Stereoselective synthesis of opine-type secondary amine carboxylic acids by a new enzyme opine dehydrogenase
Use of recombinant enzymes

Yasuo Kato, Hideaki Yamada, Yasuhisa Asano *

Biotechnology Research Center, Faculty of Engineering, Toyama Prefectural University, 5180 Karokawa, Kosugi, Toyama 939-03, Japan
Received 12 September 1995; accepted 19 October 1995

Abstract

The substrate specificity of the recently discovered enzyme, opine dehydrogenase (ODH) from Arthrobacter sp. strain 1C for amino donors in the reaction that forms secondary amines using pyruvate as a fixed amino acceptor is examined. The enzyme was active toward short-chain aliphatic (S)-amino acids and those substituted with acyloxy, phosphonooxy, and halogen groups. The enzyme was named N-[1-(R)-(carboxyl)ethyl]-(S)-norvaline: NAD⁺ oxidoreductase (L-norvaline forming). Other substrates for the enzyme were 3-aminobutyric acid and (S)-phenylalaninol. Optically pure opine-type secondary amine carboxylic acids were synthesized from amino acids and their analogs such as (S)-methionine, (S)-isoleucine, (S)-leucine, (S)-valine, (S)-phenylalanine, (S)-alanine, (S)-threonine, (S)-serine, and (S)-phenylalaninol, and α-keto acids such as glyoxylate, pyruvate, and 2-oxobutyrate using the enzyme, with regeneration of NADH by formate dehydrogenase (FDH) from Moraxella sp. C-1. The absolute configuration of the nascent asymmetric center of the opines was of the (R) stereochemistry with > 99.9% e.e. One-pot synthesis of N-[1-(R)-(carboxyl)ethyl]-(S)-phenylalanine from phenylpyruvate and pyruvate by using ODH, FDH, and phenylalanine dehydrogenase (PheDH) from Bacillus sphaericus, is also described.

Keywords: Enzymatic synthesis; Opine dehydrogenase; Dehydrogenase; Phenylalanine dehydrogenase; Formate dehydrogenase; Arthrobacter sp.

1. Introduction

NAD⁺-dependent amino acid dehydrogenase (EC 1.4.1) catalyzes reversible amination-deamination reactions between (S)-amino acids and α-keto acids [1]. We have used phenylalanine dehydrogenase and other amino acid dehydrogenases to enzymatically synthesize optically pure natural and unnatural amino acids from their corresponding α-keto acids [2–5].

Opine-type secondary amine dicarboxylic acids are useful chiral intermediates of angiotensin converting enzyme (ACE) inhibitors such as enalapril and lysinopril [6–8]. In order to extend the use of enzymes in stereoselective synthesis, we screened for an enzyme catalyzing the reversible oxidation-reduction of opine-type secondary amine dicarboxylic acids and isolated the bacterial producer. Arthrobacter sp. strain...
1. We purified and characterized an NAD$^+$-dependent secondary amine dicarboxylic acid dehydrogenase, and named it opine dehydrogenase (ODH) [9]. We then cloned and sequenced the $odh$ gene, and overproduced the enzyme for use in synthesis, since the enzyme is only induced by opine-type secondary amine dicarboxylic acids, the preparation of which is rather laborious [10]. *Escherichia coli* JM109/pODH1 expresses about 6.6-fold higher activity of the enzyme per liter of culture than the wild type *Arthrobacter* sp. strain 1C without the addition of the chemically synthesized growth substrate, N-[1-(R)-(carboxyl)ethyl]-(S)-phenylalanine.

Optically active secondary amine dicarboxylic acids have been chemically synthesized as follows: (i) reductive condensation reaction of $\alpha$-keto acids or their esters and amino acid derivatives using sodium cyanoborohydride [11], Raney-Ni [12], or catecholborane [13], (ii) SN$_2$ reaction of optically active 2-halo [14] and 2-trifluoromethanesulfonyloxy esters [15] with amino acid derivatives. However, these methods generally require protection of the functional groups and the stereoselectivity is not always high. We chose ODH to apply to the synthesis of secondary amine dicarboxylic acids without protection of the substrates.

In this report, we clarified the substrate specificity for amino donors of *Arthrobacter* ODH with several unnatural amino acids and amino compounds. We applied the enzyme to the synthesis of secondary amine dicarboxylic acids from (S)-amino acids and $\alpha$-keto acids with a regeneration of NADH by formate dehydrogenase (FDH) [2–5] (Fig. 1). A secondary amine carboxylic acid from (S)-phenylalaninol and pyruvate was also synthesized. Optically pure N-[1-(R)-(carboxyl)ethyl]-(S)-phenylalanine was synthesized in one-pot reaction from phenylpyruvate and pyruvate using ODH, FDH, and phenylalanine dehydrogenase (PheDH) from *Bacillus sphaericus* [3] (Fig. 2).

