Gene Cloning and Sequence Determination of Leucine Dehydrogenase from *Bacillus stearothermophilus* and Structural Comparison with Other NAD(P)$^+$-Dependent Dehydrogenases†

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ABSTRACT: The gene for leucine dehydrogenase (EC 1.4.1.9) from *Bacillus stearothermophilus* was cloned and expressed in *Escherichia coli*. The selection for the cloned gene was based upon activity staining of the replica printed *E. coli* cells. A transformant showing high leucine dehydrogenase activity was found to carry an about 9 kilobase pair plasmid, which contained 4.6 kilobase pairs of *B. stearothermophilus* DNA. The nucleotide sequence including the 1287 base pair coding region of the leucine dehydrogenase gene was determined by the dideoxy chain termination method. The translated amino acid sequence was confirmed by automated Edman degradation of several peptide fragments produced from the purified enzyme by trypsin digestion. The polypeptide contained 429 amino acid residues corresponding to the subunit (M_r 49 000) of the hexameric enzyme. Comparison of the amino acid sequence of leucine dehydrogenase with those of other pyridine nucleotide dependent oxidoreductases registered in a protein data bank revealed significant sequence similarity, particularly between leucine and glutamate dehydrogenases, in the regions containing the coenzyme binding domain and certain specific residues with catalytic importance.

Leucine dehydrogenase (EC 1.4.1.9) is an NAD$^+$-dependent oxidoreductase which catalyzes the reversible deamination of L-leucine and several other aliphatic L-amino acids to their keto analogues. It occurs ubiquitously in *Bacillus* species (Ohshima et al., 1978a) and functions catabolically in the bacterial metabolism of branched-chain L-amino acids (Zink & Sanwal, 1962). Hermier et al. (1970a,b) suggested that the enzyme of *B. subtilis* plays an important role in spore germination in cooperation with alanine dehydrogenase (EC 1.4.1.1). We have purified the enzyme to homogeneity from *Bacillus sphaericus* (Soda et al., 1971) and studied its physicochemical and enzymological properties (Ohshima et al., 1978a,b; Ohshima & Soda, 1984). The enzyme has a regular hexagonal structure of identical subunits as revealed by small-angle X-ray scattering (Hiragi et al., 1982). Recently, we also found the occurrence of the enzyme in cells of a thermophilic bacterium, *Bacillus stearothermophilus*, and purified it to show its enzymological properties (Ohshima et al., 1985). The enzyme is more resistant to both thermal and chemical denaturation than that from the mesophile *B. sphaericus*.

Although a large number of NAD(P)$^+$-dependent dehydrogenases have been sequenced to date and three-dimensional structures of several enzymes have been elucidated in atomic detail, very little structural information is available for those acting on amino acids. Glutamate dehydrogenases (EC 1.4.1.2–4) from various sources are the only enzymes for which mechanistic features of amino group dehydrogenation have been correlated with structure (Smith et al., 1975). Because sequence comparison between glutamate and other amino acid dehydrogenases is expected to be useful for identification of functional residues involved in catalysis and binding of substrates or the cofactor, we have begun work to clone and sequence the leucine dehydrogenase gene. Since thermostable enzymes are generally advantageous for structural studies, we used *B. stearothermophilus* as the donor of the gene for leucine dehydrogenase.

In this paper we report the cloning and efficient expression of the leucine dehydrogenase gene in *Escherichia coli*, a simple and effective purification method of the enzyme based on its thermostability, and its complete amino acid sequence. We present herein evidence that reveals the significant structural correlation between glutamate and leucine dehydrogenases.

Experimental Procedures

Strains and Media. *B. stearothermophilus* IFO 12550 and *E. coli* C600 $r_K^-m_K^-$ *thi thr leu* were used as a donor strain of the gene and the host strain for plasmid construction, respectively. Transformed *E. coli* cells were grown on L broth (1% polypeptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose; pH 7.2) supplemented with 2% agar and appropriate antibiotics. Antibiotic concentrations used for the selection of transformants were 25 μg/mL ampicillin and 15 μg/mL tetracycline. For enzyme production, bacteria were grown at 37 °C in a medium (pH 7.2) containing 1.5% polypeptone, 0.1% glycerol, 0.01% yeast extract, 0.5% NaCl, 0.01% meat extract, 0.1% KH_2PO_4, 0.2% K_2HPO_4, and $MgSO_4 \cdot 7H_2O$.

