Evidence for Lysine 80 as General Base Catalyst of Leucine Dehydrogenase*

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To elucidate the functional role of the lysyl residue highly conserved in NAD(P)*-dependent amino acid dehydrogenases, Lys-80 of leucine dehydrogenase from Bacillus stearothermophilus has been mutated into Ala, Arg, or Gln. All of the mutant enzymes had markedly reduced activities in the oxidative deamination, whereas the Michaelis constants for substrate and coenzyme did not change significantly upon the mutation, except for a 10-30-fold increase in $K_m$ values for $\alpha$-keto-iso-caproate in the Ala and Gln mutants. The pH profiles of kinetic parameters of the mutants considerably differed from those of the wild type, in which two isozymic groups with $pK_a$ values of 8.9 and 10.7 must be unprotonated for catalysis and protonated for substrate binding, respectively. Combined with the analyses of solvent isotope effect and inhibition by substrate analogs, these results unequivocally show that the $\epsilon$-amino group of Lys-80 participates in catalysis as a general base, assisting the nucleophilic attack of a water molecule to the substrate $\alpha$-carbon atom. Furthermore, the Ala mutant was markedly stimulated by primary amines depending on the $pK_a$ and molecular volume, suggesting that in the Ala mutant the added amines can partially replace the general base function of Lys-80 in the wild type enzyme.

Leucine dehydrogenase (EC 1.4.1.9), which belongs to a family of NAD(P)*-dependent oxidoreductases acting on amino acids, catalyzes the reversible deamination of L-leucine and some other branched chain L-amino acids to their keto analogs, serving in the first step of bacterial catabolism of these amino acids (1). The enzyme, occurring mainly in Bacillus species (2), has been purified from Bacillus sphaericus (3) and Bacillus stearothermophilus (4) and characterized for its enzymological properties including the B-type stereospecificity of the hydride transfer. The gene encoding 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 46,900 and corresponds to a subunit of the hexameric enzyme. Sequence comparison with other amino acid dehydrogenases, including glutamate (6-15), phenylalanine (16, 17), and alanine (18) dehydrogenases, revealed significant identities in the catalytic and coenzyme binding domains (5, 19). The reaction catalyzed by the enzyme has been proposed to proceed according to the ordered bi-ter mechanism, in which NAD* and L-leucine are bound and NADH, $\alpha$-keto-iso-caproate, and $\alpha$-hydroxyisocaproate are released in that order (2).

Recently, the X-ray crystallographic structure of NAD*-linked glutamate dehydrogenase from Clostridium symbiosum was solved to 1.96 Å resolution (20), and the molecular basis for the stereospecificity of the hydride transfer was proposed as well as the charge-site model involving a cluster of 3 conserved lysyl residues, which are situated close to the B face of the nicotinamide ring of the bound cofactor and presumably interact with the negative charges of substrate (glutamate/$\alpha$-ketoglutarate). Although Lys-128 of NADP*-dependent glutamate dehydrogenase from E. coli, corresponding to Lys-125 of the 3 conserved lysyl residues in the clostridial enzyme, has already been shown by site-directed mutagenesis to participate in either direct or indirect interactions with substrate and in catalysis (21), the precise assignment of the functional roles of the conserved residues remains to be further studied. More recently, Singh et al. (22) proposed the catalytic mechanism of the early steps in the oxidative deamination of glutamate dehydrogenase based on the novel transient-state kinetic analysis of the bovine liver enzyme and deduced the roles of the 3 lysyl residues.

We have identified an active-site lysyl residue (Lys-80) of B. stearothermophilus leucine dehydrogenase by modification with pyridoxal phosphate (19). Lys-80 of leucine dehydrogenase corresponds to Lys-125 of the clostridial glutamate dehydrogenase (and its equivalents in other glutamate dehydrogenases) and is contained in a glycine-rich sequence conserved in all of the amino acid dehydrogenase sequences reported so far. In the present study, to elucidate the functional role of Lys-80 of leucine dehydrogenase, we have substituted Ala, Gln, or Arg for Lys-80 by site-directed mutagenesis and analyzed the kinetic properties of the mutant enzymes. Here we provide the evidence that the $\epsilon$-amino group of Lys-80 participates in catalysis as a general base by promoting the nucleophilic attack of a water molecule to the $\alpha$-carbon atom of the putative imine intermediate. The assigned function of Lys-80 in leucine dehydrogenase corresponds well with the proposed catalytic role of the lysyl residue in the conserved glycine-rich sequence of glutamate dehydrogenases. The marked stimulation by primary amines of the Lys-80 $\rightarrow$ Ala mutant leucine dehydrogenase is also reported.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—A HindIII-KpnI fragment (about 2 kilobase pairs) excised from pBLeuDH, which contains the entire coding
Role of Lys-80 in Leucine Dehydrogenase

