BIOORGANIC CHEMISTRY 19, 29–39 (1991)

Enzyme-Catalyzed Asymmetric Synthesis of (*S*)-2-Amino-4-phenylbutanoic Acid and (*R*)-2-Hydroxy-4-phenylbutanoic Acid

CURT W. BRADSHAW,* C.-H. WONG,*,[1] WERNER HUMMEL,† AND MARIA-REGINA KULA†

* *Department of Chemistry, Research Institute of Scripps Clinic, La Jolla, California 92037; and*
† *Institute of Enzyme Technology, KFA Jülich, Heinrich-Heine University, Dusseldorf, Federal Republic of Germany*

Received August 21, 1990

This paper describes enzymatic procedures for the synthesis of (*S*)-2-amino-4-phenylbutanoic acid ((*S*)-APBA) and (*R*)-2-hydroxy-4-phenylbutanoic acid ((*R*)-HPBA), building blocks of angiotensin converting enzyme inhibitors. 2-Oxo-4-phenylbutanoic acid was converted to (*S*)-APBA and (*R*)-HPBA catalyzed by L-phenylalanine dehydrogenase from *Rhodococcus sp. M4* and D-lactate dehydrogenase from *Leuconostoc mesenteroides*, respectively. Both reactions require NADH and the cofactor was regenerated from NAD catalyzed by formate dehydrogenase or glucose dehydrogenase. Studies on the substrate specificity, stability, and substrate inhibition of phenylalanine dehydrogenase and comparison of the two regeneration systems for the synthesis of target molecules are also included.

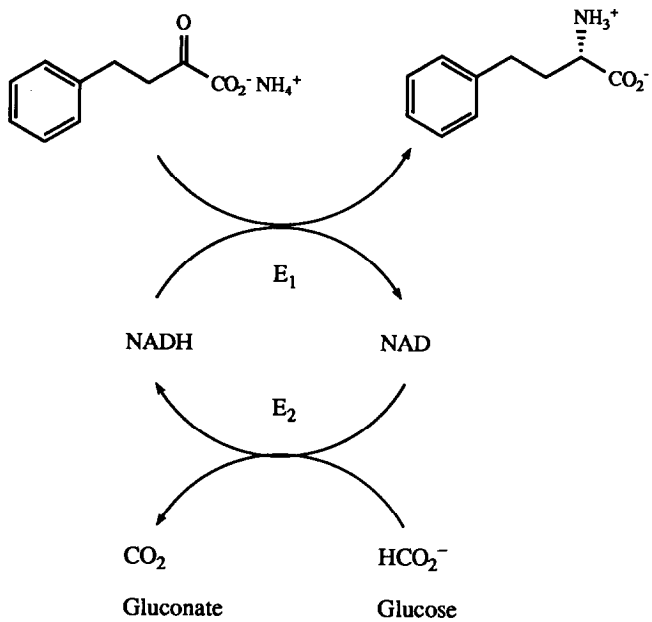
INTRODUCTION

Catalysis by dehydrogenases has been well utilized for the synthesis of chiral compounds (*1*). The enzymatic reduction of prochiral carbonyl groups can readily yield chiral alcohols, hydroxy acids, or amino acids from alcohol dehydrogenases, hydroxy acid dehydrogenases, or amino acid dehydrogenases, respectively. For example, the hydroxy acid dehydrogenases, D- and L-lactate dehydrogenase, have both demonstrated practicality for organic synthesis (*2*). Several amino acid dehydrogenases have been shown to be synthetically useful, including L-leucine (*3*) and L-phenylalanine dehydrogenase (PheDH) (*4*).

Initial studies on the substrate specificity of PheDH from *Rhodococcus* strain M4 revealed opportunities for the synthesis of a number of unusual amino acids (*4a*). In the present study, we report the substrate specificity and inhibition of PheDH and the application of PheDH to the synthesis of L-homophenylalanine ((*S*)-2-amino-4-phenylbutanoic acid) from 2-oxo-4-phenylbutanoic acid (Scheme 1). L-Homophenylalanine is a vital component of angiotensin converting enzyme and renin inhibitors (*5*). Angiotensin converting enzyme has been intensely studied as a medicinal target for the treatment of hypertension and heart failure (*6*). Strategies for the synthesis of L-homophenylalanine include selective crystalliza-

[1] To whom correspondence should be addressed.

0045-2068/91 $3.00



SCHEME 1. E_1 = phenylalanine dehydrogenase; E_2 = formate dehydrogenase or glucose dehydrogenase.

tion of diastereomeric salts (*7*), enzymatic resolution of racemic derivatives (*5b, 8*), chemical syntheses (*9*), and enzymatic transamination of 2-oxo-4-phenylbutanoic acid (*10*).

