Carbon isotope effects in the studies of the mechanism of action of tyrosine phenol-lyase

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Abstract $^{12}C/^{14}C$ kinetic isotope effects on the hydrolytic cleavage of tyrosine to phenol and ammonium pyruvate catalyzed by *Citrobacter freundii* tyrosine phenol-lyase have been determined in positions 2, 3 and ring-1' of L-tyrosine. The competitive method with dual-label approach was applied with 3',5'-ring ^3H as remote label. The results revealed the change of the effect on carbon atom in position 2 during the reaction course from the high normal values to low inverse values. On the other hand, the effect values on 3 and ring-1' position remained constant during the reaction course. The discussion of these results regarding the reaction mechanism is presented.

Key words $^{12}C/^{14}C$ kinetic isotope effects • tyrosine phenol-lyase

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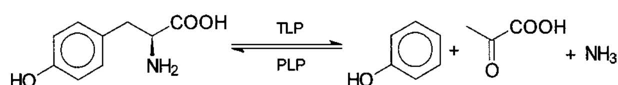
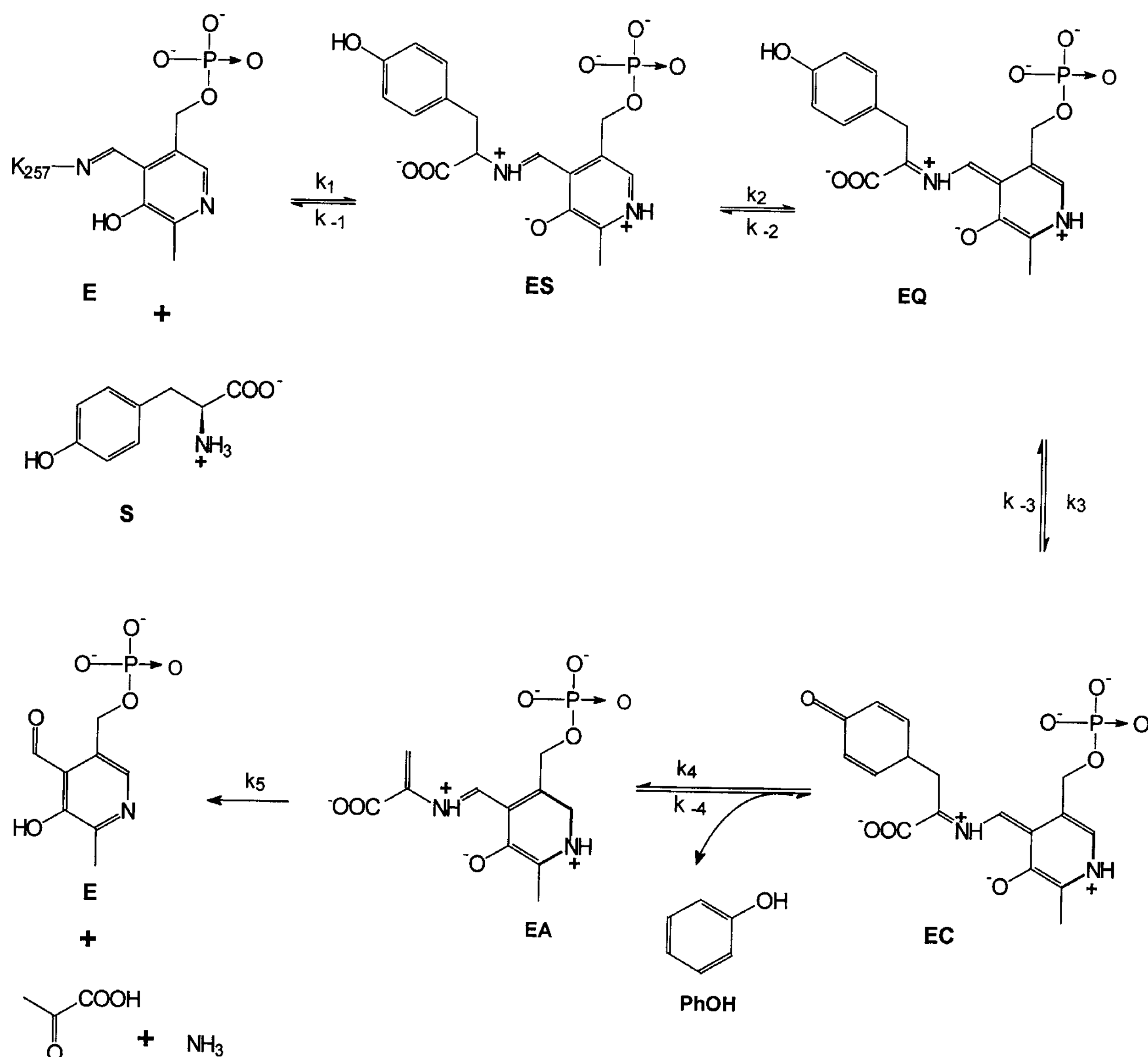
Introduction

