Leucine Dehydrogenase from *Bacillus stearothermophilus*: Identification of Active-Site Lysine by Modification with Pyridoxal Phosphate

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We have constructed an efficient expression plasmid for the leucine dehydrogenase gene previously cloned from *Bacillus stearothermophilus*. The recombinant enzyme was overproduced in *Escherichia coli* cells to a level of more than 30% of the total soluble protein upon induction with isopropyl β-D-thiogalactopyranoside. The enzyme could be readily purified to homogeneity by heat treatment and a single step of ion-exchange chromatography. The purified enzyme was inactivated in a time-dependent manner upon incubation with pyridoxal 5'-phosphate (PLP) followed by reduction with sodium borohydride. The inactivation was completely prevented in the copresence of L-leucine and NAD+. Concomitantly with the inactivation, several molecules of PLP were incorporated into each subunit of the hexameric enzyme. Sequence analysis of the fluorescent peptides isolated from a proteolytic digest of the modified protein revealed that Lys80, Lys91, Lys206, and Lys265 were labeled. Among these residues, Lys80 was predominantly labeled and, in the presence of L-leucine and NAD+, was specifically protected from the labeling. Furthermore, a linear relationship of about 1:1 was observed between the extent of inactivation and the amount of PLP incorporated into Lys80. A slightly active mutant enzyme, in which Lys80 is replaced by Ala, was not inactivated at all by incubation with PLP, showing that the inactivation is correlated with the labeling of only Lys80. Lys80 is conserved in the corresponding regions of all the amino acid dehydrogenase sequences reported to date. These results suggest that Lys80 is located at the active site and plays an important role in the catalytic function of leucine dehydrogenase.

Leucine dehydrogenase [EC 1.4.1.9] is an NAD+-dependent oxidoreductase that catalyzes the reversible deamination of L-leucine and some other branched-chain L-amino acids to their keto analogues. The enzyme, occurring mainly in *Bacillus* species (1), functions catalytically in the bacterial metabolism of branched-chain L-amino acids (2), and has been purified to homogeneity from *B. sphaericus* (3) and *B. stearothermophilus* (4). The gene coding for the *B. stearothermophilus* enzyme has been cloned, sequenced, and expressed in *Escherichia coli* (5). The polypeptide deduced from the nucleotide sequence is composed of 429 amino acid residues with a calculated molecular weight of about 47,000 and corresponds to a subunit of the hexameric enzyme, whose structure has been revealed by a small-angle X-ray scattering study (6). The reaction catalyzed by leucine dehydrogenase proceeds according to the ordered bi-ter mechanism, in which NAD+ and L-leucine are bound and NH2-κ, α-keto-iso-caproate, and NADH are released in that order (1, 4).

Although the three-dimensional structures of several NAD(P)+-dependent dehydrogenases have been elucidated in detail, none of those acting on amino acids has been analyzed by X-ray crystallography. Further, little is known about the molecular mechanisms of amino acid dehydrogenase catalysis. In order to shed light on the active-site structure and reaction mechanism of amino acid dehydrogenases, we have first constructed an efficient expression plasmid for the cloned gene coding for *B. stearothermophilus* leucine dehydrogenase, and then have attempted to identify an active-site lysine residue by modification with pyridoxal 5'-phosphate (PLP). In this paper, we present evidence that Lys80 conserved in the Gly-rich consensus sequence is an active-site residue in leucine dehydrogenase, presumably being involved in the catalytic function.

**EXPERIMENTAL PROCEDURES**

**Construction of Overexpression Plasmid**—The gene coding for leucine dehydrogenase of *B. stearothermophilus* was previously cloned into the *SalI* site of pBR322, the constructed plasmid being designated as pICD2 (5). Since the expression level of the enzyme in *E. coli* cells carrying pICD2 was not high, a new overexpression plasmid was constructed using Bluescript II (Stratagene) as a vector.