DNA Cloning, Mapping, and Sequencing. The chromosomal DNA was isolated from *B. stearothermophilus* according

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to the published procedures (Saito & Miura, 1963). Plasmids were isolated by a modification of the method of Oka (1978); after removal of RNA by gel filtration on a Bio-Gel A-5m (Bio-Rad Laboratories) column (1.0 × 40 cm), covalently closed circular plasmids were purified by cesium chloride–ethidium bromide density-gradient centrifugation. A rapid small-scale procedure was used to screen transformed strains for plasmids (Kado & Liu, 1981). *Ban*I restriction endonuclease was obtained from Toyobo (Osaka, Japan), and other restriction enzymes were from Takara Shuzo (Kyoto, Japan). Products of restriction endonuclease digestion were separated by electrophoresis on a 0.7% agarose slab gel in 89 mM Tris–borate buffer (pH 8.3) containing 2.5 mM ethylenediaminetetraacetate (EDTA) and 0.5 μg/mL ethidium bromide.

Chromosomal DNA (12 μg) from *B. stearothermophilus* was partially digested with *Sal*I, and the resultant fragments were ligated into the *Sal*I site of pBR322 (6 μg) with T4 DNA ligase (Takara Shuzo). The ligated mixture was used directly for transformation (Mandel & Higa, 1970). To identify the clones producing leucine dehydrogenase, the replica printing method developed by Raetz (1975) was modified as follows: colonies of transformants grown on the L broth plates at 37 °C for 16 h were transferred onto a Toyo No. 5C filter paper disc (8-cm diameter). The filter paper was treated with lysozyme and EDTA as indicated (Raetz, 1975) and then transferred to a Petri dish containing 1.5 mL of a solution composed of 20 mM NaN_3, 10 mM KF, and 1 mM Na_2HAsO_4 in 4 mM Tris-HCl buffer (pH 8.0). After incubation at room temperature for 20 min, the filter paper was rapidly frozen in liqud N_2 followed by thawing in a water bath. This treatment was repeated twice. The filter paper was dried at 70 °C for 10 min and soaked in 1.5 mL of a reaction mixture for leucine dehydrogenase assay at 55 °C (50 mM glycine–KOH buffer, pH 10.5, 50 mM L-leucine, 0.0625 mM NAD^+, 0.064 mM phenazine methosulfate, and 0.24 mM nitroblue tetrazolium). The colonies producing leucine dehydrdogenase appeared as blue spots on the replica disc.

DNA sequence was determined by the dideoxy chain termination method (Messing, 1983) with the M13 *E. coli* host strain JM 109, the sequencing vectors M13 mp18 and mp19, and a M13 sequencing kit purchased from Takara Shuzo. 2′-Deoxy-7-deazaguanosine triphosphate (Takara Shuzo) was substituted for dGTP to reduce compression artifacts (Mizusawa et al., 1986). Several regions of the sequence were determined also by selective priming with the oligonucleotides synthesized on an Applied Biosystems DNA synthesizer, Model 381A.

Enzyme and Protein Assays. Leucine dehydrogenase was assayed essentially according to the method of Ohshima et al. (1978a). The reaction mixture (0.8 mL) contained 100 μmol of L-leucine, 2.5 μmol of NAD^+, 120 μmol of glycine–KCl–KOH buffer (pH 10.7), and enzyme. In a blank, L-leucine was replaced by water. Incubation was carried out at 55 °C in a cuvette of 1-cm light path. The reaction was started by addition of enzyme and was followed by measurement of the initial change for a few minutes in the absorbance at 340 nm with a Union Giken SM-401 spectrophotometer (Hirakata, Japan). One unit of the enzyme is defined as the amount of enzyme that catalyzes the appearance of 1 μmol of NADH, whose molar absorption coefficient is 6220 M^{-1} cm^{-1}. Specific activity is expressed as units per milligram of protein. Protein was determined by the method of Lowry et al. (1951) with crystalline bovine serum albumin as a standard.

Enzyme Purification from E. coli Clone Cells. All operations were performed at 0–5 °C, and 10 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol was used throughout the purification.

Step 1. Cells of *E. coli* C600 carrying pICD1 (see below) were cultured in 20 L of media for 12 h, harvested by centrifugation, and washed twice with 0.85% NaCl. The washed cells (100 g wet wt) were suspended in 200 mL of 10 mM buffer and subjected to sonication at 0 °C for 10 min with a 19-kHz Kaijo Denki oscillator (Tokyo, Japan). After centrifugation at 40000*g* for 30 min, the supernatant solution (230 mL) was dialyzed against 500 volumes of the buffer for 13 h. The precipitate formed during dialysis was removed by centrifugation.

