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Structure of a Loop-Deleted Variant of 3-Isopropylmalate Dehydrogenase from *Thermus thermophilus*: an Internal Reprieve Tolerance Mechanism

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Abstract

A loop-deleted mutant form of 3-isopropylmalate dehydrogenase from *Thermus thermophilus* was constructed to investigate the relationship between the flexibility of the structure and the thermostability of the enzyme. The structure of the mutant enzyme was determined by X-ray crystallography and was found to be almost the same as that of the native enzyme with a reduced temperature factor. Although the mutant protein had lost the flexible loop, its function and thermostability had remained unchanged. This phenomenon can be explained by an internal reprieve tolerance mechanism.

1. Introduction

During evolution, the primary structure of a given enzyme may have varied to allow the enzyme to exhibit high catalytic activity in response to changes in the environment, such as changes in optimal temperature, pH, ionic strength. Various proteins from thermophiles are thought to have acquired their characteristic resistance to high temperature as one example of such protein engineering in nature (Oshima, 1990). During the evolutionary process amino-acid residues must be replaced step by step; more dramatic changes would cause the immediate disappearance of an enzyme's specific function. In particular, a 'soft' structure, such as a loop region is easily mutated to allow a change in the character of an enzyme.

3-Isopropylmalate dehydrogenase (E.C. 1.1.1.85; IPMDH) is an enzyme in the leucine biosynthetic pathway. It catalyzes the dehydrogenation and decarboxylation in the conversion of 3-isopropylmalate (IPM) to 2-oxoisocaproate, with the accompanying reduction of NAD^+ (Yamada *et al.*, 1990). Several genes for IPMDH's have been cloned from eubacteria (Sekiguchi, Suda, Ishii, Nosoh & Tsuda, 1987; Imai, Sekiguchi, Nosoh & Tsuda, 1987; Kawaguchi, Inagaki, Kuwata & Tano, 1993), extreme thermophiles (Kagawa *et al.*, 1984; Kirino & Oshima, 1991; Kirino, Aoki, Aoshima, Hayashi & Oshima, 1994), and eukaryotes (Sekiguchi *et al.*, 1986). The three-dimensional structure of IPMDH from an extreme thermophile, *Thermus thermophilus* HB8 was determined at 2.2 Å resolution (Imada, Sato, Tanaka, Katsube & Oshima, 1991). The enzyme is composed of two isomorphic subunits that are associated with each other by hydrophobic interactions between four α-helices (Fig. 1*a*). Each subunit is separated into the two domains, designated the subunit–subunit interface domain and distal domain (Fig. 1*b*; Kirino *et al.*, 1994). The analysis of IPMDH revealed the presence of some flexible regions with high temperature factors or poor electron-density distribution. A loop region from residues Phe75 to Ile84, located near the *d* α-helix, had a higher temperature factor than other regions, which suggested that this loop might be very mobile and might play an important role in heat stability. This loop is used to locate the *d* α-helix at the putative binding site of the substrate, as deduced from a comparison of primary and tertiary structures with isocitrate dehydrogenase (ICDH) (Eguchi, Wakagi & Oshima, 1988; Thorsness & Koshland, 1987; Dean, Lee & Koshland, 1989; Laporte & Koshland, 1983; Hurley *et al.*, 1989; Hurley, Dean, Thorsness, Koshland & Stroud, 1990; Hurley, Dean, Sohl, Koshland & Stroud, 1990; Hurley, Dean, Koshland & Stroud, 1991) (Fig. 2). The various observations imply that the deletion of two residues in this loop has a critical effect upon the choice of substrate by each enzyme.

A loop-deleted variant of IPMDH from *T. thermophilus* was constructed to examine the importance of the flexible loop. While only two amino acids (Arg82 and Lys83), were deleted from the loop upstream of the *d* α-helix (Pro86 through Lys95), 11 amino acids (Asp79 through Thr88) were replaced by the corresponding nine amino acids (Gly83 through Gln91) from yeast IPMDH in order to prevent a dramatic conformational change near the deleted residues (Fig. 2). In spite of the deletion, the mutant protein was found to have catalytic activity. In the present report we describe the structure of the loop-deleted IPMDH as determined at 2.2 Å resolution by X-ray crystallography. A comparison of the structure with that of the wild-type enzyme explains the importance of the flexible loop in the thermostability of the enzyme.