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Abbreviations: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; kbp, kilobase pair(s); PLP, pyridoxal 5'-phosphate; PTH, phenylthiohydantoin.
Briefly, the 0.89-kbp SalI fragment encoding the C-terminal half of the enzyme was excised from pICD2 and inserted into the XhoI site in Bluescript II. The 1.3-kbp XhoI-NeoI fragment from pICD2 was then inserted into the SalI and NeoI sites of the above plasmid to produce the final plasmid designated as pHLeuDH (Fig. 1).

**Enzyme and Protein Assays**—The standard assay mixture (1.0 ml) contained 0.1 M sodium carbonate buffer (pH 10.5), 10 mM L-leucine, and 1.25 mM NADH. The reaction was started by the addition of the enzyme, and the increase in absorbance at 340 nm was continuously monitored with a spectrophotometer at 55°C (5). One unit of the enzyme is defined as the amount of enzyme that produces 1 μmol of NADH per min under the above conditions. Specific activity is expressed as units per milligram of protein. Protein concentration of the purified enzyme was derived from the absorbance at 280 nm. The absorption coefficient (A mg/ml = 0.851) at 280 nm determined by amino acid analysis was used throughout.

**Enzyme Purification**—*E. coli* JM109 cells carrying pHLeuDH were grown at 37°C for 12 h in 1 liter of Luria broth (1.0% tryptone, 1.0% NaCl, and 0.5% yeast extract, pH 6.8) containing 50 μg/ml sodium ampicillin and 0.5 mM IPTG. Cells harvested by centrifugation were washed with 500 ml of 0.85% NaCl.

**Step 1:** The washed cells (about 4 g, wet weight) were suspended in 20 ml of 10 M potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol (buffer A) supplemented with 0.1 M phenylmethylsulfonyl fluoride. After the cells were disrupted in a French pressure cell, the resultant solution was centrifuged at 46,000 × g for 30 min.

**Step 2:** The supernatant solution (21 ml) was incubated at 70°C for 30 min, then cooled on ice, and centrifuged as above to remove the denatured proteins. The supernatant was dialyzed at 4°C for 6 h against 5 liters of buffer A.

**Step 3:** The enzyme solution was applied to a column (2.5 x 20 cm) of DEAE-Toyopearl 650M (Toyobo) pre-equilibrated with buffer A. Proteins were eluted with a 1-liter linear gradient of KCl (0.1-0.2 M) in the same buffer. The active fractions were pooled (about 150 ml) and concentrated by ultrafiltration through an Amicon PM-10 membrane to about 10 ml. The homogeneity of the preparation was examined by SDS-PAGE with a 12.5% polyacrylamide gel (7).

**Modification with PLP**—The purified enzyme (56.5 μM) was incubated at 30°C with various concentrations (0.1-10 mM) of PLP in 50 mM HEPES buffer (pH 7.8) and the enzyme activity was determined fluorophotometrically using the enzyme that produces 1 μmol of NADH per min as a standard, and the amount of PLP incorporated into the enzyme was measured fluorophotometrically after denaturation of the enzyme protein (0.1 M HEPES buffer (pH 7.8) containing 8 M urea). The fluorescence emission at 395 nm due to the reduced phosphopyridoxyl-lysine moiety was measured upon excitation at 330 nm using known amounts of PLP as a standard, which was modified with excess N-α-acetyllysylserine (Sigma) followed by NaBH₄ reduction.

**Isolation of Labeled Peptides**—The enzyme (50 nmol) modified with 2 mM PLP for 30 min and reduced with NaBH₄, as described above, was carboxymethylated, extensively dialyzed against water, and lyophilized. The protein was suspended in 0.1 ml of 0.1 M ammonium bicarbonate buffer (pH 8.0) containing 4 M urea, and then diluted with 0.1 ml of 0.1 M ammonium bicarbonate. N-Tosyl-L-phenylanilinochloromethylketone-treated trypsin (Millipore) was added to the turbid solution in a 1:50 (mol/mol) ratio of protease to substrate. Digestion was performed at 37°C for 24 h with a second addition of trypsin, similar to the first, after a period of 12 h. The peptides were separated on a Gilson HPLC system equipped with a Cosmosil C₄, reverse-phase column using a solvent system of 0.1% trifluoroacetic acid (A) and 0.088% trifluoroacetic acid containing 60% acetonitrile (B). A 60-min linear gradient from 0 to 80% B was used to elute peptides at a flow rate of 1.0 ml/min. The absorbance at 215 nm and the fluorescence (excitation at 330 nm and emission at 395 nm) of effluents were continuously monitored. The amount of PLP bound to each labeled peptide was calculated from the area of fluorescent peaks using known amounts of the reduced PLP-N-α-acetyllysylserine as a standard.