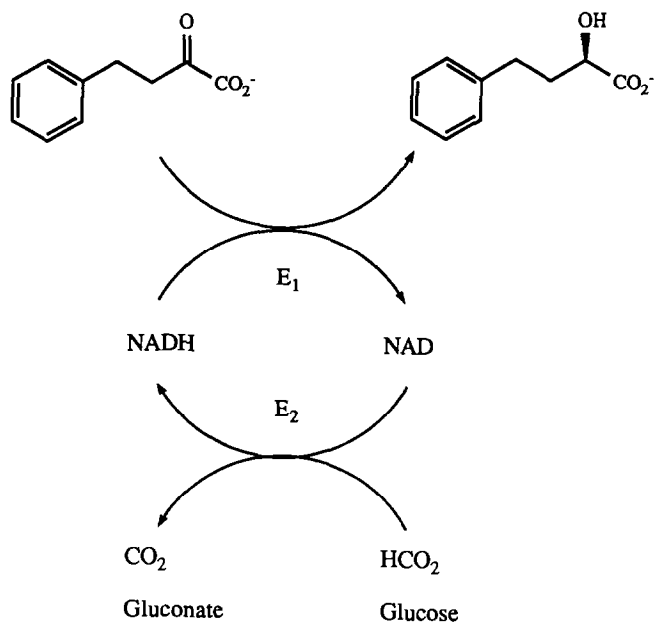
We also report the synthesis of (*R*)-2-hydroxy-4-phenylbutanoate from 2-oxo-4-phenylbutanoic acid catalyzed by D-lactate dehydrogenase from *Leuconostoc mesenteroides* (Scheme 2). (*R*)-2-Hydroxy-4-phenylbutanoic acid is also a useful intermediate for the synthesis of angiotensin converting enzyme inhibitors (*11*). The chiral hydroxy acid has been prepared via crystallization of diastereomeric salts (*12*), enzymatic resolution of derivatives (*11a, 13*), and various chemical methods (*14*). The present results are the first direct enzymatic reduction of 2-oxo-4-phenylbutanoic acid to obtain (*R*)-2-hydroxy-4-phenylbutanoic acid.

Both enzymatic reactions require NADH and regeneration of the nicotinamide cofactor was accomplished readily with the established formate/formate dehydrogenase (*15*) or glucose/glucose dehydrogenase (*16*) systems.

EXPERIMENTAL

Materials and Methods

D-Lactate dehydrogenase (*Leuconostoc mesenteroides*) (EC 1.1.1.28), cofactors, and buffers were obtained from Sigma. Glucose dehydrogenase (*Bacillus* species) (EC 1.1.1.47) and formate dehydrogenase (yeast) (EC 1.2.1.2) were pur-



SCHEME 2. E_1 = D-lactate dehydrogenase; E_2 = formate dehydrogenase or glucose dehydrogenase.

chased from Amano and Boehringer Mannheim, respectively. L-Phenylalanine dehydrogenase (*Rhodococcus sp.* M4) was prepared as described previously (*4*). NMR spectra were recorded on a Bruker AM-300 spectrometer. Assays were performed using a Beckmann DU-70 spectrophotometer. Melting points were determined with a Thomas Hoover unimelt capillary melting point apparatus. Kinetic data were analyzed with the aid of the Enzfitter program available from Biosoft.

Synthesis of 2-Oxo-4-Phenylbutanoic Acid

Ethyl-2-oxo-4-phenylbutanoic acid (190 mmol; 39.14 g), prepared from 2-phenylethylmagnesium bromide and diethyl oxalate (*17*), was stirred overnight in 400 ml 1 N NaOH at 50°C. The aqueous layer was acidified to pH 1 and extracted 3 × 150 ml with ethyl acetate. The ethyl acetate layers were combined, dried over sodium sulfate, and evaporated to yield a residue which was recrystallized from toluene to give 179 mmol (32 g) of the title compound, 95% yield. Melting point, 41–43°C; NMR ($CDCl_3$) ^1H:2.97 ppm (t, 2H), 3.28 ppm (t, 2H), 7.23 ppm (m, 5H), 8.16 ppm (bs, 1H); ^{13}C:29.0, 39.6, 126.9, 128.7, 129.0, 140.1, 161.0, 195.4 ppm (literature mp 44°C (*17*)).