**Lys-80 → Ala:** 5'-GACCCTTGCCCCTGCCC-3'

**Lys-80 → Arg:** 5'-GACCCTTGCCCCTGCCC-3'

**PRIMERS 1-3**

These primers were synthesized with an Applied Biosystems DNA synthesizer model 381. Synthesis and selection of mutant DNA were performed by the method of Kunkel et al. (23) using a commercial kit (Muta-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 (24). The 1-kilo-base pair HindIII-Sall fragment containing the mutant site was excised from the double-stranded M13mp19 phage DNA, ligated into the HindIII-Sall site of pBluescript, and transformed into E. coli JM109 cells.

**Purification of the Wild Type and Lys-80 Mutant Enzymes—** E. coli JM109 cells carrying pBluescript or the mutant plasmid were grown at 37 °C for 12 h in 1 liter of Luria broth (1.0% tryptone, 1.0% NaCl, and 0.6% yeast extract) containing 50 µg/ml sodium ampicillin and 0.5% yeast extract and 0.6% glucose. Cells harvested by centrifugation were washed with 500 ml of 0.85% NaCl. The wild type and mutant enzymes were purified to homogeneity by the procedure reported previously (19).

**CD Measurements—** CD spectra of the wild type and mutant enzymes were measured at 25 °C in 10 mM potassium phosphate buffer, pH 7.2, with a Jasco model J-600 spectropolarimeter. The protein concentration was 0.2 mg/ml in a 2.0-cm light path cuvette for measurement in the wavelength region above 250 nm and 0.1 mg/ml in a 0.1-cm light path cuvette for measurement in the wavelength region below 250 nm. Thermal stabilities were analyzed by monitoring the protein unfolding process by heat following the changes in ellipticity at 222 nm. All measurements were carried out in 10 mM potassium phosphate buffer, pH 7.2, at a protein concentration of 0.5 mg/ml. The temperature was raised at a constant rate of 1 °C/min through a thermostatic cell holder and was measured directly in the cuvette with a Rikagaku Kogyo model DP-500 microthermometer.

**Enzyme Assay and Steady-state Kinetic Analysis—** The standard assay mixture for the oxidative deamination contained 0.1 M sodium carbonate, pH 10.5, 10 mM L-leucine, and 1.25 mM NAD⁺ in a total volume of 1.0 ml. The reaction was started by the addition of the enzyme, and the change in absorbance at 340 nm was monitored continuously with a spectrophotometer at 25 °C. To obtain steady-state kinetic parameters, initial velocities in the oxidative deamination were measured with five different concentrations of NAD⁺ at four fixed concentrations of L-leucine.

**RESULTS**

**Purification and Physical Characterization of Lys-80 Mutant Enzymes of Leucine Dehydrogenase—** Lys-80 of *B. stearothermophilus* leucine dehydrogenase was replaced with Ala, Gin, or Arg by site-directed mutagenesis using the synthetic oligonucleotides as primers.***

Prior to the analysis of pH dependence of the kinetic properties, the following three oligonucleotide primers were designed to be complementary to this template DNA and to contain appropriate mismatching bases (asterisked) in the codon for Lys-80 (underlined).

**Effect of Primary Amines on the Lys-80 → Ala Mutant Enzyme—** The effect of added primary amines on the oxidative deamination of Lys-80 → Ala was examined by measuring the initial rate, kobs (s⁻¹), of the reaction at 25 °C in the assay mixture (1.0 ml) containing 0.8 mM Lys-80 → Ala mutant enzyme, 0.1 M CAPS-tetramethylammonium hydroxide, pH 10.5, 10 mM L-leucine, 1.25 mM NAD⁺, and various concentrations (10–50 mM) of 11 different primary amines, which had been titrated in advance to pH 10.5 with tetramethylammonium hydroxide; pH 10.5 with tetramethylammonium hydroxide; and 0.5 M tetramethylammonium chloride. The rate constants, k₉ (M⁻¹·s⁻¹), corrected for the concentration of a free base form of each amine at a given pH, were calculated by linear regression of the kobs values to Equation 5 (27), where k₉ is the observed rate constant in the absence of amines.

For generalization, the k₉ values were then fitted to a multiple linear form (Equation 6) with the pKₐ values and solvent-excluded molecular volumes (V) of amines, taken from Jencks and Regenstein (28) and Toney and Kirsch (27), as independent variables, and C as the constant term.

