Leucine Dehydrogenase from Corynebacterium pseudodiphtheriticum: Purification and Characterization

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Leucine dehydrogenase [EC 1.4.1.9] was purified to homogeneity from Corynebacterium pseudodiphtheriticum ICR 2210. The enzyme consisted of a single polypeptide with a molecular weight of about 34,000. Stepwise Edman degradation provided the N-terminal sequence of the first 24 amino acids, and carboxypeptidase Y digestion provided the C-terminal sequence of the last 2 amino acids. Although the enzyme catalyzed the reversible deamination of various branched-chain L-amino acids, L-valine was the best substrate for oxidative deamination at pH 10.9 and the saturated concentration. The enzyme, however, had higher reactivity for L-leucine, and the $k_cJ/k_m$ value for L-leucine was higher than that for L-valine. The enzyme required NAD+ as a natural coenzyme. The NAD+ analogs 3-acetylpyridine-NAD+ and deamino-NAD+ were much better coenzymes than NAD+. The enzyme activity was significantly reduced by sulfhydryl reagents and pyridoxal 5'-phosphate. D-Enantiomers of the substrate amino acids competitively inhibited the oxidation of L-valine.

NAD+ -dependent leucine dehydrogenase (Leu DH) [EC 1.4.1.9] catalyzes the reversible deamination of branched-chain and straight-chain L-amino acids. It occurs not only in the endospore-forming bacteria, bacilli4,2) and clostridia,3) but also in the nonsporing bacteria Corynebacterium pseudodiphtheriticum, C. sepedonicum, and Alcaligenes faecalis.2) Although the enzymes have been purified to homogeneity and characterized from B. subtilis,4) B. sphaericus,2) B. cereus,3) B. steatothermophilus,6) and B. caldolyticus,7) little attention has been paid to the enzymes from the nonsporing bacteria. The activity staining of the enzyme after disc gel electrophoresis of a crude extract from C. pseudodiphtheriticum ICR 2210 gave a stained band which was different in mobility from that of the hexameric Leu DH from B. sphaericus ICR 3525. Moreover, the Corynebacterium enzyme had its highest activity with L-valine as a substrate. NADP+ -dependent valine dehydrogenase (Val DH) [EC 1.4.1.8] was first reported in pea shoots,8,9) while the NAD+ -dependent Val DH activity was found in Streptomyces fradiae.10) Recently, NAD+ -dependent Val DH has been purified to homogeneity and characterized from St. fradiae,11) St. aureofaciens,12) and St. cinnamonensis.13) To learn whether the Corynebacterium enzyme belongs to the group of Leu DH or Val DH, we characterized the enzyme and found that the enzyme belongs to the group of Leu DH.

We describe here the purification and characterization of LeuDH from C. pseudodiphtheriticum.

Materials and Methods

Materials. NAD+, NADP+, and NADH were obtained from Kojin Biochemicals, Tokyo; amino acids from Nacalai Tesque, Kyoto; NAD+ analogs and α-keto acids (sodium salt) from Sigma Chemicals Co., St. Louis; DEAE-cellulose from Serva, Heidelberg; 5'-AMP-Sepharose 4B and Mono Q anion exchange column HR

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Abbreviations: Leu DH, leucine dehydrogenase; Val DH, valine dehydrogenase; SDS, sodium dodecyl sulfate; FPLC, fast-protein liquid chromatography.
5/5 (0.5 x 5 cm) from Pharmacia Fine Chemicals, Uppsala; TSK gel G3000SW from Tosoh, Tokyo; and marker proteins for molecular weight measurements from Oriental Yeast, Osaka. Hydroxyapatite was prepared by the method of Tiselius et al.14) Other chemicals were of analytical grade.

Microorganism and conditions for cell growth. C. pseudodiphtheriticum ICR 2210 was grown in a medium containing 1.5% peptone, 0.2% K2HPO4, 0.2% KH2PO4, 0.2% NaCl, 0.01% MgSO4·7H2O, and 0.01% yeast extract. The pH of the medium was adjusted to 7.2 with 4 M NaOH. The bacteria were grown aerobically in 2-l flasks containing 750 ml of the medium on a reciprocal shaker at 30°C for 12 hr. The cells, harvested by centrifugation, were washed twice with 0.85% NaCl solution.