Synthesis of L-Homophenylalanine

2-Oxo-4-phenylbutanoic acid (7.22 mmol; 1.285 g), 8 mmol (0.544 g) sodium formate, and 0.75 mmol (0.116 g) dithiothreitol were dissolved in 120 ml H_2O with

the addition of concentrated ammonium hydroxide to maintain the pH at 7.8. This solution was degassed with argon. NAD (20 mg), 25 U formate dehydrogenase, and 75 U phenylalanine dehydrogenase were dissolved in an aliquot of the solution, enclosed in a previously washed dialysis bag (SpectraPor 2 MWCO 12–14,000), and placed in the degassed solution. The solution was stirred at room temperature in an inert atmosphere at pH 7.8 maintained by the addition of 1 N NH_4OH. Three days later, an additional 60 U of both enzymes was added. Another 4 days later when the consumption of base ceased, the formate dehydrogenase retained 40% and the phenylalanine dehydrogenase had 10% of the original activity. Upon removal of the dialysis bags, the solution was acidified to pH 5.5 and stored at 4°C. The resulting white precipitate was isolated by filtration to give 0.815 g of product, 63% yield. The precipitate was resuspended in 50 ml water (pH 5.5) and stored at 4°C. Pure product precipitated upon standing, 48% yield; $[\alpha]_D^{20}$, +48° (c 1, 1 N HCl); NMR (D_2O) ^1H:2.25 ppm (m, 2 H), 2.82 ppm (m, 2H), 4.1 ppm (t, 1 H), 7.36 ppm (m, 5H) (literature $[\alpha]_D^{20}$, = +48 (c 1, 1 N HCl) (*10a*)).

Synthesis of (R)-2-hydroxy-4-phyenylbutanoic Acid

2-Oxo-4-phenylbutanoic acid (4.7 mmol; 0.84 g) was dissolved in 80 ml water containing 5.4 mmol (0.367 g) sodium formate, 30 mg NAD, and 0.5 mmol (0.08 g) dithiothreitol at pH 7.5. An aliquot of this degassed solution was used to dissolve 5000 U D-lactate dehydrogenase and 45 U formate dehydrogenase. The enzymes were enclosed in a previously washed dialysis tube (SpectraPor2 MWCO 12–14,000) and suspended in the solution. The reaction was kept under an argon atmosphere and the pH maintained at 7.5 with continuous addition of 1 N HCl. The lactate dehydrogenase retained 65% of the original activity and the formate dehydrogenase had 40% of the original activity when the reaction was finished (7 days). The pH of the solution was adjusted to 3 with concentrated HCl and extracted 3 × 30 ml with ethyl acetate. The organic layers were combined and dried over sodium sulfate and evaporated to dryness to yield 4 mmol (0.72 g) of product, 85% yield. The white solid was further purified with recrystallization from toluene. NMR ($CDCl_3$) ^1H:1.95–2.25 ppm (m, 2H), 2.77 ppm (t, 2H), 4.24 ppm (q, 1H), 6.0 ppm (bs, 1H), 7.22 ppm (m, 5H); ^{13}C:31.0 ppm, 35.7 ppm, 69.6 ppm, 126.2 ppm, 128.49 ppm, 128.54 ppm, 140.71 ppm, 179.66 ppm; mp, 113–115°C; $[\alpha]_D^{20}$, −9.4° (c = 1, EtOH) (literature mp 114–116°C (*14b*)).

Alternatively, the NADH could be regenerated with the use of glucose dehydrogenase. Using the same procedure as above with 5 mmol glucose and 60 U glucose dehydrogenase with 0.3 M NaCl in the buffer, instead of formate/formate dehydrogenase, the reaction was complete in 7 days. The D-lactate dehydrogenase retained 40% of the original activity and the glucose dehydrogenase had 60% remaining. Product isolation was accomplished via identical means with the same yield (81%) and without complications from the gluconic acid or glucose.

Comparison of the Stability of Enzymes for Cofactor Regeneration

Stability of soluble enzymes. The following solutions were stirred at room temperature and assayed periodically for decrease in enzyme activity over time: (a) 1

mg glucose dehydrogenase in 4 ml 0.1 M Tris buffer, pH 7.0; (b) 1 mg glucose dehydrogenase in 4 ml 0.1 M Tris buffer containing 0.3 M NaCl, pH 7.0; (c) 3.5 mg formate dehydrogenase in 4 ml 0.1 M Tris buffer, pH 7.0; (d) 25 U D-lactate dehydrogenase in 4 ml 25 mM Tris buffer, pH 7.3, with various concentrations of NaCl. The assays were performed as described previously (*19*).