**Amino Acid Composition and Sequence Analysis**—Amino acid composition was determined, after hydrolysis with 6 N HCl in an evacuated tube, with a Hitachi 835 amino acid analyzer using o-phthalaldehyde. An authentic N-ε-lysyllysine was prepared from N-α-acetyllysylserine by the procedure reported previously (9). The amino acid sequence was determined with an Applied Biosystems Model 477A protein sequencer linked with an Applied Biosystems Model 120A PTH analyzer.

**Determination of Apparent Dissociation Constant**—A solution (500 μl) containing various concentrations of PLP (0.2-1.0 mM) in 50 mM HEPES buffer (pH 7.8) and the enzyme (23 μM) was preincubated at 25°C for 30 min in a 0.7-ml cuvette. The same solution without the enzyme was placed in a reference cell, and the difference absorption spectra were measured in the 340-500 nm region with a recording spectrophotometer. The formation of a Schiff base between the 4-formyl group of PLP and an ε-amino group(s) of lysine residue was monitored by following the increase in absorbance at 434 nm. The apparent dissociation constant (K app) was calculated by using the following equation (10):

\[ \Delta A = \Delta A_{\text{max}} \cdot [\text{PLP}]_0 / (K_{\text{app}} + [\text{PLP}]_0) \]

where [PLP]₀ is the initial concentration of PLP, ΔA is the increase in absorbance at 434 nm, and \( \Delta A_{\text{max}} \) (2.55 × 10⁶ M⁻¹) is the maximum increase in absorbance observed when all the enzyme molecules are presumed to be complexed with PLP.

**NADH Binding Assay**—The binding of NADH with the enzyme was measured by the gel permeation method (11). The enzyme (32 μM), unlabelled or labeled with 2 mM PLP for 30 min as above, was incubated with 50 μM NADH and applied to a column (5 x 200 mm) of Sephadex G-50 (fine) previously equilibrated with 10 mM potassium phosphate buffer (pH 7.8) containing 50 μM NADH. The enzyme was
eluted with the same buffer at a constant flow rate of 0.4 ml/min, and the absorbance at 340 nm was continuously monitored to determine the amount of NADH bound to the enzyme from the area of the trough.

**Site-Directed Mutagenesis**—The HindIII–KpnI fragment (2.0 kbp) from pBLeuDH, which contains the entire coding region for leucine dehydrogenase, was subcloned into phage M13 mp19 RF DNA. E. coli BW313 (dut− ung−) cells were transfected with the M13 phage, and the single-stranded phage DNA containing uracil was purified from the culture supernatant. An oligonucleotide primer, 5′-TGACCGTT-CCGCCCCCG-3′, was designed to be complementary to this single-stranded template DNA and to contain appropriate mismatching bases (asterisked) in the codon for Lys80 (underlined), and was synthesized with an Applied Biosystems DNA synthesizer Model 381. Synthesis of mutant DNA and selection were performed by the method of Kunkel et al. (12), using a commercial kit (Mutan-K; Takara Shuzo). The sequence of nucleotides 1–666 corresponding to the region from the translational initiation codon ATG to the SalI site in the mutant gene obtained was confirmed by the dideoxy chain termination method (13). The 1.1-kbp HindIII–SalI fragment containing the mutated site was excised from the double-stranded M13 mp19 phage DNA, ligated into the HindIII–SalI site of pBLeuDH, and transformed into E. coli JM109 cells. The Ala-for-Lys80 mutant enzyme produced was purified to homogeneity by a similar procedure to that for the wild-type enzyme.

