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Note

Overproduction and Substrate Specificity of 3-Isopropylmalate Dehydrogenase from *Thiobacillus ferrooxidans*

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We constructed an overexpression system in *Escherichia coli* of the *leuB* gene coding for 3-isopropylmalate dehydrogenase in *Thiobacillus ferrooxidans*. *E. coli* harboring the plasmid we constructed, pKK leuB1, produced 17-fold the enzyme protein of the expression system previously used for purification. The substrate specificity of the enzyme was analyzed with synthetic (2*R*, 3*S*)-3-alkylmalates. The 3-isopropylmalate dehydrogenase of *Thiobacillus ferrooxidans* had broad specificity toward the alkylmalates.

Key words: *Thiobacillus ferrooxidans*; 3-isopropylmalate dehydrogenase; *leuB*; overproduction; substrate specificity

3-Isopropylmalate dehydrogenase (EC 1.1.1.85), a key enzyme in leucine biosynthesis, catalyzes the oxidative decarboxylation of the substrate 3-isopropylmalate to 2-oxoisocaproate simultaneously with dehydrogenation. The enzyme has been found in a wide variety of bacteria and the amino acid sequences of the enzyme from various microorganisms including yeasts are similar.[1-10] The catalytic mechanism of 3-isopropylmalate dehydrogenase may resemble that of bacterial isocitrate dehydrogenase. The X-ray crystal structure of *Thermus thermophilus* 3-isopropylmalate dehydrogenase[11-13] is like that of *Escherichia coli* isocitrate dehydrogenase.[14,15] Most of the residues that seem from crystallographic results to be key to enzyme function are found in both enzymes.

We previously cloned the *leuB* gene coding for the 3-isopropylmalate dehydrogenase of an acidophilic chemolithotrophic bacterium, *Thiobacillus ferrooxidans*, in *E. coli*[16] and purified the enzyme to homogeneity from *E. coli* cells harboring a recombinant plasmid, pTFL101, containing the *leuB* gene.[17] The properties of the enzyme are similar to those of the *Salmonella typhimurium* enzyme,[18] except for substrate specificity. The *T. ferrooxidans* 3-isopropylmalate dehydrogenase utilizes malate as a substrate as well as 3-isopropylmalate.[17] The enzymes from an extreme thermophile, *T. thermophilus*,[19] and thermoacidophilic archaeon, *Sulfolobus* sp. strain 7,[20] also utilize the various 3-alkylmalates with broad substrate specificity.

The purification of the *T. ferrooxidans* 3-isopropylmalate dehydrogenase from the *E. coli* transformant was not enough for further investigation, for example, crystallography, because the *E. coli* was grown in minimal medium at a low cell yield. Because of the high demand for 3-isopropylmalate dehydrogenase, we undertook to construct an overexpression system. In this paper, we describe an construction of the overexpression plasmid and report the substrate specificity of the 3-isopropylmalate dehydrogenase from *T. ferrooxidans* purified from *E. coli* using the overexpression system.

The *T. ferrooxidans leuB* structural gene was amplified by PCR with the synthetic oligonucleotides 5′-GGAGAATTCATGAAAAAAATGGCC-3′ and 5′-ATCCCCGGGTCAATCCTTCAGGTT-3′. The underlined sequences are restriction sites of *Eco*RI and *Sma*I, respectively. The amplified fragment was blunted with DNA polymerase I and phosphorylated with ATP and polynucleotide kinase. The phosphorylated fragment was ligated with pKK223-3 (Pharmacia LKB Biotechnology Inc.), which had been digested with *Sma*I, and then dephosphorylated. The ligated plasmid was digested with *Eco*RI and ligated with itself, giving pKK leuB1. *E. coli* JA221 harboring pKKleuB1 had high 3-isopropylmalate dehydrogenase activity: 17-fold that of *E. coli* HB101 harboring pTFL101 (Table I). Earlier, we purified the enzyme by three kinds of column chromatography, DEAE-Toyopearl 650 M, butyl-Toyopearl 650 M (Tosoh Corp.), and hydroxyapatite.[17] However, with this overexpression system, purification was done with only two kinds of column chromatography, DEAE-Toyopearl 650 M and Q-Sepharose Fast Flow (Pharmacia LKB Biotechnology Inc.) (data not shown).

