Articles

Enantioselective Synthesis of (S)-Amino Acids by Phenylalanine Dehydrogenase from *Bacillus sphaericus*: Use of Natural and Recombinant Enzymes

Yasuhisa Asano,* Akiko Yamada, Yasuo Kato, Kaori Yamaguchi, Yasuo Hibino, Kenji Hirai, and Kiyosi Kondo

Sagami Chemical Research Center, Nishi-Ohnuma 4-4-1, Sagamihara, Kanagawa 229, Japan

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The substrate specificity of phenylalanine dehydrogenase (L-phenylalanine: NAD^+ oxidoreductase, deaminating (EC 1.4.1.–), PheDH) from *Bacillus sphaericus* SCRC-R79a in the reductive amination reaction was examined with various natural and synthetic 2- and 3-oxo acids and their analogues. The enzyme was active toward 3-substituted pyruvic acids with bulky substituents. Optically pure (*S*)-phenylalanine and other (*S*)-amino acids were quantitatively synthesized from their oxo analogues by using PheDH, with a regeneration of NADH by formate dehydrogenase from *Candida boidinii* No. 2201. Recombinant PheDH overproduced in *Escherichia coli* JM109/pBPDH1-DBL was effectively used for the syntheses of (*S*)-amino acids from unnatural substrates. (*S*)-Phenylalanine could be continuously synthesized for about 1 month with the enzymes, packed in a dialysis tube. It is calculated that 1 molecule of PheDH catalyzed the synthesis of 4.8×10^8 molecules of (*S*)-phenylalanine, and 2.4×10^5 times the weight of (*S*)-phenylalanine than that of the enzyme. Acetone-dried cells of *B. sphaericus* SCRC-R79a and *C. boidinii* No. 2201 were also effective for (*S*)-phenylalanine synthesis, providing a simple microbial method of synthesis.

Introduction

The production of (*S*)-phenylalanine as a starting material for the artificial sweetener aspartame has been a target of industrial development.[1] Several enzymatic processes of (*S*)-phenylalanine synthesis have been reported: *S*-specific hydrolysis of benzylhydantoin,[2] amination of *trans*-cinnamic acid,[3] transamination from an amino donor to phenylpyruvate,[4] and two-step conversion starting from acetamidocinnamic acid via phenylpyruvate.[5] The recent success in the development of an efficient method of phenylpyruvic acid synthesis by double carbonylation of benzyl chloride in the presence of a cobalt catalyst[6] has prompted us to seek a new enzymatic method to synthesize (*S*)-phenylalanine from phenylpyruvate. We have screened and isolated bacterial producers of a new enzyme phenylalanine dehydrogenase and characterized crystalline enzymes in detail from *Sporosarcina ureae* SCRC-R04[7,8] and *Bacillus sphaericus* SCRC-R79a,[8] as well as a homogeneous one from *Bacillus badius* IAM 11059.[9] The *pdh* genes have been cloned and sequenced, and overproduction of the *Bacillus* enzymes has been achieved.[9-11] *Escherichia coli* JM 109/pBPDH1-DBL expresses about 120-fold higher activity of PheDH (7200 units/L) than the wild type *B. sphaericus* SCRC-R79a per liter culture.[10,11] The enzyme from *B. sphaericus* SCRC-R79a was chosen to study the application of the enzyme to the synthesis of various (*S*)-amino acids, because it is very stable and shows broader substrate specificity than other PheDH's, acting on (*S*)-tyrosine as well as (*S*)-phenylalanine.[8]

We aimed to synthesize (*S*)-amino acids using PheDH with the regeneration of NADH by formate dehydrogenase (Figure 1).[8,10,12,15] Firstly, we clarified the substrate specificity of PheDH from *B. sphaericus* SCRC-R79a with synthetic 2-oxo acids and their analogues. Secondly, we applied the enzyme to the syntheses of (*S*)-amino acids

Table I. Substrate Specificity of PheDH from *B. sphaericus* SCRC-R79a[a]

substrate (10 mM)	rel act.,[b] %
(4-hydroxyphenyl)pyruvate	100
phenylpyruvate	74
(4-vinylphenyl)pyruvate	38
(4-fluorophenyl)pyruvate	29
2-oxo-4-(methylthio)butyrate	8.1
2-oxo-3(*RS*)-phenylvalerate	6.5
2-oxoisocaproate	5.7
2-oxobutyrate	4.6
2-oxoisovalerate	4.0
2-oxo-3(*RS*)-(4-fluorophenyl)butyrate	3.7
2-oxo-3(*R*)-phenylbutyrate	3.5
2-oxo-4-phenylbutyrate	2.5
2-oxo-3(*RS*)-methylvalerate	2.1
2-oxo-5-phenylvalerate	2.0
2-oxo-3(*RS*)-(3-methylphenyl)butyrate	0.74
2-oxononanoate	0.54
2-oxo-3-(2-naphthalenyl)propionate	0.34
2-oxo-3(*RS*)-phenyl-4-methylvalerate	0.07