Step 2. The pH of the supernatant solution (250 mL) was adjusted to 5.4 with 1 M acetate buffer (pH 5.0). After incubation at 70 °C for 30 min and then cooling in an ice–water bath, the solution was returned to pH 7.2 with 1 M NaOH and centrifuged.

Step 3. The supernatant solution was applied to a DEAE-Toyopearl 650M (Toyo Soda, Tokyo, Japan) column (2.7 × 80 cm) equilibrated with 10 mM buffer. After the column was washed with 2 L of the buffer containing 0.16 M KCl, the enzyme was eluted with the buffer supplemented with 0.18 M KCl at a flow rate of 100 mL/h. The active fractions were pooled (1 L) and concentrated to about 50 mL by ultrafiltration through a Diaflow membrane (Bio Engineering, Tokyo, Japan).

Step 4. The enzyme solution was applied in five portions to a column (2.5 × 106 cm) of Sephadex G-200 (Pharmacia) equilibrated with the buffer containing 0.18 M KCl and eluted at a flow rate of 12 mL/h with the same buffer. The active fractions were pooled and concentrated by ultrafiltration to about 10 mL.

Amino Acid Analysis. Amino acid analysis was performed according to the method of Spackman et al. (1958) with a Hitachi Model 835 amino acid analyzer. The enzyme was dialyzed against 5 mM potassium phosphate buffer (pH 7.2) before lyophilization. The enzyme (500 μg) was hydrolyzed at 110 °C with 6 N HCl in evacuated and sealed tubes for 24, 48, and 72 h. The hydrolysates were evaporated to dryness under reduced pressure and subjected to amino acid analysis in duplicate. Half-cystine was determined as cysteic acid after performic acid oxidation and hydrolysis. Tryptophan and tyrosine were determined spectrophotometrically by the method of Edelhoch (1967).

N- and C-Terminal Sequence Analyses. The purified enzyme (about 2 nmol in 1 mM potassium phosphate buffer, pH 7.2) was used directly for the N-terminal sequence analysis by automated Edman degradation with an Applied Biosystems 470A gas–liquid-phase protein sequencer. The phenylthiohydantoin (PTH) amino acids were separated and identified by an on-line PTH analyzer 120A (Applied Biosystems) with a PTH-C_{18} column.

The C-terminal amino acid residues were analyzed by carboxypeptidase Y digestion (Klemm, 1984). The enzyme (1.2 mg/0.1 mL; 24.5 nmol of subunits) was denatured with 0.1% sodium lauryl sulfate and digested at 37 °C with 0.8 nmol of carboxypeptidase Y (Oriental Yeast) in 1 mL of 0.2 M pyridine–acetate buffer (pH 6.7). At various intervals, 0.1-mL samples were withdrawn and mixed with 0.05 mL of 5.5 N HCl. The amino acids released in the supernatant were measured by automated amino acid analysis.

Trypsin Digestion, Isolation of Peptide Fragments, and Peptide Sequencing. After precipitation of the purified leucine dehydrogenase (3 mg) with 5% trichloroacetic acid and washing with water, the precipitated protein was digested at

Table I: Purification of Leucine Dehydrogenase from *E. coli* C600 Carrying pICD1

step	vol (mL)	total protein (mg)	total act. (units)	sp act. (units/mg)	yield (%)
crude extract	230	9000	27 000	3.0	100
heat treatment	250	1450	26 200	18.0	97
DEAE-Toyopearl 650M	50	244	23 500	96.0	87
Sephadex G-200	10	133	14 900	112.0	70

37 °C with 0.03 mg (1:100 w/w) of bovine pancreas trypsin (TPCK treated, Cooper) for 12 h in 0.1 M ammonium bicarbonate buffer (pH 8.5). The clear solution obtained was acidified with 1% trichloroacetic acid to stop the digestion and dried by centrifugal evaporation. The tryptic peptides were separated with an ULTRON N-C_{18} reversed-phase column (4.6 × 150 mm, Shinwa Kako, Kyoto, Japan) in an LKB Ultrochrome GTi high-performance liquid chromatography (HPLC) system (Bromma, Sweden). A 40-min linear gradient from 0 to 56% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid was used to elute peptides at a flow rate of 0.6 mL/min. Peptides which were separated well from other small peptides were further purified by HPLC under similar chromatographic conditions as above except that 0.1% (v/v) heptafluorobutyric acid was used instead of 0.1% trifluoroacetic acid and a 30-min linear gradient from 16 to 48% acetonitrile was employed. The tryptic peptides isolated (0.6–1.5 nmol) were sequenced by automated Edman degradation.