The *T. ferrooxidans* 3-isopropylmalate dehydrogenase utilizes malate as a substrate as well as 3-isopropylmalate.[17] The substrate specificity of the enzyme was analyzed with (2*R*, 3*S*)-3-alkylmalates synthesized as described previously.[21] Table II lists the kinetic constants K_m and k_{cat} for several alkylmalates of *T. ferrooxidans* 3-isopropylmalate dehydrogenase. All of these alkylmalates served as a substrate of the enzyme, so its substrate specificity seemed to be broad. The K_m value decreased with increasing hydrophobicity[22] of the alkyl group at position 3 up to 3-ethylmalate and increased afterwards, and k_{cat}/K_m increased up to 3-ethylmalate and decreased afterwards. This result coincides with those for enzymes from *T. thermophilus*[19] and *Sulfolobus* sp.[20] The relationship between the cata-

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Table I. 3-Isopropylmalate Dehydrogenase Activity of *E. coli* Transformants

Strain (plasmid)	Medium	IPTG[a] (1 mM)	Specific activity[b] (units/mg)	Total activity[c] (units)
JA221 (pKKleuB1)	LB	+	8.56	810
JA221 (pKKleuB1)	LB	−	5.65	514
JA221 (pKKleuB1)	minimal	−	6.74	150
JA221 (pTFL101)	minimal	−	2.53	49.6
JA221 (pTFL101)	LB	+	0.12	7.71
HB101 (pTFL101)	minimal	−	2.84	48.4

[a] Isopropyl-β-D-thiogalactopyranoside.
[b] Specific activity is defined as μmol of NADH formed per milligram of protein per minute.
[c] Total activity is defined as activity per 100 ml of culture.

Table II. Kinetic Parameters for the Catalysis of (2*R*, 3*S*)-3-Alkylmalate by *Thiobacillus ferrooxidans* 3-Isopropylmalate Dehydrogenase

Substrate	π[a]	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
malate[b]	0	7.7	5.0	0.65
3-methylmalate	0.50	0.050	28	560
3-ethylmalate	1.00	0.013	38	2900
3-isopropylmalate	1.30	0.015	28	1900
3-*tert*-butylmalate	1.98	0.074	2.7	36
3-isoamylmalate	2.30	0.11	20	180

[a] The π values are the Hansch constants for the alkyl substituents.[22]
[b] Malate has only one asymmetric carbon atom.

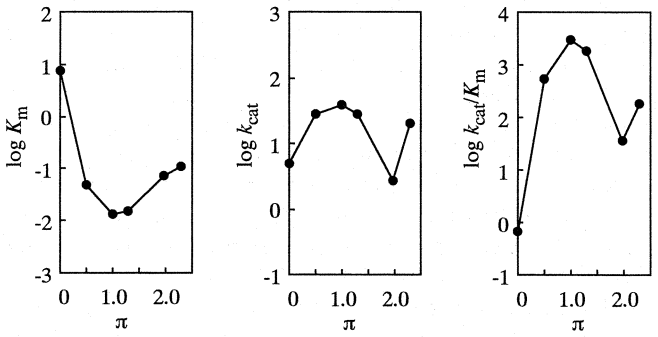

Fig. 1. Relationship between the Hydrophobicity of the Alkyl Group and K_m, k_{cat}, and k_{cat}/K_m for the Catalysis of Alkylmalate by 3-Isopropylmalate Dehydrogenase.

The data were replotted from the values in Table II. Energies are in kcal/mol. The π values are the Hansch constants for the alkyl substituents.[22]

lytic properties and the hydrophobicity of the alkyl group is shown in Fig. 1. These results suggest that the reaction was generally independent of the hydrophobicity. The *T. ferrooxidans* 3-isopropylmalate dehydrogenase had broad substrate specificity toward alkylmalates, but it had no activity with isocitrate,[17] which has a negatively charged carboxymethyl group instead of an alkyl group. This finding suggests a chemical difference in the recognition sites of the 3-isopropylmalate dehydrogenase and the isocitrate dehydrogenase for the substituent at C-3 of malate.

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