[a] Conditions to measure the activity are described in the Experimental Section. [b] The following compounds were inert as substrates: benzoylformate, ethyl phenylpyruvate, ethyl 2-oxo-4-phenylbutyrate, ethyl 3-oxo-4-phenylbutyrate, 3-oxo-4-phenylbutyrate, 2-oxo-phenylpropanol, 2-oxo-3-phenylnonanoate, and 2-oxo-3-methyl-3-(4-propylphenyl)propionate.

* Present address: Toyama Prefectural University, 5180 Kurokawa, Kosugi, Toyama 939-03. Telephone: 0766-56-7500. Fax: 0766-56-6182.

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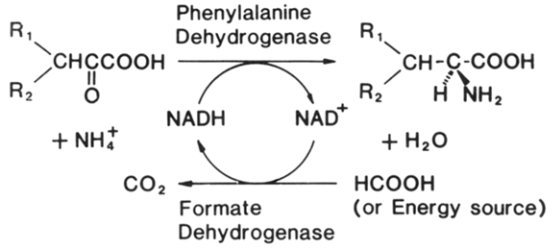

Figure 1. Synthesis of (*S*)-amino acids from their 2-oxo analogues by PheDH with a regeneration of NADH by formate dehydrogenase.

using natural and recombinant enzymes. Thirdly, (*S*)-phenylalanine was synthesized in two ways: continuously with a dialysis tube containing the enzymes, and with acetone-dried cells of *B. sphaericus* SCRC-R79a and *Candida boidinii* No. 2201.

Results and Discussion

Substrate Specificity of *B. sphaericus* PheDH. The relative rates of the reductive amination of 2-oxo acids and their analogues catalyzed by PheDH from *B. sphaericus* SCRC-R79a are shown in Table I. Benzoylformate, 2-oxo esters such as ethyl phenylpyruvate and ethyl 2-oxo-4-phenylbutyrate,[13] 3-oxo esters such as ethyl 3-oxo-4-phenylbutyrate, 3-oxo acids such as 3-oxo-4-phenylbutyrate, and 2-oxo alcohols such as 2-oxo-3-phenylpropanol were inactive as substrates. On the other hand, phenylpyruvate analogues substituted at the phenyl ring were relatively good substrates. The enzyme utilized the compounds substituted at the 3-position of pyruvic acid with a longer or a bulkier group, although the relative velocity of the reductive amination reaction was low. Substitution at the 3-position of phenylpyruvate with a bulkier group, such as hexyl, greatly lowered the reaction velocity.

Synthesis of (S)-Amino Acids from Their 2-Oxo Analogues. On the basis of the results of the studies on the substrate specificity of the enzyme in the reductive amination of 2-oxo acids, various optically pure (*S*)-amino acids were quantitatively synthesized by using PheDH and formate dehydrogenase.[10,14,15] The relative rates in Table

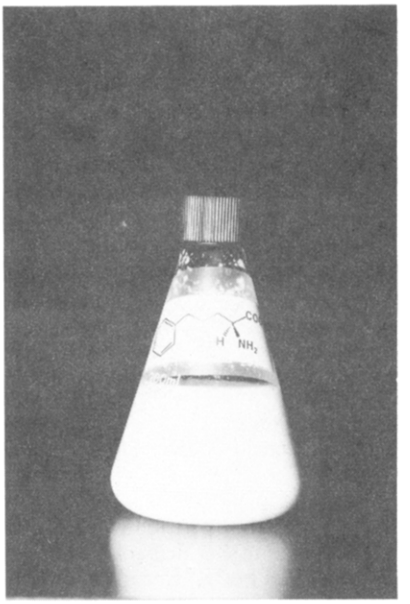

Figure 2. Enzymatic synthesis of (*S*)-2-amino-5-phenylvaleric acid.

