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Molecular and Phylogenetic Characterization of Isopropylmalate Dehydrogenase of a Thermoacidophilic Archaeon, *Sulfolobus* sp. Strain 7

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The archaeal *leuB* gene encoding isopropylmalate dehydrogenase of *Sulfolobus* sp. strain 7 was cloned, sequenced, and expressed in *Escherichia coli*. The recombinant *Sulfolobus* sp. enzyme was extremely stable to heat. The substrate and coenzyme specificities of the archaeal enzyme resembled those of the bacterial counterparts. Sedimentation equilibrium analysis supported an earlier proposal that the archaeal enzyme is homotetrameric, although the corresponding enzymes studied so far have been reported to be dimeric. Phylogenetic analyses suggested that the archaeal enzyme is homologous to mitochondrial NAD-dependent isocitrate dehydrogenases (which are tetrameric or octameric) as well as to isopropylmalate dehydrogenases from other sources. These results suggested that the present enzyme is the most primitive among isopropylmalate dehydrogenases belonging in the decarboxylating dehydrogenase family.

3-Isopropylmalate dehydrogenase (IPMDH; EC 1.1.1.85) is the third enzyme in the leucine biosynthetic pathway and catalyzes the oxidative decarboxylation of *threo*-D_S-3-isopropylmalate to 2-oxoisocaproate (32). Genes coding for the enzyme have been cloned and sequenced from various bacteria (eubacteria) and eukarya (eukaryotes). IPMDHs so far investigated are NAD dependent and homodimeric (20, 39).

It has been suggested that the catalytic mechanism of IPMDH is similar to that of bacterial homodimeric NADP-dependent isocitrate dehydrogenase (ICDH; EC 1.1.1.42) a key enzyme in the tricarboxylic acid cycle. The latter catalyzes the oxidative decarboxylation of *threo*-D_S-isocitrate to oxoglutarate (9). The primary structures of IPMDHs are homologous to those of the homodimeric bacterial NADP-dependent ICDHs (42). In addition, IPMDHs show sequence similarities to mitochondrial NAD-dependent ICDHs. The eukaryotic NAD-dependent ICDHs are either heterotetrameric (e.g., $\alpha 2\beta\gamma$ for the mammalian mitochondrial enzymes) (33, 41) or heterooctameric (e.g., $\alpha 4\beta 4$ for the yeast mitochondrial enzyme) (21), consisting of subunits homologous to each other.

Recently, the X-ray crystal structure of *Thermus thermophilus* HB8 IPMDH has been determined (13, 15, 19). The structure resembles that of *Escherichia coli* NADP-dependent ICDH (12, 14). In particular, both enzymes are unique in lacking the Rossmann fold motif which is frequently found in NAD(P)-dependent enzymes. In addition, most of the functional key residues proposed by the crystallographic studies are conserved among IPMDHs, bacterial NADP-dependent ICDHs, and mitochondrial NAD-dependent ICDHs (42). The structural evidence strongly supports the idea (13, 42) that these enzymes originated from a gene duplication that preceded the divergence of the three domains of life.

To clarify the phylogenetic relationship among IPMDHs and ICDHs, the structural and functional data of archaeal enzyme are absolutely required, since *Archaea* (archaebacteria) comprises the third independent domain of life, which is only distantly related to both the *Bacteria* and the *Eukarya* (38). Recently, we have isolated and preliminarily characterized IPMDH from an extreme thermoacidophilic archaeon, *Sulfolobus* sp. strain 7 (40). The archaeon (originally named *Sulfolobus acidocaldarius* 7) grows optimally at 80°C and pH 2.5. Biochemistry and molecular biology of this microorganism have been extensively studied; many enzymes such as IPMDH, ATPase, ferredoxin, NADH dehydrogenase, and so on have been isolated and characterized from the archaeon, and the phylogenetic relationship of the organism with other organisms has been discussed based on the biochemical properties and sequences of the enzymes (5, 16–18, 37, 40). In the present report, the archaeal *leuB* gene encoding IPMDH of *Sulfolobus* sp. strain 7 was cloned and sequenced. Moreover, the *leuB* gene was overexpressed in *E. coli*, and the recombinant IPMDH was characterized. On the basis of the structural and functional data, the phylogenetic implication among IPMDH and ICDH was discussed.

MATERIALS AND METHODS

Strains, media, and chemicals. The thermoacidophilic archaeon, *Sulfolobus* sp. strain 7, was cultivated aerobically and heterotrophycally at pH 3 and 80°C as described previously (16). *E. coli* JM109 and BL21 were used as hosts for DNA manipulation and gene expression, respectively. LB broth containing ampicillin (100 μg/ml) was used for *E. coli* cultivation. Isopropyl-β-D-thiogalactopyranoside (IPTG, 4 μg/ml) was added to the medium when required.

D,L-3-Isopropylmalate was purchased from Wako Pure Chemical (Tokyo, Japan). Other D-3-alkylmalates were kind gifts from Dr. Kakinuma (Tokyo Institute of Technology). NAD and NADP were from Oriental Yeast (Tokyo, Japan). All other reagents used in this study were from Wako Pure Chemical (Tokyo, Japan) and were of the purest grade available.