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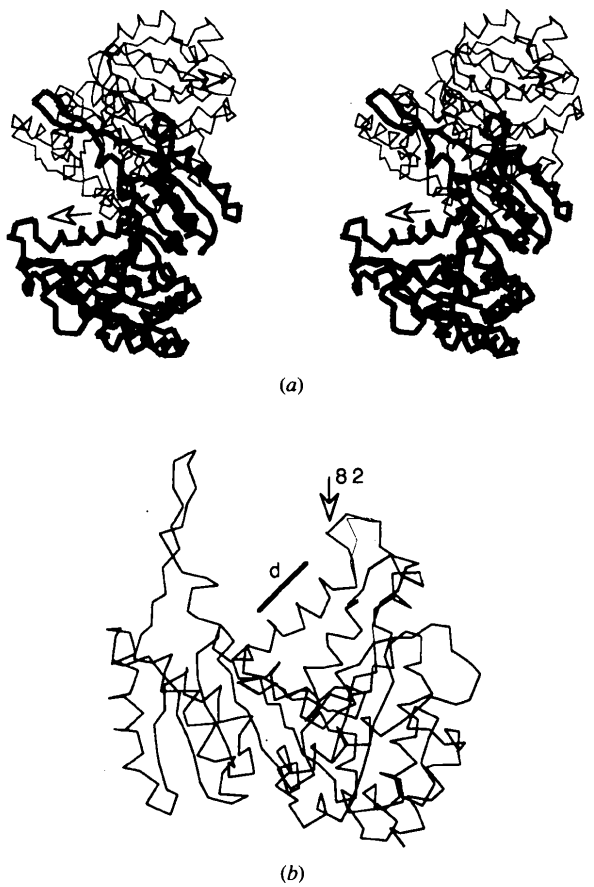
2. Materials and methods

2.1. *Purification of the enzyme*

The loop-deleted IPMDH was constructed by site-directed mutagenesis (Kunkel, 1985) using the oligonucleotide 5′-GGA AAG AAG CCC CTG CTC CGG-GCG GAC GGA TCC CGT GCC CCA CTT GGG CCC-3′. The mutant gene was expressed in *E. coli* JA221 as described elsewhere (Yamada *et al.*, 1990). The enzyme was purified by the previously described method with minor modifications (Miyazaki, Eguchi, Yamagishi, Wakagi & Oshima, 1992; Miyazaki & Oshima, 1993). About 40 mg of enzyme were isolated from 30 g wet weight of cells.

2.2. *Crystallization*

In order to compare the structure of the mutant enzyme with that of the wild-type enzyme under the same conditions, 20 m*M* phosphate buffer at pH 7.6 was used as the crystallization buffer. The solution in which the enzyme was dissolved was replaced by the phosphate buffer by the dialysis method. The concentration of the protein was adjusted to 10 mg ml^{-1} for crystallization by ultrafiltration with a YM-10 membrane (Amicon, Beverly, MA, USA). The hanging-drop vapor-diffusion method was employed for crystallization (Onodera *et al.*, 1994; Sakurai *et al.*, 1992). Droplets were made on the cover glass by mixing 5 µl of the solution of protein and 5 µl of reservoir solution, which contained ammonium sulfate of various concentrations from 0.9 to 1.4 *M*. Droplets were equilibrated against the corresponding reservoir solutions at 298 K. Crystals were grown for about 2 weeks, and the typical dimensions of the resultant crystals were 0.5 × 0.5 × 1.0 mm.



(*a*)

(*b*)

Fig. 1. (*a*) The stereo Cα-trace of the IPMDH dimer. Each of the thick and thin lines represents a subunit of the dimer. The arrows indicate the junctions between *d* α-helix and the loop region. (*b*) Superposed monomers of native and loop-deleted IPMDH. The thick lines and thin lines represent native and loop-deleted IPMDH, respectively. The arrow, thick bar, and N indicate the sites of deletions in the loop-deleted IPMDH, *d* α-helix, and N-terminal, respectively.

2.3. *X-ray diffractometry*

Three-dimensional X-ray diffraction data were collected on an R-AXIS IIc system (Rigaku, Tokyo, Japan), on the assumption that the cell dimensions of the present crystals were the same as most of the intact crystals of the wild type. The morphology of the crystals of the mutant protein closely resembled that of the wild type. Before the collection of all reflections, several still photographs were successfully processed. They were used to provide refined cell parameters and the orientation of the lattice by application of the *PROCESS* program (Rigaku). The crystals belonged to the $P3_221$ space group, with cell dimensions of $a = b = 78.45$, and $c = 158.07$ Å. These values deviated from those of the wild type by less than 0.2%. The protocol and results of data collection are summarized in Table 1. The merging and scaling of the data were performed by the method of Fox & Holmes (1966). The data up to 1.9 Å resolution were collected with an R_{merge} of 3.79% in the present study.

