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Reaction pathway of tryptophanase degrading D-tryptophan

Short Communication

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Summary. Tryptophanase, which has the very strict stereospecificity to L-tryptophan under ordinary condition, becomes active to D-tryptophan in highly concentrated diammoniumhydrogen phosphate solution. The reaction process of D-tryptophan degradation is studied in terms of kinetics. Diammoniumhydrogen phosphate acts on tryptophanase as activator below 3.1 M, and as noncompetitive inhibitor over it. Additionally, the pathway of the reaction is provided on the basis of kinetic parameters.

Keywords: Amino acids – Tryptophanase – D-tryptophan – Diammoniumhydrogen phosphate – Kinetic analysis – Stereospecificity

Abbreviations: TPase: tryptophanase, L-Trp: L-tryptophan, D-Trp: D-tryptophan, DAP: diammoniumhydrogen phosphate, PLP: pyridoxal 5′-phosphate

Introduction

Stereospecificity of enzyme is very rigid. This property which enzyme obtained in early metabolism has excludes only D type of amino acids and has made asymmetric biological world. The enzymological first step to elucidate its mechanism is to study the difference between the reaction with L type and D type on the same enzyme. It, however, is difficult to acquire enzyme active to both D type and L type, judging from three point theory (Popjak, 1970). We have studied whether the stereospecificity to optical isomers is changeable. The substrate specificity of enzyme is established by not only amino acid sequence of enzyme but also environment around the enzyme. The interaction of the enzyme with the environment influences its stereospecificity, too. If an appropriate condition is given to the environment, enzyme active to both D type and L type will be possibly obtained in the native state without modifying amino acid on the primary sequence. Our recent

paper showed that TPase degraded D-Trp in a saline solution including concentrate DAP and the removal of DAP reversibly returned TPase to the original one (Shimada et al., 1996). However, the reason why TPase cannot decompose D-Trp under ordinary condition and become active to D type in the presence of DAP has remained open. The characterization of an active site for D-Trp degradation is required to clarify it. Kinetic analysis is one of the effective ways to acquire detailed information on the active site. We report the process of D-Trp degradation in terms of kinetics. A pathway of the reaction and the equation of its initial velocity is provided on the basis of kinetic parameters.

Material and methods

Enzyme and reagents

TPase was purchased from Sigma Chem. Co. (St. Louis, USA), fractionated by a pH gradient provided with an isoelectric focusing apparatus, BIORAD ROTOFOR CELL (BioRad, California, USA) to be separated from other contaminants prior to experiment. This purification procedure was repeated twice. The TPase solution was prepared in 36 μg/ml of protein concentration, exhibiting a specific activity of 17 μmol/min/mg and Km of 0.33 mM with L-tryptophan at 37°C and pH 8.3, and was a single band on SDS-polyacrylamide gel electrophoresis. D-Trp was purchased from Peptide Inst. Inc. (Osaka, Japan). Other reagents were obtained from Wako Pure Chem. Co. (Osaka, Japan).

Rate measurements

Reaction mixture was prepared in the required combination of DAP and D-Trp concentration with a fixed concentration of TPase. The reaction mixtures contained, in 2.0 ml, various amounts of D-Trp (245 ~ 980 μM) and DAP (1.2 ~ 4.3 M), 380 μM PLP, and 0.1 M potassium phosphate buffer, pH 7.8. The reaction was initiated by adding 67 μl of a solution containing 2.4 μg (0.04 units at 37°C) of TPase, and mixing immediately. TPase reacted with D-Trp at 37°C for 4 hr. Indole into which D-Trp was decomposed increased linearly with time. The reaction was terminated by adding n-butanol with the extraction of indole. Indole was separated from other products with centrifugation and colored with Ehrlich's reagents. Indole was spectroscopically examined at λ = 570 nm. Initial velocities represented indole formed during one minute after the addition of 1 mg of TPase. Each combination of DAP and D-Trp concentrations was assayed in triplicate. All kinetic data were analyzed by averaging the initial velocities and generating Lineweaver-Burk plots of the averages. Kinetic parameters (e.g. K_m, V_m) were determined from least-squares analysis.