Molecular cloning and sequencing of the *Sulfolobus* sp. *leuB* gene. The DNA manipulations and hybridizations followed the methods in the literature (34). Based on the N-terminal amino acid sequence of IPMDH of *Sulfolobus* sp. strain

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7 (40), an oligonucleotide mixture, 5′-ACIAT(T/C)TCIGGICCIATICC(A/G)TCICC(T/C)-TGIAT-3′, was synthesized as a probe. The probe was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Chromosomal DNA of *Sulfolobus* sp. strain 7 was completely digested with some restriction enzymes, and Southern hybridization using the probe was carried out at 37°C for 12 h in 6× NET (1× NET contains 150 mM NaCl, 15 mM Tris-HCl, and 1 mM EDTA) supplemented with 0.4% polyvinylpyrrolidone, 0.4% Ficoll 400, 0.4% bovine serum albumin, 0.2% sodium dodecyl sulfate (SDS), 200 μg of sheared herring sperm DNA per ml, and the labeled probe (2×10^7 cpm/ml). Filters were washed twice at 50°C for 20 min with 0.5× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate) containing 0.1% SDS and exposed to X-ray film. The *Eco*RI-digested chromosomal DNA of *Sulfolobus* sp. strain 7 was ligated to *Eco*RI-digested pUC19, with which *E. coli* JM109 was transformed. Colony hybridization was carried out under the same conditions described for Southern hybridization. One of the plasmids isolated by the screening procedure was named pU7E.

The *leuB* gene and its flanking region were sequenced by the dideoxy chain termination method (35).

Expression of the *Sulfolobus* sp. *leuB* gene in *E. coli*. A *Nde*I recognition site was introduced around the start codon of the *Sulfolobus* sp. *leuB* gene by PCR with synthetic oligonucleotides, 5′-GGGCATATGGGCTTT-ACTGTTGCTT-3′ and 5′-GCCCTACTTTTGCTATTCTCTC-3′. The sequence of the amplified fragment was verified by the dideoxy chain termination method (35). A 0.3-kbp *Nde*I-*Sma*I fragment containing the 5′-terminal region of *Sulfolobus* sp. *leuB* gene was prepared by digesting the PCR-amplified fragment with *Nde*I and *Sma*I. A 1.1-kbp *Sma*I-*Eco*RI fragment containing the 3′-terminal region of *Sulfolobus* sp. *leuB* gene was prepared by digesting pU7E with *Sma*I and *Eco*RI. The plasmid pE7-SB6 was constructed by inserting these two fragments into plasmid pET-17b (Novagen) previously digested with *Nde*I and *Eco*RI.

E. coli BL21 harboring pE7-SB6 was cultured in LB broth containing ampicillin at 37°C. IPTG was added to the medium at late-log phase of the cell growth, and cultivation was continued for 3 h after the induction.

Purification of the recombinant *Sulfolobus* sp. IPMDH. *E. coli* cells (10 g) expressing the *Sulfolobus* sp. *leuB* gene were suspended in 20 ml of buffer A (20 mM potassium phosphate buffer, pH 7.8, containing 0.5 mM EDTA) and disrupted by sonication. The supernatant obtained after ultracentrifugation was treated at 83°C for 20 min and denatured proteins were discarded by centrifugation. The remaining supernatant was mixed with an equal volume of buffer A containing 2.0 M ammonium sulfate and loaded onto a Butyl-Toyopearl 650M column (Tosoh) equilibrated in buffer A containing 1.0 M ammonium sulfate. Bound *Sulfolobus* sp. IPMDH was eluted from the column by a linear gradient of 0.7 M to 0.3 M ammonium sulfate. Pooled fractions were passed through a DEAE-Sepharose Fast Flow column (Pharmacia) which had been preequilibrated with buffer A.

Purity of the recombinant enzyme was verified by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE). The amino acid sequence of the *Sulfolobus* sp. IPMDH was determined with a gas-phase peptide sequencer (model 470; Applied Biosystems, Foster City, Calif.).

Enzyme assay and heat stability analysis of *Sulfolobus* sp. IPMDH. Our standard enzyme assay followed the method of Yoda et al. (40) with a small modification. Activity was measured in a steady-state experiment in 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate (HEPES) buffer, pH 8.0, containing 5.0 mM $MgCl_2$, 100 mM KCl, 5.0 mM NAD, and 1 mM D,L-3-isopropylmalate, by recording the production of NADH at 340 nm.

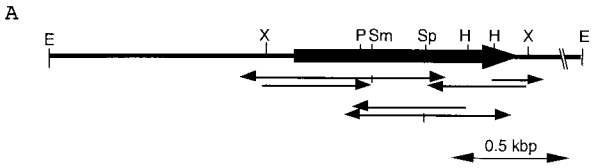
Michaelis constants (K_m) and catalytic constants (k_{cat}) for several D-3-alkylmalates and isocitrate were determined in the presence of 5.0 mM NAD. K_ms for NAD and NADP were determined in the presence of 1.0 mM D-3-isopropylmalate.