Computer Search for Sequence Similarities. A search of the National Biomedical Research Foundation (NBRF) protein sequence data bank (George et al., 1986) for sequence similarities was carried out on a FACOM M-380Q computer with a SEQHP algorithm (local homology alignment; Goad & Kanehisa, 1982) of the Integrated Database and Extended Analysis System (IDEAS) for nucleic acids and proteins.

Results and Discussion

Cloning of B. stearothermophilus Leucine Dehydrogenase Gene. The *B. stearothermophilus* leucine dehydrogenase gene was isolated from a plasmid pool containing *Sal*I fragments of *B. stearothermophilus* chromosomal DNA, which had been ligated into the unique *Sal*I site in the tetracycline-resistance gene of pBR322. We selected the gene with the *E. coli* C600 strain as a host using an activity staining procedure on the replica plate. Recombinant *E. coli* cells producing leucine dehydrogenase were expected to show the blue color of the reduced nitroblue tetrazolium; *E. coli* has no leucine dehydrogenase activity. In order to avoid nonspecific color development caused by respiratory chain enzymes from the host strain, the procedure involved the treatment with respiratory chain inhibitors such as NaN_3 and KF. Of approximately 2700 Amp^rTc^s transformants obtained, two colonies turned blue on the replica plate. The plasmids isolated from both of the putative leucine dehydrogenase expressing transformants were designated pICD1 (8.95 kilobase pairs, kbp) and pICD2 (6.6 kbp). The cell extracts from both the clones showed much higher leucine dehydrogenase activity (about 3.5 units/mg of protein) than those from *B. stearothermophilus* (0.07 unit/mg). Thus, the enzyme expressed in *E. coli*–pICD1 cells amounted to about 3% of the total soluble protein on the basis of the specific activity of the purified enzyme.

Digestion with *Sal*I restriction endonuclease yielded three fragments of 2.45, 1.4, and 0.85 kbp from the plasmid pICD1 and two fragments of 1.4 and 0.85 kbp from pICD2 in addition to the 4.36-kbp fragment of pBR322. Southern blot analysis indicated that these foreign DNA fragments were derived from the *B. stearothermophilus* chromosomal DNA. By evaluation of the electrophoretic patterns of both the plasmids after single and double digestion with restriction endonucleases, the plasmid pICD2 was confirmed to be a plasmid lacking the 2.45-kbp *Sal*I fragment of pICD1. Figure 1 shows the restriction map of pICD2 containing the leucine dehydrogenase gene. Subcloning study of pICD1 and pICD2 showed that the two small *Sal*I fragments (1.4 and 0.85 kbp) are necessary for the expression of leucine dehydrogenase (data not shown).

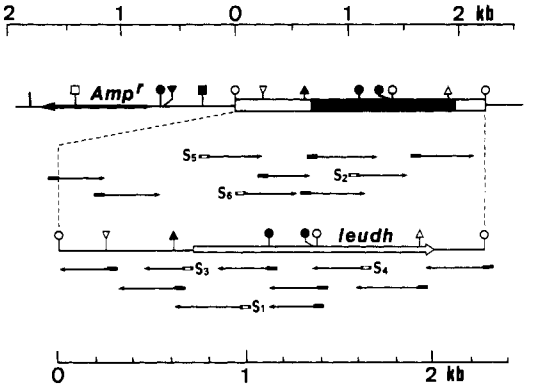

FIGURE 1: Cleavage map of pICD2 and DNA sequencing scheme for the 2.26-kbp *Sal*I fragment. The single line represents pBR322 DNA. The open bar represents the genomic insert, and the shaded region represents the coding region for the leucine dehydrogenase gene. Restriction endonucleases are *Sal*I (○), *Eco*RI (●), *Pst*I (□), *Bam*HI (■), *Hin*cII (△), *Ban*I (▲), *Xho*I (▽), and *Hin*dIII (▼). The restriction fragments were subcloned into M13 vectors by directional cloning, and the resulting single-stranded templates were sequenced by the dideoxy chain termination method with either the universal (solid bars) or site-specific sequencing primers (S_1 to S_6, open bars).