The abbreviations used are: MES, 2-(N-morpholino)ethanesulfonic acid; Bicine, and CAPS and tritiated to the desired pH values with NaOH. The ionic strengths of buffers were maintained at 0.1 by the addition of an appropriate amount of NaCl calculated according to Ellis and Morrison (26). For determination of Kₛ for a keto acid analog, the buffer in the reductive amination was 1 M NH₄Cl titrated with NH₄OH to the desired pH values, and the ionic strengths were adjusted to 1.0 with NaCl. Maximal activity (V) was expressed as turnover numbers (s⁻¹) using a calculated molecular weight of 46,900/subunit (5), each having one active site. The variations with pH of the values for V, Kₛ, and V/K were analyzed by fitting the data to Equations 1, 2, and 3 using the programs HABELL, HBBELL, and BELL, respectively (25).

\[
\log y = \log \left( \frac{C}{1 + [H^+] / K_b} \right) \quad (1)
\]

\[
\log y = \log \left( \frac{C}{1 + K_b / [H^+]} \right) \quad (2)
\]

\[
\log y = \log \left( \frac{C}{1 + K_b / [H^+] + K_a / [H^+]} \right) \quad (3)
\]

In these equations, y represents the value of V, V/K, or 1/K. For each particular pH, C is the pH-independent value of the parameter, and Kₛ and K₉ are acid dissociation constants associated with ionizable groups on the acid limb and alkaline limb, respectively, of pH profiles. The enthalpies of ionization (ΔHᵢₒₐₜ) for the ionizable groups were calculated by fitting apparent pK values obtained at different temperatures (T in Kelvin scale) to Equation 4, where R is the gas constant, 1.987 cal mol⁻¹ deg⁻¹.

\[
\Delta H_{iₒₐₜ} = 2.303RT + C \quad (4)
\]

\[
\log k_a = \log k_{9,[amine]}_{total} + \log 1 + (1/[H^+]) \quad (5)
\]

\[
\log k_a = \delta(pK_a) + \delta'(V) + C \quad (6)
\]

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\[
\log k_a = \delta(pK_a) + \delta'(V) + C \quad (6)
\]
procedure as the wild type enzyme (19), which involves heat treatment (at 70 °C for 30 min) and a single step of ion exchange chromatography (DEAE-Toyopearl). All of the wild type and Lys-80 mutant enzymes were eluted from the ion exchange column at the same salt concentration, 160 mM KCl, indicating similar overall available charges on the wild type and mutant proteins.

CD spectra of the wild type and three Lys-80 mutant enzymes purified were measured to see whether or not global conformational changes were brought about by the amino acid replacement of Lys-80. The spectra of mutant enzymes, after correction at the same protein concentration, overlapped almost completely with that of the wild type enzyme in the wavelength region of 200–250 nm with a mean residue ellipticity (θ) of about −13,200° at 222 nm. In the wavelength region of 250–320 nm, which manifests ellipticities of aromatic residues, the spectra of mutant enzymes except for Lys-80 → Ala are also nearly identical with that of the wild type protein (θ ≈ −63° at 276 nm). This suggests that the replacement of Lys-80 with Ala, Arg, or Gln does not affect the secondary structure content of the enzyme, and therefore conformational changes caused by the mutation of Lys-80 are very minor, if any. However, the slight increase in ellipticity of the Lys-80 → Ala mutant protein in the region of 260–290 nm (θ ≈ −82° at 276 nm) indicates that the replacement of Lys-80 with the smaller residue Ala leads to a slight conformational change of nearby aromatic residues.

Conformational stabilities of the wild type and Lys-80 mutant proteins were analyzed by monitoring the thermal unfolding process following the changes in ellipticity at 222 nm. Tm values, the temperatures at the midpoint of the unfolding transition, calculated according to the method of Pace et al. (29), of the wild type and Lys-80 → Ala, Lys-80 → Arg, and Lys-80 → Gln mutant proteins were 79.9, 74.7, 78.6, and 78.7 °C, respectively. The replacement of Lys-80 with Arg or Gln had little effect on the conformational stability of the enzyme protein, although that with Ala slightly reduced the conformational stability.

The pH stabilities of the wild type and Lys-80 mutant enzymes were also examined by measuring the remaining activity after incubation at 25 °C for 30 min in 0.1 M HEPES, pH 7.0–7.5; TAPS, pH 8.0–8.5; CHES, pH 9.0–10.0; CAPS, pH 10.5–11.0; or potassium phosphate, pH 11.0–12.0. The wild type enzyme and all of the mutant enzymes lost no activity on incubation in the pH range 7.0–11.2, showing that they have similar pH stabilities.