Stability of enzymes under reaction conditions. Enzymes enclosed in dialysis tubes (SpectraPor2 MWCO 12–14,000) were stirred in the following solutions at room temperature and assayed periodically for the loss of enzyme activity over time: (a) 1 mg NAD, 4 mg 2-oxo-4-phenylbutanoic acid, 5 mg glucose, 100 U D-lactate dehydrogenase, 10 ml 5 mM Hepes buffer, pH 7.3; (b) 1 mg NAD, 4 mg 2-oxo-4-phenylbutanoic acid, 5 mg glucose, 100 U D-lactate dehydrogenase, 10 ml 5 mM Hepes buffer containing 0.3 M NaCl, pH 7.3; (c) 1 mg NAD, 4 mg sodium formate, 15 U formate dehydrogenase, 10 ml 5 mM Hepes, pH 7.3. Aliquots of the enzyme solution were removed periodically and assayed by monitoring the change of absorbance at 340 nm (ε NADH = 622 liter/mol mm) with the following substrate concentrations in 50 mM Tris, pH 8.5: (a) 60 mM glucose and 2 mM NAD; (b) 75 mM formate and 6 mM NAD; (c) 40 mM pyruvate and 0.5 mM NADH.

Determination of Kinetic Parameters

Phenylalanine dehydrogenase. Assays were performed by combining aliquots of each of the following solutions and equilibrating in a cuvette at 25°C: 700 mM ammonium chloride in 100 mM Tris buffer, pH 8.5; 1.3 mM NADH (or alternatively 4 mM NAD) in the buffer; and 6 mM of the α-oxoacids (or 3 mM of various amino acids) in the buffer. The reaction was started by addition of enzyme (final volume 1 ml) and monitored spectrophotometrically. The initial velocities were determined based on the change of absorbance at 340 nm where $\varepsilon = 6.22\ \text{mM}^{-1}\ \text{cm}^{-1}$ for NADH. Michaelis constants were obtained from double reciprocal plots. The substrate inhibition data were fitted to the following equation with the aid of the Enzfitter program from Biosoft which is a nonlinear least squares fit (*20*):

$$v = \frac{V_{\max} AB}{K_{ia}K_b (1 + B/K_i) + K_a B (1 + B/K_i) + K_b A + AB},$$

where A is the concentration of NADH, B is the concentration of ketone, K_a is the Michaelis constant for NADH, K_b is the Michaelis constant for ketone substrate, K_{ia} is the dissociation constant for EA and K_i is the dissociation constant of the EB complex.

D-Lactate dehydrogenase. Various amounts of the following solutions were combined in a cuvette for standard assays: 100 mM triethanolamine buffer, pH 7.6; 1.34 mM NADH in the buffer; and 3.26 mM 2-oxo-4-phenylbutanoic acid or 3.91 mM pyruvate in the buffer. After equilibration at 25°C, an aliquot of the enzyme solution was added to the cuvette to yield a total volume of 1 ml. The change in absorbance at 340 nm was recorded for determination of initial velocities in the same manner as above.

TABLE 1

Substrate Specificity of PheDH for the Reductive Amiantion of Ketoacid Substrates

Substrate	K_m	V^a_{rel}	Ref.
Phenyl pyruvate	160 μM	100	(*4a*)
2-Oxo-4-pheynlbutanoate	117 μM	72	
2-Keto-4-methylmercaptobutanoate	2.1 mM	33	(*4a*)
4-Hydroxyphenylpyruvate	2.4 mM	5	(*4a*)
Indole pyruvate	7.7 mM	3	(*4a*)
Ethyl-2-oxo-4-phenylbutanoate		0	

[a] Relative velocities were determined at substrate concentrations three times the K_m value. Specific activity of the partially purified enzyme is 1300 U/mg (*4a*) where one unit is defined as 1 μmol NADH consumed per minute.

RESULTS AND DISCUSSION

The substrate specificity of phenylalanine dehydrogenase indicates possibilities for the synthesis of several unusual amino acids (Table 1). L-Phenylalanine derivatives with modification at the aromatic group can be prepared from the corresponding 2-keto acid. Our limited study seems to indicate that the homoanalogs of L-phenylalanine (L-homophenylalanine) and other amino acids with related structure (L-methionine) are good substrates for the enzyme. Various 2-oxo-4-substituted butanoates may be converted to the corresponding L-α-amino acids. The enzyme does not accept D-amino acids, esters, or amides and surprisingly does not accept L-histidine or L-phenylglycine as substrates.

As an example, L-homophenylalanine was selected for a representative synthesis based upon the value of the product. The synthesis of 2-oxo-4-phenylbutanoic acid could be easily accomplished on a 40-g scale. Inhibition of the enzyme (pheDH) was observed at high concentrations of substrate. The initial velocity data for 2-oxo-4-phenylbutanoic acid obtained with excess NH_4^+ were thus fitted to an equation for the determination of substrate inhibition (see Experimental) on the basis of an ordered mechanism (Fig. 1). The calculated substrate inhibition constant (K_i) for 2-oxo-4-phenylbutanoic acid is 230 μM. No product inhibition was observed. It is obvious that 2-oxo-4-phenylbutanoic acid must be kept at about 200 μM over the reaction period in order to have the maximum reaction rate. The initial velocity data for phenylpyruvate, however, did not show any substrate inhibition (Fig. 2).