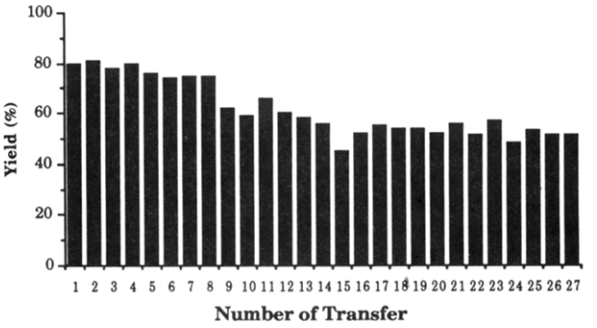

Figure 3. Continuous synthesis of (*S*)-phenylalanine. A dialysis tube containing PheDH from *B. sphaericus* SCRC R-79a and formate dehydrogenase from *C. boidinii* No. 2201 was transferred to a new reaction medium daily for 20 times from the beginning, and every other day from the 21st to 27th changes. The reaction was continued for 34 days.

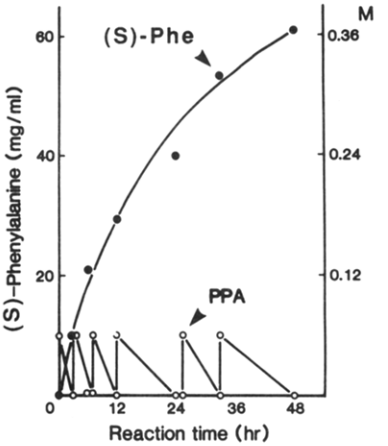

Figure 4. Synthesis of (*S*)-phenylalanine by acetone-dried cells of *B. sphaericus* SCRC R-79a and *C. boidinii* No. 2201. Details of the conditions are described in the Experimental Section; (●) (*S*)-phenylalanine; (○) sodium phenylpyruvate (PPA).

I show the initial velocities in the reductive amination reaction and indicate how much PheDH is required to synthesize various (*S*)-amino acids at the same reaction velocity as in the (*S*)-phenylalanine synthesis. Table II shows the yield of the (*S*)-smino acid thus synthesized. The products from 2-oxo-3(*RS*)-methylvalerate and 2-

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(13) Apparent oxidation of NADH as a decrease of absorbance at 340 nm was spectrophotometrically observed, when ethyl phenylpyruvate and ethyl 2-oxo-4-phenylbutyrate was used as substrates. However, the corresponding free amino acids were identified as the products, proving that a spontaneous hydrolysis of the esters occurred prior to the NADH oxidation.
(14) The equilibrium constant (K_{eq} = [phenylpyruvate][NADH]-[NH_4^+][H^+]/[(*S*)-phenylalanine][NAD^+][H_2O]) of *B. sphaericus* PheDH-catalyzed reaction has been calculated to be 2.0×10^{-14} at pH 8.0–10.38, with a conventional water concentration ([H_2O] = 1).[15] The equilibrium of the enzyme reaction strongly favors the synthesis of (*S*)-phenylalanine.
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Table II. Synthesis of (S)-Amino Acids from 2-Oxo Acids by Using PheDH and Formate Dehydrogenase[a]

substrate	product	yield, %
phenylpyruvate	(S)-phenylalanine	>99[b]
(4-hydroxyphenyl)-pyruvate	(S)-tyrosine	>99[c]
(4-fluorophenyl)pyruvate	(S)-(4-fluorophenyl)alanine	>99[d]
2-oxo-4-phenylbutyrate	(S)-2-amino-4-phenylbutyric acid	99[e]
2-oxo-5-phenylvalerate	(S)-2-amino-5-phenylvaleric acid	98[f]
2-oxo-3-methyl-3-phenylpropionate	(S)-2-amino-3(RS)-methyl-3-phenylpropionate	98[g]
2-oxononanoate	(S)-2-aminononanoic acid	99[h]