Steady-state Kinetic Analysis—Leucine dehydrogenase from _B. sphaericus_ has been shown to catalyze the reaction according to the ordered bi-ter mechanism, in which NAD+ and L-leucine are bound and NH2-α-keto-iso-caproate, and NADH are released in that order (2). In the present study, we confirmed by steady-state kinetic analysis that the reaction by the recombinant _B. stearothermophilus_ enzyme is also fully consistent with proceeding through the ordered bi-ter mechanism (data not shown). However, in the product inhibition analysis in which L-leucine is supposed to show uncompetitive inhibition with respect to the keto acid in the reverse reaction, noncompetitive inhibition was observed, suggesting that L-leucine also binds to the free enzyme to form, for example, a dead-end complex.

Steady-state kinetic parameters of the wild type and Lys-80 mutant enzymes were measured at 25 °C by systematic variations of substrate and coenzyme concentrations (Table 1). In the oxidative deamination, the kmax values of the mutant enzymes were only 0.2–1.6% of that of the wild type. In contrast, those values in the reductive amination were quite variable among the Lys-80 mutant enzymes; Lys-80 → Ala showed nearly 90% of the wild type activity, whereas kmax of Lys-80 → Arg was comparable in its activity to the oxidative deamination (0.6% of the wild type activity). The considerable variation in kmax values of the reductive amination among the mutant enzymes is because of the difference in degree of stimulation of the mutant enzymes by one of the substrates, ammonia, as described below.

The Km values for NAD+ and NADH are essentially unchanged in the wild type and Lys-80 mutant enzymes, suggesting that the replacement of Lys-80 with other residues has little effect on the affinity for the nicotinamide coenzyme. In contrast, significant changes in Km values were observed for substrate; Lys-80 → Ala and Lys-80 → Gln showed the values for α-keto-iso-caproate 11- and 28-fold, respectively, larger than that of the wild type, whereas Lys-80 → Arg had a Km for the keto acid essentially identical to that of the wild type. The Km value for L-leucine of only Lys-80 → Gln is about three times larger than that of the wild type, but those of the other two mutants are even smaller than that of the wild type. These results suggest that the presence of a basic group (an amino or guanidino group) at residue 80 is preferable for efficient binding of the keto acid, whereas the basic group may be unimportant for binding of the amino acid. The presence of the bulky, neutral carboxamide group in Lys-80 → Gln leads to the increase in Km values for amino and keto acids probably by both steric and charge effects. The Km value for ammonia of the wild type enzyme in the reductive amination was determined to be 74.8 ± 9.7 mM, but those values of the Lys-80 mutant enzymes could not be determined because of the marked stimulation by ammonia, the initial rates being unsaturable with increasing concentrations (up to 0.2 M) of ammonia (see below).

**Kinetic Isotope Effect**—To see the kinetic isotope effect in the oxidative deamination, in which an imine intermediate would be first formed from L-leucine by oxidation with the bound NAD+ followed by the nucleophilic attack of a water molecule at the α-carbon atom of the putative imine as will be discussed later, the reaction was performed in both H2O and D2O for the wild type and Lys-80 → Ala mutant enzymes under the standard assay conditions except for varying the concentrations (1.5–10 mM) of L-leucine. The kcat values of the wild type enzyme were 39.1 s−1 in H2O and 29.7 s−1 in D2O, giving a kinetic isotope effect of 1.32, whereas those of the Lys-80 → Ala mutant enzyme were 1.17 s−1 in H2O and 0.368 s−1 in D2O, giving a kinetic isotope effect of 3.18. The large deuterium isotope effect of the solvent shows that the nucleophilic attack of a water molecule is a rate-limiting step in the oxidative deamination particularly for the Lys-80 → Ala mutant enzyme.

**Inhibition by Keto Acid Analogs**—Since the Lys-80 → Ala mutant enzyme showed a specific increase in the Ks value for only α-keto-iso-caproate, we analyzed inhibition of the wild type and that particular mutant by keto acid analogs in the reductive amination (Table 1). Iso-caproate, an analog without a carbonyl group, inhibited the reductive amination competitively with α-keto-iso-caproate for both the wild type and Lys-80 → Ala mutant enzymes. The Ks values for iso-caproate of both enzymes were considerably larger than the Ks value of the wild type enzyme for α-keto-iso-caproate (see Table 1), and the difference between the Ks values of the wild type and mutant enzymes is insignificant as compared with the 11-fold difference between the Ks values of both enzymes for α-keto-iso-caproate. Therefore, the α-carbonyl group in the keto acid substrate is playing a role in lowering the Ks value of the wild type enzyme but not of Lys-80 → Ala. Thus, it is suggested
Role of Lys-80 in Leucine Dehydrogenase