2. Experimental

2.1. Materials

$^1$H- and $^{13}$C-NMR spectra were recorded in D$_2$O using a JEOL JNM-EX400 spectrometer (Tokyo, Japan), with tetramethylsilane as the internal standard. Mass spectra were recorded on a JEOL JMS-AX500 mass spectrometer (Tokyo, Japan) under fast atom bombardment (FAB) conditions. Optical rotations were recorded on a Horiba SEPA-200 polarimeter (Kyoto, Japan). DEAE-Toyopearl 650 M,
Butyl-Toyopearl 650 M, and HPLC column ODS-80Ts were purchased from Tosoh Corp. (Tokyo, Japan), ion-exchange resins DIAION SK1B and SA20A were from Mitsubishi Chemicals (Tokyo, Japan), and the HPLC column Crownpak CR(+) was from Daicel Chem. Ind. Ltd. (Tokyo, Japan). All other chemicals were from commercial sources and used without further purification.

2.2. Enzyme preparations

Amounts of ODH were purified from E. coli JM109/pODH1 up to 45.8 units/mg as described previously [9,10], by means of ammonium sulfate fractionation, DEAE-Toyopearl and Butyl-Toyopearl column chromatography. Amounts of FDH were purified from E. coli JM109/pMxFDH2 [16,17] up to 4.1 units/mg in a similar manner. Amounts of PheDH were purified to homogeneity from E. coli JM109/pBPDH1-DBL [3] as described [4].

2.3. Enzyme assay

ODH activity in the reductive secondary-amine forming reaction was assayed at 25°C by measuring the oxidation of NADH at 340 nm in a reaction mixture (1 ml) containing 100 μmol of Tris-HCl (pH 8.0), 10 μmol of sodium pyruvate, 0.1 μmol of NADH, 10 μmol of amino compound, and the enzyme (0.5–3 units) with a Hitachi U-3210 spectrophotometer (Tokyo, Japan). A linear change in absorbance for the initial 10 s was used for the calculation. One unit of the enzyme was defined as the amount of the enzyme that catalyzed the consumption of 1 μmol of NADH per min. FDH [16] and PheDH [3] activities were measured as described. One unit of these enzymes was defined as the amount that catalyzed the formation of 1 μmol of NADH in the oxidation reaction at pH 7.5 with sodium formate and at pH 10.5 with (S)-phenylalanine as substrates for FDH and PDH, respectively. Protein was assayed by measuring the absorbance at 280 nm.

2.4. General procedure for the secondary amine-forming reaction from (S)-amino acids and pyruvate or glyoxylate

The reaction mixture contained 0.5 mmol of (S)-amino acid, 0.75 mmol of sodium salt of α-keto acid, 0.75 mmol of sodium formate, 25 μmol of NAD⁺, 0.5 mmol of Tris–HCl (pH 8.5), ODH (48 units), and FDH (40 units) in a total volume of 5 ml. The reaction mixture was incubated at 30°C for 16 h and the disappearance of (S)-amino acid was confirmed using a Hitachi L-8500 amino acid analyzer (Tokyo, Japan). The reaction mixture was boiled for 10 min and centrifuged to remove denatured protein. The supernatant was applied to a column of DIAION SK-1B (H⁺) and eluted with 1 N NH₄OH. The effluent was evaporated to dryness, applied to a column of DIAION SA-20A (Cl⁻) and eluted with 1 N formic acid. The eluate was concentrated and lyophilized.

2.5. Determination of enantiomeric excess (e.e.) and absolute configuration of synthesized secondary amine carboxylic acids

The compounds synthesized (10 mg) by the secondary amine-forming reaction were oxidized with 1% KMnO₄ as described by Hatanaka et al. [18]. Amino acids formed were purified from the reaction mixture by paper chromatography (Advantec 51B, 20 × 20 cm, Tokyo, Japan) using 70% n-propanol as the developing solvent. The amino acids were analyzed using a Waters HPLC system (Millipore Corp., Bedford, MA, USA) with a Crownpak CR(+) (4.6 × 150 mm) at a flow rate of 0.5 ml/min using aqueous HClO₄ (pH 1.0) as the mobile phase at 4°C to determine the optical purity and absolute configuration.

2.6. N-[1-(R)-(Carboxyl)ethyl]-(S)-methionine

This was synthesized from (S)-methionine and pyruvate. Yield, 15.7% (17.3 mg) based on (S)-methionine (75 mg). ¹H-NMR (D₂O) δ ppm.
3.969–4.037 (m, 1+1 H), 2.652–2.741 (m, 2H), 2.199–2.252, (m, 2H), 1.557 (d, 3H, J = 7.3 Hz); 13C-NMR (D2O) Δppm 171.48, 170.73, 58.14, 55.86, 30.02, 29.63, 14.69, 14.62; FAB-MS: m/z 221 (rel. int. 33%, M + H), 171.48, 170.73, 58.14, 55.86, 30.02, 29.63, 14.69, 14.62; FAB-MS: m/z 221 (rel. int. 33%, M + H), 193 (100); [α]D + 8.3° (c = 1.0, H2O).

