resulting in the activation entropy being relatively smaller.

We thus performed the following kinetic studies on the enzymatic decarboxylation of three representative compounds (**1**, **2**, **3** in Fig. 1) at various temperatures in order to understand the conformation of the substrates at the transition state: 1) The activation entropy ($\Delta S^{\ddagger}$) for IDA (**1**) was compared with those of other substrates. 2) The activation enthalpy ($\Delta H^{\ddagger}$) for the enzymatic decarboxylation was compared with that of a non-enzymatic reaction. The results are reported here to show that IDA binds to the active-site pocket of the enzyme, thus making an *entropy trap*. Based on the obtained activation parameters, the relationship between the active-site structure of the enzyme with its catalytic function is discussed.



Scheme 1. AMDase-catalyzed decarboxylation reaction. Proposed negatively charged intermediates binding to the active site are shown in the parenthesis.

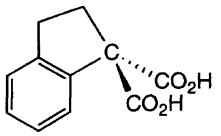
Results and Discussion

To obtain the activation parameters for three substrates (**1**, **2**, **3**), Arrhenius plots were prepared by measuring the k_{cat} values at various temperatures (Fig. 2).[6] The results of the calculations of $\Delta S^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta G^{\ddagger}$ are given in Table 1.

The activation entropy ($\Delta S^{\ddagger}$) for indane-1,1-dicarbox-

Table 1. Kinetic and Activation Parameters for AMDase-Catalyzed Decarboxylation

	Indane-1,1-dicarboxylic acid	*ortho*-Chlorophenyl malonic acid	Phenylmalonic acid
$k_{cat}(s^{-1})$	1.9	934	250
K_m(mM)	0.92	12.9	11.9
$\Delta S^{\ddagger}$(cal mol^{-1} K^{-1})	−27.6	−36.8	−38.5
$\Delta H^{\ddagger}$(kcal mol^{-1})	8.9	2.4	2.7
$\Delta G^{\ddagger}$(kcal mol^{-1})	17.1	13.3	14.1



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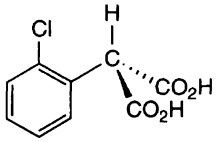

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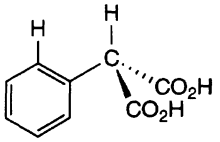

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Fig. 1. Representative compounds for kinetic studies. Indane-1,1-dicarboxylic acid (**1**) is a conformationally restricted substrate. *ortho*-Chlorophenylmalonic acid (**2**) and phenylmalonic acid (**3**) are freely rotating substrates.

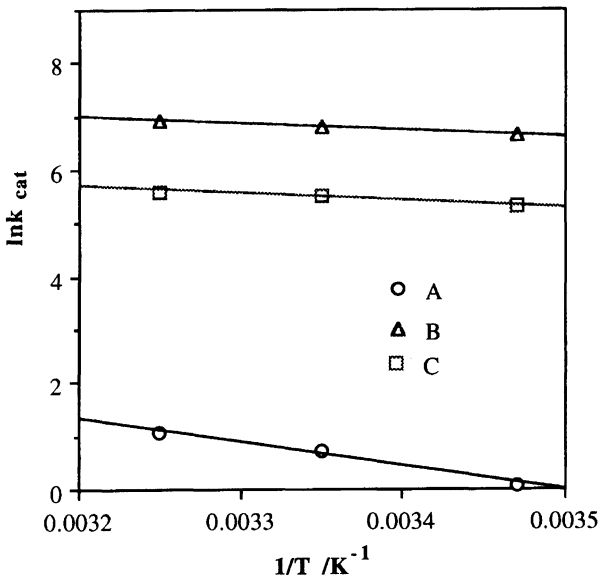

Fig. 2. Arrhenius plots of rate constants (k_{cat}) in AMDase-catalyzed decarboxylation of three substrates (**1**, **2**, **3**). A; indane-1,1-dicarboxylic acid, B; *ortho*-chlorophenylmalonic acid, C; phenylmalonic acid.