Table II: Amino Acid Composition of Leucine Dehydrogenase from *B. stearothermophilus*

amino acid	predicted	observed	amino acid	predicted	observed
Asn	19		Cys	4	4
Asx		50	Val	25	28
Asp	25		Met	13	14
Thr	24	18	Ile	35	33
Ser	16	12	Leu	24	24
Gln	13		Tyr	18	20
Glx		49	Phe	13	13
Glu	32		His	8	9
Pro	14	11	Lys	25	25
Gly	41	43	Arg	27	25
Ala	52	50	Trp	1	1

Purification of Leucine Dehydrogenase from E. coli Carrying pICD1. When the crude extract from *E. coli*–pICD1 cells was heated at 70 °C for 30 min under acidic conditions (pH 5.4), the specific activity increased markedly without significant loss of total enzyme activity (see Table I) due to the precipitation of most of the impure proteins derived from *E. coli*. The enzyme was further purified by two chromatographic procedures, and a summary of the purification is shown in Table I. The final preparation was found to be homogeneous by disc gel electrophoresis and had a specific activity of 112, which is comparable to that of the enzyme purified from *B. stearothermophilus* (Ohshima et al., 1985). The overall purification procedure of the enzyme is very efficient as compared with the previous purification from the original strain (Ohshima et al., 1985)

Amino Acid Composition. The amino acid composition of the enzyme is summarized in Table II. The predominant

FIGURE 2: DNA sequence of the gene and the deduced amino acid sequences for leucine dehydrogenase from *B. stearothermophilus*. Twenty peptides (12 N-terminal residues and 19 tryptic peptides, which are numbered as T-1 to T-19 in the order of elution on HPLC) for which sequences were determined from the purified gene product by automated Edman degradation are underlined. The C-terminal sequence identified by the carboxypeptidase Y digestion is also indicated.

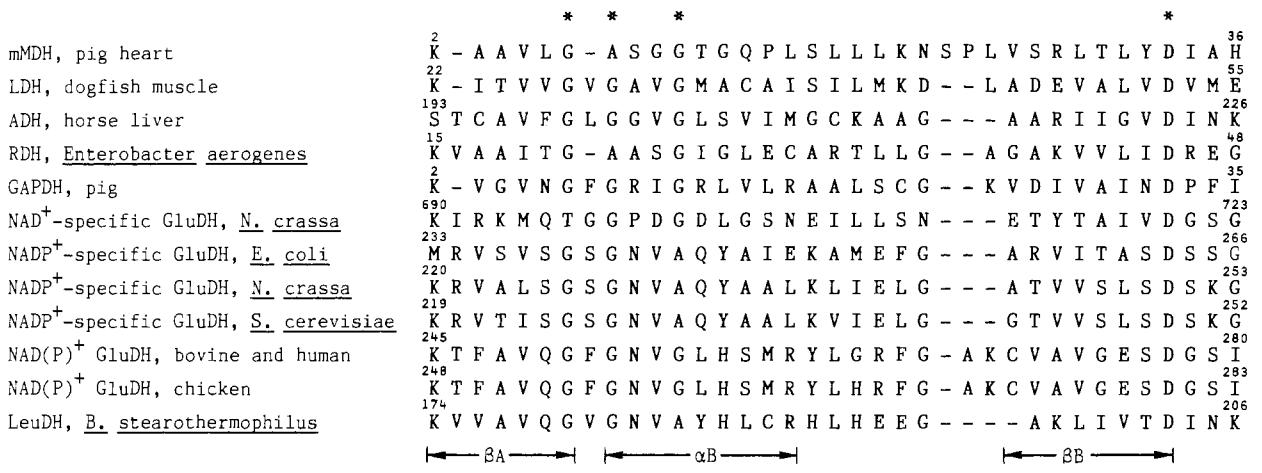

FIGURE 3: Sequence comparison of the βA–αB–βB regions of several pyridine nucleotide dependent dehydrogenases. Conserved residues which are thought to be important for coenzyme binding are indicated by asterisks. The sequence numbers of the first and last residues involved in this region are shown above each sequence. Abbreviations: mMDH, mitochondrial malate dehydrogenase; LDH, lactate dehydrogenase; ADH, alcohol dehydrogenase; RDH, ribitol dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GluDH, glutamate dehydrogenase; LeuDH, leucine dehydrogenase.

residues of the enzyme protein were glutamic acid, aspartic acid, glycine, and alanine, whereas only a few half-cystine and tryptophan residues were found in the enzyme. The analytic data are in good agreement with the amino acid composition predicted from the DNA sequence of the enzyme gene.

