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Kinetic analysis on the substrate specificity of 3-isopropylmalate dehydrogenase

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Substrate specificity of 3-isopropylmalate dehydrogenase is analyzed using a series of synthetic (*2R,3S*)-3-alkylmalates. Each analog with hydrogen, methyl, ethyl, isopropyl, isobutyl, *tert*-butyl, and isoamyl group on C-3 functions as a substrate, implying a broad substrate specificity of the enzyme toward alkylmalates. The incremental binding energy of the isopropyl group of 3-isopropylmalate to the enzyme is estimated to be 3.55 kcal/mol, the rather small value supporting the broad specificity. Although the enzyme shows a broad specificity toward the alkylmalates, it does not show activity with isocitrate which has a negatively charged carboxymethyl group instead of the alkyl groups.

Analog; Hydrophobicity; 3-Isopropylmalate dehydrogenase; Substrate specificity

1. INTRODUCTION

3-Isopropylmalate dehydrogenase (EC 1.1.1.85, IPMDH) catalyzes the oxidative decarboxylation of (*2R,3S*)-3-isopropylmalate to 2-oxoisocaproate in the leucine biosynthetic pathway. We have cloned [1] and sequenced [2] the gene from an extreme thermophile, *Thermus thermophilus* HB8, and characterized the enzymatic properties [3]. X-ray crystallographic analysis was also conducted at the high resolution of 2.2 Å [4]. A stereospecific synthesis of the substrate has recently been developed [5] and the reaction mechanisms of the enzyme have been elucidated using chemically isotope-labeled isopropylmalates. The hydride-accepting site of the nicotinamide ring during the enzyme reaction has been identified as A (or proR position) by using deuterated isopropylmalate [6]. It was also confirmed that the configuration at the C-3 position of the substrate is retained throughout the decarboxylation by using another labeled isopropylmalate [7].

In this paper, we describe the kinetic analysis of the substrate specificity of *T. thermophilus* IPMDH using a series of (*2R,3S*)-3-alkylmalates.

2. EXPERIMENTAL

T. thermophilus IPMDH was prepared as previously described [3]. (*2R,3S*)-3-Alkylmalates were synthesized as described elsewhere (Kakinuma, K. et al., submitted for publication). Kinetic constants were determined in 50 mM sodium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (pH 7.8), containing 100 mM KCl, 5.0 mM $MgCl_2$, and 5.0 mM NAD in a total volume of 500 μl at 60°C. The concentration of analogs were 400–2,000 μM for malate; 3.33–10 μM for methylmalate, isoamylmalate, and *tert*-butylmalate; and 2.86–6.67 μM for ethylmalate, isobutylmalate, and isopropylmalate, respectively. Initial velocities were determined by monitoring the formation of NADH at absorbance 340 nm on a spectrophotometer. Values of k_{cat} were the means of three experiments.

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3. RESULTS AND DISCUSSION

All the alkylmalates were active as a substrate of IPMDH, implying a broad substrate specificity of the enzyme toward alkylmalates. The kinetic constants K_m, k_{cat}, and k_{cat}/K_m of various alkylmalates are summarized in Table I. The K_m value decreases up to isopropylmalate and increases afterwards; the k_{cat} value decreases depending on the increase of the hydrophobicity; and k_{cat}/K_m increases up to ethylmalate and decreases afterwards. These results suggest that the reaction generally occurs (Fig. 1) independently of the hydrophobicity of the alkyl group.

The change of binding energy ($\Delta\Delta G_B$) of an alkyl group R of an alkylmalate, RM (relative to the hydrogen at C-3 of malate, HM), can be estimated by comparing the values of k_{cat}/K_m for the enzyme-catalyzed reactions using the relationship [8]:

$$\Delta\Delta G_B = -RT \ln(k_{cat}/K_m)_{HM}/(k_{cat}/K_m)_{RM}$$

(where R is the gas constant and T the absolute temperature). Application of this equation gives a value of 3.55 kcal/mol for the reaction of isopropylmalate. This small value supports the broad specificity of the enzyme.

