HYDROGEN KINETIC ISOTOPE EFFECTS IN THE STUDIES OF THE MECHANISM OF ACTION OF L-TYROSINE PHENOL LYASE

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Summary

The kinetic and solvent isotope effects have been determined in the reaction of decomposition of L-tyrosine catalyzed by enzyme TPL (EC 4.1.99.2). The numerical values of isotope effects were obtained using non competitive and competitive combined with internal radioactive standard methods.

Keywords: Deuterium, enzyme, isotope effects , L-tyrosine, tritium.

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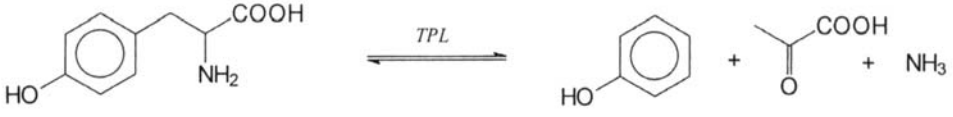
Introduction

The bacterial enzyme tyrosine phenol lyase (EC 4.1.99.2), TPL, catalyzes the reversible decomposition of L-tyrosine, L-Tyr, to phenol, pyruvate and ammonia[1] (Scheme 1).



Scheme 1. Reaction catalyzed by the enzyme TPL

This enzyme also decomposes L-serine, L-cysteine, as well as their O(S) derivatives and is useful for synthesis of wide range of L-Tyr derivatives[2] and for neutralization of waste[3]. In literature there are described many studies concerning the mechanism of action of TPL. Most of them were performed using stopped flow spectroscopy[4] and the studies with using of mutants[5, 6] or chemically modified[7] enzyme. There are also some kinetic isotope effects (KIE) data[8-10] but no systematic studies on this field were attempted. The aim of our reserch is to determine values of KIE for all positions involved in course of reaction depicted in Scheme 1. For this studies we used the compounds of L-Tyr, which have been synthesized as described elsewhere[11-13]. A part of obtained data is presented in this paper.

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Experimental

1. Non competitive method: In the typically assayed medium concentrations of the components were as follows: 0.1 M potassium phosphate buffer (pH 8.3), 0.2 M KCl, 1 mM of dithiothreitol, 50 μM of 5'-pyridoxal phosphate, PLP, (cofactor), 13.4 U/ml of L-lactid acid dehydrogenase, LDH, (EC 1.1.1.27), 0.1 mM of NADH, 0.25 U/ml of TPL from *Citrobacter freundii* and various amounts of L-Tyr (from 120 μM to 4 mM). High excess of NADH and LDH quickly converts pyruvate formed (Scheme 1) into L-lactate and NAD[8, 10]. In this way the degree of decomposition of L-Tyr via intermediate pyruvate is easily detected spectrophotometrically at 340 nm wavelength. The assays were carried out at RT. Lineweaver-Burke plots were used to determine inversed maximal velocity ($1/V_{max}$) and ratio of Michaelis constant per maximal velocity (K_m/V_{max}).

2. Competitive method: The concentrations of component in assayed medium were similar as mentioned above only with the difference that concentration of NADH and TPL are equal to 1 mM and 0.4 U/ml respectively. To this medium [1-^{14}C]-L-Tyr (sp. activity of 6.88 MBq/mmole) and [2-^{3}H]-L-Tyr (sp. activity of ^{3}H-isotopomer was 12-50 fold higher than ^{14}C-one) were added up to conc. 0.5 mM. Internal radioactive standard method assumes using $^{3}H/^{14}C$ ratio instead of specific activity of ^{3}H-labeled L-Tyr, therefore, the determination of KIE is much more precise. KIE assays was carried out at RT. During the reaction fractions were taken in preset times. The products were separated on ion exchange column (Amberlit IR 120, H^+ form, 60×5 mm) and their radioactivities were measured on LSC. The degree of conversion was determined using ^{14}C-activity of the product and substrate. Yankwich-Tong equation[14] was used to calculate KIE.