2.7. N-[1-(R)-(Carboxyl)ethyl]-(S)-isoleucine

This was synthesized from (S)-isoleucine and pyruvate. Yield, 56.7% (57.6 mg) based on (S)-isoleucine (65 mg), 1H-NMR (D2O) Δppm 4.18 (q, 1H, J = 7.2 Hz), 4.02 (d, 1H, J = 3.9 Hz), 2.062–2.124 (m, 1H), 1.556–1.624 (m, 1H), 1.584 (d, 3H, J = 7.4 Hz), 1.370–1.443 (m, 1H), 1.010 (d, 3H, J = 6.9 Hz), 0.959 (t, 3H, J = 7.3 Hz); 13C-NMR (D2O) Δppm 174.93, 173.88, 65.76, 58.69, 38.92, 28.79, 16.75, 16.35, 13.95; FAB-MS: m/z 204 (rel. int. 100%, M + H), 158 (88), 132 (34), 112 (29), 86 (43), 69 (23), 44 (82); [α]D + 19.1° (c = 1.0, H2O).

2.8. N-[1-(R)-(Carboxyl)ethyl]-(S)-leucine

This was synthesized from (S)-leucine and pyruvate. Yield, 68.2% (69.2 mg) based on (S)-leucine (65 mg), 1H-NMR (D2O) Δppm 4.18 (q, 1H, J = 7.3 Hz), 3.996–4.029 (m, 1H), 1.860–1.910 (m, 1H), 1.682–1.816 (m, 1+1 H), 1.592 (d, 3H, J = 7.3 Hz), 0.963 (d, 6H, J = 6.9 Hz); 13C-NMR (D2O) Δppm 175.07, 175.04, 60.59, 58.03, 41.42, 27.41, 24.84, 23.9, 16.84; FAB-MS: m/z 204 (rel. int. 100%, M + H), 158 (77), 132 (34), 112 (29), 86 (77), 70 (42), 44 (100); [α]D + 9.3° (c = 1.0, H2O).

2.9. N-[1-(R)-(Carboxyl)ethyl]-(S)-valine

This was synthesized from (S)-valine and pyruvate. Yield, 58.9% (55.7 mg) based on (S)-valine (55 mg), 1H-NMR (D2O) Δppm 4.148 (q, 1H, J = 7.3 Hz), 3.913 (d, 1H, J = 4.4 Hz), 2.326–2.405 (m, 1H), 1.576 (d, 3H, J = 7.3 Hz), 1.116 (d, 3H, J = 6.9 Hz), 1.046 (d, 3H, J = 7.3 Hz); 13C-NMR (D2O) Δppm 175.04, 174.14, 67.93, 58.91, 32.32, 21.18, 19.73, 16.26; FAB-MS: m/z 190 (rel. int. 100%, M + H), 144 (77), 98 (37), 72 (34), 44 (56); [α]D + 9.3° (c = 1.0, H2O).

2.10. N-[1-(R)-(Carboxyl)ethyl]-(S)-phenylalanine

This was synthesized from (S)-phenylalanine and pyruvate. Yield, 29.4% (34.8 mg) based on (S)-phenylalanine (80 mg), 1H-NMR (D2O) Δppm 7.329–7.439 (m, 5H), 4.215 (dd, 1H, J = 4.4, 6.4 Hz), 3.927 (q, 1H, J = 7.3 Hz), 3.313 (d, 2H, J = 6.4 Hz), 1.487 (d, 3H, J = 7.3 Hz); 13C-NMR (D2O) Δppm 175.58, 174.61, 137.36, 132.22, 132.15, 130.87, 63.66, 58.60, 38.53, 16.92; FAB-MS: m/z 260 (rel. int. 24%, M + H), 238 (40), 192 (46), 146 (30), 120 (38), 91 (69), 44 (100); [α]D + 7.7° (c = 1.0, H2O).

2.11. meso-N-[1-(Carboxyl)ethyl]-alanine

This was synthesized from (S)-alanine and pyruvate. Yield, 61.1% (49.2 mg) based on (S)-alanine (45 mg), 1H-NMR (D2O) Δppm 4.181 (q, 2H, J = 6.9 Hz), 1.596 (d, 6H, J = 6.9 Hz); 13C-NMR (D2O) Δppm 175.29, 57.42, 17.32; FAB-MS: m/z 162 (rel. int. 56%, M + H), 116 (48), 90 (30), 70 (35), 44 (100); [α]D + 0° (c = 1.0, H2O).

2.12. N-[1-(R)-(Carboxyl)ethyl]-(S)-threonine

This was synthesized from (S)-threonine and pyruvate. Yield, 71.1% (67.9 mg) based on (S)-threonine (60 mg), 1H-NMR (D2O) Δppm 4.209–4.285 (m, 1+1 H), 3.930 (d, 2H, J = 6.8 Hz), 1.582 (d, 3H, J = 6.8 Hz); 13C-NMR (D2O) Δppm 172.95, 68.89, 68.03, 59.07, 22.39, 16.35; FAB-MS: m/z 192 (rel. int. 100%, M + H), 146 (38), 120 (21), 100 (20), 44 (100); [α]D + 14.4° (c = 1.0, H2O).