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LD IPMDH   74 GPKWGTGS--VRPEQGLLSLRKS  94
IPMDH Sc   79 GPKWGTGS--VRPEQGLLKIRKE  99
      Tt   74 GPKWDGLPRKISpetgllslrkS  96
      Ec   78 GPKWEHLPPDQQPERGALLPLRK 100
ICDH  Sc  101 GPLATPIG--KGHRSLNLTLRKT 121
      Tt   85 GPLETPVG--YGEKSANVTLRKL 105
      Ec  101 GPLTTPVG--GGIRslnvalrqe 121
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Fig. 2. The aligned amino-acid sequences of the loops and *d* α-helix regions of IPMDH and ICDH. The loop-deleted (LD) IPMDH is shown in the top line. Sequences are shown from *Thermus thermophilus* (Tt; Kagawa *et al.*, 1984; Miyazaki & Oshima, 1993), *Escherichia coli* (Ec; Kirino *et al.*, 1994; Thorsness & Koshland, 1987), and *Saccharomyces cerevisiae* (Sc; Andreadis, Hsu, Hermodson, Kohlhaw & Schimmel, 1984; Froman, Tait & Rodriguez, 1984; Cupp & McAlister-Henn, 1991), together with the available details of the secondary structure. Bacterial ICDH is two residues shorter than IPMDH in this region. Numbers indicate the residue numbers for the first and last residues shown. A deletion is represented by a dash (–). Small characters represent *d* α-helix, determined by X-ray crystallography. The numbers indicate the numbers of the first and the last residues of the known secondary structure.

Table 1. *Summary of data collection on R-AXIS IIc and statistics*

Resolution limit (Å)	2.2
Mosaic spread (°)	0.5
No. of observed reflections	44526
No. of independent reflections	27190
Completeness (%)	76.0
R_{merge}* (%)	
Full reflections	3.35
Partial reflections	4.32
Total reflection	3.79

* $R_{merge} = \sum\sum|Ii(h)/Gi - \langle I(h)\rangle|/\sum\sum\langle I(h)\rangle$, where Gi is the inverted scale factor, $Ii(h)$ is the diffraction intensity of the symmetry-equivalent reflections and $\langle I(h)\rangle$ is the mean value of $\langle Ii(h)\rangle$.

Table 2. *Final refinement statistics*

Resolution (Å)	2.20
No. of observed reflections ($F > 3\sigma$)	20014
R factor	0.17
Bond r.m.s. deviation (Å)	0.01
Angle r.m.s. deviation (°)	1.41

2.4. *Determination of the structure and refinement*

Since the present enzyme was isomorphous with the wild-type enzyme, the structure of the mutant enzyme was determined directly from $[2F_o$(mutant) $- F_c$(wild type)$]\varphi c$ and $[F_o$(mutant) $- F_c$(wild type)$]\varphi c$ difference electron-density maps, calculated by *X-PLOR* (Brünger, Karplus & Petsko, 1989; Brünger, Karplus & Erickson, 1990). An initial model was built by replacing the 11 amino acids of the wild-type enzyme by alanine residues and removing all solvent molecules found on the molecular surface. After the model had been refined by *X-PLOR* to an R factor of 0.21, the 11 residues were replaced by the correct residues by inspecting the $[2F_o - F_c]$ map with the *FRODO* program (Jones, 1978). Successive refinements reduced the R factor to 0.17 between 5.0 and 2.2 Å resolution (Table 2). The final structure was evaluated by construction of a Ramachandran plot (Ramachandran & Sassiekharan, 1968) and a Luzzati plot (Luzzati, 1952). The obtained final model has been deposited in Protein Data Bank.*