In the representative synthesis, the reductive amination of 2-oxo-4-phenylbutanoic acid proceeds in 63% yield. After a period of 7 days, phenylalanine dehydrogenase retained 10% of the original activity and formate dehydrogenase had 40% of original activity under reaction conditions. The enzymes were placed in a dialysis tube to allow for free passage of substrates, cofactors, and products, while retaining the enzymes (*2a*). L-Homophenylalanine was isolated and purified at pH 5.5. The product was optically pure as determined by optical rotation and compared to an authentic sample and literature values.

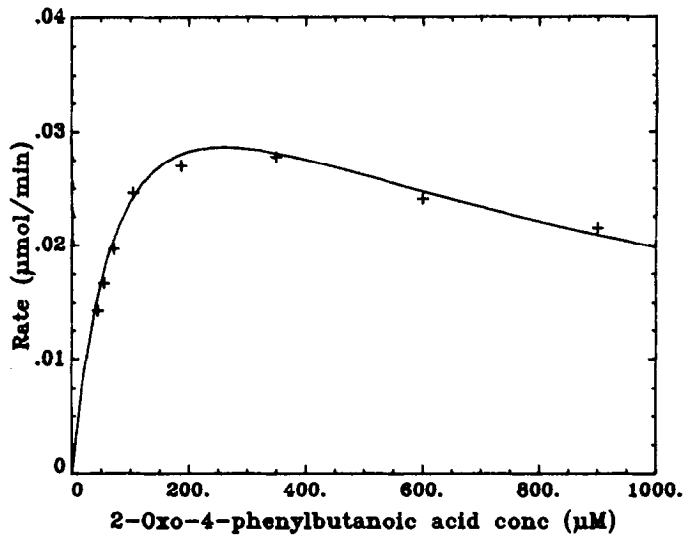


FIG. 1. Initial velocity for the reductive amination of 2-oxo-4-phenylbutanoate catalyzed by phenylalanine dehydrogenase.

An alternative approach to homophenylalanine from the same ketoacid was conversion of the ketoacid to (*R*)-HPBA followed by chemical conversion to L-homophenylalanine. The chiral hydroxyacid can also be directly converted to angiotensin converting enzyme inhibitors (*11*). D-Lactate dehydrogenase from *L. mesenteroides*, which is commercially available and inexpensive, was used for the synthesis of (*R*)-HPBA.

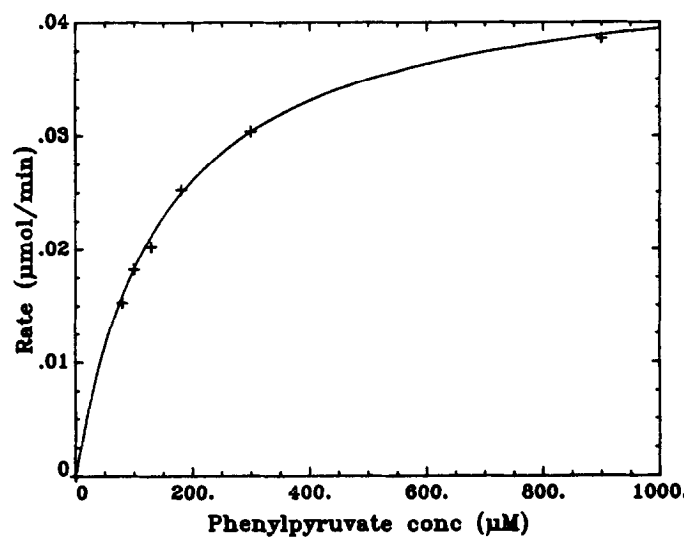


FIG. 2. Initial velocity for the reductive amination of phenylpyruvate catalyzed by phenylalanine dehydrogenase.

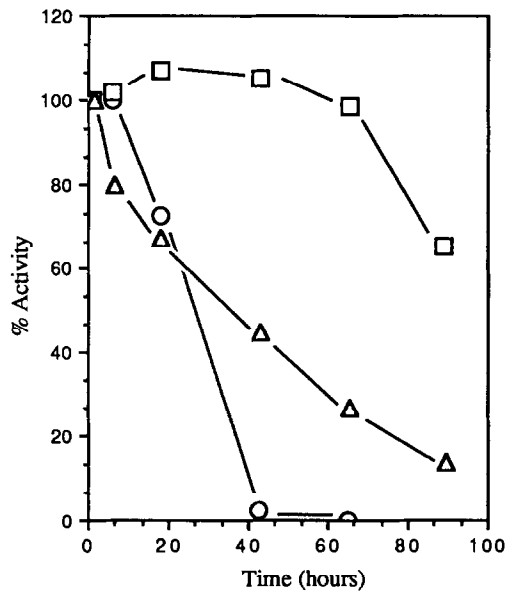


FIG. 3. Stability of soluble enzymes in 0.1 M Tris, pH 7.0 (○) Glucose dehydrogenase; (□) glucose dehydrogenase with 0.3 M NaCl; (△) formate dehydrogenase.