**RESULTS**

**Construction of Overexpression Plasmid and Purification of Leucine Dehydrogenase**—The gene coding for leucine dehydrogenase from B. stearothermophilus has already been cloned (5). However, E. coli cells transformed with the resultant plasmid pICD2 produced the enzyme corresponding to only about 3% of the total soluble protein in the cell extract (5). This was probably because the plasmid had been derived from pBR322, usually used for the purpose of gene cloning but not for gene expression. The presence of three SalI sites in pICD2 also appeared unfavorable for future site-directed mutagenesis (see below). Therefore, using an expression phagemid vector Bluescript II, we have derivatized pICD2 into pBLeuDH containing only a unique SalI site (Fig. 1).

A crude extract from E. coli JM109 cells carrying pBLeuDH thus constructed and being grown in the presence of IPTG exhibited a high-level expression of leucine dehydrogenase with a specific activity of about 35 units/mg protein [cf. 3 units/mg in the extract from E. coli C600/ pICD2 (5)]. On the basis of the specific activity of the purified enzyme (112 units/mg), the amount of the enzyme in the cell extract corresponded to more than 30% of that of the total soluble protein (see also Fig. 2). The enzyme was found to be inducibly formed by the addition of IPTG; the expression level in the absence of IPTG was less than 3% of the total soluble protein. Thus, the lac promoter in the vector Bluescript II functioned efficiently for expression of the enzyme gene placed 3′-downstream.

The high-level expression of the enzyme in E. coli led to the establishment of a very simple and rapid purification method for the recombinant enzyme, which consisted of heat treatment and a single column chromatographic step as described under “EXPERIMENTAL PROCEDURES.” The overall recovery of the enzyme in the purification was about 70%, and the homogeneity of the purified enzyme was >95% as judged by SDS-PAGE (Fig. 2). Direct Edman degradation of the purified enzyme afforded an N-terminal partial sequence of Met–Glu–Leu–Phe–Lys–Tyr–Met–Glu-Thr–Tyr, which coincided with that predicted from the DNA sequence (5).

**Inactivation by PLP**—When incubated with PLP follow-
Fig. 3. Inactivation of leucine dehydrogenase by PLP. (a) Time course. The wild-type enzyme (57 μM) (●) or the Ala-for-Lys80 mutant enzyme (57 μM) (▲) was incubated at 30°C with 2 mM PLP in 50 mM HEPES buffer (pH 7.8) in a final volume of 0.1 ml. At each indicated time, an aliquot was withdrawn and reduced with 20 mM NaBH₄, and the remaining activity was assayed. (b) Effect of substrates and coenzymes. The above inactivation mixtures were supplemented with 10 mM L-leucine plus 2 mM NAD⁺ (□), 2 mM NADH (△), 10 mM L-leucine (●), 2 mM NAD⁺ or 10 mM α-keto-isocaprate (□), or 10 mM L-glutamate plus 2 mM NAD⁺ (○). The control reaction without these additives (●) was the same as in (a).

ed by reduction with sodium borohydride, the enzyme was inactivated in a time-dependent manner, reaching a plateau after about 20 min (Fig. 3). The extent of inactivation depended on the concentration of PLP; about 90% of the original activity was lost by incubation with 4 mM PLP. In addition, the inactivation by PLP was also dependent on pH, the enzyme being inactivated most effectively around pH 8.5. The optimum pH for inactivation may reflect the pK₅ of a reactive lysine residue(s) that is essential for activity. PLP is known to react with ε-amino groups of lysine residues in proteins to form a Schiff base that can be reduced by sodium borohydride (14). Actually, the difference spectrum between the enzyme plus PLP and PLP alone exhibited an absorption maximum at 434 nm due to the formation of the Schiff base (Fig. 4a). The apparent dissociation constant for PLP of the Schiff base was determined to be 0.92 mM from the increases in absorbance at 434 nm in the difference spectra with various concentrations of PLP as described under "EXPERIMENTAL PROCEDURES." (Fig. 4b).

