Short Communication

Occurrence of D-Threonine Dehydrogenase in *Pseudomonas cruciviae*

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Although methylene blue or 2,6-dichlorophenolindophenol-dependent D-amino acid dehydrogenases have been found in various bacteria, including *Pseudomonas aeruginosa*,^{1,2)} *P. fluorescens*,³⁾ *Salmonella typhimurium*,⁴⁾ *Escherichia coli* B,^{5~7)} and *E. coli* K 12,⁸⁾ no pyridine nucleotide-dependent D-amino acid dehydrogenase has been reported. We found an NADP⁺-dependent D-threonine dehydrogenase in the crude extract of *P. cruciviae* IFO 12047.

We present here evidence for the occurrence of a novel pyridine nucleotide-dependent D-amino acid dehydrogenase that catalyzes the oxidation of 3-hydroxy group of D-threonine.

The dehydrogenase activity was determined at 30°C by measuring the rate of increase in the absorbance at 340 nm with a Shimadzu double-beam spectrophotometer UV-140-02 using a cuvette with a 1.0-cm light path. The standard reaction mixture consisted of 40 μ mol of D-threonine, 1 μ mol of NADP⁺, 200 μ mol of glycine-KCl-KOH buffer (pH 10.5), and enzyme in a final volume of 1.0 ml. Protein was measured by the method of Lowry *et al.*⁹⁾ with bovine albumin as the standard. *P. cruciviae* IFO 12047 was grown aerobically at 30°C for 24 hr in a medium (pH 7.2) containing 1.5% peptone, 0.2% K₂HPO₄, 0.2% KH₂PO₄, 0.2% NaCl, 0.01% MgSO₄·7H₂O, and 0.01% yeast extract. The washed cells were disrupted in a mortar with aluminum oxide, and extracted with 0.1 M potassium phosphate buffer (pH

7.2) containing 0.02% 2-mercaptoethanol. The supernatant solution obtained by centrifugation was dialyzed against 0.01 M potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and used as the crude extract. The crude extract was brought to 55% saturation with solid ammonium sulfate. The precipitate obtained by centrifugation was dissolved in 0.01 M potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and was dialyzed against the same buffer. To the enzyme solution was added 1.0 ml of 1.5% protamine sulfate solution (pH 7.2) per 100 mg of the protein with stirring. After 10 min, the bulky precipitate was removed by centrifugation. The enzyme solution was put on a DEAE-cellulose column equilibrated with 0.01 M potassium phosphate

FIG. 1. Amino Acid Analysis of the Reaction Product Formed from D-Threonine.

The reaction mixture (1.0 ml) containing 10 μ mol of D-threonine, 10 μ mol of NADP⁺, 100 μ mol of Tris-HCl buffer (pH 9.0), 10 μ mol of KOH, and 0.44 mg of enzyme was incubated at 30°C for 1 hr. After the addition of 0.1 ml of 50% trichloroacetic acid and centrifugation, the supernatant solution was diluted 20 times with 0.02 M HCl and 0.05 ml of the sample was put on a column (4 $\times$ 150 cm) of a Hitachi 835 automatic amino acid analyzer. A, the reaction mixture (1 hr); B, the reaction mixture (0 time); and C, authentic threonine (I), ammonia (II), histidine (III), aminoacetone (IV), and arginine (V).

buffer (pH 7.4) containing 0.01% 2-mercaptoethanol. After the column was washed with this buffer and with the buffer containing 0.15 M KCl, the enzyme was eluted with the buffer supplemented with 0.2 M KCl. The active fractions were pooled and concentrated. All operations were done at 0~5°C. This enzyme preparation was used in this experiment.

When D-threonine was incubated with the enzyme in the presence of NADP^+ at pH 9.0 (Tris-HCl buffer), the reduction of NADP^+ was observed. The reaction occurred only in the presence of D-threonine and enzyme, and proceeded linearly as a function of the amount of enzyme and incubation time. Ammonia was not formed by the reaction, when measured by a microdiffusion procedure¹⁰⁾ using Nessler's reagent¹¹⁾ and with a Hitachi 835 automatic amino acid analyzer. This shows that the enzyme does not catalyze the deamination of D-threonine, although D-amino acid oxidases,^{12~14)} dye-dependent D-amino acid dehydrogenase,³⁾ and D-serine dehydratases^{15,16)} act on D-threonine. To identify the reaction product, the reaction mixture was analyzed with a Hitachi 835 automatic amino acid analyzer. In addition to the peak of threonine, an unknown peak was observed between histidine and arginine. The retention time of this peak was in accord with that of the peak of the authentic aminoacetone (Fig. 1). Since aminoacetone reacts with acetylacetone to form 2,4-dimethyl-3-acetylpyrrole,¹⁷⁾ the reaction mixture was reacted with acetylacetone at 100°C for 10 min. The pyrrole formed was extracted with ether and chromatographed on a thin-layer cellulose plate (DC-plastikfolien cellulose, Merck, Art 5577). The developing solvent was the upper phase of the mixture, *n*-butanol-H₂O-25% NH₄OH (10:3:1, by volume). The pyrrole was visualized by spraying the Ehrlich-Hg reagent.¹⁷⁾ The pyrrole of the reaction product had an *R_f* value (0.82) very similar to that of aminoacetone. When aminoacetone was measured by the method of Urata and Granick,¹⁷⁾ it was formed only in the presence of D-threonine, NADP^+ , and

FIG. 2. D-Threonine Dehydrogenase Reaction.

enzyme, and the amount of aminoacetone increased with increasing incubation time. The results obtained show that the 3-hydroxy group of D-threonine is oxidized by the enzyme to yield D-2-amino-3-ketobutyrate, which is spontaneously decarboxylated into aminoacetone (Fig. 2).

