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Tryptophanase-catalysed degradation of D-tryptophan in highly concentrated diammonium hydrogen phosphate solution

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Summary. Tryptophanase is and is perfectly inert to D-tryptophan under ordinary conditions. However, activity that can degrade D-tryptophan into indole is observed when tryptophanase is in highly concentrated diammoniumhydrogen phosphate solution. The reaction has been so far unknown in tryptophanase metabolic pathways. Here we report the characteristic of the reaction. We also discuss its significance in relation to selection of an amino acid optical isomer from a racemic mixture.

Keywords: Amino acids – Tryptophanase – D-tryptophan – Specificity to optical isomer – Diammoniumhydrogen phosphate

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

Introduction

There are some enzymes that can only react with their corresponding substrates, while there are other enzymes that can widely react with both their substrates and analogues. Substrate specificity of enzyme is not generally rigid enough to affect only a specific substrate. On the contrary, the specificity towards a particular amino acid optical isomer is generally exclusive (Webb, 1984). If we wish to study a selection mechanism for an amino acid optical isomer, it is useful to change and control the specificity of an enzyme. We have such skilful techniques as chemical modifications or partially deletions of primary sequence for altering the specificity (Mizobata et al., 1995). But when we modify an enzyme, we need to pay attention to the point that we transform it into a form different from the original one. It is more desirable to change the specificity to optical, isomers just as enzyme is intact.

We found that the bacterial enzyme tryptophanase with the rigid specificity to L-tryptophan at ordinary condition becomes active to D-tryptophan in high saline solution. Tryptophanase is a pyridoxal 5′-phosphate

requiring lyase which eliminates indole from the β position of L-tryptophan with the concurrent formation of pyruvate and ammonium ions (Behbahani-Nejad et al., 1987). The reaction of tryptophanase with D-tryptophan has been unknown so far in its reaction pathways (Snell, 1975). The reaction gets the maximal activity at 50% saturation concentration of diammoniumhydrogen phosphate. The activity to D-tryptophan was about 5% of that to L-tryptophan. We here report the characterization of the reaction.

Materials and methods

Enzyme and reagents

TPase was purchased from Sigma Chem. Co. (St. Louis, USA). Impure proteins contained in it was immediately removed with an isoelectric focusing apparatus, BIORAD ROTOFOR CELL (BioRad, California, USA), and then the purified TPase was analyzed with SDS PAGE. It was purified so sufficiently that a condense band was only observed in a position of MW 55000. Specific activity of the purified TPase was 3.4 times as high as that of the original TPase (data unshown). The TPase preparation obtained in this way was provided to be served to experiments. L-Trp, PLP, AP and other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) and D-Trp was from Peptide Institute Inc. (Osaka, Japan). All chemicals were reagent grade. All glasswares were washed by soaking for more than 3 days in a special detergent, CLEAN 99CL (Clean Chemical Co. Ltd., Osaka, Japan), thoroughly rinsed in deionized and ceramics-distilled water, and then dried in an oven. All aqueous solutions were prepared from deionized and ceramics-distilled water.

Reaction mixture

Reaction mixture was prepared as follows. After AP was completely dissolved in boiling water, it was cooled at 37°C. Solution that AP deposited was regarded as 100% saturation concentration. The required saturation concentration from 0 to 80% was diluted and prepared with Britton-Robinson wide range buffer including PLP, D-Trp and TPase. The concentration of D-Trp was 980μM. TPase activity was prepared to 6.0×10^{-4} unit/ml. PLP concentration was 380μM unless otherwise described. Reaction mixture prepared in these procedures was adjusted to a pH of 7.8. The reaction mixture was incubated at 37°C for the required time from 0 to 6 hours, after mouths of glass tubes were tightly closed with rubber stoppers.

Measurement of TPase activity

An volume of water saturated n-butanol equal to a sample volume was vigorously mixed, and reaction was stopped. It was centrifuged at 1,000G for 10min, and then the supernatant was mixed with an equal volume of Ehrlich's reagent. Indole in the mixture was redly colored in an oven at 60°C for 30min. Absorbance of the reddish matter was spectroscopically measured at $\lambda = 570$nm. TPase activity was expressed in μM/hr calculated from a calibration curve.