Results

1. Solvent and deuterium ($^{1}H/^{2}H$) effects on [3', 5'-$^{2}H_2$]-L-Tyr (non competitive ***method).***
The result of kinetic studies on enzymatic decomposition of L-Tyr in water and deuteriated water are shown in Tables 1 and 2.

Table 1. Kinetic properties of TPL reacting with differently labeled substrates

Substrate	Solvent	V_{max} [U/μlTPL]	V_{max}/K_m [U/(mM*μlTPL)]
L-tyrosine	H_2O	3.78 ± 0.07	9.3 ± 1.4
L-tyrosine	D_2O	2.23 ± 0.19	1.02 ± 0.08
[3',5'-$^{2}H_2$]-L-tyrosine	H_2O	2.70 ± 0.12	4.10 ± 0.14

Table 2. KIE's measured for TPL with L-tyrosine in non-competitive experiments

Studied position	KIE on V	KIE on V/K
3',5'-H_2	1.40 ± 0.07	2.27 ± 0.35
solvent H/D	1.70 ± 0.15	9.12 ± 1.5

2. Tritium KIE,s ($^{1}H/^{3}H$) of hydrogen bonded to α-carbon measured in water and deuteriated water (competitive method).

These studies were performed in water and heavy water in the similar conditions as in non competitive method. A rare phenomenon was discovered – the value of KIE is increasing with conversion degree, **f**. Moreover – it changes from inverse values for low **f** to high values of KIE for higher **f.** This relationship is described by linear regression with

quite good correlation factors of 0.94 and 0.98 for water and heavy water respectively. Dot-plots of obtained data with trend lines added are shown in Fig. 1. Table 3 presents also the quantitative characteristic of data obtained; all errors were calculated for 0.05 significance level.

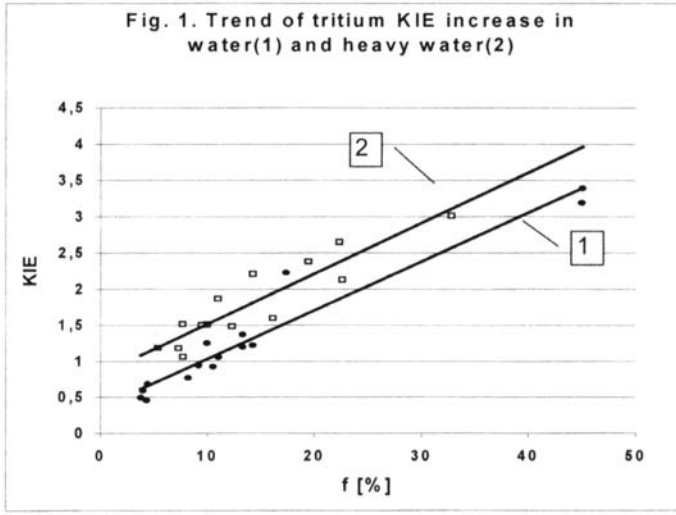

Table 3. Quantitative characteristics of $^{1}H/^{3}H$ KIEs in water and heavy water in the decomposition of L-tyrosine catalyzed by TPL

Solvent	Slope	Intercept	Error	Correlation factor
H_2O	7.22	0.794	5.6%	0.940
D_2O	6.38	0.302	3.9%	0.989

Discussion

1. $^{1}H/^{2}H$ solvent kinetic isotope effects on the reaction catalyzed by TPL.

$^{1}H/^{2}H$ solvent kinetic isotope effects on V equals to 1.70, and on V/K to 9.12. The value of KIE on V is similar to value (1.66) previously obtained for TPL from *Erwinia herbicola*[10]. Replacement of protium with deuterium may slightly change conformation of TPL's active center, and thus slow down the reaction. KIE on V/K is much higher. It means that Michaelis constant in heavy water is much greater (5.4-fold), than in water. It is due to much worse recognition of tyrosine in heavy water and significant changes in the recognition domain of TLP.