Enzyme assay. Since L-valine was the best substrate for the enzyme, L-valine was used for the assay. The standard reaction mixture for oxidative deamination contained 20 μmol of L-valine, 2 μmol of NAD+, 200 μmol of glycine-KCl-KOH buffer (pH 10.9), and enzyme in a final volume of 1.0 ml. The assay system for reductive amination consisted of 10 μmol of sodium α-ketoisovalerate, 0.2 μmol of NADH, 750 μmol of NH4Cl, 200 μmol of glycine-KCl-KOH buffer (pH 9.5), and enzyme in a final volume of 1.0 ml. Incubation was done at 30°C in a cuvette with a 1-cm light path. The reaction was started by addition of NAD+ (or NADH) and followed by measuring the initial change in absorbance at 340 nm with a Shimadzu double-beam spectrophotometer UV-140-02.

Definition of units and protein measurement. One unit of enzyme was defined as the amount of enzyme that catalyzes the appearance of 1 μmol of NADH per min in the oxidative deamination. Specific activity was expressed as units per milligram of protein. Protein was measured by the method of Lowry et al.15) with crystalline bovine serum albumin as the standard. Concentrations of the purified enzyme were estimated from the absorption at 280 nm. The absorption coefficient (A°280 at 280 nm = 10.0) was used throughout.

Enzyme purification. All the procedures were done at 0–5°C except for Mono Q column chromatography, and potassium phosphate buffer containing 0.01% 2-mercaptoethanol was used unless otherwise stated. The washed cells (about 500 g, wet weight) were suspended in 2 l of 0.1 M buffer (pH 7.2) and disrupted by sonication. The intact cells and cell debris were removed by centrifugation at 10,000 × g for 30 min. To the cell extract (2,040 ml) was added 1.0 ml of 1.5% proteamine sulfate solution (pH 7.2) per 100 mg of protein with stirring. After 10 min, the mixture was centrifuged at 10,000 × g for 20 min and the bulky precipitate was discarded. The supernatant solution (2,350 ml) was brought to 40% saturation with solid ammonium sulfate. The precipitate collected by centrifugation at 10,000 × g for 30 min was dissolved in 10 mM buffer (pH 7.4) and dialyzed against the same buffer. The inactive precipitate formed during dialysis was removed by centrifugation at 10,000 × g for 30 min. The enzyme solution (550 ml) was put on a DEAE-cellulose column (7.4 x 35.5 cm) equilibrated with 10 mM buffer (pH 7.4). After the column was washed thoroughly with the same buffer and then with the buffer supplemented with 50 mM KC1, the enzyme was eluted with the buffer containing 0.1 M KCl. The active fractions were pooled and concentrated with a Pellicon labocasette (PTGC OLG membrane, Nihon Millipore Ltd., Tokyo). The enzyme solution (83 ml) was dialyzed against 1 mM buffer (pH 7.4) containing 0.1 M KCl and was then put on two hydroxyapatite columns (3 x 18.5 cm) equilibrated with 1 M buffer (pH 7.4) containing 0.1 M KCl. The enzyme was eluted with 5 mM buffer (pH 7.4) containing 0.1 M KCl. The active fractions were collected and concentrated with an Amicon membrane filter PM 10. The enzyme solution (60 ml) was dialyzed against 10 mM buffer (pH 7.4) containing 10% glycerol and was put in 10-ml portions on a 5'-AMP-Sepharose 4B column (1.2 x 9.7 cm) equilibrated with 0.1 M buffer (pH 7.2) containing 10% glycerol. The column was developed with 10 mM buffer (pH 7.4) containing 10% glycerol. The enzyme was eluted with the same buffer after elution of contaminating proteins (Fig. 1). The active fractions were concentrated with an Amicon membrane filter PM 10 and dialyzed against 10 mM Tris–HCl buffer (pH 8.0) containing 10% glycerol. The enzyme solution (200–300 units each) was put on a Pharmacia FPLC Mono Q column HR 5/5 (0.5 x 5 cm) equilibrated with 10 mM Tris–HCl buffer (pH 8.0) containing 10% glycerol. The column was developed at room temperature at a flow