The heat stability of *Sulfolobus* sp. IPMDH was measured as follows. The enzyme dissolved in buffer A (0.2 mg/ml) was incubated at various temperatures for 10 min and immediately chilled in ice-water. Then, aggregated protein was removed by centrifugation (12,000 × *g* for 20 min), and IPMDH activity of the supernatant was measured. The heat stability of purified *T. thermophilus* HB8, *E. coli*, and *Bacillus subtilis* IPMDHs (prepared as described in references 22 and 28) was measured under the same conditions.

Analytical procedures. Sedimentation equilibrium analysis of the purified enzyme was carried out with a Beckman Optima XL-A analytical ultracentrifuge fitted with a Beckman An-60Ti analytical rotor. The molecular weight of the enzyme was calculated according to the procedure of Van Holde and Baldwin (36).

Quantitative analysis of free SH groups of *Sulfolobus* sp. IPMDH was carried out as described previously (6) with a small modification. The enzyme (0.64 mg/ml) was incubated in 50 mM HEPES buffer, pH 8.0, containing 0.5% SDS. A freshly prepared solution of 5,5′-dithiobis(2-nitro-benzoate) was added to a final concentration of 0.4 mg/ml, and the quantity of produced thionitrobanzoate was monitored at 412 nm.

Phylogenetic analysis. Composite trees between IPMDH and ICDH were constructed by using maximum parsimony or neighbor-joining methods. Sequence data used for the calculation were obtained from the GenBank and PIR databases. The amino acid sequences of IPMDHs and ICDHs were firstly aligned by using the program package Genetyx (SDC, Japan) and subsequently adjusted by hand on the basis of the secondary and tertiary structural information of the X-ray crystallographic structures of *T. thermophilus* HB8 IPMDH (13, 15, 31) and *E. coli* ICDH (12) as described in the literature (15, 42). In order to improve the reliability of the sequence comparison, we selected and used the highly conserved regions (marked with plus signs in Fig. 2) including the substrate and coenzyme binding sites as performed in the literature (8). The phylogenetic analyses were performed by using SEQBOOT, PROTPARS, PROTDIST, NEIGHBOR, and CONSENCE of the program package Phylip 3.5c (7). Evolutionary distances were estimated by using a Dayhoff amino acid comparison matrix. We generated 100 bootstrapped data sets to make a consensus tree.