ylic acid (IDA) and phenylmalonic acid were −27.6 cal mol^{-1} K^{-1} and −38.5 cal mol^{-1} K^{-1}, respectively. The difference between the two values, i.e., 10.9 cal mol^{-1} K^{-1}, shows that IDA is conformationally advantageous for enzymatic decarboxylation. In our previous paper,[4] which revealed that IDA exhibited the smallest K_m value, it was suggested that IDA was most favorably accepted by the active-site pocket of the enzyme. The present results demonstrate that the value of the activation entropy for IDA is smaller than that of phenylmalonic acid; in other words, the indane-skelton is entropically advantageous in contrast to a freely rotating substrate at the transition state. Thus, the conformation of the substrates required in the enzyme pocket is similar to that of the indane derivative, i.e., synperiplanar. From the above results, the catalytic action of the enzyme is proposed to be as follows. The active-site structure of the enzyme will contain some hydrophobic amino acids, such as phenylalanine, tyrosine and leucine.[7] When a freely rotating substrate, such as phenylmalonic acid, binds to the pocket, these hydrophobic side chains of amino acids fix the phenyl ring of the substrate via CH–π interactions[8] to form a synperiplanar conformation. In this way, the degree of freedom in the conformation of the substrate decreases. It is worth mentioning that the activation energy of *ortho*-chlorophenylmalonic acid (**2**) and a nonsubstituted compound (**3**) are almost the same, which means that the chlorine atom at the *ortho* position has only a small steric effect for the substrate to take the synperiplanar conformation. This is consistent with the results of an ab initio calculation. When the potential-energy surfaces were calculated by rotating the dihedral angle between the phenyl ring and α-hydrogen, we obtained two energy minima for compound **2**, corresponding to the synperiplanar and antiperiplanar conformation. The energy difference was less than 0.5 kcal mol^{-1}, demonstrating that the steric repulsion between the *ortho* chlorine and the α-hydrogen is negligible.[4] In the case of IDA, the conformation of which is already arranged to one that fits to the enzyme pocket, no conformational restriction is required, resulting in $\Delta S^{\ddagger}$ being smaller.

On the other hand, the activation enthalpy ($\Delta H^{\ddagger}$) for IDA was 6.2 kcal mol^{-1} higher than that of phenylmalonic acid. This result clearly shows that the presence of two electron-donating groups (methylene) at the α- and *ortho*-position disfavors the formation of a negatively charged transition state, leading to a remarkable dicrease in the k_{cat} value (Table 1). As a whole, while IDA is entropically advantageous for binding to the active-site pocket, it is disfavorable for a smooth reaction from an electronic standpoint of view.

The activation free energy ($\Delta G^{\ddagger}$) of the biocatalytic decarboxylation of phenylmalonic acid is 14.1 kcal mol^{-1}. This value is 12.6 kcal mol^{-1} lower than that of a non-enzymatic reaction involving the same substrate, the $\Delta G^{\ddagger}_{uncat}$ value be-

Table 2. Kinetic Parameters for AMDase-Catalyzed Decarboxylation

	Indane-1,1-dicarboxylic acid		*ortho*-Chlorophenyl malonic acid		Phenylmalonic acid	
Temp	k_{cat}	K_m	k_{cat}	K_m	k_{cat}	K_m
°C	s^{-1}	10^{-1} mM	10^2 s^{-1}	mM	10^2 s^{-1}	mM
15	1.0±0.1	9.3±0.5	7.6±0.6	13.5±1.0	2.1±0.2	19.6±2.0
25	1.9±0.1	9.2±0.5	9.3±0.8	12.9±0.9	2.5±0.2	11.9±1.0
35	2.7±0.2	9.0±0.5	10.0±0.9	12.2±0.9	2.6±0.2	5.8±0.5

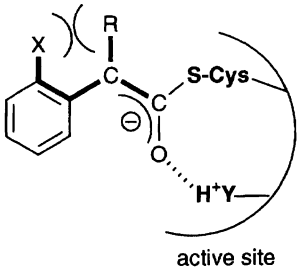

Fig. 3. Proposed structure of the enzyme-substrate complex. Negatively charged transition state would be stabilized by sulfur atom of a cysteine and a positively charged amino acid (H^+Y).

ing 26.7 kcal mol^{-1}.[9] This difference shows that the rate of AMDase-catalyzed decarboxylation is 10^9 faster than that of non-enzymatic decarboxylation. The effeciency of the enzymatic process can be estimated to be achieved by reducing $\Delta H^{\ddagger}$ value.[10] This result can be interpreted as follows. Positive charges of the amino acid side chains in the active site and thiol ester formation[11] between a cysteine residue and the substrate could stabilize the negatively charged transition state, as shown in Fig. 3.

In conclusion, a conformationally restricted substrate, of which the phenyl ring is coplanar to the substituent on the α-position, is entropically advantageous for an AMDase-catalyzed reaction. A hydrophobic pocket is supposed to be oriented so as to fix the phenyl ring of the substrate synperiplanar with the α-substituent, which is the most favorable conformation for the decarboxylation reaction. In addition, the anion stabilizing effect of the active site pocket contribute much to the excellent effeciency of the enzymatic reaction process.

