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Cloning, sequencing, and expression of the *meso*-diaminopimelate dehydrogenase gene from *Bacillus sphaericus*

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Abstract

The gene encoding the *meso*-diaminopimelate dehydrogenase of *Bacillus sphaericus* was cloned into *E. coli* cells and its complete DNA sequence was determined. The *meso*-diaminopimelate dehydrogenase gene consisted of 978 nucleotides and encoded 326 amino acid residues corresponding to the subunit of the dimeric enzyme. The amino acid sequence deduced from the nucleotide sequence of the enzyme gene of *B. sphaericus* showed 50% identity with those of the enzymes from *Corynebacterium glutamicum* and *Brevibacterium flavum*. The enzyme gene from *B. sphaericus* was highly expressed in *E. coli* cells. We purified the enzyme to homogeneity from a transformant with 76% recovery. The N-terminal amino acid of both the enzyme from *B. sphaericus* and the transformant were serine, indicating that the N-terminal methionine is removed by post-translational modification in *B. sphaericus* and *E. coli* cells.

Keywords: Diaminopimelate dehydrogenase; *Bacillus sphaericus*; Nucleotide sequence; Primary structure

1. Introduction

meso-Diaminopimelate dehydrogenase (EC 1.4.1.16) catalyzes the reversible oxidative removal of an amino group in the D-configuration from the substrate in the presence of $NADP^+$ to yield L-2-amino-6-ketopimelate [1]. It is the only $NAD(P)^+$-dependent amino acid dehydrogenase which stereospecifically deaminates an amino group with a D-stereocenter. This enzyme functions in lysine biosynthesis of *Bacillus sphaericus* [2] and *Corynebacterium glutamicum* [3]. It has been purified to homogeneity from *B. sphaericus* IFO 3525 [4], *C. glutamicum* ATCC 13032 [5], and *Brevibacterium* sp. ICR 7000 [6], and characterized enzymologically. The enzyme is absolutely specific for *meso*-2,6-diaminopimelate and is used for spectrophotometric measurement of that compound [7] and assay of diaminopimelate epimerase [8]. The enzyme is also useful for the preparation and measurement of L-2-amino-6-ketopimelate, an intermediate in bacterial lysine biosynthesis [2,9], and for the preparation of stereospecifically isotope-labeled *meso*-2,6-diaminopimelate [10].

The enzyme genes from *C. glutamicum* [11] and *Brevibacterium flavum* [12] have been sequenced. The amino acid sequences of both enzymes are highly similar. The *C. glutamicum* gene has been expressed in *Escherichia coli* cells [13]. The three-dimensional structures of the enzyme–$NADP^+$ complex [14], the enzyme–substrate complex, and an enzyme–$NADP^+$–inhibitor complex [15] of the *C. glutamicum* enzyme have been solved and refined to 2.2 Å resolution.

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These data suggest that the enzyme undergoes significant conformational changes upon binding of both $NADP^+$ and substrate, and that the *C. glutamicum* enzyme is different from the *B. sphaericus* enzyme in its hydrogen transfer [14] and kinetic mechanism [15]. To confirm the structure of the *B. sphaericus* enzyme and to investigate the amino acid residues at the catalytic site of the enzyme by site-directed mutagenesis, we cloned the *B. sphaericus* enzyme gene.

In this paper, we describe the cloning, sequencing, and expression in *E. coli* of the *meso*-diaminopimelate dehydrogenase gene from *B. sphaericus* and compare the primary structure, the hydrophobicity profile, and the predicted tertiary structure of the *B. sphaericus* enzyme with those of the enzyme from *C. glutamicum*.