Fig. 1. 5'-AMP-Sepharose 4B Column Chromatography.
The enzyme was put on a 5'-AMP-Sepharose 4B column (1.2 x 9.7 cm) equilibrated with 0.1 M potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol and 10% glycerol. The enzyme was eluted with 10 mM potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and 10% glycerol. The eluate was collected in 4.0-ml portions.
Leucine Dehydrogenase from *C. pseudodiphtheriticum*

rate of 0.5 ml per min with a 40-min linear gradient of KCl concentration (0.1–0.2 M) in the same buffer. The active fractions were combined, enriched with an Amicon membrane filter PM 10, and stored at −20°C in the presence of 50% glycerol.

**Electrophoresis.** Disc gel electrophoresis of the native enzyme was done with a 7.5% polyacrylamide gel by the method of Davis. Protein was stained with 0.04% Coomassie brilliant blue G-250 in 3.5% HClO₄. The enzyme was stained for activity with a solution (4.0 ml) containing 10 mM L-valine or L-leucine, 1 mM NAD⁺, 0.2 M Tris–HCl buffer (pH 9.0), 40 μg of phenazine methosulfate, and 400 μg of nitroblue tetrazolium salt. Disc gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) was done on 10% polyacrylamide gel by the method of Weber and Osborn. Isoelectric focusing of the enzyme in polyacrylamide gel was done as described by Righetti and Drysdale with carrier ampholytes in the pH range of 3.5 to 7.0 at 4°C.

**Estimation of molecular weight.** The molecular weight of the enzyme was estimated at room temperature by high-performance liquid chromatography on a TSK G3000SW column (0.75 x 60 cm, Tosoh) at a flow rate of 0.7 ml/min with an elution buffer consisting of 50 mM potassium phosphate buffer (pH 7.2) containing 0.2 M KCl. A calibration curve was made with the following proteins: yeast glutamate dehydrogenase (mol. wt., 290,000), pig heart lactate dehydrogenase (142,000), yeast enolase (67,000), yeast adenylate kinase (32,000), and horse cytochrome c (12,400). The molecular weight of the enzyme was also estimated by SDS-disc gel electrophoresis. Standard proteins used were catalase (mol. wt., 60,000), ovalbumin (43,000), yeast alcohol dehydrogenase (37,000), κ-chymotrypsinogen A (25,700), and myoglobin (17,200).

**Analysis of N- and C-terminal amino acid sequence.** The N-terminal amino acid sequence analysis of the enzyme was done by automated Edman degradation with an Applied Biosystems 470A gas-phase protein sequencer. The phenylthiobutyriamino acid derivatives (PTH) were identified by an on-line PTH analyzer 120A (Applied Biosystems) with a PTH-C18 column. About 0.25 nmol of the enzyme dissolved in 70% formic acid was used. The C-terminal amino acid sequence was analyzed by the carboxypeptidase Y digestion method. Carboxypeptidase Y (0.5 nmol) was added to 28.6 nmol of the enzyme in 0.4 ml of 0.1 M pyridine-acetate buffer (pH 5.6) containing 1% SDS, and incubated at 25°C. Samples (60 μl) were withdrawn and the reaction was stopped by addition of 10 μl of glacial acetic acid. After addition of 430 μl of 0.02 M HCl, the sample was centrifuged at 15,000 × g for 10 min. The supernatant solution (250 μl) was put on the column (4 x 150 mm) of an amino acid analyzer (Hitachi model 835).

**Results**

**Purification of the enzyme from *C. pseudodiphtheriticum***

The enzyme was purified about 320-fold from the crude extract by this procedure (Table I). The purified enzyme is homogeneous.

