Tritium kinetic isotope effects on enzymatic decomposition of L-tryptophan

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Abstract The tritium kinetic isotope effect on position 2 has been determined in the reaction of decomposition of L-tryptophan, L-Trp, catalyzed by enzyme TPase, (EC 4.1.99.1). The numerical values of isotope effects in the course of reaction were obtained by the competitive method using [1-^{14}C]-L-tryptophan as internal radiometric standard.

Key words carbon-14 • isotope effect • tritium • tryptophan • tryptophanase

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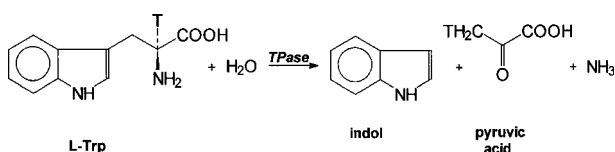E-mail: mkanska@chem.uw.edu.pl

Introduction

The enzyme tryptophanase (L-tryptophan indole lyase EC 4.1.99.1), TPase, catalyses the decomposition of L-Trp to the corresponding indole, pyruvic acid, and ammonia [12], (Scheme 1).

Under certain conditions, there is a possibility of reverse reaction, leading to the formation of L-tryptophan. This enzyme also decomposes L-serine, L-cysteine, S-methyl-L-cysteine, and is often used in the synthesis of L-tryptophan [6]. In the literature, the mechanism of TPase catalysis are well documented [4, 8]. Most of study of them were performed using stopped flow spectroscopy [7, 9, 11] and research with using mutants [10]. One technique often used in studying such a kind of reaction mechanisms is the kinetic isotopic effect method (KIE). By labelling the molecule in different positions and determining primary and secondary KIE's, it is possible to find out the number of reactive sites and to elucidate the structure of transition state [3].

The purpose of our study was to determine the KIE for the α-hydrogen atom during breaking the bond between indol moiety and the 3-carbon atom in the side chain of L-Trp in the course of decomposition reaction shown in Scheme 1. In this study, we have used an isotopomer of L-Trp, i.e., [2-^{3}H]-L-tryptophan, [2-^{3}H]-L-Trp and the second isotopomer of [1-^{14}C]-L-tryptophan, [1-^{14}C]-L-Trp, as an internal radiometric

Scheme 1. Decomposition of L-tryptophan catalysed by enzyme TPase.

Table 1. Tritium kinetic isotope effect (^{1}H/^{3}H) for hydrogen bonded to α-carbon position

Degree of conversion, f	^{1}H/^{3}H kinetic isotope effect, KIE
0.128	6.44
0.138	5.97
0.146	5.88
0.147	6.40
0.150	5.91
0.162	6.34
0.166	5.53
0.173	5.97
0.180	5.33
0.183	5.88
0.186	5.97
0.191	5.08
	Avg. 5.89 ± 0.21

standard. Synthesis of these isotopomers of L-Trp have been described before [1, 2]. The results pertaining to ^{1}H/^{3}H KIE in position 2 of L-tryptophan will be presented as well.

Results and discussion

Tritium kinetic isotope effects (^{1}H/^{3}H) of hydrogen bonded to α-carbon position of L-Trp in the course of decomposition have been measured in water at room temperature. The values of tritium kinetic isotope effects in the mentioned above reaction are given in Table 1. These effects were determined using an internal radiometric standard ([1-^{14}C]-L-Trp) and the Yankwich-Tong equation to calculate KIE values [13]. The internal radioactive standard method assumes the using of ^{3}H/^{14}C ratio instead of the specific activity of ^{3}H-labelled L-Trp, therefore, the determination of KIE is much more precise.

$$\alpha = \frac{\ln\left[1 - f\,\dfrac{(1-f)R_0}{R_f}\right]}{\ln(1-f)}$$

where: α – ^{1}H/^{3}H kinetic isotope effect; R_0 – ^{3}H/^{14}C radioactivity ratio in L-Trp at the start of reaction; R_f – ^{3}H/^{14}C radioactivity ratio in L-Trp after the f degree of conversion; f – degree of conversion.

The experimental error was assessed by the Student t-test with a confidence of 95%.

Considerably large KIE of tritium implies that its value is typical of primary KIE, and therefore, the hydrogen atom in position 2 plays a significant role in transformation of the enzyme-substrate complex into an enzyme-product complex. In this study, the kinetic isotope effect of enzymatic decomposition of L-Trp was determined for the first time using the radioactive ^{3}H

and ^{14}C isotopes. While KIE for this reaction has been previously investigated, it was relied upon stable isotopes, specifically determining the solvent isotope effects [5]. The magnitude of KIE for tritium indicates that hydrogen atom bonded with α-carbon of L-Trp is involved in the proton transfer during the decomposition of tryptophan.

Experimental

Materials

All chemicals were from Aldrich. Enzymes TPase (EC 4.1.99.1) from *Escherichia coli*, LDH (1.1.1.27) from rabbit muscle, cofactor PLP, and NADH were from Sigma. The scintillation cocktail was from Rotiszint (Germany).

Methods

The concentration of pyruvic acid was determined spectrophotometrically using a Shimadzu UV-102 CE-LV spectrometer. This indirect procedure consists of the conversion of pyruvic acid to L-lactic acid by the enzyme LDH and coenzyme NADH (Scheme 2) and determination of the concentration changes of NADH by measuring the absorbance at 340 nm [11].

The radioactivity of all samples was determined using an automatic liquid scintillation counter (LISA LSC PW470 – Raytest, Germany).

KIE assays

KIE assays was carried out at room temperature. In the catalysed reaction by TPase, the LDH/NADH couple allows to indirect determination of the concentration of pyruvic acid [11]) (Scheme 2), and the degree of decomposition of L-Trp.

For each kinetic run, the assayed sample of [2-^{3}H], and [1-^{14}C]-L-Trp was placed in an encapped vial and dissolved in 4.5 ml of 0.1 M phosphate buffer, pH 8. Radioactivity of ^{3}H-isotopomer was 3–7 fold higher than the ^{14}C-one. To this, the following reagents were added in turn:
1. 90 mg of KCl;
2. 9.5 mg D,L-dithiothreitol (HSCH$_2$(OH)CH$_2$(OH)CH$_2$SH 1,4-dithiobutan-2,3-diol);
3. 300 μl of 1 mM 5'-pyridoxal phosphate, PLP (cofactor);
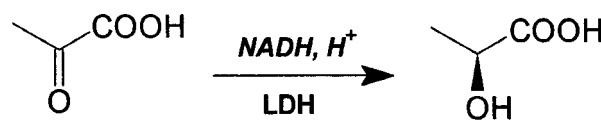
Scheme 2. Conversion of pyruvic acid into L-lactic acid by enzyme LDH.

4. 600 μl of the enzyme L-lactic dehydrogenase (LDH, EC 1.1.1.27) of activity 270 U/ml;
5. 300 μl of 2 mM NADH;
6. 300 μl of enzyme TPase (0.13 U/ml).

In the present time of the course of reaction, 1 ml samples were taken, and the degree of conversion was determined spectrophotometrically. Next, the reaction was quenched by acidifying the reaction mixture to pH 5 with glacial acetic acid.

The non-reacted L-Trp and L-lactic acid were separated on an ion exchange column (Amberlite IR 120 H$^+$ form, 60×5 mm) by elution with 0.3 M NH$_3$(aq), their radioactivities being measured on LSC. Additionally, the degree of conversion was checked using the radioactivity of the product and substrate.

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