Results and discussion

Kinetic constants

The initial velocity of TPase rises with increasing concentration of DAP from 1.2 M to 3.1 M, and four linear lines intersect each other at a point on the abscissa in Fig. 1. DAP activates TPase to degrade D-Trp into indole through TPase·DAP·D-Trp complex. The activity of D-Trp degradation is maximal at 3.1 M. Maximum velocity of D-Trp degradation, Vm, and dissociation con-

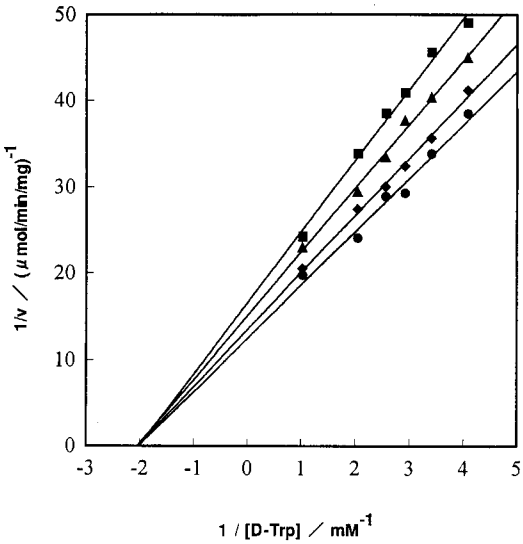

Fig. 1. TPase increasingly degrading D-Trp with increasing DAP concentration. The reciprocal of initial velocity and D-Trp concentration is respectively plotted as ordinate and abscissa. DAP concentrations are designated as follows; ■: 1.2 M, ▲: 1.9 M, ◆; 2.5 M, ●; 3.1 M

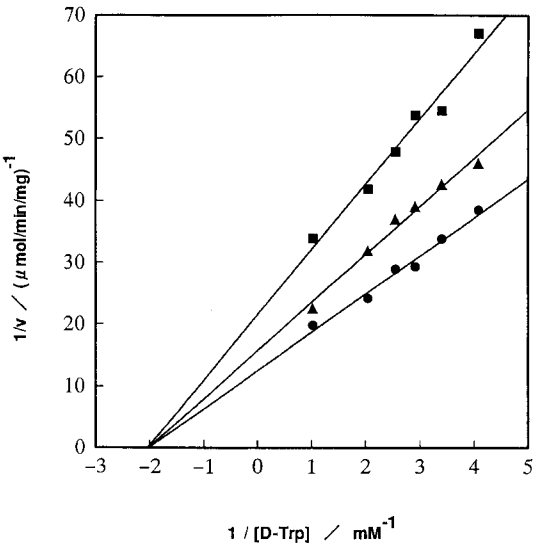

Fig. 2. DAP noncompetitively inhibiting D-Trp degradation. The reciprocal of initial velocity and D-Trp concentration is respectively plotted as ordinate and abscissa. DAP concentrations are designated as follows; ●: 3.1 M, ▲: 3.7 M, ■: 4.3 M

stant between DAP and TPase, K^{DAP}, are calculated from the intercept of each plots, and averaged. Vm is 0.1 μmol/min/mg, and K^{DAP} is 0.7 M. Substrate constant for D-Trp, $Ks^{D\text{-}Trp}$, is determined to be 0.5 mM, too. The specific activity for D-Trp is 0.6% of that for L-Trp. If an apparent Michaelis constant, Km_{app}, is defined as the concentration of D-Trp giving v = Vm/2, it is 750 μM

at 3.1 M DAP. Catalytic efficiency, Vm/Km, is convenient to compare the efficiency of enzyme reaction. The catalytic efficiency for D-Trp has 0.3% of that for L-Trp (Vm/Km = 0.1/750 = 1.3×10^{-4} ℓ/min/mg for D-Trp *versus* 17/330 = 5.2×10^{-2} ℓ/min/mg for L-Trp). On the other hand, Fig. 2 indicates that DAP over 3.1 M deactivates TPase to inhibit D-Trp degradation. Excess DAP acts on TPase as noncompetitive inhibitor. Inhibition constant, Ki^{DAP}, is derived from the intercept and gradient of each of three plots in Fig. 2, determined to be 1.4 M.