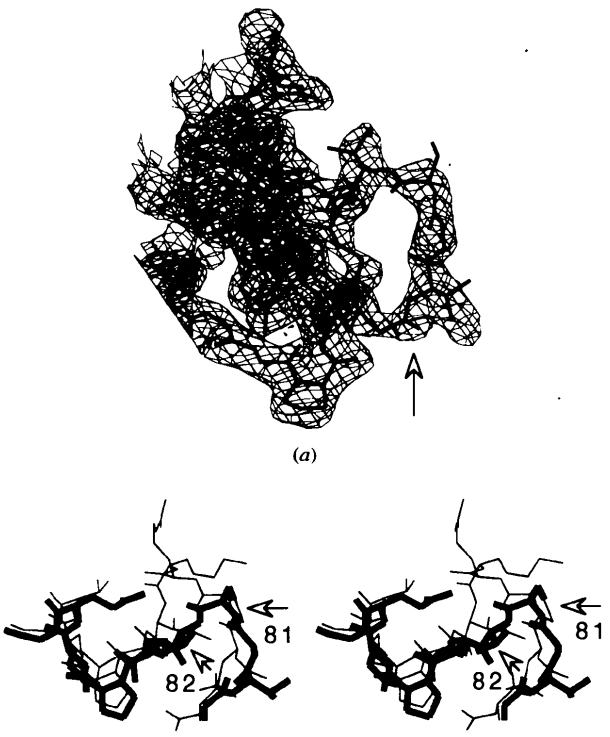
2.5. *Assays of enzymatic activity*

Kinetic parameters, K_m and k_{cat}, were determined under steady-state conditions at 333 K in 50 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate–NaOH buffer, pH 7.8, that contained 100 m*M* KCl, 5 m*M* $MgCl_2$ and NAD^+. The K_m for NAD^+ was measured in the presence of 1 m*M* 3-isopropylmalate. The initial velocity was calculated from the increase in the amount of NADH, determined by monitoring the absorbance at 340 nm. The residual activity of the enzyme was assayed by the standard method after incubation at an elevated temperature for designated period of time (Kirino *et al.*, 1994).

3. Results and discussion

The structure of the loop-deleted mutant was successfully determined at 2.2 Å resolution. The quality of the collected intensity data was better than that of the intact IPMDH, that is, the R_{merge} was 3.8% for the entire data set. The improved stabilization of the crystal was attributed to the deletion of the loop. The R factor after refinement by *X-PLOR* was reduced to 0.17 within 2.2 Å resolution and the r.m.s. deviations of the bond distances and angles were 0.2 Å and 0.2°, respectively. The Luzzati plot indicated that the overall error of the atomic positions was 0.25 Å within 2.2 Å resolution.

Amino-acid residues from Glu78 to Leu88 were clearly identified on the electron-density map, as shown in Fig. 3(*a*). A short section of the loop was observed upstream of the *d* α-helix. Although the primary structure



(*a*)

(*b*)

Fig. 3. (*a*) $[2F_o - F_c]$ map of the shortened loop region in loop-deleted IPMDH: The electron densities are represented by a basket map. The loop region has been indicated by the arrow. (*b*) Superposed models of the native and loop-deleted IPMDH. The thin lines and thick lines represent native and loop-deleted IPMDH. Arrows show the locations of Cα atoms at the two ends of the deletion.

* Atomic coordinates have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Reference: 1IDM). Free copies may be obtained through The Managing Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England (Reference: AS0687).

Table 3. *Newly established hydrogen bonds in the loop-deleted IPMDH*

Donor		Acceptor		Length (Å)
Arg83	Nη1	Glu85	Oε2	3.20
	Nη2		Oε1	2.70
	N	Gln86	Oε1	3.26

Table 4. *Kinetic parameters of the loop-deleted form of IPMDH at 333 K*

Enzyme	K_m (μM) IPM*	K_m (μM) NAD	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$) IPM	k_{cat}/K_m ($s^{-1}\mu M^{-1}$) NAD
Native	1.5	40.9	13.6	9.2	0.3
Loop-deleted	8.8	365.0	49.7	5.7	0.1

* 3-Isopropylmalate.

differed from that of the wild type as a result of the deletion of two residues and the replacement of five residues, the chain folding of the peptide bonds remained unaltered compared with the wild type, as shown in Figs. 1(*b*) and 3(*b*). Apart from the replaced residues, the root-mean-square deviation of the Cα atom was 0.01 Å from the wild-type molecule. The positions of residual atoms were also unchanged, with deviations within 0.3 Å. The Ramachandran plot indicated that most of the torsion angles had normal values and, as found in the wild-type enzyme, glycine residues and several other residues including Arg176, Asp231 and Ile284 were located beyond the accepted regions. These outliers have hydrogen bonds between Nη1 of Arg176 and Oδ1 of Asp231, between Nη2 of Arg176 and Oδ1 of Glu127, and between the backbone N atom of Ile284 and Oγ1 of Tyr333. In the vicinity of the loop-deleted region, additional hydrogen bonds were found between Arg83 and Glu85 and between the backbone N atom of Arg83 and Gln86, in spite of the similarities in the folding of the chain. The former bond was generated as a result of rotation of the Cα—Cβ bond of Arg83 by 180° and may contribute to the lower mobility of Arg83, as judged by its lower temperature factor (Fig. 4). The latter bond may be a result of the difference in bulkiness caused by the replacement of Gln by Thr at the 86th position. The lengths of the hydrogen bonds are listed in Table 3. The residues from Val72 to Lys76 appeared to adapt to the deletion by loss of folding of the chain since these residues in the mutant had higher temperature factors than those in the wild-type enzyme (Fig. 4).