2. Experimental

2.1. Materials

Primers for polymerase chain reaction (PCR) were obtained from Hokkaido System Science (Hokkaido, Japan). Plasmid pUC18, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), isopropyl-β-D-thiogalactopyranoside (IPTG), all restriction enzymes, TaKaRa Ex Taq, T4 DNA ligase, and Random Primer DNA Labeling Kit were purchased from Takara Shuzo (Kyoto, Japan). DNA Sequencing Kit (ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit) was obtained from Perkin Elmer (California, USA). Nylon membrane (GeneScreen *Plus*) was supplied by Dupont Co. (Delaware, USA). X-ray film (Fuji AIF new RX) was obtained from Fuji Film Co. (Tokyo, Japan). DEAE-cellulose was supplied by Serva (Heidelberg, Germany). Other chemicals were of analytical grade.

2.2. Bacterial strains and media

B. sphaericus IFO 3525 was used as a source of chromosomal DNA. *E. coli* JM 109 was used as a host strain for gene cloning. *B. sphaericus* was cultured at 30°C for 20 h in Luria broth (LB)(1% peptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.2). Transformants were grown in LB containing ampicillin (50 μg/ml), with or without IPTG (120 μg/ml).

2.3. Enzyme and protein assay

The standard reaction mixture for deamination consisted of 10 μmol of *meso*-2, 6-diaminopimelate, 1 μmol of $NADP^+$, 200 μmol of glycine-KCl–KOH buffer (pH 10.5), and enzyme in a final volume of 1.0 ml. The substrate was replaced by water in a blank. Incubation was done in a cuvette with a 1 cm light path. The reaction was started by addition of $NADP^+$ and monitored by measuring the initial changes in the absorbance at 340 nm with a Shimadzu UV-140–02 double beam spectrophotometer. One unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol of NADPH per min in the deamination of *meso*-2,6-diaminopimelate. Specific activity was expressed as units per mg of protein. Protein concentration was derived from the absorbance at 280 nm ($A^{1\%}_{1\,cm}$ at 280 = 7.07 [5]).

2.4. Isolation of peptides obtained by lysyl endopeptidase digestion of the enzyme

The enzyme was purified from cell extracts of *B. sphaericus* IFO 3525 according to the procedure described previously [8]. The purified enzyme (1 nmol) was dialyzed against water and lyophilized. The protein was dissolved in 20 μl of 8 M urea and incubated at 37°C for 1 h. To the solution, 60 μl of digestion buffer (Wako Pure Chemical Industries, Osaka, Japan) and 5 pmol of lysyl endopeptidase were added, and the mixture was incubated at 37°C for 12 h. The peptides were separated on a Shimadzu HPLC system with a YMC-Packed C4 column (YMC, Kyoto, Japan) using a solvent system of 0.1% trifluoroacetic acid and acetonitrile containing 0.07% trifluoroacetic acid. A 90 min linear gradient from 5 to 50% acetonitrile was used to elute peptides at a flow rate of 1.0 ml/min. The absorbance at 210 nm of the effluent was continuously monitored. The isolated peptides were lyophilized. The amino acid sequence of the purified enzyme and the sequences of the isolated peptides were analyzed with an Applied Biosystems model 492 protein sequencer linked to a phenylthiohydantoin derivative analyzer.