Protection by Coenzymes and Substrates—The protective effect by substrates and coenzymes on the enzyme inactivation by PLP was investigated to elucidate where PLP was bound. The enzyme was incubated with 2 mM PLP in the presence of 10 mM substrate or 2 mM coenzyme (Fig. 3b).

Fig. 4. Difference absorption spectrum between the enzyme plus PLP and PLP alone. (a) The difference absorption spectrum of the solution containing 0.2 mM PLP and 23 μM enzyme in 50 mM HEPES buffer (pH 7.8) against the solution without the enzyme was measured in the wavelength region from 340 to 500 nm. (b) The increases in absorbance at 434 nm in the difference spectra obtained with various concentrations of PLP were plotted against the PLP concentration. The curve is the least-squares best fit with K_app = 0.92 mM.

The enzyme was completely protected from inactivation by the concomitant presence of both L-leucine and NAD⁺. NADH also considerably protected the enzyme, whereas L-leucine alone protected it only moderately and α-keto-
Fig. 6. Stoichiometry of inactivation and labeling. The enzyme was incubated with various concentrations of PLP for 30 min or with 2 mM PLP for various times and reduced with NaBH₄. The remaining activities were plotted against the amounts of PLP incorporated into the enzyme subunit (●) or Lys80 (○) determined from the areas of the fluorescence peak of Tᵢ in HPLC of tryptic digests of the labeled enzyme (see Fig. 7). Further details are given in the text.

iso-caproate or NAD⁺ alone offered little protective effect. These results can be explained by the ordered sequential mechanism, in which L-leucine is bound to the enzyme following NAD⁺ in the oxidative deamination, and NADH is bound to the enzyme before the others in the reductive amination (1, 4). L-Glutamate, a non-substrate amino acid, gave no protection against inactivation by PLP.

Stoichiometry of Inactivation and Labeling—The enzyme was incubated with various concentrations (0.2–5 mM) of PLP for 30 min and reduced, then the excess reagents were removed by centrifugal ultrafiltration. The fluorescence derived from the pyridoxyllysine moiety, residual activities, and protein concentrations were measured. The residual activities were plotted against the amounts of PLP incorporated (Fig. 6). The line deviated greatly from linearity at high degrees of inactivation and the molar ratio of bound PLP to the enzyme subunit was far above unity. This result shows that PLP is incorporated into several lysine residues other than that (or those) related to the activity.

Identification of Labeled Residues—To identify the lysine residue(s) labeled by PLP, the enzyme was modified with PLP followed by carboxymethylation and then digested with trypsin. The digest was separated by HPLC as described under “EXPERIMENTAL PROCEDURES.” Fluorescent peptides were eluted as one major peak accompanied by three minor peaks (designated T₁–T₄, in order of elution, Fig. 7a). In contrast, the modification by PLP in the copresence of 10 mM L-leucine and 2 mM NAD⁺ yielded a labeling pattern, in which only the major peak (T₁) in the digest of the unprotected enzyme was dramatically decreased (Fig. 7b). The elution profiles were reproducible, showing that the multiple peaks were not due to incomplete digestion. After the labeled peptides containing the fluorescent phosphopyridoxyl moiety were further purified by re-chromatography on a reverse-phase column, their amino acid compositions were analyzed. All four peptides (T₁–T₄) were found to contain an amino acid that does not correspond to any natural amino acid in the automated amino acid analysis, but corresponds to the authentic N-α-pyridoxyllysine eluted just after histidine and much before arginine, although its recovery in the hydrolysates was rather low (5–30%) as compared to other amino acids.

Amino acid sequences of the four peptides were determined with a protein sequencer, and the results are summarized in Table I. The PTH derivative of N-α-pyridoxyllysine is not identifiable with a protein sequencer (15). However, when the partial sequences were compared with the complete sequence of the enzyme from B. stearothermophilus (5), all the unidentified residues corresponded to particular lysine residues. These structures were also consistent with the amino acid compositions. Therefore, it was concluded that Lys265, Lys91, Lys206, and Lys80 were the lysine residues labeled with PLP in T₁–T₄, respectively. Lys80 was predominantly labeled, and its labeling was preferentially prevented in the copresence of L-leucine and NAD⁺, suggesting that Lys80 is located at the active site of the enzyme.