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TCTAGATTGGATATATATAGCAAAGAAGACATTGAAAAATATTTACCTATAATAAAATAT    60
GAGATTGACATTATACGTAAAACTATGCCAAATGACATAAAAATTATTGAAAAAATTAAG   120
                                                      start
GAACTTATGTTACAGAAAGGGTTAAAAATTTAATTTCAAAAACCCCCCACatgGGCTTTA   180
                                                  M  G  F  T     4
CTGTTGCTTTAATACAAGGAGATGGAATTGGACCAGAAATAGTATCTAAATCTAAGAGAA   240
  V  A  L  I  Q  G  D  G  I  G  P  E  I  V  S  K  S  K  R  I    24
TATTAGCCAAAATAAATGAGCTTTATTCTTTGCCTATCGAATATATTGAAGTAGAAGCTG   300
  L  A  K  I  N  E  L  Y  S  L  P  I  E  Y  I  E  V  E  A  G    44
GTGATCGTGCATTGGCAAGATATGGTGAAGCATTGCCAAAAGATAGCTTAAAAATCATTG   360
  D  R  A  L  A  R  Y  G  E  A  L  P  K  D  S  L  K  I  I  D    64
ATAAGGCCGATATAATTTTGAAAGGTCCAGTAGGAGAATCCGCTGCAGACGTTGTTGTCA   420
  K  A  D  I  I  L  K  G  P  V  G  E  S  A  A  D  V  V  V  K    84
AGTTAAGACAAATTTATGATATGTATGCCAATATTAGACCAGCAAAGTCTATCCCGGGAA   480
  L  R  Q  I  Y  D  M  Y  A  N  I  R  P  A  K  S  I  P  G  I   104
TAGATACTAAATATGGTAATGTTGATATACTTATAGTGAGAGAAAATACTGAGGATTTAT   540
  D  T  K  Y  G  N  V  D  I  L  I  V  R  E  N  T  E  D  L  Y   124
ACAAAGGTTTTGAACATATTGTTTCTGATGGAGTAGCCGTTGGCATGAAAATCATAACTA   600
  K  G  F  E  H  I  V  S  D  G  V  A  V  G  M  K  I  I  T  R   144
GATTTGCTTCTGAGAGAATAGCAAAAGTAGGGCTAAACTTTGCATTAAGAAGGAGAAAGA   660
  F  A  S  E  R  I  A  K  V  G  L  N  F  A  L  R  R  R  K  K   164
AAGTAACTTGTGTTCATAAGGCTAACGTAATGAGAATTACTGATGGTTTATTCGCTGAAG   720
  V  T  C  V  H  K  A  N  V  M  R  I  T  D  G  L  F  A  E  A   184
CATGCAGATCTGTATTAAAAGGAAAAGTAGAATATTCAGAAATGTATGTAGACGCAGCAG   780
  C  R  S  V  L  K  G  K  V  E  Y  S  E  M  Y  V  D  A  A  A   204
CGGCTAATTTAGTAAGAAATCCTCAAATGTTTGATGTAATTGTAACTGAGAACGTATATG   840
  A  N  L  V  R  N  P  Q  M  F  D  V  I  V  T  E  N  V  Y  G   224
GAGACATTTTAAGTGACGAAGCTAGTCAAATTGCGGGTAGTTTAGGTATAGCACCCTCTG   900
  D  I  L  S  D  E  A  S  Q  I  A  G  S  L  G  I  A  P  S  A   244
CGAATATAGGAGATAAAAAAGCTTTATTTGAACCAGTACACGGTGCAGCGTTTGACATTG   960
  N  I  G  D  K  K  A  L  F  E  P  V  H  G  A  A  F  D  I  A   264
CTGGAAAGAATATAGGTAATCCCACTGCATTTTTACTTTCTGTAAGTATGATGTATGAAA  1020
  G  K  N  I  G  N  P  T  A  F  L  L  S  V  S  M  M  Y  E  R   284
GAATGTATGAGCTATCTAATGACGATAGATATATAAAAGCTTCAAGAGCTTTAGAAAACG  1080
  M  Y  E  L  S  N  D  D  R  Y  I  K  A  S  R  A  L  E  N  A   304
CTATATACTTAGTCTACAAAGAGAGAAAAGCGTTAACCCCAGATGTAGGTGGTAATGCGA  1140
  I  Y  L  V  Y  K  E  R  K  A  L  T  P  D  V  G  G  N  A  T   324
                                          stop
CAACTGATGACTTAATAAATGAAATTTATAATAAGCTAGGCtaaAAGtttttAAATCACT  1200
  T  D  D  L  I  N  E  I  Y  N  K  L  G  *                    337
ACTTTATCTATTATCGAGGTGATCTAGA                                  1228
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FIG. 1. Physical map (A) and nucleotide sequence (B) of the *Sulfolobus* sp. *leuB* gene. (A) The solid arrow indicates the *Sulfolobus* sp. *leuB* gene, and the determined sequence is indicated by thin arrows. Abbreviations: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I. (B) The nucleotide sequence and the deduced amino acid sequence of the *Sulfolobus* sp. *leuB* gene. The N-terminal amino acid sequence of the recombinant *Sulfolobus* sp. IPMDH as determined by automated Edman degradation is underlined.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession number D86857.

RESULTS AND DISCUSSION

Cloning and sequencing of the *Sulfolobus* sp. *leuB* gene. In the Southern hybridization, a 2.3-kbp positive band was detected in the *Eco*RI-digested chromosomal DNA libraries. Plasmids containing the 2.3-kbp *Eco*RI fragment from genomic DNA of *Sulfolobus* sp. were isolated by colony hybridization, and one of them was used in the present study. The restriction map of the fragment and the sequencing strategy are shown in Fig. 1A. The sequence of the 1,228-bp *Xba*I-*Xba*I fragment comprising the *Sulfolobus* sp. strain 7 *leuB* gene is

shown in Fig. 1B. The sequence exhibited an open reading frame encoding a 337-residue polypeptide. Its deduced N-terminal amino acid sequence matched with that of IPMDH previously purified from *Sulfolobus* sp. strain 7 cells (XFTVA LIQGDGIGPE [40]). No potential Shine-Dalgarno sequence complementary to the conserved 3′ end of archaeal 16S rRNA [. . .GGUGGAUCACCUCC(N)] (2, 29) could be identified upstream or downstream of the initiating codon. However, a sequence (TTTTT) typical of an archaeal transcription termination signal (2) was located four nucleotides downstream from the UAA termination codon.

The G+C content of the *Sulfolobus* sp. *leuB* gene is 34.8%, slightly lower than that of the chromosomal DNA (37%) (23). The codon usage is similar to that of other proteins from *Sulfolobus* sp. strain 7 (5). The deduced amino acid sequence of the *Sulfolobus* sp. IPMDH shares considerable similarity (36.9% amino acid sequence identity) with the *T. thermophilus* HB8 IPMDH sequence. The percent identity was calculated by using the length of the *Sulfolobus* sp. IPMDH (337 residues) as a common denominator.

The deduced molecular mass of the *Sulfolobus* sp. IPMDH calculated from the *leuB* gene sequence is 36,832 Da, which is in good agreement with the apparent size of the subunit of IPMDH previously purified from *Sulfolobus* sp. strain 7 (37 kDa) (40).