DNA Sequence of the Leucine Dehydrogenase Gene. DNA sequence of the 2.26-kbp fragment of pICD2 containing the entire leucine dehydrogenase gene was determined in both strands by sequencing the M13 clones generated from restriction fragments (Figure 1). The sequencing of extended regions was accomplished by priming of the single-stranded recombinants with six oligonucleotides newly synthesized on the basis of the farthest sequence determined. Artifacts in the reading of sequencing gels (e.g., G-compression) were successfully identified with 2′-deoxy-7-deazaguanosine triphosphate instead of deoxyguanosine triphosphate in the Sanger reaction. A single open reading frame of 1287 nucleotides starting with the codon ATG and terminating in the TAA nonsense codon at position 1288 was found (Figure 2). We identified 12 residues from the N-terminus of the enzyme by automated Edman degradation of the purified gene product: Met-Glu-Leu-Phe-Lys-Tyr-Met-Glu-Thr-Tyr-Asp-Tyr-. The N-terminal sequence coincided with that predicted from the DNA sequence. The C-terminal amino acid was determined by carboxypeptidase Y digestion. The first residue released by the digestion was isoleucine, which was calculated to be 0.87 mol/mol of subunit, followed by the release of alanine and serine, indicating the C-terminal sequence -Ser-Ala-Ile. Thus, the 1287-bp open reading frame was identified as the translational unit for leucine dehydrogenase. About 50% of the entire sequence was also confirmed by amino acid sequence analyses of 19 peptides obtained by the trypsin digestion and purification by HPLC. These peptides are shown at their positions in the amino acid sequence (Figure 2). The only inconsistency of amino acid residues between the sequence predicted from DNA and that determined by the peptide sequencing was found at position 356: Asn from DNA sequence; Asp from peptide sequence. This is probably due to a deamination which occured during the enzyme purification or the isolation of trypsin peptides. On the basis of the predicted amino acid sequence (429 residues), the molecular weight of the enzyme is calculated as 46 903, which corresponds with the value (49 000) determined by sodium lauryl sulfate gel electrophoresis of the protein (Ohshima et al., 1985).

Comparison of the Amino Acid Sequence with the NBRF Data Bank. A search of the National Biomedical Research Foundation (NBRF) protein sequence data bank (George et al., 1986) with the SEQHP algorithm of the Integrated Database and Extended Analysis System (IDEAS) for nucleic acids and proteins (Goad & Kanehisa, 1982) revealed significant sequence similarities between leucine dehydrogenase and other pyridine nucleotide dependent dehydrogenases. Although the overall similarities are 13–23% on the basis of common residues between the sequences which have been aligned by introducing gaps to maximize identities, the regions with high similarities are nonuniformly distributed through the polypeptide chain. For example, a partial sequence of about 30 residues (from Lys-174 to Asp-203) in leucine dehydrogenase was found to contain many residues identical with those in the nicotinamide coenzyme binding region of various dehydrogenases (Figure 3). The coenzyme binding domain of dehydrogenases has been shown to display a high degree of conservation of tertiary structure (Birktoft & Banaszak, 1984): it consists of a four-stranded parallel β-sheet and one α-helix with virtually identical arrangement. Some residue conservations have been noted in the sequence of this domain (Birktoft & Banaszak, 1984). One of them is a hexapeptide sequence, G-X-(G or A)-X-X-(G or A), which is highly conserved in the βA–αB region. A basic residue (usually Lys) at five to six residues before the hexapeptide and a hydrophobic residue (usually Val) at two residues before Gly at the top of the hexapeptide are also conserved in this region. Another residue that is functionally important in binding of the coenzyme is an Asp residue at the end of the βB strand, which is also found in the leucine dehydrogenase sequence at position 203. When a recent method for prediction of the occurrence of the ADP-binding $\beta\alpha\beta$-fold in proteins using an amino acid sequence fingerprint (Wierenga et al., 1986) was applied to the leucine dehydrogenase sequence, we obtained a score of 10 for the region 174–203 with a probe length of 30. These findings support the view that this region of leucine dehydrogenase participates in the binding of NAD^+ in a manner analogous to the other $NAD(P)^+$-dependent dehydrogenases.