[a] To prevent substrate inhibition, 2-oxo acids were divided into portions and added to the reaction mixture not to exceed 50 mM. The optical purity of the amino acids was determined to be 100% ee by HPLC (Waters Assoc., equipped with a chiral column, Crownpak CR (+) (Daicel Co. Ltd., Osaka, Japan)) with a solvent system of 0.01 M perchloric acid. (S)-Methionine, (S)-valine, and (S)-leucine were stoichiometrically synthesized in 0.1-mmol scale from 2-oxo-4-(methylthio)butyrate, 2-oxoisovalerate, and 2-oxoisocaproate, respectively. A reaction mixture (0.5 mL) containing 100 μmol of salt of 2-oxo acid, 5 mmol of NAD^+, 800 μmol of ammonium formate, 250 μmol of Tris·HCl, pH 8.5, 29 μg (2.5 units) of PheDH, and 0.5 units of formate dehydrogenase was incubated for 24 h at 30 °C. The yields were determined microbiologically (Snell, E. E. *Methods Enzymol.* **1957**, *3*, 477–492). A mixture of (S)-isoleucine and (S)-alloisoleucine was formed from 2-oxo-3-(RS)-methylvalerate, as determined by an amino acid analyzer (Kyowa Seimitsu, Model K-101). We thank Professor H. Yamada, Kyoto University, Japan, for his help in amino acid analysis. [b] Synthesized in 8-mmol scale and the yield determined microbiologically. The amino acid formed was purified by a column of Amberlite IRA-400 (OH^-) and crystallized from alcoholic water. Anal. Calcd for $C_9H_{11}NO_2$: C, 65.44; H, 6.71; N, 8.48. Found: C, 65.48; H, 6.71; N, 8.47. $[\alpha]^{20}_D$ = −34.1° (c = 1.72, H_2O). [c] Synthesized in 6-mmol scale and the yield determined by HPLC. Anal. Calcd for $C_9H_{11}NO_3$: C, 59.66; H, 6.12; N, 7.73. Found: C, 59.45; H, 6.11; N, 7.69. $[\alpha]^{20}_D$ = −7.33 (c = 4, 6 N HCl). [d] Synthesized in 2-mmol scale and the yield determined by HPLC. [e] Synthesized in 39-mmol scale. IR (KBr): ν_{max} 3050, 2950, 2170, 1580, 1520, 1410, 1355, 1320, 1200, 1140, 990, 870, 745, 695, 545, 490 cm^{-1}. ^1H NMR spectrum of its methyl ester: ($CDCl_3$) δ (ppm) 1.7 (s, 2 H), 1.6–2.2 (m, 2 H), 2.7 (t, 2 H), 3.4 (dd, 1 H), 3.7 (s, 3 H), 7.0–7.3 (m, 5 H). MS: m/z 179 (relative intensity 4%), 162 (12), 134 (36), 117 (20), 105 (8), 91 (100), 75 (14), 74 (13), 65 (13), 57 (7), 51 (7), 43 (5). Anal. Calcd for $C_{10}H_{13}NO_2$: C, 67.02; H, 7.27; N, 7.80. Found: C, 67.02; H, 7.31; N, 7.82. $[\alpha]^{20}_D$ = +44.7° (c = 1.04, 1 N HCl). (Literature:[24b] $[\alpha]^{20}_D$ = +45.6 (c = 1, 1 N HCl).) [f] Synthesized in 19-mmol scale. IR (KBr): ν_{max} 3050, 2950, 2600, 2150, 1610, 1590, 1515, 1410, 1330, 750, 710, 700, 660, 530 cm^{-1}. ^1H NMR spectrum of its methyl ester: ($CDCl_3$) δ (ppm) 1.7 (s, 2 H), 1.5–1.9 (m, 2 H), 1.5–1.9 (m, 2 H), 2.6 (m, 2 H), 3.4 (m, 1 H), 3.7 (s, 3 H), 7.0–7.3 (m, 5 H). MS: m/z 193 (relative intensity 92%), 148 (100), 131 (93), 105 (45), 104 (35), 91 (92), 74 (58), 56 (65). Anal. Calcd for $C_{11}H_{15}NO_2$: C, 68.37; H, 7.82; N, 7.25. Found: C, 68.12; H, 7.94; N, 7.29. $[\alpha]^{20}_D$ = +39.2° (c = 1.16, 5 N HCl–DMF). (Literature:[16] $[\alpha]^{21}_D$ = +33.5° (c = 2, 5 N HCl–DMF).) [g] Synthesized in 2-mmol scale. The amino acid formed was purified by a column of Dowex X-8 (Cl^-). ^1H NMR spectrum (400-MHz; acetone-d_6): δ (ppm) 1.3 and 1.47 (dd (mixture of diastereomeric proton of 3-methyl group), 3 H, J = 8 Hz), 3.18–3.3 (m, 1 H), 4.01, 4.09 (m, 1 H), 7.1–7.45 (m, 5 H). [h] Synthesized in 20-mmol scale and the yield determined by HPLC. IR (KBr): ν_{max} 3100, 2970, 2950, 2880, 1700, 1590, 1580, 1510, 1490, 1470, 1130, 860, 825, 590 cm^{-1}. ^1H NMR spectrum of its methyl ester: ($CDCl_3$) δ (ppm) 0.9 (t, 3 H), 1.3–1.5 (br, 12 H), 1.7 (s, 2 H), 3.4–3.6 (m, 1 H), 3.7 (s, 3 H). Anal. Calcd for $C_9H_{19}NO_2$: C, 62.39; H, 11.05; N, 8.09. Found: C, 62.34; H, 11.38; N, 8.09. $[\alpha]^{20}_D$ = +32.2° (c = 1.02, acetic acid) (lit. $[\alpha]^{26}_D$ = +33.5° (c = 1, acetic acid)[17a]; R isomer, $[\alpha]^{20}_D$ = −29.2° (c = 1.0, acetic acid)[17b]).

oxo-3(RS)-methyl-3-phenylpyruvate were identified as diastereomeric mixtures of (S)-isoleucine and (S)-alloisoleucine, and (S)-2-amino-3(RS)-methyl-3-phenylpropionic acid, respectively. The product from 2-oxo-5-phenylvalerate solidified as the reaction proceeded as shown in Figure 2.