2.13. N-[1-(R)-(Carboxyl)ethyl]-(S)-serine

This was synthesized from (S)-serine and pyruvate. Yield, 65.3% (57.8 mg) based on...
(S)-serine (50 mg). $^1$H-NMR (D$_2$O) $\delta$ppm 4.169–4.452 (m, 1 + 1 H), 4.119–4.136 (m, 2H), 1.629 (d, 3H, J = 6.4 Hz); $^{13}$C-NMR (D$_2$O) $\delta$ppm 175.09, 172.77, 63.2, 61.56, 57.77, 17.26; FAB-MS: m/z 178 (rel. int. 100%, M + H), 132 (41), 93 (39), 44 (46); [\(\alpha\)]$_D^{15}$ + 5.9° (c = 1.0, H$_2$O).

2.14. N-Carboxymethyl-(S)-isoleucine

This was synthesized from (S)-isoleucine and glyoxylate. Yield, 66.3% (62.7 mg) based on (S)-isoleucine (65 mg). $^1$H-NMR (D$_2$O) $\delta$ppm 4.058 (m, 1H), 3.989 (dd, 2H, J = 13.4, 17.1 Hz), 2.122–2.129 (m, 1H), 1.532–1.602 (m, 1H), 1.339–1.442 (m, 1H), 1.039 (d, 3H, J = 3.9 Hz), 0.962 (t, 3H, J = 7.3 Hz); $^{13}$C-NMR (D$_2$O) $\delta$ppm 173.57, 171.94, 67.83, 49.93, 38.81, 28.49, 16.9, 13.99; FAB-MS: m/z 190 (rel. int. 100%, M + H), 144 (58), 98 (10), 86 (15), 30 (24); [\(\alpha\)]$_D^{15}$ + 7.9° (c = 1.0, H$_2$O).

2.15. N-Carboxymethyl-(S)-leucine

This was synthesized from (S)-leucine and glyoxylate. Yield, 69.6% (65.8 mg) based on (S)-leucine (65 mg). $^1$H-NMR (D$_2$O) $\delta$ppm 4.075–4.165 (m, 1H), 4.012 (dd, 2H, J = 4.4, 17.1 Hz), 1.857–1.943 (m, 1H), 1.731–1.839 (m, 1 + 1 H), 0.974 (d, 6H, J = 5.8 Hz); $^{13}$C-NMR (D$_2$O) $\delta$ppm 174.71, 171.85, 61.76, 49.2, 41.15, 27.25, 24.69, 24.01; FAB-MS: m/z 190 (rel. int. 100%, M + H), 144 (78), 88 (31), 86 (43), 44 (44), 30 (64), [\(\alpha\)]$_D^{15}$ + 8.1° (c = 1.0, H$_2$O).

2.16. N-Carboxymethyl-(S)-valine

This was synthesized from (S)-valine and glyoxylate. Yield, 65.7% (57.5 mg) based on (S)-valine (55 mg). $^1$H-NMR (D$_2$O) $\delta$ppm 3.962 (dd, 2H, J = 7.1, 27.8 Hz), 3.943 (d, 1H, J = 3.9 Hz), 2.376–2.435 (m, 1H), 1.090 (dd, 6H, J = 6.3, 6.9 Hz); $^{13}$C-NMR (D$_2$O) $\delta$ppm 173.87, 172.07, 69.33, 50.05, 32.11, 20.68, 19.9; FAB-MS: m/z 176 (rel. int. 100%, M + H), 130 (80), 84 (31), 72 (22), 30 (31); [\(\alpha\)]$_D^{15}$ + 2.2° (c = 1.0, H$_2$O).

2.17. General procedure for the secondary amine-forming reaction from (S)-amino acids and 2-ketobutyrate

A reaction mixture (5 ml) containing 0.5 mmol of (S)-amino acid, 0.75 mmol of sodium 2-ketobutyrate, 0.75 mmol of sodium formate, 25 µmol of NAD$^+$, 0.5 mmol of Tris–HCl (pH 8.5), ODH (110 units), and FDH (40 units) was incubated at 30°C for 21 h and the disappearance of the (S)-amino acid was confirmed as described above. Six hours after starting the reaction, ODH (220 units), FDH (17 units) and sodium formate (1.5 mmol) was added. The reaction product was purified as described above.