2-Oxo-4-phenylbutanoic acid was found to be a substrate for D-lactate dehydrogenase with K_m 12 mM and V_{rel} 0.2, where the V_{rel} for phenylpyruvate is 10. Using the membrane reaction technique and formate dehydrogenase or glucose dehydrogenase for NADH regeneration, the reaction proceeds in high yield, 85%. The product was easily isolated from the reaction mixture via organic solvent extraction and recrystallized from toluene.

An important consideration for cofactor regeneration is the stability of the enzymes. The most successful regeneration methods to date are formate dehydrogenase and glucose dehydrogenase. Since sodium chloride has been shown to affect the stability of glucose dehydrogenase (*16*), a comparative investigation of glucose dehydrogenase and formate dehydrogenase with respect to their stability and activity in the presence of NaCl was undertaken. The stabilities of free glucose dehydrogenase and formate dehydrogenase are illustrated in Fig. 3. As indicated, glucose dehydrogenase is less stable than formate dehydrogenase in the absence of NaCl. In the presence of NaCl (0.3 M), glucose dehydrogenase, however, is much more stable than formate dehydrogenase; NaCl had no effect on the stability of formate dehydrogenase. Enclosing the enzymes in dialysis bags yields similar results (Fig. 4).

Sodium chloride does not adversely affect the activity of glucose dehydrogenase at pH 7, with no loss of activity below 2 M NaCl (Fig. 5). At pH 8.5, the enzyme exhibits decreased activity from high concentrations of sodium chloride. The activities of other enzymes are slightly inhibited as the concentration of NaCl is increased. These results indicate that glucose dehydrogenase is useful for

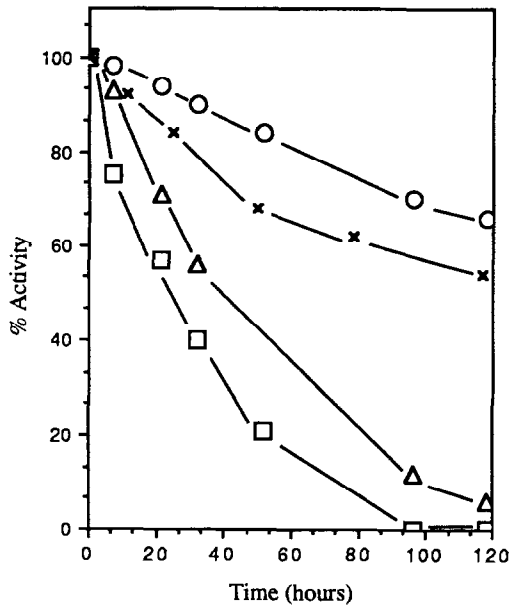


FIG. 4. Stability of enzymes under reaction conditions (see Experimental). (□) Glucose dehydrogenase; (○) glucose dehydrogenase with 0.3 M NaCl; (△) formate dehydrogenase; (×) D-lactate dehydrogenase.

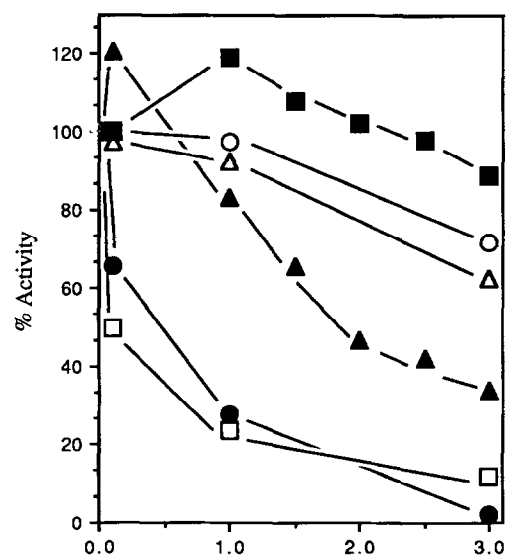


FIG. 5. Effect of NaCl on enzyme activity in 0.1 M Tris, pH 7.0 (○) L-lactate dehydrogenase; (△) D-lactate dehydrogenase; (□) horse liver alcohol dehydrogenase; (●) *Thermoanaerobium brockii* alcohol dehydrogenase; (■) glucose dehydrogenase, pH 7.0; (▲) glucose dehydrogenase, pH 8.5.