Phylogenetic analysis of the *Sulfolobus* sp. IPMDH. The deduced amino acid sequence of the *Sulfolobus* sp. IPMDH is more closely related to those of bacterial IPMDHs (*E. coli*, 33.1%; *B. subtilis*, 35.4%; *T. thermophilus* HB8, 36.9% identity) than to those of eukaryote IPMDHs (*Saccharomyces cerevisiae*, 27.2%; *Neurospora crassa*, 30.9% identity). In addition, the present enzyme is closer to mitochondrial NAD-dependent ICDHs (*S. cerevisiae* IDH1 subunit, 36.0%; *S. cerevisiae* IDH2 subunit, 38.1%; *Homo sapiens* α subunit, 40.5%; *Bos taurus* 3/4 subunit, 39.9% identity) than to the IPMDHs.

To clarify the phylogenetic relationship among these enzymes, their amino acid sequences were aligned as illustrated in Fig. 2. The distance matrix was calculated from the sequence comparison of the highly conserved regions, and a composite phylogenetic tree of the IPMDH/ICDH pair was constructed by the neighbor-joining method (Fig. 3). The same topological tree was also obtained by the maximum parsimony method (data not shown).

It has been speculated that IPMDH and ICDH would have been diverged from an ancestral enzyme by a gene duplication (13, 42). Considering division of the primary domains proposed by Woese et al. (38), it is suggested that the gene duplication and the subsequent divergence of IPMDH and ICDH might have occurred before the divergence of the domains. The results are consistent with the idea that IPMDH and ICDH are phylogenetically related and can be classified into a "superfamily," though the functions and the substrate specificities of these enzymes differ from each other. It is noteworthy that a similar relationship has been proposed for isopropylmalate isomerases (coded by the *leuC* and *leuD* genes) involved in the leucine biosynthetic pathway, and aconitase, a member of the tricarboxylic acid cycle (reviewed in reference 8).

The topology of the archaeal and bacterial IPMDHs in the composite tree follows essentially that of the 16S-rRNA tree (30). It is clear that the *Sulfolobus* sp. IPMDH represents the most divergent member of IPMDHs investigated to date. The putative root of IPMDH can be placed at the position marked with the asterisk in Fig. 3. The topology also suggests that the eukaryal IPMDH was derived from the purple bacterial IPMDH lineage. This suggests a bacterial endosymbiotic origin

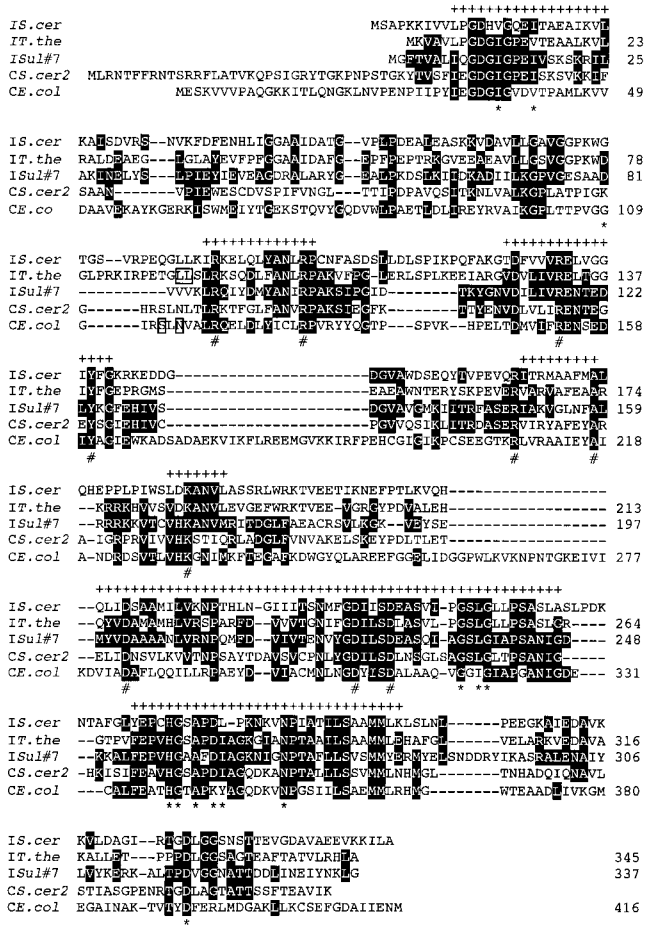

FIG. 2. Alignment of amino acid sequences of IPMDHs and ICDHs from various sources. The residues identical to those of the *Sulfolobus* sp. IPMDH sequence are boxed inversely. #, residues that interact with the malate moiety of isocitrate; ∗, residues that interact with the adenine-ribose portion of NAD in *T. thermophilus* HB8 IPMDH; +, residues used for the calculation for phylogenetic analyses. Abbreviations: I*S.cer*, *Saccharomyces cerevisiae* IPMDH; I*T.the*, *Thermus thermophilus* HB8 IPMDH; I*Sul#7*, *Sulfolobus* sp. IPMDH; C*S.cer2*, *Saccharomyces cerevisiae* NAD-dependent ICDH (IDH2); C*E.col*, *Escherichia coli* NADP-dependent ICDH.

for the eukaryal IPMDHs, as in the case reported for the phylogenetic analysis of aminoacyl-tRNA synthetases (1).