Resolution with HPLC CROWN PACK CR(+)

980μM D-tryptophan and 380μM PLP was added to AP solution of 50% saturation. They were incubated at 37°C for 6hr. 50μl of them was charged on HPLC, CROWN PACK CR(+) (Daicel Chemical Industries, Ltd., Tokyo, Japan), which was connected with

Hitachi 655 Liquid Chromatograph and UV monitor, LC monitor 635M (Tokyo, Japan). pH 2.0 $HClO_4$ as eluent was flowed at the rate of 1.0 ml/min under 100 kg/cm^2. Tryptophan was detected at λ = 280 nm. DL-Trp, which was used as marker, was prepared in the way described above except that it has no incubation of 6 hr and its concentration was 1,960 μM. It was resolved on CROWN PACK CR(+) to determine the retention time of D- or L-Trp, too. The form of optical isomer in the sample was determined by comparing with the retention time of D- or L-Trp as described previously (Yuasa et al., 1984).

Salines

Salines used in this study were KCl, NaCl, Na_2SO_4, $MgSO_4$, NH_4Cl, NH_4HO_3, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ (=AP), ammonium oxalate ($H_4NOCOCOONH_4$), ammonium citrate tribasic ($H_4NOCOCH_2C(OH)$ $(COONH_4)CHCOONH_4$), ammonium tartrate ($H_4NOOCCH(OH)CH(OH)COONH_4$). Saturated solutions were respectively diluted to 20, 40, 60 and 80% saturation concentration with Britton-Robinson wide range buffer. It was investigated whether the activity of TPase to D-Trp appeared every saturation concentration.

Results and discussion

No racemization in highly concentrated AP solution

We analyzed D-Trp which was incubated at 37°C for 6 hr in 50% saturation AP solution because there was a possibility that D-Trp chemically racemized to L-Trp in highly concentrated AP solution (De-Vrese et al., 1994). Fig. 1a,b shows two chromatograms resolved on CROWN PACK CR, HPLC. Figure 1a shows the chromatogram that resolved tryptophan racemate as marker. Retention time of D-Trp and L-Trp is respectively 18 min, 23 min. The resolution chromatogram of the D-Trp which was incubated for 6 hr at 37°C in the solution of AP is shown in Fig. 1b. The peak of L-Trp cannot been found out except one sharp peak of D-Trp. This indicates that D-Trp doesn't racemize to L-Trp in the highly concentrated AP solution.

No possibility of chemical reaction

We paid attention to a possibility that AP directly acted on D-Trp and chemically degraded D-Trp into indole (Landry et al., 1994). Removing either TPase or D-Trp from the reaction mixture, we respectively measured the indole produced in respective solutions. Indole, however, isn't detected in neither of them (▲ or ■) as shown in Fig. 2. Indole can be never produced until both TPase and D-Trp (●) coexist. This reaction isn't a chemical reaction, but an enzymatic one through TPase. Figure 1 and Fig. 2 shows that not only D-Trp doesn't racemize to L-Trp in the AP solution, but also TPase is indispensable for the reaction. AP immediately acts on TPase and collapses the system to select only L-isomer.

Effects of other salts

We studied on effects of other salines except AP, too. We dominantly selected salts containing ammonium ions. Table 1 shows that TPase has no activity to

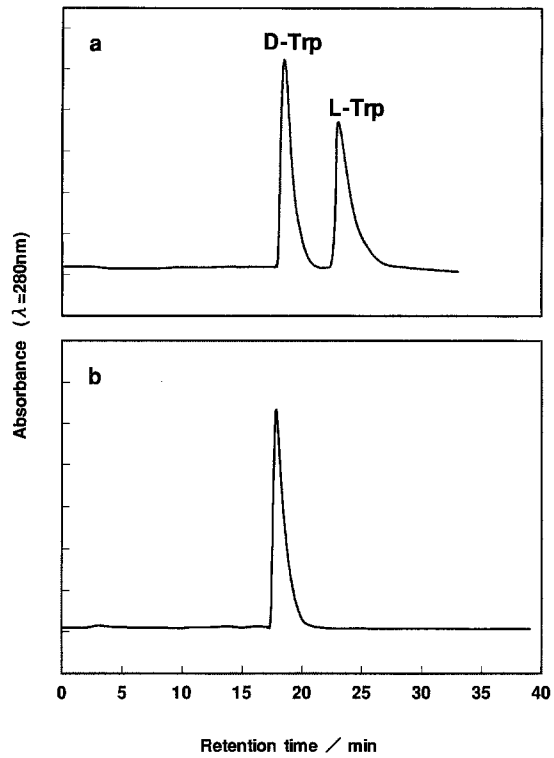

Fig. 1. Chromatogram of D-Trp in AP solution. **a** A chromatogram of DL-Trp resolved as marker. **b** A chromatogram of D-Trp which was incubated at 37°C for 6 hr in the AP solution of 50% saturation. Column used was DAICEL CROWNPAK CR(+). Eluant was $HClO_4$ of pH 2.0 and its flow rate was 1.0 ml/min