2.5. Preparation of oligonucleotide probe and hybridization

Chromosomal DNA was prepared by the method of Saito and Miura [16]. The sense (N) and antisense (C3) primers for PCR were designed from the N-terminal amino acid sequence and the amino acid sequence of peptide P-3 obtained from a lysyl endopeptidase digest of the enzyme, respectively. Sequences of the primers were 5′-GGGAATTCGTTGGTATTGTNGGNTAC(T)GGNAAC(T)-3′ (primer N) and 5′-GGGGATCCGGCATTCCTGTA(G)TGA(G)TTNGCA(G)TTA(G)AA-3′ (primer C3). The reaction mixture for PCR (50 μl) consisted of 100 pmol of each of the primers, 0.5 μmol of Tris–HCl buffer (pH 8.3), 2.5 μmol of KCl, 0.15 μmol of $MgCl_2$, 10 nmol of each dNTP, 2.5 units of DNA polymerase (TaKaRa Ex Taq), and 20 ng of the chromosomal DNA as a template. After 7 min incubation at 94°C, the reaction mixture was heated at 94°C for 1 min (for denaturation), then cooled at 55°C for 2 min (for annealing), and incubated at 72°C for 3 min (for extension). The programmed temperature shift was repeated 30 times. The amplified DNA fragment (about 0.7 kb) was digested with both *Eco*RI and *Bam*HI, and then ligated into the *Eco*RI–*Bam*HI site of pUC18. The plasmid was introduced into *E. coli* JM109 competent cells. The nucleotide sequence of the inserted DNA fragment (740 b) was determined with an Applied Biosystems 373 A DNA sequencer and a DNA Sequencing kit. A radioactive 740 b DNA probe was prepared by PCR as described above using a Random Primer DNA labeling kit and [α-^{32}P]-dCTP. The chromosomal DNA (1 μg) was digested with various restriction enzymes at 37°C overnight. The resulting DNA fragments were electrophoretically separated in a 0.7% agarose gel and blotted onto a nylon membrane. The immobilized DNA fragments were then subjected to Southern hybridization with the radioactive probe.

2.6. Cloning of the meso-diaminopimelate dehydrogenase gene

Chromosomal DNA from *B. sphaericus* was digested with both *Pst*I and *Hin*dIII at 37°C overnight and electrophoresed in a 0.7% agarose gel. Fragments of about 6 kb, which were hybridized with the radioactive probe, were collected from the gel by centrifugation using a SUPREC^{TM}-01 tube (Takara Shuzo, Kyoto, Japan) and then ligated into the *Pst*I–*Hin*dIII site of pUC18 with T4 DNA ligase. The resulting plasmid was then introduced into *E. coli* JM109 competent cells. Transformants were selected on LB agar plates containing ampicillin (50 μg/ml), IPTG (120 μg/ml), and X-Gal (100 μg/ml). A positive clone was selected from the transformants by colony PCR with single primers (primer N1, 5′-CCAGATATGGAATTAGTAGCGG-3′ and primer C1, 5′-ACTCATCGAAATAGTTCGGC-3′) [17]. We named the plasmid isolated from the positive clone pBSDAP1. The sequence of the enzyme gene in the plasmid was analyzed in both directions. The resulting nucleotide sequence data will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB030649.

To obtain a high expression strain, pBSDAP1 was digested with both *Hpa*I and *Ssp*I, and DNA fragments about 1.1 kb in size were subcloned into the *Sma*I site of pUC18. We named the constructed plasmid pBSDAP2.

2.7. Purification of the enzyme from the E. coli clone

All procedures were performed at 0–5°C, and potassium phosphate buffer containing 0.01% 2-mercaptoethanol was used, unless otherwise stated.

E. coli cells carrying pBSDAP2 were cultured aerobically at 37°C for 20 h in LB containing ampicillin (50 μg/ml) and IPTG (120 μg/ml). The cells (wet weight 3.5 g) were suspended in 30 ml of 10 mM buffer (pH 7.4) and disrupted by sonication at 4°C for 10 min. The supernatant obtained after centrifugation was dialyzed overnight against the same buffer at 4°C and used as the crude extract. To the crude extract, solid ammonium sulfate was added to 50% saturation with stirring. After 1 h, the precipitate was removed by centrifugation. The supernatant was brought to 80% saturation with solid ammonium sulfate and left for 1 h. The precipitate was collected by centrifugation and dissolved in 10 mM buffer (pH 7.4) and dialyzed overnight against the same buffer. The enzyme solution was loaded on a DEAE-cellulose column (2 cm × 10 cm) equilibrated with 10 mM buffer (pH 7.4). After the column had been washed with the same buffer and with the buffer containing 0.15 M KCl, the enzyme was eluted with the buffer containing 0.3 M KCl. The

active fractions were collected, concentrated with an Amicon ultrafiltration unit with a PM-10 membrane, and brought to 60% saturation with solid ammonium sulfate. After 1 h, precipitate was removed by centrifugation. To the supernatant, solid ammonium sulfate was added to 80% saturation with stirring. The precipitate was collected by centrifugation and dissolved in 10 mM buffer (pH 7.4). The enzyme solution was dialyzed overnight against the same buffer and stored at −20°C in the presence of 50% glycerol until use.