The topology of ICDHs in the composite tree suggests that ICDHs can be classified into two divergent groups; mitochon-

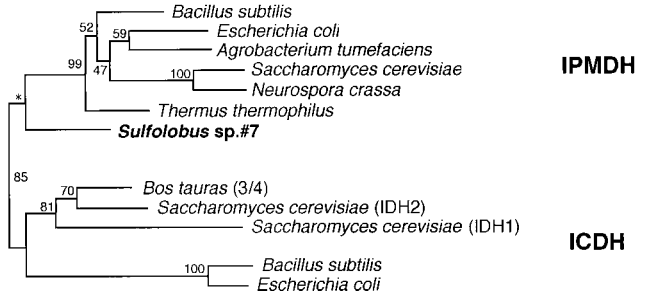

FIG. 3. Composite phylogenetic tree constructed from a simultaneous comparison of IPMDH and ICDH. The putative roots of each groups were tentatively chosen at a point between the two clusters. Numbers on selected nodes indicate bootstrap values.

TABLE 1. Purification procedure of *Sulfolobus* isopropylmalate dehydrogenase

Step	Total activity (U)	Yield (%)	Protein (mg)	Sp act (U/mg)	Purity (fold)
Crude extract			1,140		
After heating	235	100	123	1.91	1.00
Butyl-Toyopearl	157	66.8	22.7	6.93	3.63
DEAE-Toyopearl	159	67.3	10.0	15.9	8.32

TABLE 2. Kinetic constants for D-3-alkylmalate of *Sulfolobus* sp. IPMDH

Alkyl group	π^a	K_m (μM)	k_{cat} (S^{-1})	k_{cat}/K_m ($S^{-1} \times \mu M^{-1}$)
Hydrogen[b]	0	390	4.0	0.010
Methyl	0.5	3.7	3.5	0.94
Ethyl	1.0	1.1	3.1	2.7
Isopropyl	1.3	1.2	3.6	3.0
Isobutyl	1.8	1.5	3.1	2.0
tert-Butyl	2.0	2.3	0.96	0.42
Isopentyl	2.3	5.8	2.7	0.46

[a] The π values are the Hansch constants for the alkyl substituents (9).
[b] Malate has only one asymmetric carbon atom.

drial NAD-dependent ICDHs and bacterial NADP-dependent ICDHs (Fig. 3). The subunit structure of the former enzymes is more complex, either heterotetramer (33, 41) or heterooctamer (21), than the latter, which is dimeric (14). In case of the heterooctameric mitochondrial ICDH (α4β4) of *S. cerevisiae* (4, 21), the genes for the two subunits, *IDH1* and *IDH2*, would have been evolved from a single gene by a gene duplication after divergence of the *Eukarya* and *Bacteria* domains. The tree shown in Fig. 3 indicates that the *Sulfolobus* sp. IPMDH is more closely related to the eukaryal NAD-dependent ICDH than to the bacterial NADP-dependent ICDH. The tree clearly indicates that *Sulfolobus* sp. IPMDH defines the earliest divergence within the IPMDHs.

Expression and purification of *Sulfolobus* sp. IPMDH. The *Sulfolobus* sp. *leuB* gene was introduced into the expression plasmid pET-17b, and the recombinant plasmid was named pE7-SB6. *E. coli* BL21 harboring pE7-SB6 produced the recombinant IPMDH of *Sulfolobus* sp. strain 7. The purification of the recombinant enzyme is summarized in Table 1. The majority of proteins in the crude extract was readily removed by heat treatment at 83°C for 20 min, which facilitated further purification of the recombinant enzyme by two successive column chromatographies with a typical yield exceeding 65%. Approximately 10 mg of *Sulfolobus* sp. IPMDH was routinely obtained from 10 g of wet cells. Purity of the recombinant protein was verified by SDS-PAGE, which showed a single 37-kDa band on the gel (data not shown). This apparent molecular mass is consistent with that of the *Sulfolobus* sp. *leuB* gene product (36,832 Da) and is identical to that previously observed for the enzyme purified from the cells of *Sulfolobus* sp. strain 7 (40). N-terminal amino acid sequence analysis of the purified recombinant protein (underlined residues in Fig. 1B) indicates the removal of the first methionine residue, as in the case reported for IPMDH purified from the *Sulfolobus* sp. strain 7 cells (40).

Quaternary structure of *Sulfolobus* sp. IPMDH. In a previous study, we have reported a possibility that *Sulfolobus* sp. IPMDH is homotrimeric or homotetrameric, based on the gel filtration analysis (40). To confirm this, the quaternary structure of the recombinant *Sulfolobus* sp. IPMDH was subjected to sedimentation equilibrium analysis. A molecular mass of 136 ± 10 kDa was obtained by this analysis, assuming a partial specific volume of 0.749 which was calculated from the amino acid composition. The results demonstrated that the *Sulfolobus* sp. IPMDH is a homotetramer, supporting the earlier proposal (40). While prokaryotic IPMDHs and eubacterial NADP-dependent ICDH are homodimeric (12, 18, 37). The mitochondrial NAD-dependent ICDHs are either tetrameric (33, 41) or octameric (21). The unique quaternary structure of the *Sulfolobus* sp. IPMDH may reflect its phylogenetic relations to mitochondrial NAD-dependent ICDHs.