2.18. N-[1-(R)-(Carboxyl)propyl]-(S)-isoleucine

This was synthesized from (S)-isoleucine and 2-ketobutyrate. Yield, 63.9% (69.3 mg) based on (S)-isoleucine (65 mg). $^1$H-NMR (D$_2$O) $\delta$ppm 4.117 (d, 1H and q, 1H, J = 2.9 Hz), 2.054–2.161 (m, 2H), 1.999–2.062 (m, 1H), 1.566–1.634 (m, 1H), 1.036 (t, 3H, J = 7.3 Hz), 0.962 (t, 3H, J = 7.3 Hz); $^{13}$C-NMR (D$_2$O) $\delta$ppm 173.81, 173.04, 67.28, 63.88, 31.73, 24.65, 21.54, 19.28, 11.70; FAB-MS: m/z 218 (rel. int. 100%, M + H), 172 (69), 86 (25), 58 (42), 41 (28); [\(\alpha\)]$_D^{15}$ + 16.2° (c = 1.0, H$_2$O).

2.19. N-[1-(R)-(Carboxyl)propyl]-(S)-valine

This was synthesized from (S)-valine and 2-ketobutyrate. Yield, 62.7% (63.6 mg) based on (S)-valine (55 mg). $^1$H-NMR (D$_2$O) $\delta$ppm 4.084 (dd, 1H, J = 6.9, 7.3 Hz), 4.025 (d, 1H, J = 4.4 Hz), 2.394–2.439 (m, 1H), 1.974 2.093 (m, 1 + 1H), 1.143 (d, 3H, J = 6.8 Hz), 1.046 (d, 3H, J = 7.3 Hz), 1.037 (t, 3H, J = 7.8 Hz); $^{13}$C-NMR (D$_2$O) $\delta$ppm 173.96, 173.24, 67.28, 63.88, 31.73, 24.65, 21.54, 19.28, 11.70; FAB-
2.20. Asymmetric synthesis of N-[1-(R)-(carboxy)ethyl]-(S)-phenylalaninol from (S)-phenylalaninol and pyruvate

A reaction mixture (5 ml) containing 0.5 mmol of (S)-phenylalaninol, 0.75 mmol of sodium pyruvate, 0.75 mmol of sodium formate, 25 μmol of NAD⁺, 0.5 mmol of Tris-HCl (pH 8.5), ODH (110 units), and FDH (40 units) was incubated at 30°C for 26 h. The reaction product was purified by ion exchange column chromatography and acidified with hydrochloric acid followed by lyophilization. N-[1-(R)-(carboxy)ethyl]-(S)-phenylalaninol was obtained as a hydrochloride salt in a yield of 87.5% (113.5 mg). ¹H-NMR (D,O) δ ppm 7.326–7.442 (m, 5H), 4.123 (q, 1H, J = 7.4 Hz), 3.834 (m, 1H), 3.639–3.707 (m, 1 + 1H), 3.139 (dd, 1H, J = 4.8, 5.8 Hz), 3.027 (dd, 1H, J = 4.8, 8.5 Hz), 1.585 (d, 3H, J = 7.4 Hz); ¹³C-NMR (D₂O) δ ppm 175.15, 138.39, 132.17, 132.08, 130.47, 62.73, 61.91, 57.04, 36.39, 17.78; FAB-MS: m/z 204 (rel. int. 100%, M + H), 158 (81), 112 (39), 72 (43), 55 (27), 41 (22); [α]D⁰ + 5.0° (c = 1.0, H₂O).

2.21. One-pot synthesis of N-[1-(R)-(carboxy)ethyl]-(S)-phenylalanine by ODH and PheDH from phenylpyruvate and pyruvate

A reaction mixture (5 ml) containing 0.25 mmol of sodium phenylpyruvate, 0.75 mmol of sodium pyruvate, 2.5 μmol of ammonium formate, 25 μmol of NAD⁺, 0.5 mmol of Tris-HCl (pH 8.5), ODH (110 units), PheDH (35 units), and FDH (40 units) was incubated at 30°C for 26 h. The reaction product was purified by ion exchange column chromatography and acidified with hydrochloric acid followed by lyophilization. N-[1-(R)-(carboxy)ethyl]-(S)-phenylalanine was obtained as a hydrochloride salt in a yield of 87.5% (113.5 mg). ¹H-NMR (D,O) δ ppm 7.326–7.442 (m, 5H), 4.123 (q, 1H, J = 7.4 Hz), 3.834 (m, 1H), 3.639–3.707 (m, 1 + 1H), 3.139 (dd, 1H, J = 4.8, 5.8 Hz), 3.027 (dd, 1H, J = 4.8, 8.5 Hz), 1.585 (d, 3H, J = 7.4 Hz); ¹³C-NMR (D₂O) δ ppm 175.15, 138.39, 132.17, 132.08, 130.47, 62.73, 61.91, 57.04, 36.39, 17.78; FAB-MS: m/z 204 (rel. int. 100%, M + H), 158 (81), 112 (39), 72 (43), 55 (27), 41 (22); [α]D⁰ + 5.0° (c = 1.0, H₂O).