Experimental

Enzyme Purification Methods. AMDase was purified from the *E. coli* mutant DH5α-MCR, which was transformed by the gene coding AMDase.[7] The cultivation method of the mutant strain was as follows. A loopful of the *E. coli* mutant was incubated in an LB medium (50 ml) at 30 °C for 18 h. A growth culture (1 ml) was added to a fresh LB medium (100 ml) and further cultivated for 6 h. Then, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the mixture and further cultivation was continued (12 h). The grown cells (OD_{660} = 1.8) were harvested by centrifugation. Purification of the enzyme was performed at 4 °C as follows. The precipitated wet cells were suspended in a phosphate buffer (100 mM (1 M = 1 mol dm^{-3}), pH 7.0) and disrupted by an ultrasonic treatment. To the cell-free solution, ammonium sulfate was slowly added to 60 % saturation. Then, the enzyme precipitated with ammonium sulfate was dissolved and dialyzed with Tris buffer (10 mM, pH 8.0). The resulting enzyme solution was partially purified by anion-exchange chromatography using DEAE Toyopearl gel. The active fraction of the enzyme was further purified by a second chromatography using hydrophobic Butyl Toyopearl gel. The purity of the enzyme was checked by SDS PAGE and TOF mass measurement.[2] The final concentration of the purified enzyme was determined to be 2.5 mg ml^{-1} by a UV absorption method.[12]

Preparation of Substrates. Phenylmalonic acid was purchased from Tokyo Kasei Co. (Japan). *ortho*-Chlorophenylmalonic acid was prepared according to a previously reported method.[1] Indane-1,1-dicarboxylic acid (IDA) was synthesized from indane-1-carboxylic acid according to a reported method.[4] The synthesis of indane-1-carboxylic acid from indene is described below.

Indene-3-carboxylic Acid. To a solution of butyllithium (22.1 ml of 1.64 M solution in hexane, 36.2 mmol) in dry ether (80 ml) was added dropwise a solution of indene (4.2 g, 36.7 mmol) in 12 ml of ether at room temperature. The solution was stirred for 10 min, cooled to −78 °C, and poured at once into 150 g of freshly crushed solid carbon dioxide. The resulting slurry was stirred for 15 min, and then poured into 150 ml of 10% of aqueous hydrochloric acid at 0 °C. A work-up and recrystallization from ether–hexane afforded 5.35 g (91%) of indene-3-carboxylic acid as orange needles; mp 158.5—159.5 °C (lit,[13] mp 158—159 °C). The spectral data (^{1}H NMR, IR) were identical with those reported.[13] ^{1}H NMR (270 MHz, $CDCl_3$) δ = 3.47 (d, J = 2.0 Hz, 2H), 7.14—7.41 (m, 3H), 7.47 (t, J = 2.0 Hz, 1H), 7.97—8.00 (m, 1H); IR (KBr) 3150, 2900, 2725, 1690, 1590, 1375, 1260, 1205, 1100, 740, 625 cm^{-1}.

Indane-1-carboxylic Acid. A solution of indene-3-carboxylic acid (1.0 g, 6.30 mmol) in 13 ml of acetic acid was vigorously stirred for 12 h with 10% Pd–C (102 mg) under an atmosphere of hydrogen. Filtration of the catalyst followed by evaporation of acetic acid afforded indane-1-carboxylic acid as a colorless solid (840 mg, 82%). The spectral data were identical to those reported in the literature.[4] ^{1}H NMR (270 MHz, $CDCl_3$) δ = 2.20—2.44 (m, 2H), 2.79—2.90 (m, 1H), 2.98—3.09 (m, 1H), 3.98—4.02 (t, J = 7.5 Hz, 1H), 7.09—7.18 (m, 3H), 7.34—7.36 (m, 1H); IR (KBr) 2900, 1710, 1470, 1420, 1310, 1225, 935, 740, 670 cm^{-1}.

Kinetic Procedures. The general procedures were the same as those previously reported.[2] The enzymatic decarboxylations of three substrates (**1**, **2**, **3**) were performed in a Tris buffer solution (pH 8.5) at various temperatures (15, 25, and 35 °C). The initial rate at each temperature was measured by a gas-chromatographic analysis of a methylated product (methyl ester of monocarboxylic acid). The kinetic parameters (K_m and k_{cat}) were obtained by a Lineweaver-Burk analysis (Table 2).

An Arrhenius plot was prepared by measuring the k_{cat} value for each substrate, as shown in Fig. 2. The activation parameters ($\Delta S^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta G^{\ddagger}$) were calculated based on Eq. 3:

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (1)$$

$$k_{cat} = (kT/h)\cdot \exp(-\Delta G^{\ddagger}/RT) \quad (2)$$

$$\ln k_{cat} = -\Delta H^{\ddagger}/RT + \{\Delta S^{\ddagger}/R + \ln(kT/h)\} \quad (3)$$

$R = 1.986\ \mathrm{cal\,K^{-1}\,mol^{-1}}$

$k = 1.380 \times 10^{-16}\ \mathrm{erg\,K^{-1}}$

$h = 6.626 \times 10^{-27}\ \mathrm{erg\,s^{-1}}$

$(1\ \mathrm{erg} = 10^{-7}\ \mathrm{J})$

$(1\ \mathrm{cal} = 4.184\ \mathrm{J})$

This work financially supported in part by a Grant-in-Aid for Scientific Research No. 07459023 from the Ministry of Education, Science and Culture.

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