2.8. Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 10% polyacrylamide by the method of Laemmli [18] with the following standard proteins: bovine serum albumin (68 kDa), yeast alcohol dehydrogenase (37 kDa), and myoglobin (17.2 kDa).

2.9. Tertiary structure of the enzyme

The tertiary structure of the enzyme was predicted by computer modeling with the ProMod II program (SWISS MODEL, http://www. expancy.ch/swissmod/SWISS-MODEL.html) based on the X-ray diffraction pattern of the *C. glutamicum* enzyme [14].

3. Results

3.1. Cloning and sequencing of the B. sphaericus meso-diaminopimelate dehydrogenase gene

The enzyme gene was isolated from a plasmid pool containing *Pst*I–*Hin*dIII fragments of the *B. sphaericus* chromosomal DNA. We selected a transformant with the enzyme gene by the PCR method [17] from about 3000 transformants as described in Section 2. The plasmid (pBSDAP1) isolated from the positive clone had an about 6 kb fragment of *B. sphaericus* chromosomal DNA in the *Pst*I–*Hin*dIII site of pUC18. The nucleotide sequence of the fragment showed an open reading frame consisting of 978 nucleotides, starting with an ATG initial codon (Fig. 1). The putative Shine-Dalgarno sequence was located 5-bases upstream from the initiation codon. The sequence of the putative promoter region was ATTGTG and TATTAT separated by 18 bases. The first 20 amino acids (excepting the initial methionine) of the predicted amino acid sequence were identical with the N-terminal amino acid sequence of the *B. sphaericus* enzyme. The amino acid sequences of six internal peptides isolated from the lysyl endopeptidase digest of the enzyme were in good agreement with the predicted amino acid sequence (Fig. 1). The calculated molecular mass of this protein was 35,575 Da, which agreed with the apparent subunit molecular mass of 39,000 Da of the enzyme purified from *B. sphaericus*.

For the expression of the *meso*-diaminopimelate dehydrogenase gene in *E. coli*, pBSDAP2, containing the 1.1 kb *Hpa*I–*Ssp*I fragment of pBSDAP1, was constructed. The specific activity of a cell extract of *E. coli* cells harboring pBSDAP2 was 11.3 units/mg when the cells were cultured in medium without IPTG. The addition of IPTG (120 μg/ml) to the medium increased the specific activity to 20.3 units/mg. This value is 135 times higher than that of *B. sphaericus* IFO 3525 (0.15 unit/mg).

3.2. Purification of meso-diaminopimelate dehydrogenase from E. coli JM109/pBSDAP2

meso-Diaminopimelate dehydrogenase was purified to homogeneity in three steps, with a final yield of 76%. A summary of the purification is given in Table 1. The specific activity of the purified enzyme was 138 units/mg. This value is similar to that of the enzyme purified from *B. sphaericus* [4]. The apparent subunit molecular mass (39 kDa), which was estimated by SDS-PAGE, was identical with that of the enzyme purified from *B. sphaericus* (Fig. 2). The sequence of the first twenty N-terminal amino acids of the enzyme, SAIRVGIVGYGNLGRGVEFA, coincided with that of the *B. sphaericus* enzyme. The initiator methionine, which is not observed in the N-terminal sequences of either of the enzymes, may have been removed by a post-translational modification in vivo. The presence of serine as the second amino acid is predicted to enhance the cleavage of the preceding initiator methionine [19].

