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Crystallization and preliminary X-ray analysis of 3-isopropylmalate dehydrogenase from the moderate facultative thermophile *Bacillus coagulans*

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Abstract

Three crystalline forms of 3-isopropylmalate dehydrogenase from the moderate facultative thermophile *Bacillus coagulans* were obtained by hanging-drop vapor-diffusion methods. One of them, which had crystallized under slightly milder conditions than the others, was suitable for X-ray analysis. Its asymmetric unit contains one dimeric molecule and the solvent content is higher than in other protein crystals. The crystal structure was solved in a preliminary manner by the molecular-replacement technique.

1. Introduction

In leucine biosynthesis, 3-isopropylmalate dehydrogenase (IPMDH, E.C. 1.1.1.85) catalyzes an oxidative decarboxylation of 3-isopropylmalate with concomitant reduction of nicotinamide adenine dinucleotide. The enzyme from *Bacillus coagulans* (BcIPMDH) is composed of two identical subunits (Sekiguchi, Harada, Shishido & Nosoh, 1984), each of which has the molecular weight of 39 808 Da with 366 amino-acid residues (Sekiguchi *et al.*, 1986). BcIPMDH retains its enzymatic activities up to 338 K with an optimum temperature of 331 K. Compared with the enzyme from *Thermus thermophilus* (TtIPMDH) (Yamada *et al.*, 1990), BcIPMDH is less thermostable. It is interesting to note that *B. coagulans* (a moderate facultative thermophile) can proliferate over a wider range of lower temperatures than can *T. thermophilus* (an extreme thermophile). There may be some different structural mechanisms that permit the function over such a broad range of temperature.

In order to investigate the relationship between these properties and structures, it is necessary to compare the three-dimensional structures of the enzymes from different sources. We have been engaged in crystallographic studies of the two IPMDH's. At first BcIPMDH was crystallized, but the crystals contained several molecules in the asymmetric unit. After that, suitable crystals of TtIPMDH were easily obtained (Katsube, Tanaka, Takenaka, Yamada & Oshima, 1988). Although the X-ray structures of TtIPMDH (Imada *et al.*, 1991; Hurley & Dean, 1994) and its chimeric enzymes with IPMDH of *Bacillus subtilis* (Onodera *et al.*, 1994) were reported, the detailed reaction mechanism of the enzymes is still ambiguous. For the purpose mentioned above and for the establishment of the reaction mechanism, the three-dimensional structure of BcIPMDH should be revealed.

A great effort has been made to crystallize BcIPMDH in different crystalline forms by surveying a variety of conditions. It has been found that one of the three forms thus obtained is suitable for structure determination. This crystal is quite different in crystal packing from those of TtIPMDH and the chimeric enzymes.

2. Materials and methods

BcIPMDH's were produced in recombinant cells carrying a plasmid coding for the enzyme gene of *B. coagulans* (Sekiguchi *et al.*, 1984). The cultured cells were disrupted by ultrasonication. After cell debris and unbroken cells were removed by centrifugation, the supernatant (which included 2 *M* NaCl) was subjected to heat treatment at 336 K for 10 min and the denatured proteins were removed by centrifugation. The resulting supernatant (which included 0.65 *M* NaCl) was then fractionated with polyethylene glycol 6000. The precipitates between 12 and 28%(*w*/*v*) polyethylene glycol were collected by centrifugation and suspended in 20 m*M* phosphate buffer (pH 6.5) containing 0.5 m*M* dithiothreitol and 0.5 μ*M* $MnCl_2$. Subsequently, they were highly purified with column chromatography on Q-Sepharose, Bio-Gel HTP hydroxyapatite and TSK-Phenyl 5PW. The purity was confirmed by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and the activity was assayed by the method of Sekiguchi *et al.* (1984).

Crystallization conditions were surveyed by the hanging-drop vapor-diffusion method with ammonium sulfate or polyethylene glycol as precipitants in the pH range 6.0–8.5 using 50 m*M* Tris–HCl or 20 m*M* potassium phosphate buffer. In some cases, several kinds of salts or alcohols were tried to control nucleation or to prevent aggregation of crystals. At least three kinds of crystalline forms (form I, form II and form III) which differ in crystal habit were obtained. Their precession photographs were taken with Cu $K\alpha$ radiation (λ = 1.54184 Å) in order to examine their suitabilities for X-ray analyses. The density of the form I crystal was measured by the linear gradient method with *m*-xylene and bromobenzene to estimate the number of molecules in the asymmetric unit. Some form I crystals were dissolved and loaded for SDS–PAGE to identify the components.