Steady-state kinetic parameters of the wild type and Lys-80 mutant leucine dehydrogenases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$</th>
<th>$K_m$</th>
<th>$V/K$</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Deamination</td>
<td>Amination</td>
<td>NAD$^+$</td>
<td>L-Leucine</td>
</tr>
<tr>
<td>Wild type</td>
<td>50 ± 3 s$^{-1}$</td>
<td>280 ± 22</td>
<td>0.063 ± 0.007</td>
<td>5.1 ± 0.5</td>
</tr>
<tr>
<td>Lys-80 → Ala</td>
<td>0.75 ± 0.04</td>
<td>250 ± 15</td>
<td>0.14 ± 0.01</td>
<td>3.7 ± 0.5</td>
</tr>
<tr>
<td>Lys-80 → Arg</td>
<td>0.11 ± 0.01</td>
<td>1.7 ± 0.1</td>
<td>0.074 ± 0.012</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>Lys-80 → Gln</td>
<td>0.54 ± 0.13</td>
<td>66 ± 14</td>
<td>0.034 ± 0.015</td>
<td>17 ± 6</td>
</tr>
</tbody>
</table>

* Determined in the presence of 1 M ammonia.

Inhibition of the wild type and Lys-80 → Ala mutant enzymes by keto acid analogs

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Iso-caproate</th>
<th>4-Methyl-2-pentanone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pattern</td>
<td>$K_i$</td>
</tr>
<tr>
<td>Wild type</td>
<td>Competitive</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>Lys-80 → Ala</td>
<td>Competitive</td>
<td>33 ± 3</td>
</tr>
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<td></td>
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</tbody>
</table>

FIG. 1. pH dependences of steady-state kinetic parameters in the oxidative deamination. $V/K$ for L-leucine (A) and $V$ (B) of wild type enzyme (○), Lys-80 → Ala (□), Lys-80 → Gln (△), and Lys-80 → Arg (●). The units for $V$ and $V/K$ are s$^{-1}$ and M$^{-1}$·s$^{-1}$, respectively. The curves are a least squares best fit to Equation 1 or 3.

That the $\epsilon$-amino group of Lys-80 participates at least partly in binding of the keto acid by interacting with its $\alpha$-carboxyl group. On the other hand, 4-methyl-2-pentanone, an analog without the $\alpha$-carboxyl group, also inhibited the wild type enzyme competitively with $\alpha$-keto-iso-caproate, although very weakly. However, this analog behaved as a noncompetitive inhibitor for the Lys-80 → Ala mutant with a similar $K_i$ value, indicating that it also binds to the enzyme in a state different from the enzyme-NADH binary complex. The extremely large $K_i$ values for 4-methyl-2-pentanone suggest the main contribution of the $\alpha$-carboxyl group of substrate in binding with the enzyme (with a residue other than Lys-80).

pH Dependence of Kinetic Parameters—The oxidative deamination by the wild type and Lys-80 mutant enzymes was measured over a pH range from 7.5 to 11.2 using various concentrations of L-leucine and a fixed, saturating concentration (5 mM) of NAD$^+$; under these conditions, the kinetic parameters determined are those for L-leucine reacting with the enzyme-NAD$^+$ binary complex. As shown in Fig. 1A, the pH profile of log($V/K$) of the wild type enzyme was convex upward (bell-shaped) giving pK values of 8.9 ± 0.1 for a group on the acid limb and 10.7 ± 0.1 for a group on the alkaline limb (calculated by fitting the data to Equation 3). The group on the acid limb must be unprotonated, and that on the alkaline limb must be protonated for catalysis (for binding of L-leucine and/or the reaction thereafter). With Lys-80 → Ala and Lys-80 → Gln, the high pK group was unobservable, in addition to the considerable shift of pK values of the group on the acid limb toward an alkaline side ($pK = 9.4 ± 0.1$ in the Ala mutant and $pK = 9.3 ± 0.1$ in the Gln mutant, fitted to Equation 1), whereas Lys-80 → Arg had two groups with $pK = 8.1 ± 0.1$ and 11.1 ± 0.1 in the convex profile. In the pH profile of log($V$) shown in Fig. 1B, the wild type and all Lys-80 mutant enzymes had only a single ionizable group on the acid limb ($pK = 8.6 ± 0.1$ in the wild type; 9.1 ± 0.1 in Lys-80 → Ala; 9.1 ± 0.1 in Lys-80 → Gln; and 7.9 ± 0.1 in Lys-80 → Arg). Thus, the protonated group with $pK$ of 10.7 seen in the convex profile of log($V/K$) is not directly involved in the catalytic process.

To characterize the ionizable groups observed in the pH profiles of kinetic parameters, the pH dependence of log($V$/$K$) of the wild type enzyme was analyzed at three different temperatures (18, 25, and 35 °C). Then, the enthalpies of ionization ($\Delta H_{\text{ion}}$) were calculated by fitting the apparent pK values to Equation 4. The low pK group had a $\Delta H_{\text{ion}}$ value of about 14.4 kcal/mol, and the high pK group had a $\Delta H_{\text{ion}}$ value of about 12.8 kcal/mol. These results suggest that the two groups are both assigned to an $\epsilon$-amino group of lysyl residues having a large enthalpy of ionization ($\Delta H_{\text{ion}} = 10–13$ kcal/mol) (30).