TABLE 2

Substrate Specificity of PheDH for the Oxidative Deamination of Amino Acids

Substrate	K_m	V_{rel}	Ref.
L-Phenylalanine	750 μM	100	(*4a*)
L-Homophenylalanine	650 μM	33	
L-Tyrosine	3.1 mM	12	(*4a*)
L-Methionine	430 μM	4	(*4a*)
L-Tryptophan	11 mM	2	(*4a*)
p-Fluoro-L-phenylalanine	650 μM	62	
p-Chloro-L-phenylalanine	150 μM	17	
p-Bromo-L-phenylalanine	60 μM	7	
D-Tyrosine		0	
L-Histidine		0	
L-Phenylglycine		0	
L-Phenylalanine ethyl ester		0	
L-Phenylalanineamide		0	

NAD(P)H regeneration when the byproduct gluconate does not complicate product isolation and when NaCl does not inhibit the activity of the coupling enzyme. The activity of D-lactate dehydrogenase is unaffected at concentrations below 1 M, but drops to 70% activity at 3 M NaCl. On the other hand, the stability of the enzyme between 0 and 1 M NaCl is essentially the same.

In summary, both enantiomerically pure (*R*)-HPBA and (*S*)-APBA have been prepared enzymatically from 2-oxo-4-phenylbutanoate in high yields. While phenylalanine dehydrogenase is not yet commercially available, it can be isolated readily from *Rhodococcus* and represents a good method for the synthesis of L-homophenylalanine (*4*). The enzymatic synthesis yields enantiomerically pure amino acid without problems associated with resolution. Using standard techniques such as membrane reactors, phenylalanine dehydrogenase will undoubtedly provide new and unusual amino acids.

REFERENCES

1. (a) SIH, C. J., AND CHEN, C.-S. (1984) *Angew. Chem. Int. Ed. Engl.* **23,** 570–578; (b) BADER, J., GUNTHER, H., NEUMANN, S., AND THANOS, J. (1985) *Angew. Chem. Int. Ed. Engl.* **24,** 539–553; (c) Jones, J. B. (1986) *Tetrahedron* **42,** 3351–3403; (d) CHENAULT, H. K., AND WHITESIDES, G. M. (1987) *Appl. Biochem. Biotechnol.* **14,** 147–197; (e) DAVIES, H. G., GREEN, R. H., KELLY, D. R., AND ROBERTS, S. M. (1989) Biotransformations in Preparative Organic Chemistry, Academic Press, San Diego; (f) KEINAN, E., SETH, K. K., LAMED, R., GHIRLANDO, R., AND SINGH, S. P. (1990) *Biocatalysis* **3,** 57–71; (g) HUMMEL, W., AND KULA, M.-R. (1989) *Eur. J. Biochem.* **184,** 1–13; (h) WONG, C.-H. (1990) *Chemtracts-Organic Chemistry* **3,** 91–111.
2. (a) SIMON, E. S., PLANTE, R., AND WHITESIDES, G. M. (1989) *Appl. Biochem. Biotechnol.* **22,** 169–179; (b) KIM, M.-J., AND WHITESIDES, G. M. (1988) *J. Amer. Chem. Soc.* **110,** 2959–2964.
3. WANDREY, C., AND BOSSOW, B. (1986) *Biotechnol. Bioind,* **3,** 8–13.
4. (a) HUMMEL, W., SCHÜTTE, H., SCHMIDT, E., WANDREY, C., AND KULA, M.-R. (1987) *Appl. Microbiol. Biotechnol.* **26,** 409–416; (b) HUMMEL, W., SCHMIDT, E., WANDREY, C., AND KULA, M.-R. (1986) *Appl. Microbiol. Biotechnol.* **25,** 175–185.