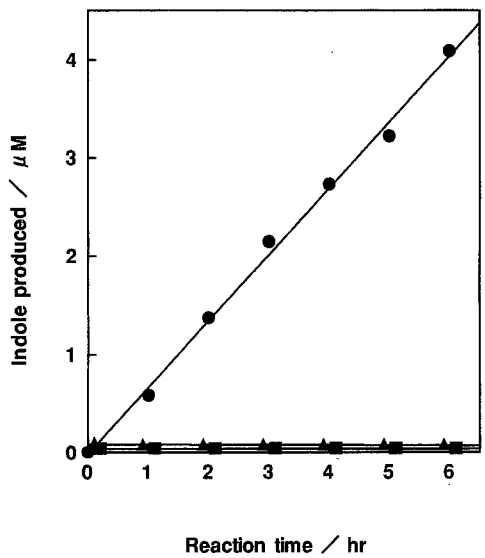

Fig. 2. No chemical degradation of D-Trp in highly concentrated AP solution. Every preparations were incubated for 6 hr at 37°C in the AP solution of 50% saturation. Either D-Trp (▲) or TPase (■) was removed from reaction mixture, respectively. Both TPase and D-Trp (●) coexist in reaction mixture

Table 1. Effect of different salines on TPase

Salines	Activity
NaCl	−
KCl	−
$MgSO_4$	−
Na_2SO_4	−
$(NH_4)_2SO_4$	+
NH_4NO_3	−
NH_4Cl	−
$(NH_4)_2HPO_4$	++
ammonium oxalate $COONH_4$ • $COONH_4$	−
ammonium citrate, tribasic $COONH_4$ • $CH(OH)$ • $CH(OH)$ • $COONH_4$	−

Each sign respectively designates the following meanings. +; active to D-Trp, ++; more active to D-Trp than +, −; inactive to D-Trp.

D-Trp at all in the saline solutions except for $(NH_4)_2HPO_4$ or $(NH_4)_2SO_4$. The effect of $(NH_4)_2HPO_4$ on the activity is about twice as active as that of $(NH_4)_2SO_4$ (data unshown), though this cause is unknown in the present day. Since the reaction doesn't occur in other salines except AP and ammonium sulfate, their proper characteristics will influence on TPase. We will need to study whether such a phenomenon is also observed in other enzymes, and to elucidate how the action of AP makes TPase lose control of a selection system of optical isomer.

Dependence on PLP and AP concentration

The reaction for D-Trp depends on PLP, which is known to be the coenzyme of TPase (Metzler et al., 1991). TPase cannot degrade D-Trp if PLP is nothing. PLP is essential to the reaction as shown in Fig. 3. PLP serves as coenzyme in aminotransferase, amino acid decarboxylase or L and D-serine dehydratase (Alexander et al., 1994). Furthermore, many racemases utilize PLP as coenzyme in its enantiomeric reactions, too. PLP seems to play an important role in the recognition of optical isomers judging from these reactions, though its mechanism is unclear in the present day.

The reaction rate of TPase rises with AP concentration in Fig. 4. It reaches the maximal rate, 2.2μM/hr at 50% saturation. On the other hand, it lowers over 50%. The initial reaction rate at 50% concentration is compared with the initial reaction rate for L-Trp at 0% AP concentration. That for L-Trp is

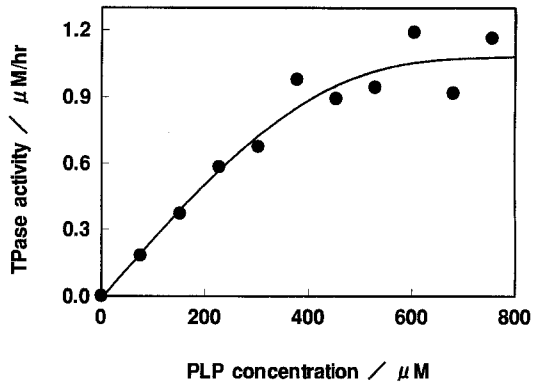

Fig. 3. Dependence on PLP. Reaction mixture was incubated at 37°C for 4 hr at 0, 76, 152, 228, 380, 456, 532, 684, 760μM of PLP. AP concentration was 50% saturation