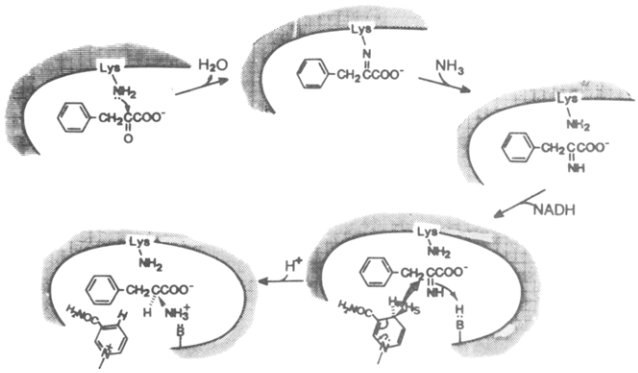

Figure 5. Proposed reaction mechanism of PheDH.

Continuous Synthesis of (S)-Phenylalanine. Very small amounts of PheDH from *B. sphaericus* SCRC-R79a and formate dehydrogenase were placed in a dialysis tube to check the durability and efficiency in a continuous synthesis of (S)-phenylalanine. As shown in Figure 3, the enzyme maintained its activity up to 28 changes. During the 34-day operation, 10.74 g (6.51×10^{-2} mol) of (S)-phenylalanine was synthesized, with 45 mg (5 units, 1.35×10^{-10} mol) of PheDH[18] and about 7.5 mg (15 units) of formate dehydrogenase[19] being used. When (S)-phenylalanine synthesis had ceased, about 60% of the formate dehydrogenase activity still remained, whereas PheDH activity was not detected. It is calculated that 1 molecule of PheDH catalyzed the synthesis of 4.8×10^8 molecules of (S)-phenylalanine, and 2.4×10^5 times the weight of (S)-phenylalanine than that of the enzyme.

Synthesis of (S)-Phenylalanine by Acetone-Dried Cells. Figure 4 shows a typical course of the reductive amination reaction of phenylpyruvate using acetone-dried cells of *B. sphaericus* SCRC-R79a and *C. boidinii* No. 2201 as catalysts. The concentration of (S)-phenylalanine reached 61.5 mg/mL with a yield of more than 99%. The reaction mixture became solidified.

When used in combination with formate dehydrogenase, PheDH from *B. sphaericus* SCRC R-79a was effective in the enantioselective syntheses of various natural and unnatural (S)-amino acids from their oxo analogues. There has been no report on the details of the substrate specificity of PheDH with the synthetic compounds, because the enzyme has become known to occur only recently, and no attempt to clone the gene of the enzyme and its overproduction have been made.[7-11,15,20] The enzyme showed relatively wide substrate specificity toward substituted pyruvic acids. However, the enzyme did not utilize ethyl esters of 2- and 3-oxo acids and 2-oxo alcohol, showing that a free carboxylic acid moiety is required to be recognized

(18) The molecular weight of the *B. sphaericus* PheDH is 332 608, composed of eight identical subunits of molecular weight 41 576 by gene sequencing, assuming that five cysteine residues are all reduced.

(19) The specific activity of the pure formate dehydrogenase from *C. boidinii* is 2.4 units/mg (Schütte, H.; Flossdorf, J.; Sahm, H.; Kula, M.-R. *Eur. J. Biochem.* **1976**, *62*, 151–160). Our preparation is 80–85% pure.

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as a substrate. The result that the enzyme utilized 2-oxo-4-phenylbutyrate and 2-oxo-5-phenylvalerate, but not 3-oxo-4-phenylbutyrate, shows that it has a definite requirement for a distance between the carbonyl carbon and the carboxyl group of the substrates.