We also examined the pH dependence of pK for iso-caproate, competing with $\alpha$-keto-caproate in the reductive amination (see above) and found that a single group with pK = 10.1 ± 0.1 (fitted to Equation 2) in the wild type and 10.5 ± 0.1 in Lys-80 → Ala is involved in binding of iso-caproate. This group corresponds to the high pK group observed in the pH profiles of log($V$/$K$) described above and probably participates in binding of the $\alpha$-carboxyl group of substrate (inhibitor) in a protonated form.

Stimulation by Primary Amines—In view of the initial rate of the Lys-80 → Ala mutant in the reductive amination being unsaturable with increasing concentrations of ammonia and the strikingly large difference in $k_{\text{cat}}$ values of the reductive amination among the three Lys-80 mutant enzymes, we presumed that the substrate ammonia might have a stimulatory effect on the mutant enzymes. Hence, we examined the effect of ammonia on the oxidative deamination of the wild type and Lys-80 mutant enzymes by assaying in the presence of various concentrations (up to 1 M) of ammonia (Fig. 2). Indeed, ammonia offered a markedly large stimulatory effect on the Lys-80 → Ala mutant enzyme. Although the stimulation was unsaturable even at 1 M ammonia, the mutant


enzyme showed 20% of the wild type enzyme activity at this concentration. The Lys-80 → Ala mutant enzyme was also activated but to a much lesser extent, and the wild type and Lys-80 → Arg mutant enzymes were activated negligibly. The $k_{\text{max}}$ value of the Lys-80 → Ala mutant in the oxidative deamination determined in the presence of 1 M ammonia is shown in Table I; the $K_w$ values for NAD$^+$ and l-leucine of the mutant enzyme were also affected considerably. The degree of stimulation by ammonia roughly corresponds with the difference in $k_{\text{max}}$ values of the mutant enzymes in the reductive amination. The discrepancy is probably because ammonia is the substrate in the reductive amination but is the product in the oxidative deamination causing the product inhibition at a high concentration. The exceptionally large stimulation of Lys-80 → Ala by ammonia suggests that this mutant with the smallest side chain has an enough space to accommodate ammonia taking the place of the ε-amino group of Lys-80. The absence of the stimulation in the wild type and Lys-80 → Arg mutant enzymes may be ascribed either to the charge-repulsion between ammonium ion and the amino or guanidino group (although the free base form appears to participate directly in the stimulation as described below) or simply to the unavailability of enough space at the active site.

The stimulatory effect on the Lys-80 → Ala mutant enzyme was further surveyed with other primary amines. In a preliminary experiment, it was verified that primary amines in a concentration range of 10-500 mM neither served as substrate in the reductive amination nor inhibited the oxidative deamination of the wild type enzyme (data not shown). The addition of various amines resulted in an acceleration in various degrees of the oxidative deamination of Lys-80 → Ala but not of the wild type. The observed first-order rate constant, $k_{\text{obs}}$, was linearly dependent on the concentration of amines; no saturation was observed even at a concentration above 400 mM, showing that amine binding is rather weak. In addition, the $k_{\text{obs}}$ values were found to be dependent on pH, indicating that the catalysis is caused by the free base form of the amine. Thus, the values of the rate constants, $k_b$, for the reaction accelerated by amines were calculated according to Equation 5 (27) and are collected in Table III, together with the $pK_w$ values and solvent-excluded molecular volumes of 11 primary amines examined.

As has been first demonstrated in the stimulation of the Lys-258 → Ala mutant enzyme of aspartate aminotransferase by exogenous amines (27), a plot of $\log(k_b)$ for the Lys-80 → Ala mutant of leucine dehydrogenase versus $pK_w$ for the five amines of nearly constant molecular volumes (64.4-74.6 Å$^3$, see Table III) exhibited good linearity (Fig. 3A, slope $\beta = 0.21 \pm 0.04$), indicating the importance of amine basicity. Similarly, a plot of $\log(k_b)$ versus molecular volumes of methyl-, ethyl-, propyl-, and butylamine having similar $pK_w$ values showed a good linear relationship (Fig. 3B, slope $\beta = (-0.022 \pm 0.002) Å^{-3}$). This indicates that a steric factor is also important in determining the rate constants for the amine-assisted reaction. Taken together, the oxidative deamination by Lys-80 → Ala is accelerated by a variety of primary amines depending on both their basicities and molecular volumes. Therefore, to generalize the two effects for various amines, $k_b$ values were fitted to a multiple linear form (Equation 6) with the $pK_w$ and solvent-excluded molecular volume ($V$) of the amine as independent variables (27). The least squares best fit of the data gave $\beta = 0.19 \pm 0.05$, $\beta' = (-0.020 \pm 0.005) Å^{-3}$, and $C = 0.02 \pm 0.53$; $\beta$ and $\beta'$ corresponded well with those obtained above, respectively, with each subset of the amines. However, these values are 2-3-fold smaller than those determined with the Lys-258 → Ala mutant of aspartate aminotransferase ($\beta = 0.39$, $\beta' = -0.055 Å^{-3}$), indicating that the degree of stimulation by amines of Lys-80 → Ala mutant of leucine dehydrogenase is smaller by a factor of 10$^2$ to 10$^3$. Nevertheless, a Brønsted type plot of $\log(k_b)$ versus ($pK_w$) demonstrated a linear relation between...
log($k_b$) and $pK_a$ (not shown), supporting the role of the ε-amino group of Lys-80 as a general base.