Reaction pathway

The reaction features that DAP acts on TPase as activator below 3.1 M but as noncompetitive inhibitor over 3.1 M should be included in a reaction pathway of TPase degrading D-Trp. A reaction model is provided on the basis of kinetic constants as shown below. Both D-Trp and DAP can attach to TPase at random, and TPase·DAP·D-Trp complex is essential to the D-Trp degradation. Preliminary experiment showed that not only D-Trp competitively inhibited TPase but also D-Trp could not be degraded at all in the absence of DAP while TPase degraded L-Trp (Kogure, 1995). This supports the existence of TPase·D-Trp complex in the reaction model. TPase can bind with D-Trp but cannot degrade it. The active site of TPase degrading D-Trp can be separated into binding site and catalytic site. The changeable stereospecificity of TPase is responsible for the interaction of TPase and DAP, though DAP binding with TPase isn't quantitatively determined. DAP which is attached to TPase will bring D-Trp into maximal contact with catalytic site, when DAP is 3.1 M. On the contrary, DAP over 3.1 M detaches D-Trp from catalytic site. DAP neither degrade nor racemize D-Trp as shown previously (Shimada et al., 1996). In addition, DAP binds with other site except the active site because noncompetitive inhibitor is considered to be independent of it. The interaction of DAP with TPase will cause small conformational change which influences the active site of TPase and makes it possible for D-Trp to contact with catalytic site.

$$
\begin{array}{lll}
 & \overset{Ks^{D\text{-}Trp}}{\rightleftharpoons} & \\
\text{TPase} & & \text{TPase}\cdot\text{D-Trp} \\
K^{DAP}\uparrow\downarrow & & \uparrow\downarrow K^{DAP} \\
 & \overset{Ks^{D\text{-}Trp}}{\rightleftharpoons} & \\
\text{TPase}\cdot\text{DAP} & & \text{TPase}\cdot\text{DAP}\cdot\text{D-Trp} \rightarrow \text{indole} + \text{TPase} + \text{DAP} \\
Ki^{DAP}\uparrow\downarrow & & \uparrow\downarrow Ki^{DAP} \\
 & \overset{Ks^{D\text{-}Trp}}{\rightleftharpoons} & \\
\text{TPase}\cdot(\text{DAP})\text{ex} & & \text{TPase}\cdot(\text{DAP})\text{ex}\cdot\text{D-Trp}
\end{array}
$$

The equation of v, initial velocity, is conducted on the basis of the reaction model. The reaction between one complex and other complex is assumed to be in rapid equilibrium. [DAP] represents the concentration of DAP. [(DAP)ex] is defined as [(DAP)ex] = 0 for [DAP] < 3.1 M, and as [(DAP)ex] = [DAP] − 3.1 M for [DAP] ≧ 3.1 M. v is given below.

$$\frac{1}{v} = \frac{1}{Vm}\left(\frac{\left[(DAP)_{ex}\right]}{Ki^{DAP}} + 1 + \frac{K^{DAP}}{[DAP]}\right) + \frac{Ks^{D-Trp}}{Vm}\left(\frac{\left[(DAP)_{ex}\right]}{Ki^{DAP}} + 1 + \frac{K^{DAP}}{[DAP]}\right)\frac{1}{[D-Trp]}$$

Conclusions

TPase has been one of the most extensively studied PLP-dependent enzymes. Nevertheless, investigations on D type of tryptophans have not been performed. Kinetic results give the significance of TPase·DAP·D-Trp complex, which is essential to gain access of D-Trp to the catalytic site through some steric structural change of TPase. DAP makes the comparison of the reaction between L type and D type possible in the state of native enzyme. This will contribute to study the selection mechanism for optical isomers in early metabolism.

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