**Table I. Purification of Leucine Dehydrogenase from *C. pseudodiphtheriticum* ICR 2210**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Total units</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>65,340</td>
<td>0.23</td>
<td>15,250</td>
<td>100</td>
</tr>
<tr>
<td>Protamine treatment</td>
<td>38,370</td>
<td>0.41</td>
<td>15,710</td>
<td>103</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>13,280</td>
<td>0.78</td>
<td>10,490</td>
<td>69</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>2,030</td>
<td>2.6</td>
<td>5,270</td>
<td>35</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>431</td>
<td>12.8</td>
<td>5,500</td>
<td>36</td>
</tr>
<tr>
<td>5’AMP-Sepharose 4B</td>
<td>57</td>
<td>23.3</td>
<td>1,330</td>
<td>8.7</td>
</tr>
<tr>
<td>Mono Q</td>
<td>13.3</td>
<td>74.0</td>
<td>981</td>
<td>6.4</td>
</tr>
</tbody>
</table>

**Fig. 2. Polyacrylamide Disc Gel Electrophoresis of the Purified Enzyme.**

(A): Purified enzyme (10 μg) was electophoresed at a current of 2.5 mA by the method of Davis.

(B): Purified enzyme was treated with 1% SDS and 0.1% 2-mercaptoethanol by the method of Weber and Osborn. The SDS-treated enzyme (5 μg) was electophoresed in the presence of 0.1% SDS at a current of 6 mA.
as judged by disc gel electrophoresis and SDS-disc gel electrophoresis (Fig. 2). The band stained for activity coincided with the protein band obtained by electrophoresis of the native enzyme.

Molecular weight and isoelectric point
The molecular weight of the enzyme was approximately 34,000 by gel filtration on a TSK G3000SW column. The value was not changed by the addition of 10% glycerol to the elution buffer. The molecular weight was also estimated to be about 34,000 by SDS-disc gel electrophoresis. These results suggested that the enzyme is composed of a single polypeptide chain. The isoelectric point of the enzyme was 5.5.

Stability
The enzyme could be stored at -20°C in 10 mM Tris- HCl buffer (pH 8.0) or potassium phosphate buffer (pH 7.4) containing 0.01% mercaptoethanol, 0.1 M KCl, and 50% glycerol for several months without apparent loss of activity. The enzyme was stable up to 35°C, when heated for 10 min in 10 mM potassium phosphate buffer (pH 7.4).

Effects of pH on the enzyme activity
The enzyme was most reactive at about pH 10.9 for the oxidative deamination of L-valine and L-leucine. The pH optima for the reductive amination of a-ketoisovalerate and a-ketoisocaproate were 9.5 and 10.0, respectively, in the presence of 0.75 mM \( \text{NH}_4\text{Cl}-\text{NH}_4\text{OH} \) buffer.

Substrate specificity
In addition to L-valine and L-leucine, which were the preferred substrates for the oxidative deamination, L-norvaline, L-isoleucine, L-allo-isoleucine, L-\( \alpha \)-aminobutyrate, L-norleucine, and DL-allylglycine served as substrates (Table II). All of the keto analogs of the substrates for the oxidative deamination served as substrates for the amination reaction (Table III). The best substrate was a-ketoisovalerate, a keto analog of valine. Ammonia was the exclusive substrate as an amino donor for the reductive amination of a-ketoisovalerate.


table II. Substrate Specificity for the Oxidative Deamination

<table>
<thead>
<tr>
<th>Substrates</th>
<th>( k_{cat} ) (sec(^{-1}))</th>
<th>( Km ) (mM)</th>
<th>( k_{cat}/Km ) (mM(^{-1}) sec(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Valine</td>
<td>41.8</td>
<td>0.57(^{a})</td>
<td>73.3</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>30.5</td>
<td>0.30(^{b})</td>
<td>101.7</td>
</tr>
<tr>
<td>L-Norvaline</td>
<td>15.5</td>
<td>0.54(^{c})</td>
<td>28.7</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>12.5</td>
<td>0.81(^{c})</td>
<td>15.4</td>
</tr>
<tr>
<td>L-( \alpha )-Isoleucine</td>
<td>11.7</td>
<td>2.2 (^{c})</td>
<td>5.32</td>
</tr>
<tr>
<td>L-( \alpha )-Aminobutyrate</td>
<td>7.52</td>
<td>2.7 (^{c})</td>
<td>2.78</td>
</tr>
<tr>
<td>L-Norleucine</td>
<td>4.18</td>
<td>2.8 (^{c})</td>
<td>1.49</td>
</tr>
</tbody>
</table>