**DISCUSSION**

The lysyl residue corresponding to Lys-80 of leucine dehydrogenase is conserved in the sequences of all amino acid dehydrogenases reported so far, irrespective of their differences in substrate and coenzyme specificities and oligomeric structures (5, 17, 19, 21, 31). The strict conservation suggests this lysyl residue occurring at the active site and bearing a functional importance common to this family of enzymes. In the present study, we have replaced Lys-80 of leucine dehydrogenase with Ala, Arg, or Gln by site-directed mutagenesis. Evidence for similar overall structures among the Lys-80 mutant enzymes prepared was provided by their similarities to the wild type enzyme in available charges on the protein surface, global conformations reflected in the CD spectra, and conformational and pH stabilities. Therefore, the profound differences in kinetic properties were the results of specific effects on the catalytic function produced by changing the side chains and not of global structural alterations.

The pH profiles of log(V) and log(V/K) for L-leucine (Fig. 1) revealed the involvement in the wild type enzyme of two ionizable groups with $pK_a$ values of about 8.9 and 10.7, which must be unprotonated for catalysis and protonated for binding of substrate, respectively. The two groups were both assigned to amino groups of the protein on the basis of the large enthalpies of ionization ($\Delta H_{ion}$). Taking into consideration of the pronounced change in $K_{m}$ value of the Lys-80 mutant enzymes and not in $K_{m}$ values for L-leucine and NAD$^+$ in the oxidative deamination (Table 1), the low $pK_a$ group is ascribable to the ε-amino group of Lys-80, whereas the high $pK_a$ group may be that of a lysyl residue other than Lys-80. Thus, Lys-80 appears to be mainly involved in catalysis but not in binding of substrate.

The unusually low $pK_a$ value of Lys-80 is consistent with its high reactivity in the chemical modification with pyridoxal phosphate (19), like the corresponding Lys of glutamate dehydrogenase. This unusual $pK_a$ value is consistent with its role as a general base.

The $pK_a$ value of Lys-80 is much decreased but still significantly high for the mutants of an active-site residue, strongly suggesting that Lys-80 does not play a direct role in catalysis. Rather, it is conceivable that the ε-amino group of Lys-80 serves as a general base to raise the nucleophilicity of a water molecule attacking the α-carbon atom of the imino acid intermediate putatively formed following the initial oxidation of 1-leucine by NAD$^+$ (see below). Thus, even without this basic group (i.e. in the Lys-80 → Ala and Lys-80 → Gln mutant enzymes), the reaction would proceed at a reduced rate depending on the concentration of the free base OH$^-$ in the solvent, which is a nucleophile stronger than H$_2$O. Therefore, we interpret that the $pK_a$ of 9.3–9.4 seen in the pH-log(V/K) profiles of the two mutant enzymes (Fig. 1) is not of a real group of the enzyme but could be caused by the compensation of the increase in rate constants by the decrease in the high $pK_a$ group unobservably. The general base function of Lys-80 via a water molecule is compatible with the fairly large solvent isotope effect on $k_{cat}$ of the Lys-80 → Ala mutant.

The guanidino group of arginine has a $pK_a$ value of about 12.5, which is higher by 2 pH units than that of the free ε-amino group of lysine ($pK_a = 10.5$); the former would thus have the basicity 2 orders of magnitude higher than the latter at the same pH. If the amino group of Lys-80 serves as a general base, the Lys-80 → Arg mutant enzyme with the lowered $pK_a$ group (7.9–9.1, Fig. 1) must therefore have much higher catalytic activity than the wild type. However, this was not the case (Table I). Although the reason for the low activity of Lys-80 → Arg mutant is unknown at present, it seems likely that the slight difference in the side chain length or bulkiness of Lys and Arg is more critical than their basicities for the group to act as a general base.