Although the catalytic domains of dehydrogenases are generally diverse (Birktoft & Banaszak, 1984), one functionally important residue has been identified in the catalytic domains of glutamate dehydrogenases: a highly reactive Lys residue in the Gly-rich region (Piszkiewicz et al., 1970), which probably functions in catalysis by forming a Schiff base adduct

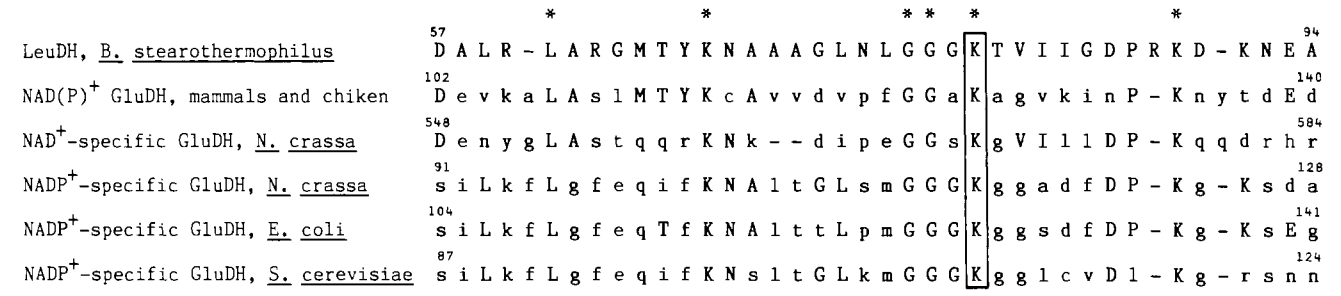

FIGURE 4: Comparison of an N-terminal partial sequence of leucine dehydrogenase with the catalytic regions of glutamate dehydrogenases. The sequences were aligned by introducing gaps (hyphens) to maximize identities. The sequence numbers of the first and last residues are shown above each sequence. The Lys residues in the Gly-rich regions are boxed, and conserved residues among all the sequences listed are shown by an asterisk. Residues in the glutamate dehydrogenase sequences, which are identical with those in the leucine dehydrogenase sequence, are shown by upper-case letters. GluDH, glutamate dehydrogenase; LeuDH, leucine dehydrogenase.

with α-ketoglutarate (Smith et al., 1975). This residue is conserved in all the glutamate dehydrogenase sequences (Moon & Smith, 1973; Moon et al., 1973; Blumenthal et al., 1975; Julliard & Smith, 1979; Haberland & Smith, 1980; McPherson & Wootton, 1983; Valle et al., 1984; Moye et al., 1985). In the N-terminal half of the leucine dehydrogenase sequence, we also found a Lys residue at position 80 in a conserved region with the glutamate dehydrogenase sequences (Figure 4). It is noteworthy that the G-G-(G or A or S)-K sequence in both the leucine and glutamate dehydrogenases is located about 100–120 residues apart from the N-terminal side of the coenzyme binding regions described above. Several other residues are also strongly conserved, suggesting that this region constitutes the catalytic domain of leucine dehydrogenase. Although the catalytic importance of the Lys-80 of leucine dehydrogenase has not been established, we have shown the presence of a reactive Lys residue in the *B. sphaericus* leucine dehydrogenase that can be labeled with pyridoxal 5′-phosphate at pH 8.0 and is protected from the labeling by NADH (Ohshima & Soda, 1984).

On the basis of the observed sequence similarities, it is tempting to propose that leucine and glutamate dehydrogenases have evolved from a common progenitor enzyme. While both enzymes are dehydrogenases acting on amino acid substrates, leucine dehydrogenase has been found only in the bacteria that show no glutamate dehydrogenase activity (Ohshima et al., 1978a; Smith et al., 1975). Both the polypeptide chain length and the hexameric subunit arrangement of leucine dehydrogenase are similar to those of glutamate dehydrogenases except for the *Neurospora crassa* NAD^+-specific enzyme, which has a tetrameric structure of subunits with more than twice the size of other glutamate dehydrogenases (Austen et al., 1980). The overall sequence similarities (18–23%) between leucine dehydrogenase and $NADP^+$-specific glutamate dehydrogenases are similar to those (18–21%) between leucine dehydrogenase and the glutamate dehydrogenases with dual coenzyme specificity. Thus, it is likely that the genes encoding leucine and glutamate dehydrogenases diverged from each other in the very early evolutionary stage before the emergence of two types of glutamate dehydrogenases.