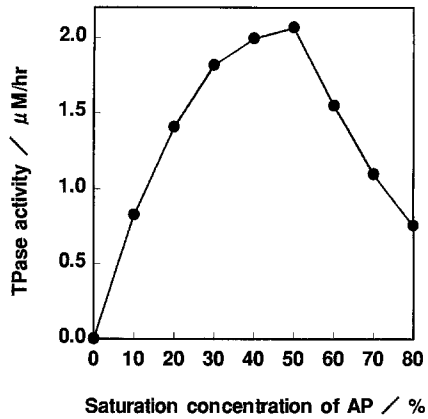

Fig. 4. Dependence on AP concentration. Reaction mixture was incubated at 37°C for 4 hr at 0, 10, 20, 30, 40, 50, 60, 70, 80% saturation of AP. PLP concentration was 380μM

calculated to be 44.7μM/hr (Shimada et al., 1992). The activity for D-Trp is about 5% of that for L-Trp. The reaction will be analyzed with ease for the elucidation of its mechanism if the activity is enhanced. It is essential for its promotion to make circumstances that TPase can more easily react with D-Trp. It is requisite to investigate whether other salts except for AP can realize such an environment. Such a bell curve as Fig. 4 is similar to pH curve seen in many enzyme reactions. If we make the reaction correspond to pH curve, we will perceive that AP functions as an activator below 50% saturation but as inhibitor over 50% saturation (Vanoni et al., 1994). Kinetical analyses for TPase in highly concentrated AP solution will be needed to demonstrate this working hypotheses though we are under investigation.

The mechanism on D-Trp degradation containing other salts in TPase is obscure in the present day. Nevertheless, experimental facts from this study attract an enantiomeric interest on enzyme evolution (Isoyama et al., 1984). AP seems to serve as molecule that fills up a great gap between D-isomer and

L-isomer on enzyme. AP may give the key to solve the riddle of L-amino acids selection in origins of life. Our results indicate the importance of the interaction between enzyme and the environment around it in the selection mechanism of amino acid optical isomers.

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References

Alexander FW, Sanmeier E, Mehta PK, Christen P (1994) Evolutionary relationships among pyridoxal 5′-phosphate-dependent enzymes: regio-specific alpha, beta and gamma families. Eur J Biochem 219: 953–960

Behbahani-Nejad I, Dye JL, Suelter CH (1987) Tryptophanase from *Escherichia coli* B/1t7-A. Methods Enzymol 142: 414–422

De-Vrese M, Middendorf K, Hagemeister H (1994) Prevention of amino acid racemization during guanidination: prerequisite for measurement of protein digestibility by homoarginine labeling. Z Ernahrungswiss 33: 310–312

Isoyama M, Ohoka H, Kikuchi H, Shimada A, Yuasa S (1984) In vitro protein synthesis using D-amino acids and its evolutionary significance. Orig Life 14: 439–446

Landry J, Delhaye S (1994) The tryptophan contents of wheat, maize and barley grains as a function of nitrogen content. J Cereal Science 18: 259–266

Metzler CM, Viswanath R, Metzler DE (1991) Equilibria and absorption spectra of tryptophanase. J Biol Chem 266: 9374–9381

Mizobata T, Kawabata Y (1995) The folding characteristics of tryptophanase from *Escherichia coli*. J Biochemistry 117: 384–391

Shimada A, Nakamura I (1992) Degradation of D-tryptophan under high salt concentration. Viva Origino 20: 147–162

Snell EE (1975) Tryptophanase: structure, catalytic activities, and mechanism of action. Adv Enzymol Relat Areas Mol Biol 42: 287–333

Vanoni MA, Accornero P, Carrera G, Curti B (1994) The pH-dependent behavior of catalytic activities of Azospirillum brasilense glutamate synthase and iodoacetamide modification of the enzyme provide evidence for a catalytic Cys-His ion pair. Arch Biochem Biophys 309: 222–230

Webb EC (1984) Enzyme nomenclature. Academic Press, Inc., Florida, p 20

Yuasa S, Itoh M, Shimada A (1984) Resolution of amino acids by a native-cellulose column. J Chromatogr Sci 22: 288–292

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