Table 1

<table>
<thead>
<tr>
<th>Amino donor</th>
<th>Kₘ (mM)</th>
<th>Vₘₐₓ (units/mg)</th>
<th>Vₘₐₓ/Kₘ (units/mg/mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S)-Norvaline</td>
<td>2.17</td>
<td>607</td>
<td>280</td>
<td>100</td>
</tr>
<tr>
<td>(S)-L-2-Aminobutyric acid</td>
<td>20.0</td>
<td>519</td>
<td>26.0</td>
<td>83.7</td>
</tr>
<tr>
<td>(S)-Norleucine</td>
<td>3.72</td>
<td>523</td>
<td>141</td>
<td>72.5</td>
</tr>
<tr>
<td>β-Chloro-(S)-alanine</td>
<td>3.19</td>
<td>362</td>
<td>113</td>
<td>53.1</td>
</tr>
<tr>
<td>o-Acetyl-(S)-serine</td>
<td>5.94</td>
<td>294</td>
<td>49.5</td>
<td>35.8</td>
</tr>
<tr>
<td>(S)-Methionine</td>
<td>4.10</td>
<td>-</td>
<td>-</td>
<td>23.9</td>
</tr>
<tr>
<td>(S)-Isoleucine</td>
<td>6.20</td>
<td>-</td>
<td>-</td>
<td>22.5</td>
</tr>
<tr>
<td>(S)-Valine</td>
<td>3.00</td>
<td>-</td>
<td>-</td>
<td>21.9</td>
</tr>
<tr>
<td>(S)-Phenylalanine</td>
<td>8.70</td>
<td>222</td>
<td>25.5</td>
<td>21.7</td>
</tr>
<tr>
<td>(S)-Leucine</td>
<td>2.90</td>
<td>-</td>
<td>-</td>
<td>21.2</td>
</tr>
<tr>
<td>(S)-Alanine</td>
<td>5.10</td>
<td>-</td>
<td>-</td>
<td>16.0</td>
</tr>
<tr>
<td>o-Phospho-(S)-serine</td>
<td>8.69</td>
<td>153</td>
<td>17.6</td>
<td>15.8</td>
</tr>
<tr>
<td>(S,S)-2,3-Diaminopropionic acid</td>
<td>11.9</td>
<td>105</td>
<td>8.82</td>
<td>9.67</td>
</tr>
<tr>
<td>(S)-Phenylglycine</td>
<td>28.3</td>
<td>82.2</td>
<td>2.90</td>
<td>4.40</td>
</tr>
<tr>
<td>(S,S)-3-Aminobutyric acid</td>
<td>7.73</td>
<td>6.34</td>
<td>0.846</td>
<td>0.685</td>
</tr>
<tr>
<td>o-Phospho-(S)-threonine</td>
<td>9.01</td>
<td>6.54</td>
<td>0.726</td>
<td>0.640</td>
</tr>
<tr>
<td>(S)-Phenylalaninol</td>
<td>46.0</td>
<td>18.2</td>
<td>0.396</td>
<td>0.631</td>
</tr>
</tbody>
</table>

a The reaction with various amine-containing compounds was tested in 1 ml of the same reaction mixture as described in Section 2 except that the substrate concentration was varied from 10 to 100 mM.
b The following compounds were inert as substrates: 4-amino-3-hydroxybutyric acid, 4-aminobutyric acid, 6-aminohexanoic acid, (S)-phenylalanine methyl ester, (S)-phenylglycine methyl ester, (S)-norvaline methyl ester, (S)-alanine methyl ester, (S)-phenylalanine amide, (S)-phenylglycine amide, (S)-alanine amide, (S)-isoleucinol, (S)-valinol, (S)-alaninol, (S)-serinol, 1-amino-2,3-propanediol, 1-amino-2-propanol, (2S,3S)-2-amino-1-phenyl-1,3-propanediol, 3-amino-4-phenyl-4-butanol, ethanolamine, 2-amino-2-methyl-1,3-propanediol, methyamine, ethylamine, isopropylamine, butylamine, benzylamine, 3-aminopentane, 2-phenylethylamine, methoxyamine, dimethylamine, hydrine, phenylhydrazine, and hydroxyamine.
c Assayed with pyruvate concentrations fixed at 10 mM.
d Reported values [9].
and FDH (40 units) was incubated at 30°C for 22 h. Seven hours after starting the reaction, sodium phenylpyruvate (0.25 mmol), ODH (110 units), FDH (35 units), PheDH (35 units), and ammonium formate (2.5 mmol) was added. The reaction product was purified as described above. 

\[ ^1\text{H-NMR (D}_2\text{O)} \delta_{	ext{ppm}} 7.332-7.435 (m, 5H), 4.215 (dd, 1H, J = 4.4, 6.4 Hz), 3.929 (q, 1H, J = 7.3 Hz), 3.311 (d, 2H, J = 6.4 Hz), 1.489 (d, 3H, J = 7.3 Hz); ^{13}\text{C-NMR (D}_2\text{O)} \delta_{	ext{ppm}} 175.57, 174.65, 137.39, 132.25, 132.21, 130.89, 63.66, 58.63, 38.50, 16.91; \text{FAB-MS: m/z 260 (rel. int. 18\%), M + H}, 238 (38), 192 (40), 146 (19), 120(22), 91 (70), 44 (100); [\alpha]_{D}^{20} +7.7^\circ (c = 1.0, \text{H}_2\text{O}).