\(^{a}\) Inert: L-alanine, L-arginine, L-aspartate, L-glutamate, L-histidine, L-proline, L-serine, L-threonine, L-tryptophan, D-valine, D-leucine, D-isoleucine, D-methionine, D-norvaline, D-norleucine, D-alanine, D-\( \alpha \)-aminobutyrate, D-aspartate, D-glutamate, D-lysine, D-serine, D-threonine, D-tryptophan, DL-homoserine, and 4-aza-L-\( \alpha \)-leucine.

\(^{b}\) The \( Km \) was obtained from the secondary plots of intercepts versus reciprocal concentrations of the substrate.

\(^{c}\) The apparent \( Km \) was calculated from Lineweaver-Burk plots.

Table III. Substrate Specificity for the Reductive Amination

<table>
<thead>
<tr>
<th>Substrates</th>
<th>( k_{cat} ) (sec(^{-1}))</th>
<th>( Km ) (mM)</th>
<th>( k_{cat}/Km ) (mM(^{-1}) sec(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-Ketoisovalerate</td>
<td>167</td>
<td>0.56(^{a})</td>
<td>298</td>
</tr>
<tr>
<td>DL-a-Keto-( \beta )-methylvalerate</td>
<td>134</td>
<td>1.25(^{a})</td>
<td>107</td>
</tr>
<tr>
<td>a-Ketoisocaproate</td>
<td>119</td>
<td>0.4 (^{a})</td>
<td>298</td>
</tr>
<tr>
<td>a-Ketovalerate</td>
<td>109</td>
<td>0.45(^{b})</td>
<td>242</td>
</tr>
<tr>
<td>( \alpha )-Ketobutyrate</td>
<td>92</td>
<td>1.4 (^{b})</td>
<td>65.7</td>
</tr>
<tr>
<td>( \alpha )-Ketocaproate</td>
<td>82</td>
<td>0.67(^{a})</td>
<td>122</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>3.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a-Ketoglutarate</td>
<td>0</td>
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\(^{a}\) The \( Km \) was obtained from the secondary plots of intercepts versus reciprocal concentrations of the substrate.

\(^{b}\) The apparent \( Km \) was calculated from Lineweaver-Burk plots.

Kinetics
The initial velocity was analyzed for the oxidative deamination by the method of Cleland.\(^{20}\) Double reciprocal plots of the initial velocity versus L-valine concentrations in the presence of various fixed concentrations of
Fig. 3. Double Reciprocal Plots of Initial Velocity versus L-Valine Concentration at a Series of Fixed Concentrations of NAD$^+$. The concentrations of NAD$^+$ were: 1, 0.05 mM; 2, 0.075 mM; 3, 0.1 mM; 4, 0.2 mM; and 5, 0.5 mM. The insert shows secondary plots of the intercepts versus the fixed NAD$^+$ concentrations.

NAD$^+$ gave intersecting straight lines (Fig. 3). This shows that the reaction proceeds through a sequential mechanism.\textsuperscript{20} The $K_m$ values for L-valine and NAD$^+$ were calculated to be 0.57 mM and 85 $\mu$m from the secondary plots of intercepts versus reciprocal concentrations of the other substrates. Kinetic analysis of the reductive amination was also done to find the $K_m$ values. The double reciprocal plots of velocities versus $\alpha$-ketoisovalerate concentrations at several fixed concentrations of NADH and a constant concentration of ammonia (0.2 M) gave straight lines intersecting on the abscissa. At a constant concentration of NADH (0.23 mM), the double reciprocal plots of velocities versus $\alpha$-ketoisovalerate concentrations at several fixed concentrations of ammonia gave similar intersecting straight lines. However, with $\alpha$-ketoisovalerate at a saturating concentration (10 mM), the double reciprocal plots of velocities versus NADH concentrations at several different concentrations of ammonia gave parallel lines. These observed kinetic patterns indicate a sequential ordered mechanism, where $\alpha$-ketoisovalerate binds to the enzyme between NADH and ammonia.\textsuperscript{20} The $K_m$ values for NADH, $\alpha$-ketoisovalerate, and ammonia were 81 $\mu$m, 0.56 mM, and 25 mM, respectively, from the secondary plots.