Kinetic constants of the wild-type and loop-deleted IPMDH for the substrate and NAD^+ are shown in Table 4. Although both the K_m and k_{cat} values for the substrate and NAD^+ increased in the mutated protein, the value of k_{cat}/K_m remained unchanged. This observation agrees well with the similarity between the two structures, and with the fact that the heat stability of the mutant protein, as measured by the standard method, was also the same as that of the wild type, as shown in Fig. 5. However, the complex of the enzyme and substrate seems less stable than that of the wild-type enzyme and substrate, since the increase in K_m corresponds to an accelerated rate of dissociation of the enzyme and the substrate.

Because the flexibility of the loop region had been restricted by the deletion of two amino acids, the optimal fit between the enzyme and substrate was destroyed. However, the mutant enzyme recognized isopropylmalate correctly and failed to interact with isocitrate. Thus, the enzyme was able to recognize the substrate correctly without the Arg82 and Lys83 residues.

An internal reprieve mechanism can be invoked to explain the heat stability of IPMDH. It is postulated that an enzyme molecule can adopt an altered active form as a result of its flexibility. Hence, the enzyme can avoid complete loss of activity. In the case of loop-deleted IPMDH, the replacement of a loop region, as a unit, decreased the flexibility of the loop. However, as shown in Fig. 4, a new flexible region appeared, in which the temperature factor was higher. The region acts to relieve stress, because the high temperature factor is the sum of

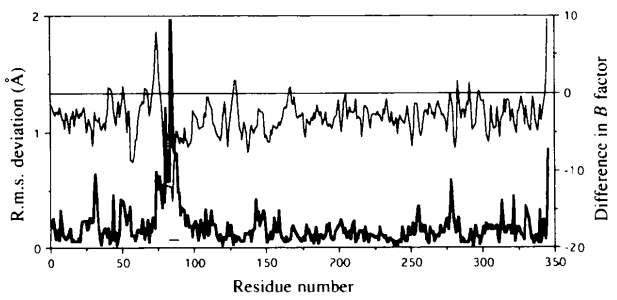


Fig. 4. Root-mean-square deviations between native and loop-deleted IPMDH (thick line) and the difference in temperature-factor (B_{native} minus B_{mutant}) distribution (thin line). A small horizontal bar represents the region of residues 80–86.

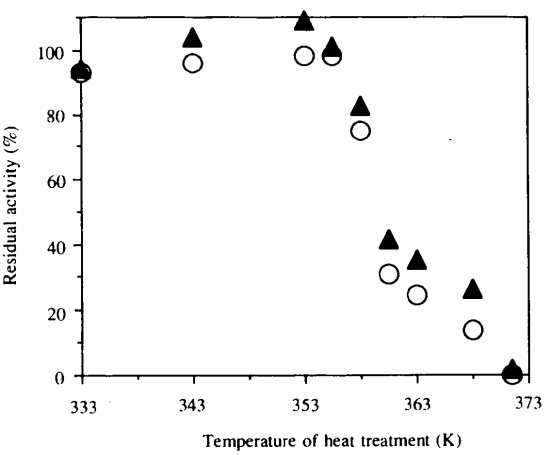


Fig. 5. The residual activity after heat treatment of native (○) and loop-deleted (▲) IPMDH.

the new constraints imposed by the new structure together with the establishment of hydrogen bonds. In chimeric IPMDH's, many amino-acid substitutions did not affect the original folding of the enzyme (Onodera *et al.*, 1994).

While protein engineering allows us to produce any variant of an enzyme at the primary sequence level, we have to develop strategies for generating an enzyme with a particular function. Even though the affinity of the loop-deleted IPMDH for IPM was reduced, the enzyme retained the ability to recognize the substrate correctly as well as, unexpectedly, its thermostability. The present study indicates that the full length of the flexible loop is not a prerequisite for activity. We had anticipated that the reduced flexibility of the loop might enhance the thermostability. However, we failed to detect any enhancement in the loop-deleted enzyme. More research is required to characterize the relationship between the structure of the enzyme and its function.

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