Tyrosine phenol-lyase (TPL) catalyzes the reversible hydrolysis of tyrosine to phenol and ammonium pyruvate (Scheme 1) [9]. Pyridoxal 5'-phosphate is a cofactor of the enzyme. TPL has been found in various bacteria, mostly in *Enterobacteriaceae* and also in some arthropods. The enzyme catalyzes β-elimination of a number of 3-substituted amino acids with good leaving groups, as, for example, *O*- and *S*-derivatives of serine and cysteine, 3-chloroalanine [7]. TPL also racemizes alanine [6] and exchanges α-proton with a solvent in some other amino acids [4]. The enzyme catalyzes reverse reaction of the synthesis of tyrosine and its derivatives starting form various phenol and pyruvate derivatives [11].

The mechanism of TPL action consists of several steps [9], main transitions are drawn in Scheme 2. The first step is the formation of external aldimine – the Schiff base between tyrosine and PLP, which is followed by the abstraction of α-proton leading to the quinoid intermediate. Subsequently, ring-1' carbon is protonated and the phenol moiety is converted to cyclohexadie-



Scheme 1. Decomposition of L-tyrosine catalyzed by enzyme tyrosinase phenol-lyase, TPL.



Scheme 2. Mechanism of action of tyrosine phenol-lyase.

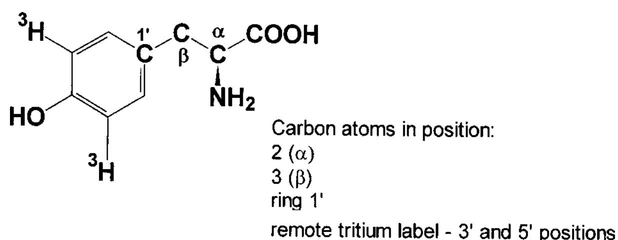
none, which is a sufficiently good leaving group to undergo β-elimination as a phenol moiety. After β-elimination, the Schiff base between α-aminoacrylate and PLP undergoes hydrolysis to E pyruvate and ammonia.

Although the molecular mechanism of the reaction is quite well known, the reaction has not been studied in the further extents of the reaction course. Kinetic isotope effects are a good method to perform such studies. We decided to study $^{12}C/^{14}C$ kinetic isotope effects in the *Citrobacter freundii* TPL-catalyzed reaction. The enzyme cleaves a carbon-carbon bond between aromatic and aliphatic moiety, which is a unique property in the living world. Our studies on carbon kinetic isotope effects may reveal some interesting phenomena concerning the studied reaction; in this paper we focused on the carbon-carbon bonds cleavage occurring during TPL catalysis.

To determine the effect values, radiochemistry was applied along with the use of a competitive method with a dual-label approach.

Experimental

The concentrations of components in the reaction medium were: 0.1 M potassium phosphate buffer (pH 8.3), 0.2 M KCl, 1 mM D,L-dithiothreitol, 50 mM PLP, 13.4 U/ml of L-lactic dehydrogenase from rabbit muscle (EC 1.1.1.27; Sigma), 1 mM NADH and 0.4 U/ml TPL from *Citrobacter freundii*. To this medium [2-^{14}C]-L-tyrosine (specific radioactivity of 0.69 MBq/mmol [1]), or [3-^{14}C]-L-tyrosine (specific radioactivity of 40.3 kBq/mmol [2]) or [1'-^{14}C]-L-tyrosine (specific radioactivity of 0.50 kBq/mmol [5]) and [3',5'-$^{3}H_2$]-L-tyrosine (as a remote label; Sigma; total radioactivity of ^{3}H-isotopomer was 10–20 fold higher than ^{14}C-one) were added up to concentration ca. 0.5 mM. Reaction was carried out at room temperature. During the reaction fractions were taken in present times. The products were separated on an ion exchange column (Amberlit IR120, H^+ form, 60 mm × 5 mm) and their radioactivities were measured using Wallac 1409, Raytest, and liquid scintillation cocktail from Rotiszint.



Scheme 3. Atoms of interest in this work.

The degree of conversion was calculated using ^{3}H activity of the product and substrate. The Yankwich-Tong equation [10] was used to calculate KIE.