3. Results and discussion

3.1. Substrate specificity of Arthrobacter opine dehydrogenase

ODH utilizes hydrophobic (S)-amino acids such as (S)-methionine, (S)-isoleucine, (S)-valine, (S)-phenylalanine, and (S)-leucine etc. as amino donors [9], although the substrate specificity of unnatural-type amino acids have not been examined. We screened for a substrate among several amino compounds, which can act as amino donors in the secondary amine-forming reaction, using pyruvate as the fixed amino acceptor. Among the unnatural types ω-amino acids tested, short-chain neutral amino acids such as (S)-2-aminobutyric acid, (S)-norvaline, and (S)-norleucine, and aromatic ring containing (S)-phenylglycine were good substrates for the enzyme. 3-Chloro-(S)-alanine, 2,3-diaminopropanionic acid, as well as serine and threonine derivatives such as ω-phospho-(S)-serine, ω-phospho-(S)-threonine, and ω-acetyl-(S)-serine also acted as substrates. Other amino compounds such as ω-amino acids, amino acid esters and amides, amino alcohols, organic amines, hydroxylamines, and hydrazines were inactive as substrates.

Kinetic studies were performed to determine the Michaelis constant (K_m) and the maximum reaction velocity (V_max). As shown in Table 1, the presence of a hydrophilic group, such as an amino or phosphonooxy group in the substrate, caused a decrease of the V_max value, but not of the K_m value. The V_max/K_m value for (S)-phenylglycine was one-tenth of that for (S)-phenylalanine. We showed that (S)-methionine was the most suitable amino donor in the secondary amine-forming reaction and the relative activity compared with that of (S)-phenylalanine was 110% [9]. This study showed that the relative activity for (S)-norvaline was four times higher than that for (S)-methionine. Therefore, the enzyme should be called opine dehydrogenase (N-[1-(R)-(carboxyl)ethyl]-(S)-norvaline: NAD+ oxidoreductase (L-norvaline forming)). The β-amino alcohol, (S)-phenylalaninol, and the β-amino acid, 3-aminobutyric acid, were active as substrates but their relative activities were very low.

The enzyme showed a relatively wide substrate specificity for several (S)-α-amino acids as amino donors, whereas it did not utilize other amino compounds, including amino acid esters and amides, indicating that a free carboxylic acid moiety is needed for recognition as an amino donor. However, (S)-phenylalaninol, which has no carboxylic acid moiety, exceptionally acted as an amino donor for the enzyme. This finding will help elucidate the reaction mechanism of the enzyme. On the other hand, amino alcohols corresponding to aliphatic (S)-amino acids such as (S)-isoleucine, (S)-valine, (S)-alanine, and (S)-serine were inert as substrates, whereas the amino acids were good substrates. The reason for the requirement of an aromatic ring in the substrate remains unknown. Probably the affinity of the aromatic moiety for the enzyme compensated for the loss of a carboxylic acid in (S)-phenylalaninol.

3.2. Synthesis of secondary amine dicarboxylic acids from (S)-amino acids and α-keto acids

Several opine-type secondary amine dicarboxylic acids were synthesized from natural-type
(S)-amino acids and α-keto acids with NADH regeneration by FDH. (S)-Methionine, (S)-isoleucine, (S)-leucine, (S)-valine, (S)-phenylalanine, (S)-alanine, (S)-threonine, and (S)-serine were used as amino donors and glyoxylic, pyruvic, and 2-ketobutyric acids were used as amino acceptors. As shown in Table 2, several secondary amine dicarboxylic acids were synthesized almost quantitatively. However, isolated yields were low because of losses during purification. The diastereomeric excess of the product from pyruvate and 2-ketobutyrate was over 99.9% according to high-resolution 1H- and 13C-NMR analysis. To confirm the absolute configuration, KMnO4 oxidation, followed by HPLC analysis, proceeded as described in Section 2. When the secondary amine dicarboxylic acid was synthesized from (S)-isoleucine and pyruvate, the amino acids obtained from the oxidation reaction were optically pure (S)-isoleucine and (R)-alanine. In a similar manner, the absolute configurations of the new asymmetric centers of the synthesized secondary amine dicarboxylic acids were all of the (R) stereochemistry with > 99.9% enantiomeric excess (% e.e.). This one step enzymatic synthesis seems to be a most simple method for the stereoselective synthesis of opine-type secondary amine dicarboxylic acids without protection of the substrates, because chemical procedures generally require five to eight steps [10–15].