### Coenzyme Specificity

The enzyme required NAD$^+$ as a natural coenzyme for oxidative deamination and NADP$^+$ was virtually inert as a coenzyme. Some analogs of NAD$^+$ served as a coenzyme (Table IV). 3-Acetylpyridine-NAD$^+$ and deamino-NAD$^+$ were much better coenzymes than NAD$^+$.

### Inhibitors

The enzyme was inhibited strongly by p-chloromercuribenzoate (1 $\mu$m), HgCl$_2$ (10 $\mu$m), and 4,4'-dithiopyridine (0.1 mM). Cu$^{2+}$ inhibited it slightly (10%, at 1 mM). The other metal ions (1 mM) such as Mg$^{2+}$, Co$^{2+}$, and Mn$^{2+}$ were not inhibitory. EDTA, $\alpha,\alpha'$-dipyridyl, NaF, NaN$_3$, and Na$_2$SO$_4$ has no effect on the oxidative deamination of L-valine. D-Enantiomers of the substrate amino acids inhibited the reaction competitively against L-valine: the $K_i$ was 3.3 mM for D-valine, 1.3 mM for D-leucine, 2.6 mM for D-norvaline, 5.7 mM for D-$\alpha$-aminobutyrate, 7.6 mM for D-alloisoleucine, and 9.3 mM for D-isoleucine. When the enzyme was incubated with 1 mM pyridoxal 5'-phosphate in 0.2 M potassium phosphate.
Table V. Comparison of N-Terminal Amino Acid Sequence of C. pseudodiphtheriticum Leucine Dehydrogenase with Those Reported for B. cereus, B. caldolyticus, B. stearothermophilus, and Cl. thermoaceticum Leucine Dehydrogenases

<table>
<thead>
<tr>
<th></th>
<th>Gly-Val-Phe-Thr-His-Val-Asp-Phe-</th>
<th>Met-Thr-Leu-Glu-Ile-Phe-Glu-Tyr-Leu-Glu-Lys-</th>
<th>Met-Glu-Leu-Phe-Gln-Tyr-Met-Glu-Lys-</th>
<th>Met-Glu-Leu-Phe-Lys-Tyr-Met-Glu-Thr-</th>
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<tbody>
<tr>
<td>C. pseudodiphtheriticum</td>
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<tr>
<td>B. cereus</td>
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<td>B. caldolyticus</td>
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<td>B. stearothermophilus</td>
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<td>Cl. thermoaceticum</td>
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buffer (pH 8.0) at 30°C for 30 min and then assayed at pH 8.0 in the presence of 0.2 mM pyridoxal 5'-phosphate, the enzyme activity decreased to 34% of that of the native enzyme.

N-Terminal and C-terminal amino acid sequences

The first 24 amino acids at the N-terminus of the enzyme were identified by Edman degradation as shown in Table V (JIPID Accession PT0076). The C-terminal amino acid sequence was -Gly-Leu.