5. (a) JOHNSON, A. L., PRICE, W. A., WONG, P. C., VAVALA, R. F., AND STRUMP, J. M. (1985) *J. Med. Chem.* **28,** 1596–1602; (b) HAYASHI, K., NUNAMI, K., KATO, J., YONEDA, N., KUBO, M., OCHIAI, T., AND ISHIDA, R. (1989) *J. Med. Chem.* **32,** 289–297.
6. (a) ONDETTI, M. A., AND CUSHMAN, D. W. (1981) *J. Med. Chem.* **24,** 355–361; (b) ABRAMS, W. B., DAVIES, R. O., AND FERGUSON, R. K. (1984) *Fed. Proc.* **43,** 1314–1321.
7. (a) MIYAZAWA, Y., OISHI, N., AND MAEHARA, K. Japan. Kokai Tokkyo Koho JP 63 63, 646; (b) MYAZAWA, Y., OOISHI, N., AND MAEHARA, K. Japan. Kokai Tokkyo Koho JP 63, 145, 256
8. (a) SCHUTT, H., SCHMIDT-KASTNER, G., ARENS, A., AND PREISS, M. (1988) *Biotechnol. Bioeng.* **28,** 420–433; (b) KOSUI, N., SHIMOHIGASHI, Y., WAKI, M., KATO, T., AND IZUMIYA, N. (1981) *Mem. Fac. Sci. Kyushu, Univ. Ser. C.* **13,** 89–96; (c) TANAKA, A., AND IZUMIYA, I. (1958) *Bull. Chem. Soc. Japan.* **31,** 529–532; (d) TANAKA, A. (1958) *Fukuoka Igaku Zasshi* **49,** 3546–3554; (e) NABE, K., NISHIDA, Y., NAKAMICHI, K., AND NISHIMOTO, S. Japan Kokai Tokkyo Koho JP 63, 273, 498; (f) KELLER, R., AND MUELLER, W. Eur Pat. Appl. EP 310,012.
9. (a) WILLIAMS, R. M., SINCLAIR, P. J., ZHAI, D., AND CHEN, D. (1988) *J. Amer. Chem. Soc.* **110,** 1547–1557; (b) HASHIMOTO, Y., AOYOGI, H., WAKI, M., KATO, T., AND IZUMIYA, N. (1983) *Int. J. Peptide Protein Res.* **21,** 11–15.
10. (a) SENUMA, M., NAKAMICHI, K., NABE, K., NISHIMOTO, S., AND TOSA, T. (1989) *Appl. Biochem. Biotechnol.* **22,** 141–150; (b) TOSA, T., NABE, K., NISHIDA, Y., NAKAMICHI, K., YONEDA, N., HAYASHYI, M., AND NUMANAMI, K. Japan. Kokai Tokkyo Koho JP 60, 156, 394; (c) ASANO, Y., IWASAKI, G., AND KONDO, S. Japan. Kokai Tokkyo Koho JP 01 47,386.
11. (a) IWASAKI, G., KIMURA, R., NUMAO, N., AND KONDO, K. (1989) *Chem. Pharm. Bull.* **37,** 280–283; (b) MONSERAT VIDAL, C. Span. ES 550,548
12. NERDEL, RACHEL, H. (1956) *Chem. Ber.* **89,** 671–77.
13. WANG, Y. F., CHEN, S. T., LIU K. C., AND WONG, C.-H. (1989) *Tetrahedron Lett.* **30,** 1917–1920.
14. (a) MINAMIKAWA, H., HAYAKAWA, S., YAMADA, T., IWASAWA, N., AND NARASAKA, K. (1988) *Bull. Chem. Soc. Jpn.* **61,** 4379–4383; (b) YANAGISAWA, H., ISHIHARA, S., ANDO, A., KANAZAKI, T., MIYAMOTO, S., KOIKE, H., IIJIMA, Y., OIZUMA, K., MAYSUSHITA, Y., AND HATA, T. (1988) *J. Med. Chem.* **30,** 1984–1991; (c) HASHIMOTO, M., AOKI, M., AND OSANAI, Y. Japan. Kokai Tokkyo Koho JP 62, 212, 329; (d) FLYNN, G. A., BEIGHT, D. W. Eur Pat. Appl. EP 280,285; (e) TAKAHASHI, S., YAMADA, K., FUKAI, M. Japan. Kokai Tokkyo Koho JP 01 79,133 Japan. Kokai Tokkyo Koho JP 01 79,134.
15. (a) WICHMANN, R., WANDREY, C., BUCKMANN, A. F., AND KULA, M.-R. (1981) *Biotechnol. Bioeng.* **23,** 2789–2802; (b) SHAKÈD, Z., AND WHITESIDES, G. M. (1980) *J. Amer. Chem. Soc.* **102,** 7104–7105.
16. WONG, C.-H., DRUECKHAMMER, D. G., AND SWEERS, H. M. (1985) *J. Amer. Chem. Soc.* **107,** 4028–4031.
17. The ethyl ester was prepared according to BLACKLOCK, T. J., SHUMAN, R. F., BUTCHER, J. W., SHEARIN, W. C., BUDAVARI, J., AND GRENDA, V. J. (1988) *J. Org. Chem.* **53,** 836–844.
18. ANATOL, J., AND MEDÈTE, A (1971) *Synthesis* **10,** 538–539.
19. BERGMEYER, H. U. (Ed.) Methods of Enzymatic Analysis Verlag Chemie: Weinheim, 1983.
20. SEGEL, I. H. (1975) Enzyme Kinetics pp 819–826, Wiley–Interscience. New York.