Diffraction data of the form I crystal were collected by the oscillation method on an R-AXIS IIc (Sato *et al.*, 1992) with Cu $K\alpha$ radiation generated at 40 kV and 100 mA against a rotating anode. 41 frames were processed by the program *PROCESS*. The intensities recorded on imaging plates were evaluated up to 4.0 Å resolution for the present preliminary study, though the crystal diffracted beyond it. 33 251 data in total were reduced to 11 493 independent reflections with an R_{merge} of 0.092 and the completeness of 87.4%.

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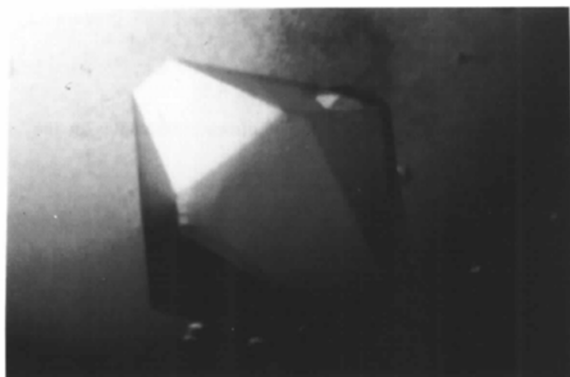
3. Results and discussion

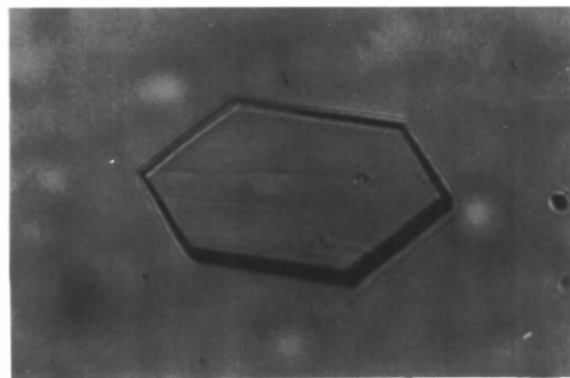
The crystallization conditions for the three forms are given in Table 1, together with their crystal data determined from precession photographs. Fig. 1 shows these crystals. The form I crystal, which grew up to a maximum size of 0.7 × 0.7 × 1.0 mm during 2 or 3 months under slightly milder conditions than the others, has the typical shape of a hexagonal bipyramid reflecting the crystal symmetry. This form coincidentally belongs to the same crystal system as crystals of TtIPMDH, but the unit-cell dimensions and diffraction patterns are quite different, suggesting a different crystal packing. The densities of the form I crystal and its solvent region are measured to be 1.15 and 1.07 mg ml^{-1}, respectively, from which the number of dimeric molecules in the asymmetric unit is estimated to be one. No other macromolecules in the crystal were confirmed by SDS–PAGE. Therefore, the V_M value is 4.6 Å^3 Da^{-1}, which corresponds to a solvent content of 73%. Although this value is out of the range reported by Matthews (1968), such higher solvent contents are also found in the crystals of glycolate oxidase (V_M = 5.0 Å^3 Da^{-1}, V_{solv} = 75%) (Lindqvist & Brändén, 1980), catalytic domain of dihydrolipoyl transacetylase (V_{solv} = 73%) (Mattevi *et al.*, 1992) and kallikrein A–trypsin inhibitor complex (V_M = 4.5 Å^3 Da^{-1}) (Chen & Bode, 1983). On the other hand, the form II and form III crystals, which are plate-shaped crystals, were easily obtained in 2 or 3 d. These crystals have the different space groups in the same crystal system, but differ from the form I crystal. The numbers of the dimeric molecules in the asymmetric unit were estimated to be three to four for both forms from the proposed range of V_M.

Since the form I crystal diffracts at higher resolution than the others (Table 1), the intensity data of the form I crystal were collected to solve the structure. The primary structure of BcIPMDH (Sekiguchi *et al.*, 1986) has a sequence identity of 50% to that of TtIPMDH (Kagawa *et al.*, 1984). Therefore, it is plausible to assume that the tertiary structure is similar to that of TtIPMDH. Molecular replacement was applicable to solve the crystal structure of the form I crystal. A polyalanine structure for the dimeric enzyme, which was constructed from the atomic coordinates of TtIPMDH (1IPD) deposited in Brookhaven Protein Data Bank (Bernstein *et al.*, 1977), was used as a probe molecule. In space group of $P3_121$, the program *AMoRe* (Navaza, 1994) gave a unique solution with a crystallographic R factor of 0.444 (Table 2) and a reasonable molecular packing. The structure was further confirmed by the omit-map method. Several electron-density maps showed no significant peaks outside the molecular envelope, indicating no macromolecules in the solvent region.