Sulfolobus sp. IPMDH contains two cysteine residues, Cys167 and Cys185. Because one of them, Cys185, is conserved in several mitochondrial NAD-dependent ICDHs (all subunits of the monkey enzyme [26], 3/4 subunit of the bovine enzyme [41], and γ subunits of the pig [11] and the rat [27] enzymes), it is possible that these Cys residues form an intersubunit disulfide bond, contributing to the formation of their quaternary structure. To investigate this possibility, a quantitative analysis of free SH groups was performed. The result showed the existence of 1.9 SH groups per subunit of the *Sulfolobus* sp. IPMDH, indicating the absence of either intra- or inter-subunit disulfide bonds.

Kinetic properties of *Sulfolobus* sp. IPMDH. Table 2 summarizes the kinetic constants, K_m and k_{cat}, for several D-3-alkylmalates of *Sulfolobus* sp. IPMDH at 70°C. All alkylmalates investigated in Table 2 served as a substrate of the archaeal IPMDH, implying its broad substrate specificity. On the other hand, isocitrate did not serve as a substrate of the enzyme. In terms of the catalytic efficiency, k_{cat}/K_m, isopropylmalate is the best substrate. The relationship between catalytic properties and hydrophobicity of the alkyl group at position 3 is illustrated in Fig. 4. Changes of catalytic properties of the *Sulfolobus* sp. enzyme in accordance of alkylmalates used as the substrate resemble those of the *T. thermophilus* HB8 enzyme as shown in the figure. This suggests the structural similarity of the substrate binding pocket between these two enzyme.

In the case of *T. thermophilus* HB8 IPMDH, 12 residues which build up the binding pocket have been proposed (19). Ten of them (indicated in Fig. 2) interact with the malate moiety of the substrate and are conserved not only in all IPMDHs but also in ICDHs (12). Two other residues (Leu90 and Leu91 in *T. thermophilus* HB8 IPMDH, boxed in Fig. 2) have been expected to interact with the isopropyl group of isopropylmalate, determining the substrate specificity (19). These two leucine residues were generally conserved among conventional IPMDHs. In the case of *Sulfolobus* sp. IPMDH, these two residues are replaced by Val83 and Val84, respectively, which probably play the same role in the substrate recognition. In *E. coli* NADP-dependent ICDH, the corresponding residues are polar (Ser113 and Asn115, boxed in Fig. 2) and interact electrostatically with the carboxymethyl group of isocitrate (12).

Recently the complete genome sequence of an archaeal organism, *Methanococcus jannaschii*, has been reported (3), which included two putative ICDH genes (MJ1596 and MK0720 in GenBank). Of these, the MJ0720 sequence had the putative binding sequence (77-AADVIV-82) for isopropyl group of D-3-isopropylmalate (79-AADVVV-84 in *Sulfolobus* sp. IPMDH), suggesting a possibility that this enzyme may be IPMDH rather than ICDH. Because this speculation needs to

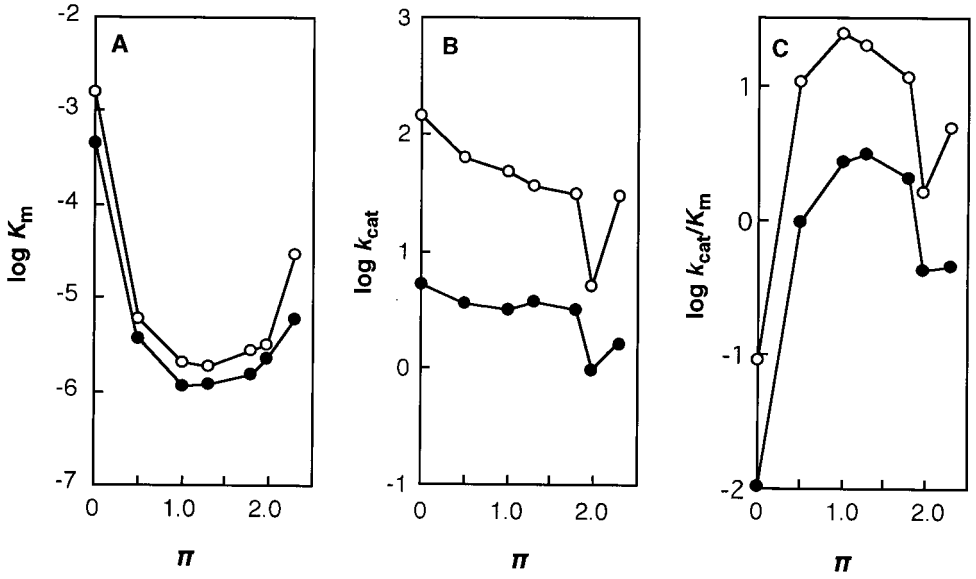

FIG. 4. Comparison of the catalytic properties of *Sulfolobus* sp. IPMDH toward 3-alkylmalates and those of *T. thermophilus* HB8 IPMDH. K_m (A), k_{cat} (B), and k_{cat}/K_m (C) were compared. Closed circles, *Sulfolobus* sp. IPMDH; open circles, *T. thermophilus* HB8 IPMDH (25). π values, the Hansch constants for the alkyl group (10).

be confirmed further at the protein level, these enzymes are not considered in the present study.