In some microorganisms, the oxidation of alkylmalates have been reported to be associated with the activity of IPMDH for its nature of the broad substrate specificity [9–13]. *T. thermophilus* IPMDH also shows

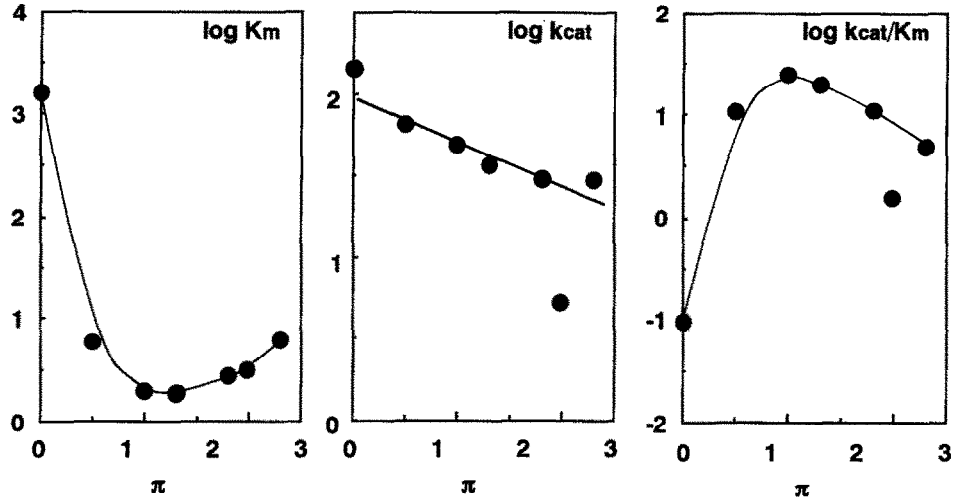

Fig. 1. The relationship between the hydrophobicity of the alkyl group and K_m, k_{cat}, and k_{cat}/K_m for the catalysis of alkylmalate by IPMDH replotted from the values in Table I. Energies are in kcal/mol. The π values are the Hansch constants for the alkyl substituents [15].

a broad specificity toward alkylmalates in vitro, and consequently, the enzyme is expected to react not only to isopropylmalate but also to methylmalate or ethylmalate in vivo.

Isocitrate has a structural similarity with isopropylmalate, HOOC(HO)CHCH(X)COOH, in which X represents the CH_2COOH of isocitrate and the $CH(CH_3)_2$ of isopropylmalate. Isocitrate dehydrogenase (EC 1.1.1.42), which is involved in the tricarboxylic acid cycle and also plays a role in glutamate biosynthesis, catalyzes a chemically equivalent reaction to IPMDH. In spite of the similarities of the substrates and the enzymes [14], isocitrate was completely inactive against IPMDH ($k_{cat} < 0.2$ s^{-1}). IPMDH showed a broad substrate specificity toward the alkylmalates, however, it does not show activity with isocitrate which has a negatively charged carboxymethyl group instead of the alkyl group. This may imply a significant chemical difference in the recognition site of the two enzymes towards the substituent at C-3 of malate.

Table I

Kinetic parameters for the catalysis of (*2R,3S*)-alkylmalate by IPMDH

Alkyl group	π[a]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
Hydrogen[b]	0	1,562	142	0.0907
Methyl	0.50	5.94	62.7	10.6
Ethyl	1.00	1.99	48.6	24.4
Isopropyl	1.30	1.84	36.0	19.6
Isobutyl	1.80	2.71	30.4	11.2
tert-Butyl	1.98	3.18	5.10	1.61
Isoamyl	2.30	6.13	29.7	4.84

[a] The π values are the Hansch constants for the alkyl substituents [15].
[b] Malate has only one asymmetric carbon atom.

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