A possible mechanism of the reaction catalyzed by PheDH is shown in Figure 5. This is based on the studies of the substrate specificity described above and on the following observations. We have proved in the previous paper that the *pro-S* hydrogen of the 4-position of the reduced pyridine ring of NADH is incorporated into the product (*S*)-phenylalanine in the reductive amination reaction (B-stereospecific).[8] Studies on the steady-state kinetics of the PheDH reaction revealed that the reaction proceeds in the sequential mechanism,[8] i.e., after all three substrates, phenylpyruvate, ammonia, and NADH, bind to the enzyme, the product (*S*)-phenylalanine is released. A nucleophilic residue[21] attacks the carbonyl carbon of the phenylpyruvate, yielding an enzyme-bound imine, which is then replaced by ammonia. A free imino acid formed in the enzyme[22] is then attacked by a hydride from the *pro-S* hydrogen of NADH from the *Re* face of the imino acid, yielding (*S*)-phenylalanine. In the active-site pocket, the orientation of phenylpyruvate is fixed with the carboxyl group as an anchor, as evidenced by the fact that only optically pure *S* isomers of amino acids are produced, and the enzyme does not catalyze the reductive amination of neutral 2-oxo-3-phenylpropanol and the 2-oxo esters. The enzyme has a wide pocket which accommodates the hydrophobic substituent of the substrate: various 3-substituted pyruvic acid analogues with bulky groups could be relatively good substrates. Such a wide substrate specificity is not reported in PheDH's,[7-9,20] even among other NAD(P)$^+$-dependent amino acid dehydrogenases.[23] It was also revealed that the enzyme does not differentiate the configuration of the substitutent at the 3-position of pyruvic acid; for example, a diastereomeric mixture of (*S*)-2-amino-3(*RS*)-methyl-3-phenylpropionate was synthesized from 2-oxo-3(*RS*)-methyl-3-phenylpropionate.[15]

This study has extended the use of PheDH and formate dehydrogenase. (*S*)-2-Amino-4-phenylbutanoic acid ((*S*)-homophenylalanine)[24] and other unnatural (*S*)-amino acids could be efficiently synthesized. The solubility of these (*S*)-phenylalanine homologues, such as (*S*)-tyrosine, (*S*)-2-amino-4-phenylbutyric acid, (*S*)-2-amino-5-phenylvaleric acid, etc., are so low that they are easily separated in crystalline forms from the reaction mixture by filtration. The filtered enzyme solution can be used for further repeated synthesis. (*S*)-Phenylalanine could be synthesized by using the purified enzymes, the purified enzymes in a dialysis tube, and the acetone-dried cells. To avoid the multistep purification procedure of the enzymes,[8] the use of the enzymes in whole cells is preferable. The use of living cells tends to lower the enantioselectivity, because of the presence of other enzyme systems that cause side reactions, as can be seen in the reduction of ketones.[25] In our case, (*S*)-phenylalanine could be synthesized when acetone-dried cells[26] were employed as catalysts, into which NAD$^+$ and NADH could permeate easily as substrates, after the acetone treatment. Virtually no enzyme activity to racemize and oxidize the enantiomers of phenylalanine or to degrade phenylpyruvate was detectable in the cells. It is thus convenient to use the acetone-dried cells in the synthesis, because these cells are powders, and their preparation and storage are simple and easy.

Membranes are efficiently used in the enzyme-catalyzed syntheses of optically active compounds on laboratory[27] and industrial[28] scales. This technique can be applied for the repeated use of the enzyme(s) in the synthesis, especially in case smaller amounts of enzyme(s) are available. The *B. sphaericus* PheDH proved to be very stable in the synthesis, with high efficiency.[29] The enzyme was very stable in the continuous synthesis operated over a month. This durability contrasts with the relatively unstable leucine dehydrogenase from *B. sphaericus* IFO3525.[30] The use of recombinant DNA technology has further extended the use of PheDH, namely, in the synthesis of (*S*)-amino acids from poorer substrates. The less the reactivity of the enzyme for certain unnatural compounds, the more the enzyme is required to achieve the same velocity as in the natural substrate. To synthesize 3.6 g of (*S*)-2-amino-5-phenylvaleric acid with 5760 units of PheDH, for example, the wild type *B. sphaericus* SCRC-R79a must be cultivated on a 100-L scale, while the culture of *E. coli* transformant JM109/pBPDH-DBL may be as little as 800 mL, assuming a quantitative recovery of the enzyme in the purification procedures.