3.3. Synthesis of N-[1-(R)-(carboxyl)ethyl]-(S)-phenylalaninol from pyruvate and (S)-phenylalaninol

Based on the substrate specificity of the enzyme, the reductive condensation between pyruvate and the α-amino group of (S)-phenylalaninol was investigated. The reaction proceeded smoothly using three-fold more ODH than that used to synthesize amine dicarboxylic acids, and it quantitatively yielded a secondary amino acid according to HPLC. The compound was proven to be diastereomerically pure N-[1-(R)-(carboxyl)ethyl]-(S)-phenylalaninol.

3.4. One-pot synthesis of N-[1-(R)-(carboxyl)ethyl]-(S)-phenylalanine using ODH and PheDH

We showed that ODH acts on pyruvate but not on phenylpyruvate [9], whereas PheDH acts

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>α-Keto acid</th>
<th>Glyoxylate</th>
<th>Pyruvate</th>
<th>2-Ketobutyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Yield (%)</td>
<td>Yield (%)</td>
<td>Yield (%)</td>
</tr>
<tr>
<td>(S)-Methionine</td>
<td></td>
<td>&gt; 99</td>
<td>&gt; 99.9</td>
<td>&gt; 99.9</td>
</tr>
<tr>
<td>(S)-Isoleucine</td>
<td></td>
<td>&gt; 99</td>
<td>&gt; 99.9</td>
<td>&gt; 99.9</td>
</tr>
<tr>
<td>(S)-Leucine</td>
<td></td>
<td>&gt; 99</td>
<td>&gt; 99.9</td>
<td>&gt; 99.9</td>
</tr>
<tr>
<td>(S)-Valine</td>
<td></td>
<td>&gt; 99</td>
<td>&gt; 99.9</td>
<td>&gt; 99.9</td>
</tr>
<tr>
<td>(S)-Phenylalanine</td>
<td></td>
<td>&gt; 99</td>
<td>&gt; 99.9</td>
<td>&gt; 99.9</td>
</tr>
<tr>
<td>(S)-Alanine</td>
<td></td>
<td>&gt; 99</td>
<td>&gt; 99.9</td>
<td>&gt; 99.9</td>
</tr>
<tr>
<td>(S)-Threonine</td>
<td></td>
<td>&gt; 99</td>
<td>&gt; 99.9</td>
<td>&gt; 99.9</td>
</tr>
<tr>
<td>(S)-Serine</td>
<td></td>
<td>&gt; 99</td>
<td>&gt; 99.9</td>
<td>&gt; 99.9</td>
</tr>
</tbody>
</table>

Table 2
Synthesis of opine-type secondary amine dicarboxylic acids from (S)-amino acids and α-keto acids using opine dehydrogenase and formate dehydrogenase

* The yields were determined by HPLC on a ODS-80Ts column at 200 nm with a mobile phase of 40% acetonitrile containing 20 mM of HClO4 at a flow rate of 1.0 ml/min using their standard curves.

* Determined by 400 MHz 1H- and 13C-NMR.

* Determined by HPLC as described in Section 2.

* The obtained compound was in the meso-form.
on phenylpyruvate but not on pyruvate [3]. Based on these findings, we attempted the one-pot synthesis of N-[1-(R)-(carboxyl)ethyl]-(S)-phenylalanine from phenylpyruvate and pyruvate by using ODH and PheDH (Fig. 2). Fig. 3 shows the effect of the phenylpyruvate concentration in the reaction mixture. At any phenylpyruvate concentration, the secondary amine dicarboxylic acid was formed quantitatively within 2 h, but the yield was decreased by further incubation when the substrate concentration was over 100 mM. We therefore set the concentration of phenylpyruvate to 50 to 75 mM. The concentration of ammonium formate was then varied while the concentration of phenylpyruvate was kept constant at 50 mM. As shown in Fig. 4, the duration of the reaction time led to a decrease in yield when the ammonium formate concentration was low (up to 125 mM). The concentration of ammonium formate was set to 375 to 500 mM.

Thus, the reaction conditions were optimized as summarized in Section 2 and the secondary amine dicarboxylic acid was synthesized at a yield of 98%. We identified the compound as optically pure N-[1-(R)-(carboxyl)ethyl]-(S)-phenylalanine.

There are no reports describing the enzyme-catalyzed in vitro asymmetric synthesis of opine-type secondary amine carboxylic acids. When used in combination with FDH, ODH effectively synthesized them. The reductiveamination reaction of the enzyme proceeded D-(R)-stereospecifically. Generally, the known NAD(P)⁺ dependent amino acid dehydrogenases, which appear to share a reaction mechanism similar to that of ODH, are all L-stereospecific [1-5], except for NADP⁺-dependent meso-α,ε-diaminopimelate D-dehydrogenase [19]. The sequence and three-dimensional structural homology among amino acid dehydrogenases indicates the existence of an enzyme superfamily related by divergent evolution [20,21]. A comparison of the structure of ODH with other amino acid dehydrogenases would provide insight to the catalytic, structural, and evolutionary relationships among members of the dehydrogenase families.
References