(*a*)

(*b*)



(*c*)

Fig. 1. Different crystalline forms of BcIPMDH. (*a*) Form I, (*b*) form II and (*c*) form III.

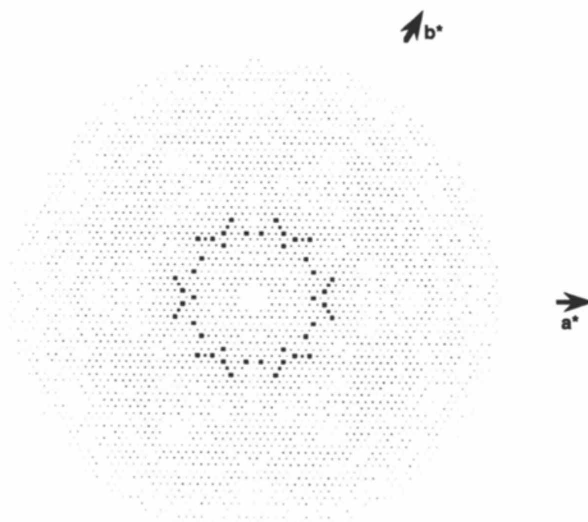


Fig. 2. A pseudoprecession plot of the *hk*0 zone of a form I crystal, calculated using the program *HKLVIEW* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). the outer edge of the pattern shows 3.0 Å resolution.

Table 1. *Crystallization conditions, crystal data and X-ray diffraction of the three crystalline forms of BcIPMDH*

	Form I		Form II	Form III
Crystallization conditions	Condition 1	Condition 2		
Protein concentration (mg ml^{-1})	10	10	10	12.5
Buffer	50 m*M* Tris-HCl	50 m*M* Tris-HCl	50 m*M* Tris-HCl	20 m*M* K phosphate
Precipitant*	1.0 *M* AS	12% PEG	2.0 *M* AS	1.8 *M* AS
Additive	—	0.5 *M* NaCl	4% MPD	—
pH	8	8	8	6.5
Temperature (K)	298	298	293, 298	277, 298
Crystal data				
Space group	$P3_121$		$P2_12_12_1$	$P2_12_12$
Cell dimensions (Å)	$a = 114$		$a = 135$	$a = 135$
	$b = 114$		$b = 241$	$b = 241$
	$c = 195$		$c = 115$	$c = 122$
Z†	6		12, 16‡	12, 14, 16‡
X-ray diffraction				
Resolution (Å)	3.0		3.5	4.0

* AS, ammonium sulfate; PEG, polyethylene glycol 2000; MPD, 2-methyl-2,4-pentanediol. † Number of dimeric molecules in the unit cell. ‡ Possible values estimated from the proposed range of V_M.

Table 2. *Results of molecular replacement*

Calculations were carried out by the program *AMoRe* (Navaza, 1994) using the 30–4.0 Å resolution data. Cc is the correlation coefficient, (α, β, γ) are eulerian angles and (Tx, Ty, Tz) is translation vector. A reasonable solution is shown in bold.

Rotation function Solution	α	β	γ	Cc
S1	**50.4**	**73.0**	**229.2**	**20.2**
S2	71.3	80.4	335.3	19.7
S3	36.0	90.0	146.5	14.2

Transition function Solution	Tx	Ty	Tz	Cc	*R* factor
($P3_121$, rotation S1)					
S1-1	**0.53313**	**0.42396**	**0.37384**	**59.5**	**0.444**
S1-2	0.86650	0.09344	0.41308	49.5	0.486
S3-3	0.19944	0.75724	0.18860	49.1	0.479
($P3_221$, rotation S1)					
S1-1	0.52968	0.89482	0.03995	44.7	0.490
($P3_121$, rotation S2)					
S2-1	0.84928	0.87865	0.37297	40.1	0.512
($P3_121$, rotation S3)					
S3-1	0.52393	0.19922	0.26451	38.5	0.513

As the possibility of solving the structure of BcIPMDH arose, intensity data of the form I crystal were re-measured at higher resolution using a Weissenberg camera with synchrotron radiation at the Photon Factory in Tsukuba (Sakabe, 1991). The significant diffraction data extended to at least 3.0 Å resolution as shown in Fig. 2. Model building and refinement are in progress. BcIPMDH has the optimum pH at 8.3 for its enzymatic activity. The form I crystals were obtained in such alkaline state (pH 8.0). It is expected that the form I crystal gives us some structural information for the active state of the enzyme.

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