2. 3',5'-$^{1}H_2/^{2}H_2$ kinetic isotope effects

3',5'-$^{1}H/^{2}H_2$ KIE in the reaction catalyzed by TPL on V and V/K are 1.40 and 2.27, respectively. The effect on V is a significant secondary one. It is according to the literature data[5, 6], which do not estimate it to be highly important for catalysis. The effect of V/K is much higher. It means, that Michaelis constant for [3',5'-$^{2}H_2$]-L-tyrosine in the reaction of TPL increases 1.62-fold comparing to non-labeled L-tyrosine. It shows great importance of orto (vs hydroxyl group) hydrogen atoms of phenol moiety to the

process of recognition of the substrate. KIE method had never been used before to prove this phenomenon.

3. 2-$^{1}H/^{3}H$ kinetic isotope effects in water and in heavy water

These effects increase linearly with conversion, as shown on schemes 2 and 3. It is important to notice that the slope of the line of dependance KIE vs f is slightly higher for $^{1}H/^{3}H$ effect in water (7.22), than in heavy one (6.38). In contrary, intercepts (KIEs estimated at 0% conversion) differ significantly; much higher value was obtained for the experiments in water (0.794), whereas corresponding value for heavy water was 0.302.

The change of KIE with conversion in the reaction catalyzed by TPL has never been observed yet (competitive method hasn't been attempted in the studies on hydrogen effects on this reaction at all). This phenomenon suggests, that during the reaction a mechanism of action changes significantly. Similar situation was observed in the studies on L-phenylalanine ammonia lyase[15]

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References

1. Brot N, Smit Z, Weissbach H. *Arch. Biochem Biophys.*, **1965**; *112:* 1-6
2. Nagasawa N, Utagawa T, Goto J, Kim C J, Tani Y, Kumagai H, Yamada H. *Eur. J. Biochem.*, **1981;** *117:* 33-40
3. Lloyd-George I, Chang T M. *Biomater. Artif. Cells Immobilization Biotechnol.*, **1993;** *21:* 323-333
4. Chen H, Phillips R S. *Biochemistry*, **1993;** *32:* 11591-11599
5. Phillips R S, von Tersch R. L, Secundo F. *Eur. J. Biochem.*, **1997;** *224:* 658-663
6. Sundararaju B, Antson A A., Phillips R S, Demidkina T V, Barbolina M V, Gollnick P, Dodson G, Wilson K S. *Biochemistry*, **1997;** *36:* 6502-6510
7. Kumagai H, Kitama S, Yamada H, Ueno T, Fukami H. *Pap. Meet. Agric. Chem. Soc. Jap.*, Kyoto, 1975.
8. Kiick D M, Phillips R S. *Biochemistry*, **1988;** *27:* 7333-7338
9. Axelsson B S, Bjurling P, Matsson O, Langstrom B. *J. Am. Chem. Soc.*, **1992;** *114:* 1502-1503
10. Fadeev N G, Spirina S N, Demidkina T V, Phillips R S. *J. Chem. Soc. Perkin Trans. 2,* **1996;** 2001-2004
11. Augustyniak W, Kański R, Kańska M. *J Label. Compd. Radiopharm.,* **2001;** *44:* 553-560
12. Augustyniak W, Suchecki P, Jemielity J, Kański R, Kańska M. *J Label. Compd. Radiopharm.,* **2002;** *45:* 559-567
13. Augustyniak W, Kański R, Kańska M. (in press in this issue)
14. Tong J Y, Yankwich P E. *J. Phys. Chem.*, **1964;** *61:* 540-543
15. Lewandowicz A, Jemielity J. Kanska M, Zon J, Paneth P. *Arch. Biochem. Biophys.,* **1999;** *370:* 216-221