3.3. Sequence similarity of the enzyme

The amino acid sequence deduced from the nucleotide sequence of the enzyme gene was compared

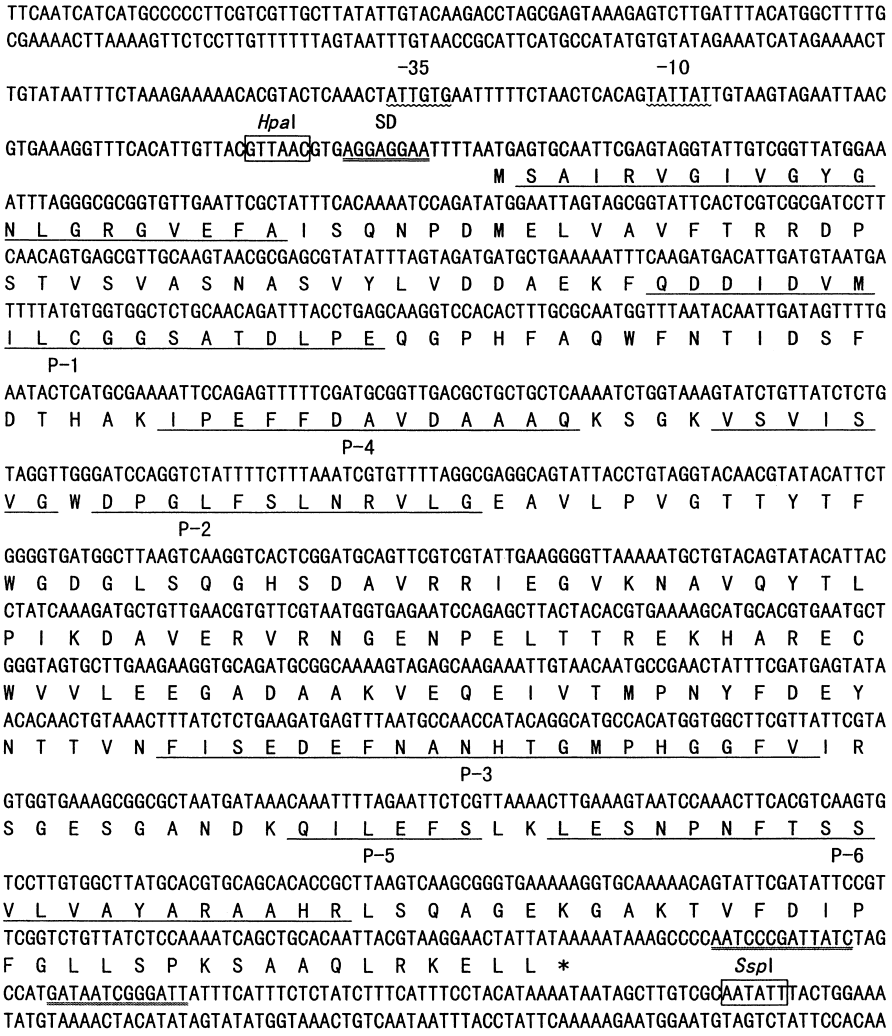

Fig. 1. Complete nucleotide sequence of the *meso*-diaminopimelate dehydrogenase gene and the deduced amino acid sequence of the enzyme. The amino acid sequences that were found by Edman degradation of the *B. sphaericus* enzyme and the peptides isolated from the lysyl endopeptidase digest of the *B. sphaericus* enzyme are shown by a single underline. The putative promoter sequences were shown by waved underlines. The putative Shine-Dalgarno (SD) sequence and the location of a terminator-like structure are indicated with double underlines.