The marked stimulation of Lys-80 → Ala mutant by exogenous primary amines further substantiates the general base function of the ε-amino group of Lys-80. The stimulation was dependent on both basicities and molecular volumes of the amines. The largest $k_b$ value was obtained with ammonia having the smallest molecular volume (23.2 Å$^3$) and a $pK_a$ value (9.2) close to that ($pK_a = 8.9$) assigned to the ε-amino group of Lys-80. The absence of the amine stimulation in the wild type and Lys-80 → Arg mutant enzymes also suggests that the space allowed for accommodating exogenous amines is very limited at the active site.

The stimulation by exogenous amines of mutant enzymes in which amino acids with small side chains have replaced active-site lysyl residues was first demonstrated by Toney and Kirsch (27) for the virtually inactive Lys-258 → Ala mutant enzyme of E. coli aspartate aminotransferase, and they designated the new approach to discern the roles of active-site residues as “chemical rescue.” Following this, Smith and Hartman (54) reported the chemical rescue of Lys-191 → Cys mutant of ribulosebisphosphate carboxylase/oxygenase by exogenously added aminomethanesulfonate. As described above, the degree of stimulation of Lys-80 → Ala mutant of leucine dehydrogenase is smaller by a factor of 10$^2$–10$^3$ than that reported for the Lys-258 → Ala mutant of aspartate aminotransferase. This difference can be attributed to the significantly high $k_{cat}$ value of the Lys-80 → Ala mutant of leucine dehydrogenase in the absence of amines (1.6% of the wild type in the oxidative deamination, see Table I) as compared with the almost inactive Lys-258 → Ala mutant of aspartate aminotransferase (10$^{-6}$ of the wild type activity) (27) and probably correlates with the indirect participation of the ε-amino group of Lys-80 in catalysis, in contrast to the direct proton-abstracting role of Lys-258 in the transamination. From the $K_m$ value of the wild type leucine dehydrogenase in the oxidative deamination (50 s$^{-1}$, Table I) compared with the $k_{cat}$ value of Lys-80 → Ala mutant for ethylamine (4.56 M$^{-1}$s$^{-1}$, Table III), which replaces most of the volume lost by replacement of Lys with Ala, the ε-amino group of Lys-80 is estimated to have an effective molarity equivalent to 11 M free base of ethylamine.

The considerable increase in $K_m$ values of Lys-80 → Gln and Lys-80 → Ala mutants for α-keto-isocaproate as compared with those of the wild type and Lys-80 → Arg mutant enzymes (Table I) suggests that the presence of a positive charge in residue 80 is needed for efficient binding of keto acid substrate in the reductive amination. Conversely, the large $K_m$ values for isocaproate of the wild type and Lys-80 → Ala mutant enzymes show that the presence of the α-carbonyl group in the monocarboxylic acid is required for effective binding with the enzyme. Thus, Lys-80 may be primarily involved in binding of the keto acid substrate in the reductive amination. This contrasts with its catalytic participation in the oxidative deamination without significant contribution to binding of the amino acid substrate. By analogy with the proposed role of the corresponding lysyl residues (Lys-126) in the bovine liver NADP+-linked glutamate dehydrogenase (35), the amino group being in the protonated form may interact electrostatically with the α-carbonyl oxygen of keto acid. However, judging from the extremely large $K_m$ values for 4-methyl-2-pentanone without α-carboxyl group (Table II),
Role of Lys-80 in Leucine Dehydrogenase

**FIG. 4. A proposed chemical mechanism of leucine dehydrogenase.**

The reaction proceeds with free ammonia attacking the \( \alpha \)-carbonyl carbon because of the electron deficiency produced by the interaction with Lys-80. The \( \epsilon \)-amino group of Lys-80 acts in this case as a general acid providing a proton to the carbonyl oxygen to form the \( \alpha \)-carbinolamine intermediate. After proton transfer from the amino group to the hydrosyl group, loss of a water molecule results in the formation of the imine intermediate, reduction of which by NADH then yields the amino acid.

Based on the findings reported here and referred to the references, we propose the following chemical mechanism for leucine dehydrogenase involving Lys-80 (Fig. 4). In the oxidative deamination, the \( \alpha \)-carboxyl group of \( \alpha \)-keto-iso-caproate to the protonated amino group results in the formation of the imine intermediate, reduction of which by NADH then yields the amino acid. The basic feature of this mechanism is that the conserved lysyl residue is involved as a general acid providing a proton to the carbonyl oxygen because of the electron deficiency produced by the interaction with Lys-80. The \( \epsilon \)-amino group of Lys-80 acts in this case as a general acid providing a proton to the carbonyl oxygen.

**REFERENCES**