The cloning and DNA sequence determination of the gene for thermostable leucine dehydrogenase from *B. stearothermophilus* has been accomplished. The high expression of the gene in *E. coli* will facilitate isolation of the enzyme in quantities sufficient for detailed mechanistic and crystallographic studies. In addition, the availability of the cloned and sequenced gene allows us further structural studies of amino acid dehydrogenases by site-directed mutagenesis.

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Dihydrofolate Synthetase and Folylpolyglutamate Synthetase: Direct Evidence for Intervention of Acyl Phosphate Intermediates†

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ABSTRACT: The transfer of ^{17}O and/or ^{18}O from (COOH-^{17}O or -^{18}O) enriched substrates to inorganic phosphate (P_i) has been demonstrated for two enzyme-catalyzed reactions involved in folate biosynthesis and glutamylation. COOH-^{18}O-labeled folate, methotrexate, and dihydropteroate, in addition to [^{17}O]-glutamate, were synthesized and used as substrates for folylpolyglutamate synthetase (FPGS) isolated from *Escherichia coli*, hog liver, and rat liver and for dihydrofolate synthetase (DHFS) isolated from *E. coli*. P_i was purified from the reaction mixtures and converted to trimethyl phosphate (TMP), which was then analyzed for ^{17}O and ^{18}O enrichment by nuclear magnetic resonance (NMR) spectroscopy and/or mass spectroscopy. In the reactions catalyzed by the *E. coli* enzymes, both NMR and quantitative mass spectral analyses established that transfer of the oxygen isotope from the substrate ^{18}O-enriched carboxyl group to P_i occurred, thereby providing strong evidence for an acyl phosphate intermediate in both the FPGS- and DHFS-catalyzed reactions. Similar oxygen-transfer experiments were carried out by use of two mammalian enzymes. The small amounts of P_i obtained from reactions catalyzed by these less abundant FPGS proteins precluded the use of NMR techniques. However, mass spectral analysis of the TMP derived from the mammalian FPGS-catalyzed reactions showed clearly that ^{18}O transfer had occurred.

The poly(γ-glutamyl) conjugates constitute the predominant intracellular forms of the vitamin folic acid; their physiological roles have been the subject of several recent reviews (Covey, 1980; McGuire & Bertino, 1981; Kisliuk, 1981; Cichowicz et al., 1981; McGuire & Coward, 1984). Conversion to the polyglutamylated form is catalyzed by the enzyme folylpolyglutamate synthetase (FPGS;[1] eq 1). FPGS from two mammalian sources, namely, the hog and rat liver, are available in small quantities but have been well studied (Cichowicz & Shane, 1987; McGuire et al., 1980). In bacteria capable of folate biosynthesis, the step preceding synthesis of poly(γ-glutamates) is the addition of the first glutamate residue to 7,8-dihydropteroate (H_2Pte), catalyzed by dihydrofolate synthetase (DHFS; eq 2). In both *Corynebacterium* (Shane, 1980a,b, 1982) and *Escherichia coli* (Ferone & Warskow, 1981, 1983; Ferone et al., 1983), DHFS and FPGS activities copurify through a number of protein fractionation steps at a constant ratio of specific activities. The bifunctional *E. coli* enzyme has been isolated in large quantities from a transformant containing the cloned FPGS/DHFS gene (*folC*) (Bognar et al., 1985), and the sequence of the *folC* gene has been determined (Bognar et al., 1987). Although kinetic properties and substrate specificities of mammalian and bacterial FPGS differ, all forms of FPGS investigated so far catalyze an ATP-dependent addition of glutamate residues to a variety of folates and antifols (eq 1). It has been postulated,

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[1] Abbreviations: pABA, *p*-aminobenzoic acid; DHFS, dihydrofolate synthetase (EC 6.3.2.12); FPGS, folylpolyglutamate synthetase (EC 6.3.2.17); PteGlu, pteroylglutamic acid (folic acid); H_2Pte, 7,8-dihydropteroic acid; MTX, methotrexate (4-NH_2,10-CH_3PteGlu); TMP, trimethyl phosphate; Tfa, trifluoroacetyl; TFA, trifluoroacetic acid; CPG_1, carboxypeptidase G_1; CPG_2, carboxypeptidase G_2; S value, NMR shift value or $\Delta\delta$.

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