Table 1
Purification of *meso*-diaminopimelate dehydrogenase from *E. coli* cells harboring pBSDAP2

Steps	Total portein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	1040	13500	13	100
Ammonium sulfate (50–80% saturation)	387	12500	32.3	93
DEAE-cellulose	143	11900	83.2	88
Ammonium sulfate (60–80% saturation)	76	10300	135	76

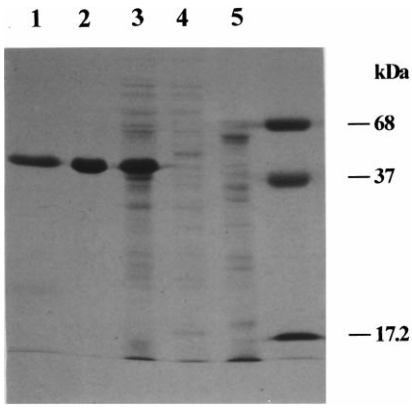

Fig. 2. SDS-PAGE of *meso*-diaminopimelate dehydrogenase purified from *E. coli* JM109/pBSDAP2. The purified enzyme (5 μg of protein) and cell extracts (10 μg of protein) were subjected to SDS-PAGE (10% polyacrylamide) with standard protein markers: serum albumin (68 kDa), yeast alcohol dehydrogenase (37 kDa), and myoglobin (17.2 kDa). Lane 1, the enzyme purified from *B. sphaericus*; lane 2, the enzyme purified from *E. coli* JM109/pBSDAP2; lane 3, cell extract of *E. coli* JM109/pBSDAP2; lane 4, cell extract of *E. coli* JM109; and lane 5, cell extract of *B. sphaericus*.

with those of *meso*-diaminopimelate dehydrogenases from other sources (Fig. 3). The sequences of the *meso*-diaminopimelate dehydrogenases from *B. sphaericus*, *C. glutamicum*, and *B. flavum* showed a high similarity to each other: the overall sequence identity was 50% between the enzymes from *B. sphaericus* and *C. glutamicum* or *B. flavum*, and 99% between those from *C. glutamicum* and *B. flavum*. The regions showing high similarity are distributed throughout the polypeptide chain.

4. Discussion

We succeeded in cloning and expression of the *meso*-diaminopimelate dehydrogenase gene of *B. sphaericus* in *E. coli* cells and analyzed its complete nucleotide sequence. Up to date, the nucleotide sequences of *meso*-diaminopimelate dehydrogenase genes from two bacteria, *C. glutamicum* and *B. flavum*, have been analyzed and their amino acid sequences deduced from their nucleotide sequences (Fig. 3).

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BS  MSAIRVGIVGYGNLGRGVEFAISQNPDMELVAVFTRRDPSTVSVASNASVYLVDDAEKFQ
CG  MTNIRVAIVGYGNLGRSVEKLIAKQPDMDLVGIFSRR----ATLDTKTPVFDVADVDKHA
BF  MTNIRVAIVGYGNLGRSVEKLIAKQPDMDLVGIFSRR----ATLDTKTPVFDVADVDKHA

BS  DDIDVMILCGGSATDLPEQGPHFAQWFNTIDSFDTHAKIPEFFDAVDAAAQKSGKVSVIS
CG  DDVDVLFLCMGSATDIPEQAPKFAQFACTVDTYDNHRDIPRHRQVMNEAATAAGNVALVS
BF  DDVDVLFLCMGSATDIPEQAPKFAEFACTVDTYDNHRDIPRHRQVMNEAATAAGNVALVS

BS  VGWDPGLFSLNRVLGEAVLPVGTTYTFWGDGLSQGHSDAVRRIEGVKNAVQYTLPIKDAV
CG  TGWDPGMFSINRVYAAAVLAEHQQHTFWGPGLSQGHSDALRRIPGVQKAVQYTLPSEDAL
BF  TGWDPGMFSINRVYAAAVLAEHQQHTFWGPGLSQGHSDALRRIPGVQKAVQYTLPSEDAL

BS  ERVRNGENPELITTREKHARECWVVLEEGADAAKVEQEIVTMPNYFDEYNTTVNFISEDEF
CG  EKARRGEAGDLTGKQTHKRQCFVV-ADAADHERIENDIRTMPDYFVGYEVEVNFIDEATF
BF  EKARRGEAGDLTGKQTHKRQCFVV-ADAADHERIENDIRTMPDYFVGYEVEVNFIDEATF