Experimental Section

Materials. ^{1}H NMR spectra were recorded on a 90-MHz spectrometer with Me_4Si as a reference. DEAE-Toyopearl and Butyl-Toyopearl were purchased from Tosoh Corp. (Tokyo, Japan). (*S*)-Phenylalanine and other natural amino acids were assayed microbiologically by using *Pediococcus acidilactici* (formerly *Leuconostoc mesenteroides*) ATCC 8042, with assay media purchased from Takara Kosan (Tokyo, Japan). Formate dehydrogenase was purified from *C. boidinii* (formerly *Kloeckera* sp.) No. 2201[31] (AKU 4705), which had been obtained from Professor Y. Tani of the Department of Agricultural Chemistry, Kyoto University, Japan, up to 2 units/mg according to the method of Kato et al.[32] *B. sphaericus* SCRC-R79a and *E. coli*

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Cultivation of Microorganisms and Preparation of Acetone-Dried Cells. *B. sphaericus* SCRC-R79a was cultivated aerobically at 30 °C for 20 h in a 2-L flask containing 500 mL of a medium composed of 1.0% (*S*)-phenylalanine, 1.0% peptone (Kyokuto, Tokyo), 0.5% yeast extract (Oriental yeast, Tokyo), 0.2% K_2HPO_4, 0.1% NaCl, and 0.02% $MgSO_4 \cdot 7H_2O$ in tap water, adjusted to pH 7.0.[8] *C. boidinii* No. 2201 was cultivated in a medium containing 1.3% (w/v) methanol as described by Tani et al.[31] The 1.3-kb fragment of the *pdh* gene encoding PheDH with endogenous promoter and ribosomal recognition sequences has been inserted into pUC9 to give pBPDH1-DBL.[10,11] *E. coli* JM109/pBPDH1-DBL was cultivated with shaking at 37 °C for 12 h in LB medium, pH 7.5, supplemented with 50 μg/mL ampicillin.[33]

Enzyme Assay and Definition of Units. NAD^+-dependent PheDH activity was assayed at 25 °C by measuring reduction of NAD^+ at 340 nm in a cuvette placed in the beam of a 1-cm light path, with a double-beam spectrophotometer.[8] The reaction mixture contained 100 μmol of glycine–KCl–KOH buffer, pH 10.4, 2.5 μmol of NAD^+, 10 μmol of (*S*)-phenylalanine, and enzyme solution in a total volume of 1.0 mL. The substrate specificity in the reductive amination reaction was measured in a reaction mixture (1.0 mL) containing 200 μmol of NH_4Cl–NH_4OH buffer, pH 9.0, 10 μmol of salt of 2-oxo acid, 0.1 μmol of NADH, and the enzyme. A linear absorbance change at least for the initial 30 s was monitored, and that for the initial 5 s was used for the calculation. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of NADH in the oxidative deamination. Phenylpyruvate was determined by a color formation with 2,4-dinitrophenylhydrazine.[34] Protein was determined according to the method of Lowry.[35]

Purification of PheDH from *B. sphaericus* SCRC-R79a and *E. coli* JM109/pBPDH1-DBL. PheDH samples were prepared in crystalline form from *B. sphaericus* SCRC R-79a[8] and in homogeneous form from *E. coli* JM109/pBPDH1-DBL[10,11,36] as described previously, by a procedure involving ammonium sulfate fractionation, DEAE-Toyopearl, Butyl-Toyopearl, and Sephadex G-200 column chromatographies.

Preparation of 2-Oxo-4-phenylbutyrate, 2-Oxo-5-phenylvalerate, and 2-Oxononanoate.[37] Grignard reagents prepared from (2-bromoethyl)benzene, 1-bromo-3-phenylpropane, and 1-bromoheptane were added to diethyl oxalate in dry THF to give ethyl 2-oxo-4-phenylbutyrate (^1H NMR ($CDCl_3$) δ (ppm) 1.2 (t, 3 H), 1.6 (s, 2 H), 1.9 (m, 2 H), 2.7 (t, 2 H), 3.4 (m, 1 H), 4.2 (q, 2 H), 7.1–7.4 (m, 5 H)), ethyl 2-oxo-5-phenylvalerate (^1H NMR ($CDCl_3$) δ (ppm) 1.3 (t, 3 H), 1.9 (m, 2 H), 2.6 (t, 2 H), 2.8 (t, 2 H), 4.3 (q, 2 H), 7.0–7.4 (m 5 H)), and ethyl 2-oxononanoate (^1H NMR ($CDCl_3$) δ (ppm) 0.9 (t, 3 H), 1.3–1.5 (br, 8 H), 1.4 (t, 3 H), 1.6 (br, 2 H), 2.8 (t, 2 H), 4.3 (q, 2 H)), in 73, 73, and 68% yields, respectively. The ethyl esters were hydrolyzed in 10% concentrated HCl in acetic acid at 50 °C for 3 h. The reaction mixture was worked up by an usual procedure, and the oxo acids were crystallized by addition of *n*-hexane. 2-Oxo-4-phenylbutyric acid, 2-oxo-5-phenylvaleric acid, and 2-oxononanoic acid were obtained from their esters in 58, 93, and 89% yields, respectively.