Discussion

Leu DH and Val DH are functionally analogous, because they catalyze the oxidative deamination of branched-chain and straight-chain L-amino acids and the reductiveamination of their keto analogs. These enzymes, however, are different in the preference for the substrate in the oxidative deamination: the best substrate of Leu DH is L-leucine, but that of Val DH is L-valine. Leu DHs from bacilli and clostridia and Val DHs from streptomyces have been characterized. However, these enzymes from nonsporing bacteria had not been purified and characterized. We characterized the enzyme that catalyzes the reversible deamination of branched-chain L-amino acids from a nonsporing aerobic bacterium, C. pseudodiphtheriticum ICR 2210. L-Valine was the best substrate for the deamination at pH 10.9 and the saturated concentration. Defining the enzyme on the basis of its substrate specificity, the Corynebacterium enzyme might be named Val DH. It, however, showed a higher reactivity for L-leucine (73% relative to L-valine) and L-ketoisocaproate (71% relative to L-ketoisovalerate), but L-leucine and L-ketoisocaproate were poor substrates for Val DHs from streptomyces. Comparison of the $k_{cat}/K_m$ values for L-leucine and L-valine (Table II) suggested that the most favorable substrate of the Corynebacterium enzyme was L-leucine. Moreover, at physiological pH, L-leucine was a better substrate than L-valine. The Corynebacterium enzyme was NAD⁺ specific; this is similar to all Leu DHs studied so far. Val DH from streptomyces could use NDAP⁺ as a coenzyme, though the reactivity was lower than NAD⁺ (8.4 to 10% relative to NAD⁺). Thus, we named the Corynebacterium enzyme Leu DH.

The Corynebacterium enzyme is unique in the sense that it is a monomer, while Leu DHs from the endospore-forming bacteria are composed of six or eight identical subunits and Val DH from streptomyces is a decamer, a tetramer, or a dimer. This is the first example of a monomeric Leu DH. Among the amino acid dehydrogenases studied so far, the monomeric structure has been reported only for alanine dehydrogenase from halophilic bacteria. Although the
N-terminal amino acid sequence was highly conserved in the Leu DHs from the endospore-forming bacteria, that of the Corynebacterium enzyme differed from those of the Bacillus\(^3,5,7,23\) and Clostridium\(^3\) Leu DHs (Table V). Some homology, however, was observed between them: identical residues occur at positions 3, 12–14, 19, 23, and 24. These facts suggest that the Corynebacterium enzyme is distantly related to the other Leu DHs. The B. stearothermophilus gene for Leu DH has been cloned and sequenced.\(^23\) Considerable DNA homology was seen between the nucleotide-binding domains of Leu DH and NAD\(^+\)-dependent glutamate dehydrogenase.\(^23\) Cloning and DNA sequencing of the Corynebacterium enzyme gene will provide insights into the primary and secondary structures and will define the nucleotide-binding and catalytic domains of the enzyme. The N-terminal amino acid sequence should facilitate the synthesis of an appropriate oligonucleotide for the use as a probe in cloning the Corynebacterium enzyme gene.

The Corynebacterium enzyme was inhibited by pyridoxal 5'-phosphate. The inhibition is probably due to the formation of a Schiff base between a lysine residue of the enzyme and the 4-formyl group of pyridoxal 5'-phosphate as reported for the Bacillus enzyme\(^24\) and glutamate dehydrogenase.\(^25\) The Corynebacterium enzyme was also inhibited by sulfhydryl reagents and \(\alpha\)-enantiomers of the substrates. In this regard, the enzyme is analogous to the Leu DH from B. sphaericus.\(^2\) Kinetic study of the Corynebacterium enzyme showed that the \(K_m\) for NH\(_3\) is 25 mm, which is different from that of the B. sphaericus enzyme (200 mm)\(^2\) and the B. cereus enzyme (220 mm).\(^3\) but is similar to that of the B. subtilis enzyme (13 mm).\(^4\) Leu DH in B. subtilis has a catabolic function\(^26\) and is important in spore germination.\(^27\) Although the physiological role of the enzyme in C. pseudodiphtheriticum has not yet been established, the enzyme may function in the catabolism of branched-chain \(\alpha\)-keto acids, because this bacterium can not use commonly used carbohydrates, but can use amino acids as a carbon source.\(^28\) Formation of NADH and branched-chain \(\alpha\)-keto acids is important for energy production and for the synthesis of branched-chain fatty acids which are needed for membrane phospholipids. The biochemical characterization of a mutant defective in Leu DH synthesis will be required for understanding of the physiological function of the enzyme.

References