BS  NANHTGMPHGGFVIRSGESGANDKQILEFSLKLESNPNFTSSVLVAYARAAHRLSQAGEK
CG  DSEHTGMPHGGHVITTGDTGGFN-HTVEYILKLDRNPDFTASSQIAFGRAAHRMKQQGQS
BF  DAEHTGMPHGGHVITTGDTGGFN-HTVEYILKLDRNPDFTASAQIAFGRAAHRMKQQGQS

BS  GAKTVFDIPFGLLSPKSAAQLRKELL
CG  GAFTVLEVAPYLLSPENLDDLIARDV
BF  GAFTVLEVAPYLLSPENLDDLIARDV
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Fig. 3. Alignment of the amino acid sequences of *meso*-diaminopimelate dehydrogenases. Amino acid sequence of the *B. sphaericus* enzyme (BS) was compared with those of the *C. glutamicum* enzyme (CG), and the *B. flavum* enzyme (BF). Common residues in these dehydrogenases are shown by white letters in black boxes.

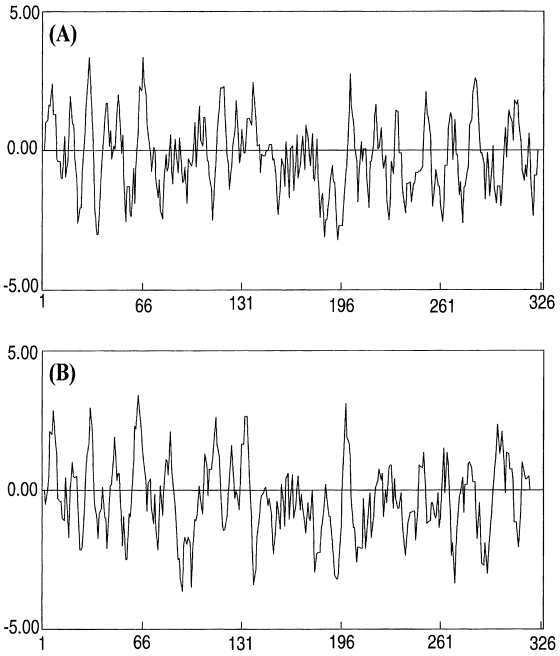

Fig. 4. Comparison of hydrophobicity profiles between the *B. sphaericus* enzyme (A) and the *C. glutamicum* enzyme (B). The averages of the Kyte and Doolittle value [29] of every five adjacent residues are plotted. Hydrophobic regions deflect upward (positive values). Numbers on the bottom indicate amino acid positions.

The amino acid sequences from these two bacteria are almost the same (99% identity). The *B. sphaericus* enzyme shows 50% sequence identity with the *C. glutamicum* enzyme. The hydrophobicity profiles of the *B. sphaericus* enzyme and the *C. glutamicum* enzyme have similar patterns on the whole, but obvious differences are found in the several regions (Fig. 4).

All $NAD(P)^+$-dependent dehydrogenases for which a tertiary structure has been determined possess a βαβ-fold that binds with the adenine nucleotide moiety of $NAD(P)^+$ [20,21]. The GXGXXG(A) sequence is conserved in this fold. Amino acid dehydrogenases such as glutamate dehydrogenase [22–24], leucine dehydrogenase [25], phenylalanine dehydrogenase [26], and alanine dehydrogenase [27] have this sequence in the middle region of their primary structure. The sequence, however, was seen in the N-terminal region of *meso*-diaminopimelate dehydrogenase, suggesting that the $NADP^+$ binding site is located in the N-terminal region. This has been confirmed by X-ray crystallography of the *C. glutamicum* enzyme [14].