Preparation of Other Substrate Analogues. Ethyl 3-oxo-4-phenylbutyrate was synthesized in 75% yield from monoethyl malonate[38] and phenylacetyl chloride, according to the method described by Wierenga and Skulnick.[39] Hydrolysis of the ester by the same procedure as described above yielded 3-oxo-4-phenylbutyric acid: ^1H NMR ($CDCl_3$) δ (ppm) 3.5 (s, 2 H), 3.8 (s, 2 H), 7.1–7.5 (m, 5 H), 8.8 (s, 1 H). 1-Diazo-2-oxo-3-phenylpropane was synthesized in 64% yield from phenylacetyl chloride and diazomethane:[40] ^1H NMR ($CDCl_3$) δ (ppm) 3.6 (s, 2 H), 5.1 (s, 1 H), 7.1–7.4 (m, 5 H). The diazo compound was hydrolyzed with H_2SO_4 in dioxane[41] to give 2-oxo-3-phenylpropanol:[42] ^1H NMR ($CDCl_3$) δ (ppm), 3.6 (s, 2 H), 3.6–3.8 (br, 1 H), 4.2 (s, 2 H), 7.1–7.4 (m, 5 H). 2-Oxo-3-(2-naphthalenyl)propionic acid was prepared from 2-naphthaleneacetic acid and diethyl oxalate according to a published procedure.[43] The preparation of (4-vinylphenyl)pyruvic acid and 3-substituted phenylpyruvic acid analogues will be reported elsewhere. Other 2-oxo acids or their salts were purchased from commercial sources.

Reductive Amination of 2-Oxo-5-phenylvalerate. A reaction mixture (100 mL) containing 4 mmol of sodium 2-oxo-5-phenylvalerate, 20 mmol of ammonium formate, 8 mmol of NH_4OH–NH_4Cl buffer, pH 8.5, 0.1 mmol of NAD^+, 5760 units of recombinant PheDH, and 240 units of formate dehydrogenase was incubated at 30 °C for 60 h. Three millimoles of the sodium salt of the oxo acid was added to the reaction mixture every 10 h for 5 repetitions. At 40 h, 20 mmol of ammonium formate was added.

Continuous Synthesis of (*S*)-Phenylalanine. PheDH (45 μg, 5 units) from *B. sphaericus* SCRC R-79a and formate dehydrogenase (7.5 mg, 15 units) were placed in a dialysis tube (18/32, Viskase Sales Corp.) and soaked in a reaction mixture containing 4 mmol of phenylpyruvate, 50 μmol of NAD^+, 12 mmol of ammonium formate, and 2.5 mmol of Tris·HCl, pH 8.5, in a total volume of 50 mL. The tube containing the enzymes was incubated at 30 °C and periodically transferred to a fresh reaction mixture of the same composition. (*S*)-Phenylalanine was determined by the microbiological method.

Synthesis of (*S*)-Phenylalanine by Acetone-Dried Cells. Acetone-dried cells of *B. sphaericus* SCRC-R79a and *C. boidinii* No. 2201 were prepared essentially as described by Izumi et al.[26] A typical example of the preparation is as follows: *C. boidinii* No. 2201 cells harvested from 6 L of culture were washed once with 0.01 M potassium phosphate buffer, pH 7.0, and suspended in 80 mL of the same buffer. To the cell suspension was added 400 mL of acetone which had been cooled to −20 °C. The cells were filtered, washed with the same volume of cold acetone, evacuated for 30 min, and then stored at −20 °C until use. Acetone-dried cells from *B. sphaericus* SCRC-R79a (2.0 g) and *C. boidinii* No. 2201 (1.3 g) per liter culture were typically obtained. A reaction mixture (30 mL) containing 1.85 mmol of sodium phenylpyruvate, 15 μmol of NAD^+, 12 mmol of ammonium formate, 1.5 mmol of Tris·HCl, pH 8.5, and acetone-dried cells of *C. boidinii* No. 2201 (300 mg) and *B. sphaericus* SCRC-R79a (300 mg) was incubated at 30 °C for 48 h. At 3, 7, 12, 25, and 30 h after the start of the reaction, the same amount of sodium phenylpyruvate (1.85 mmol) was added. At 12 h, 5.6 mmol of ammonium formate was added. (*S*)-Phenylalanine was determined by the microbiological method.

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