Aminoacetone is formed by an L-threonine dehydrogenase reaction with the intermediate formation of L-2-amino-3-ketobutyrate.¹⁸⁾ The half-life of 2-amino-3-ketobutyrate is less than 1 min at pH 7.0 and 37°C.¹⁸⁾ L-Threonine dehydrogenase activity has been found in cell-free extracts of microorganisms^{19~21)} and mammalian tissues.^{17,22,23)} The enzyme has been purified to homogeneity from *E. coli* K 12,²⁴⁾ chicken liver,²⁵⁾ and goat liver.²⁶⁾ L-Threonine dehydrogenase is NAD^+ -specific, and NADP^+ is inactive as a coenzyme. The enzyme from *E. coli* K 12 acts on D-*allo*-threonine in addition to L-threonine.²⁴⁾ D-Threonine, however, is not a substrate.

D-Threonine dehydrogenase of *P. cruci-viae* was specific for D-threonine, and the other three isomers of threonine were not substrates. The following hydroxy acids and amino acids were also inert: DL-3-hydroxy-*n*-butyrate, DL-3-hydroxypropionate, DL-2-hydroxy-*n*-butyrate, DL-4-hydroxy-*n*-butyrate, DL-lactate, D-serine, and L-serine. The enzyme had an optimum reactivity at about pH 10.5. NAD^+ showed a slight coenzyme activity (4.5% of the activity obtained with NADP^+). The *K_m* values for D-threonine and NADP^+ were 2.1 mM and 18 μM , respectively. Thus, there is good evidence for the occurrence of a novel NADP^+ -dependent dehydrogenase catalyzing the oxidation of D-threonine in the crude

extract of *P. cruciviae* IFO 12047. This is the first pyridine nucleotide-dependent amino acid dehydrogenase shown to work specifically on a D-amino acid.

Further work is needed to elucidate both the enzymological and physicochemical properties of the enzyme, and its physiological functions.

REFERENCES

- 1) V. P. Marshall and J. R. Sokatch, *J. Bacteriol.*, **95**, 1419 (1968).
- 2) A. J. Bater and W. A. Venables, *Biochim. Biophys. Acta*, **468**, 209 (1977).
- 3) K. Tsukada, *J. Biol. Chem.*, **241**, 4522 (1966).
- 4) J. Wild, W. Walczak, K. Krajewska-Cryniewicz and T. Klopotoski, *Mol. Gen. Genet.*, **128**, 131 (1974).
- 5) R. P. Raunio and W. T. Jenkins, *J. Bacteriol.*, **115**, 560 (1973).
- 6) R. P. Raunio, L. D. Straus and W. T. Jenkins, *J. Bacteriol.*, **115**, 567 (1973).
- 7) P. J. Olsiewski, G. J. Kaczorowski and C. Walsh, *J. Biol. Chem.*, **255**, 4487 (1980).
- 8) F. C. H. Franklin and W. A. Venables, *Mol. Gen. Genet.*, **149**, 229 (1976).
- 9) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 10) E. J. Conway and A. Byrne, *Biochem. J.*, **27**, 419 (1933).
- 11) J. F. Tompson and G. R. Morrison, *Anal. Chem.*, **23**, 1153 (1951).
- 12) H. J. Sallach and L. A. Fahien, "Metabolic Pathways," 3rd Ed., Vol III, ed. by D. M. Greenberg, Academic Press, New York, 1969, pp. 1~94.
- 13) A. E. Bender and H. A. Krebs, *Biochem. J.*, **46**, 210 (1950).
- 14) M. Dixon and K. Kleppe, *Biochim. Biophys. Acta*, **96**, 368 (1965).
- 15) R. Labow and W. G. Robinson, *J. Biol. Chem.*, **241**, 1239 (1966).
- 16) D. Dupourque, W. A. Newton and E. E. Snell, *J. Biol. Chem.*, **241**, 1233 (1966).
- 17) G. Urata and S. Granick, *J. Biol. Chem.*, **238**, 811 (1963).
- 18) W. G. Laver, A. Neuberger and J. J. Scott, *J. Chem. Soc.*, **1959**, 1438.
- 19) D. McGilvray and J. G. Morris, *Biochem. J.*, **112**, 657 (1969).
- 20) J. M. Turner, *Biochem. J.*, **104**, 112 (1967).
- 21) A. Neuberger and G. H. Tait, *Biochem. J.*, **84**, 317 (1962).
- 22) M. L. Green and W. H. Elliott, *Biochem. J.*, **92**, 537 (1964).
- 23) D. Hartshorne and D. M. Greenberg, *Arch. Biochem. Biophys.*, **105**, 173 (1964).
- 24) S. A. Boylan and E. E. Dekker, *J. Biol. Chem.*, **256**, 1809 (1981).
- 25) Y. Aoyama and Y. Motokawa, *J. Biol. Chem.*, **256**, 12367 (1981).
- 26) M. Ray and S. Ray, *J. Biol. Chem.*, **260**, 5913 (1985).