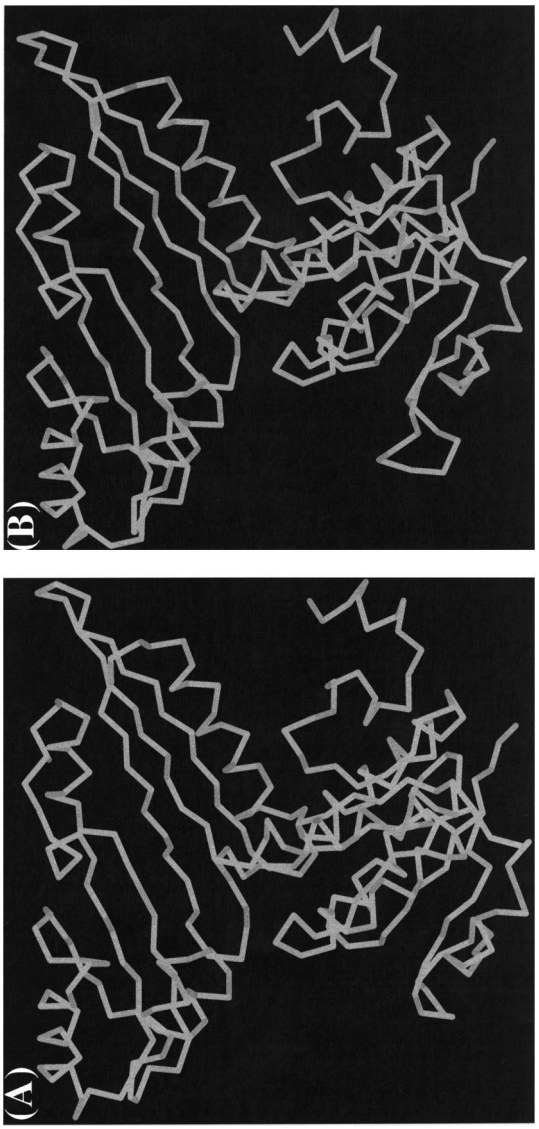

Fig. 5. Comparison of tertiary structures between the *B. sphaericus* enzyme (B) and the *C. glutamicum* enzyme (A). The tertiary structure of the *B. sphaericus* enzyme was predicted by computer modeling.

In this respect, *meso*-diaminopimelate dehydrogenase is unique among the $NAD(P)^+$-dependent amino acid dehydrogenases.

The tertiary structure of the *B. sphaericus* enzyme, as predicted from the primary structure using computer modeling, was quite similar to that of the *C. glutamicum* enzyme (Fig. 5). The structure of

meso-diaminopimelate dehydrogenase is also similar to the structures of glutamate dehydrogenase [22] and leucine dehydrogenase [25], which are B-type dehydrogenases transferring the *pro-S* hydrogen. Scapin et al. [14] reported that the *C. glutamicum meso*-diaminopimelate dehydrogenase was an A-type dehydrogenase, which transferred the *pro-R* hydrogen, on the basis of the conformation of the bound $NADP^+$ in the enzyme–$NADP^+$ complex, though the *B. sphaericus* enzyme is a B-type dehydrogenase [5]. The nicotinamide ring adopts the *anti*-conformation in the NAD^+–phenylpyruvate–enzyme complex of the *Rhodococcus* phenylalanine dehydrogenase, which is a B-type dehydrogenase [26]. However, in the NAD^+–β-phenylpropionate–enzyme ternary complex, the nicotinamide ring is rotated by approximately 180° about the glycoside bond to yield the *syn*-conformation for B-type dehydrogenases. The position of the reactive group (the *pro-S* or *pro-R* hydrogen) of nicotinamide is dependent upon the ligand occupying the substrate-binding site [28]. Thus, the conformation of the bound $NADP^+$ in the enzyme–$NADP^+$ complex does not indicate that of the nicotinamide ring during catalysis. Structural study of the $NADP^+$–substrate–enzyme ternary complex is necessary if we are to understand the stereospecificity of the hydrogen transfer of *meso*-diaminopimelate dehydrogenase.

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