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TABLE I. Structures of labeled peptides. One letter abbreviations are used. X represents an unidentified residue that was concluded to be the labeled lysine residue. Details of the sequence determinations are given in the text. Positions are in the complete sequence of leucine dehydrogenase from B. stearothermophilus (5).

<table>
<thead>
<tr>
<th>Peak</th>
<th>Sequence</th>
<th>Positions</th>
<th>Labeled site</th>
</tr>
</thead>
<tbody>
<tr>
<td>T_1</td>
<td>V·I·A·G·S·A·N·N·Q·L·X·E·P·R</td>
<td>255-268</td>
<td>K265</td>
</tr>
<tr>
<td>T_2</td>
<td>K·D·X·N·E·A·M·F·R</td>
<td>89-97</td>
<td>K91</td>
</tr>
<tr>
<td>T_3</td>
<td>L·I·V·T·D·I·N·X·E·V·V·A·R</td>
<td>199-211</td>
<td>K206</td>
</tr>
<tr>
<td>T_4</td>
<td>N·A·A·A·G·L·N·L·G·G·G·X·T·V·I·G·D·P·R</td>
<td>69-88</td>
<td>K80</td>
</tr>
</tbody>
</table>

Fig. 8. Comparison of the leucine dehydrogenase partial sequence containing Lys80 with corresponding sequences of other amino acid dehydrogenases. Lys80 of leucine dehydrogenase is shown by an asterisk, and conserved residues are boxed. Abbreviations: LeuDH, leucine dehydrogenase; PheDH, phenylalanine dehydrogenase; GluDH, glutamate dehydrogenase; and AlaDH, alanine dehydrogenase.

Lys80—The enzyme was modified with PLP to various extents by changing the incubation time, and the amount of PLP incorporated into Lys80 was determined after digestion with trypsin and separation by HPLC of the fluorescent peptide (T_4) containing Lys80. The amounts calculated from the areas of fluorescence due to peak T, and corrected per mol of enzyme subunit were plotted against the residual activities after the modification. As shown in Fig. 6, a straight line was obtained, extrapolation of which to 0% remaining activity gave a value of approximately 0.9 for the molar amount of PLP incorporated into Lys80 per mol of enzyme subunit. This result indicates that the modification with PLP of Lys80 is directly related to the loss of enzyme activity and suggests that Lys80 is an active-site residue, presumably playing an essential role in the catalysis. The other labeled lysine residues (Lys91, Lys206, and Lys265) may be located at the surface of the enzyme molecule and are functionally unimportant.

**DISCUSSION**

PLP has been used to modify reactive lysine residues in various proteins including several NAD(P)^+‐dependent dehydrogenases (16–22), though the modifications are not necessarily active-site-directed (23). The inactivation by PLP of leucine dehydrogenase from B. sphaericus has also been reported, but the modified lysine residue(s) has not been identified (24). The present study has shown that the recombinant enzyme from B. stearothermophilus is effectively inactivated by PLP with concomitant modification of four different lysine residues. Lys80 was predominantly labeled and was selectively protected from the modification in the presence of substrate and coenzyme. Furthermore, the binding to Lys80 of about 1 mol of PLP per mol of enzyme subunit corresponded to the complete loss of activity. These results lead to the conclusion that only Lys80 is located at or near the active site of the enzyme and the other three lysine residues may be located at the surface of the enzyme molecule without having direct roles in the enzymic functions.