Sulfolobus sp. IPMDH showed the coenzyme binding affinity to NAD (K_m = 150 μM) whereas it disfavored NADP (K_m = 3900 μM). This is consistent with the strict conservation of 11 amino acid residues (marked with asterisks in Fig. 2) that interact with the adenosine-ribose portion of NAD in *T. thermophilus* HB8 IPMDH (13, 19); only one residue (Val15 in *T. thermophilus* HB8 IPMDH) is replaced by a homologous residue, Ile17, in the *Sulfolobus* sp. enzyme. It is notable that a residue corresponding to Asp278 of *T. thermophilus* HB8 IPMDH, which plays a key role in discriminating the coenzyme specificity (13, 24), is also conserved in *Sulfolobus* sp. IPMDH (Asp262). The sequence alignment shown in Fig. 2 suggests that the aspartate residue is conserved in all NAD-dependent enzymes (IPMDHs and mitochondrial NAD-dependent ICDH), whereas it is replaced by Lys in bacterial NADP-dependent ICDHs (Fig. 2). The difference in the coenzyme specificity of two types of ICDHs coincides with their phylogenetic divergence (Fig. 3).

Heat stability of *Sulfolobus* sp. IPMDH. The remaining activity after heat treatment is shown in Fig. 5A. The temperature for 50% inactivation is 94°C, suggesting that *Sulfolobus* sp. IPMDH possesses the highest heat stability among IPMDHs (*T. thermophilus* HB8, 87°C; *E. coli*, 59°C; *B. subtilis*, 45°C) reported so far. The temperature dependence of the enzymatic

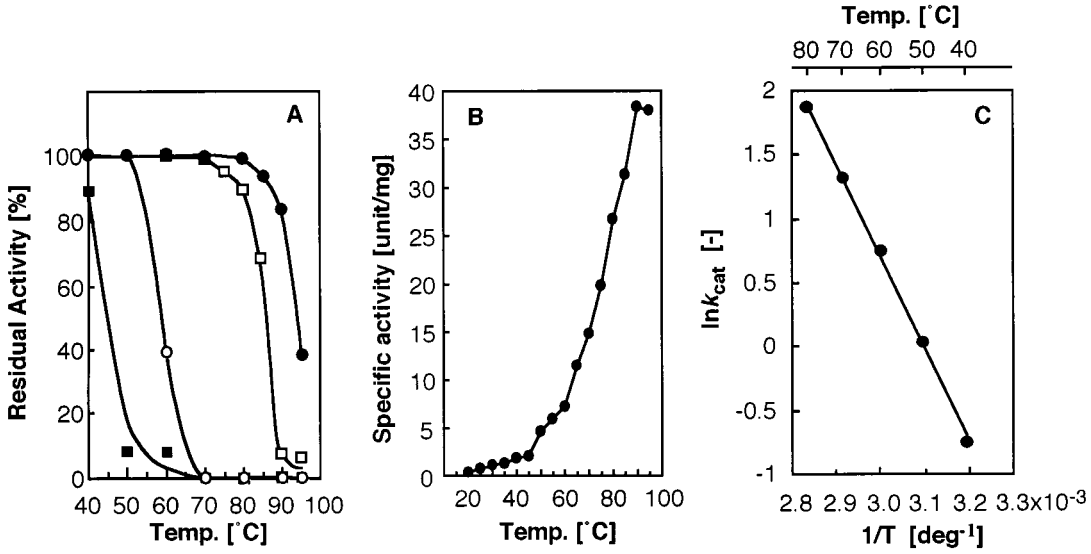

FIG. 5. Thermostability of the recombinant *Sulfolobus* sp. IPMDH. (A) residual activity after heat-treatment. Closed circles, *Sulfolobus* sp. IPMDH; open squares, *T. thermophilus* HB8 IPMDH; open circles, *E. coli* IPMDH; closed squares, *B. subtilis* IPMDH. (B) Temperature dependence of the enzymatic activity. (C) Arrhenius plot for the reaction.

activity shows that the maximum activity of *Sulfolobus* sp. IPMDH is at 95°C (Fig. 5B). The temperature dependence of the catalytic constant (k_{cat}) gave a linear relationship in an Arrhenius plot at least up to 80°C (Fig. 4C). Apparent activation energy of the catalytic reaction of *Sulfolobus* sp. IPMDH was calculated to be 62.8 kcal/mol. This archaeal enzyme is an interesting protein in view of protein conformational stability because the primary sequence of the enzyme is closer to that of the mesophilic, mitochondrial NAD-dependent ICDHs rather than the thermophilic, heat-stable *T. thermophilus* HB8 IPMDH.

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