By comparison of the sequence of B. stearothermophilus leucine dehydrogenase with those of proteins registered in the National Biomedical Research Foundation (NBRF) protein sequence data bank (25), we have found that Lys80 in leucine dehydrogenase is conserved in the corresponding regions of all other amino acid dehydrogenases sequenced to date (Fig. 8); glutamate dehydrogenases from bovine liver (26), chicken liver (27), human liver (28), E. coli (29, 30), Neurospora (31, 32), and yeast (33), phenylalanine dehydrogenases from B. sphaericus (34), Sporosarcina ureae (35), and Thermoactinomyces intermedius (36), and alanine dehydrogenases from B. sphaericus and B. stearothermophilus (37). It is noteworthy that this lysine residue occurs in a Gly-rich tetrapeptide sequence, (G or H)-G-(G or A or S)-K (38), the flanking regions of which are also highly conservative (Fig. 8). This finding suggests that
the region constitutes a part of the catalytic site in amino acid dehydrogenases.

Before undertaking the chemical modification with PLP, we tested the affinity labeling reagents, adenosine polyphosphoryloxidys, developed in this laboratory (see Ref. 39 for a recent review), for specific labeling of the NAD⁺-binding site in the leucine dehydrogenase (unpublished results). Such reagents have been successfully used for identification of the nucleotide-binding sites in various proteins, including rabbit muscle adenylate kinase (40, 41), E. coli glycogen synthase (42), and E. coli H⁺-ATPase (43).

However, for inactivation of leucine dehydrogenase, millimolar concentrations of the affinity labeling reagents were needed to attain the same level of inactivation as with PLP. Such concentrations are much higher than those generally employed for affinity labeling (micromolar order). In addition, the reagents showed no inhibition in the assay containing an eight-times-higher concentration of the reagent than that of NAD⁺. These preliminary results suggest that the NAD⁺-binding site of leucine dehydrogenase has little affinity for adenosine polyphosphoryloxidys.

A recent study using adenosine diphosphopyridoxal for affinity labeling of glucose 6-phosphate dehydrogenase from Leuconostoc mesenteroides showed that the lysine residues modified by the reagent are probably located at the substrate-binding site rather than at the NAD⁺-binding site (44).

The binding of NADH by the enzyme was not influenced by modification with PLP, suggesting that the active-site Lys80 is not located near the cofactor-binding site and that the modification with the bulky phosphopyridoxyl moiety does not affect the local conformation around the cofactor-binding region. NADH, however, considerably protected the enzyme from inactivation by PLP. The substrate 1-leucine or the cofactor NAD⁺ alone offered little protective effect, but the enzyme was protected almost completely from inactivation by their copresence (i.e., in the reactive ternary complex). These results can be interpreted on the basis of the ordered sequential mechanism of the leucine dehydrogenase reaction, in which NAD⁺ is bound first to the free enzyme followed by 1-leucine binding to the enzyme having undergone a conformational change. The ε-amino group of Lys80 is probably unreactive with PLP in this ternary complex or in the binary complex with NADH, both of which would have conformations different from that of the free enzyme. It is therefore assumed that Lys80 is located at or near the substrate-binding site and its modification with PLP results in the loss of enzymic activity.

On the basis of chemical modification with PLP, Smith et al. (45) proposed that an active-site lysine residue in glutamate dehydrogenase (corresponding to Lys80 of leucine dehydrogenase) functions in catalysis by forming a Schiff base adduct with its substrate α-ketoglutarate. However, an alternative mechanism for the glutamate dehydrogenase reaction, in which an active-site lysine residue with an unusually low pKₐ value probably participates in the reaction as a general acid-base catalyst without forming the Schiff base, has also been proposed (46).

To elucidate the catalytic role of Lys80 in leucine dehydrogenase, we have replaced it not only with Ala but also with some other amino acid residues. All the Lys80 mutant enzymes showed minuscule but detectable activities when measured under the standard assay conditions. This apparently conflicts with the suggestion in the present study that Lys80 is an indispensable residue at the active site. However, we have found that the reaction rate by these mutant enzymes is logarithmically enhanced with the increase of solvent pH (proportionally to [OH⁻]) and is also markedly enhanced by the addition of various primary amines with different pKₐ values. These results supporting the acid-base mechanism for the leucine dehydrogenase reaction will be reported shortly.

REFERENCES

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