Results

Kinetic isotope effect determination methodology and design

All of the $^{12}C/^{14}C$ kinetic isotope effects were determined using the competitive method, i.e. the carbon isotopes fractionation between the products and substrates was measured. Such approach allows only to determine effects on maximal velocity/Michaelis constant ratio. Specific radioactivities of the starting substrate and products were determined to measure the fractionation. Dual-label methodology was applied to determine the amount of the specified compounds as the value proportional to the radioactivity of the remote label. In this work, ^{3}H in position 3',5' of the tyrosine ring was the remote label (Scheme 3). Previously, it has been shown in our group that $^{1}H/^{3}H$ kinetic isotope effect on this position is nearly negligible (1.003 ± 0.007) [8]; thus the position *ortho* in the phenolic moiety of tyrosine is isotopically insensitive (i.e. does not introduce any large isotope effect to the system) and can be used as the remote label. The kinetic isotope effect values were corrected for the low $^{1}H/^{3}H$ effects on the remote position; the geometric mean rule (which is applicable for kinetic isotope effects) was used in this corrections. In addition to the specific radioactivities of the starting substrate and products, the conversion degree was determined using the remote label as internal standard. These 3 values were an input to the Yankwich-Tong equation [10], which allows to calculate the value of kinetic isotope effects:

$$k/{}^{*}k = \frac{\ln\left(1 - f\frac{R_0}{R_p}\right)}{\ln(1-f)}$$

where $k/{}^{*}k$ is the kinetic isotope effect value, R_0 is the specific radioactivity of the starting substrate, R_p is the specific radioactivity of the product, and f is the conversion degree.

Prior to the measurement of the specific radioactivities, the products and recovered substrate were separated on the ion-exchange resin. To secure the stability of pyruvate during separation, L-lactic dehydrogenase and NADH in the excess were added to the reaction mixture. The additives convert pyruvate to stable L-lactate. Since the excess of dehydrogenase and NADH is used, the conversion of pyruvate is quick comparing to the reaction catalyzed by TPL; thus lactate formation does not introduce any significant isotope fractionation comparing to the reaction catalyzed by tyrosine phenol-lyase.

α-$^{12}C/^{14}C$ kinetic isotope effect

The results of kinetic isotope effect determination in position 2 are shown in Table 1. To fully describe the effect, the data regarding reaction time and concentrations of TPL, PLP and substrate are also shown.

During the reaction course, the kinetic isotope effect value decreases from the normal ones that should have been anticipated for primary kinetic isotope effect in the enzymatic reaction (1.038) to extremely low values (0.882). The decrease effect is clearly seen during each experiment, therefore the experimental data are grouped for each experiment in Table 1. Such a behavior is quite tough to explain. It is a well known fact that primary kinetic isotope effects are always normal (i.e., higher than 1) and there are hardly known carbon secondary kinetic isotope effects lower than 0.97 in enzymatic reactions. Thus, the obtained results have to be explained on a different basis.

Table 1. α-$^{12}C/^{14}C$ kinetic isotope effect values

f	$k/{}^{*}k$	t [ks]	PLP [μM]	TPL [nM]	S [μM]
0.241	0.988	3.60	52.3	34.4	174
0.284	0.979	5.40	52.3	34.4	174
0.321	0.962	7.38	52.3	34.4	174
0.375	0.919	9.90	52.3	34.4	174
0.087	0.984	0.90	49.8	32.7	244
0.092	0.939	1.68	49.8	32.7	244
0.113	0.947	2.82	49.8	32.7	244
0.108	0.902	3.67	49.8	32.7	244
0.127	0.918	5.51	49.8	32.7	244
0.147	0.916	7.81	49.8	32.7	244
0.086	1.004	0.60	37.3	32.3	205
0.085	0.981	1.20	37.3	32.3	205
0.085	0.944	1.80	37.3	32.3	205
0.085	0.913	3.00	37.3	32.3	205
0.105	0.899	4.80	37.3	32.3	205
0.127	0.902	7.81	37.3	32.3	205
0.091	1.038	1.26	49.7	32.6	162
0.118	1.010	2.40	49.7	32.6	162
0.136	1.007	3.60	49.7	32.6	162
0.143	0.986	5.40	49.7	32.6	162
0.108	0.929	9.07	49.7	32.6	162
0.120	0.882	14.40	49.7	32.6	162

Table 2. β-$^{12}C/^{14}C$ kinetic isotope effect values

f	*k*/**k*	*t* [ks]	PLP [μM]	TPL [nM]	S [μM]
0.098	1.065	5.22	49.5	33.4	268
0.204	1.038	1.80	46.2	30.4	176
0.218	1.039	8.52	50.3	33.0	225
0.249	1.040	2.94	50.3	33.0	225

The decrease of α-$^{12}C/^{14}C$ kinetic isotope effect depends not only on the conversion degree, but also on some other factors, since the trend of effect vs. conversion is not reproduced among experiments.

β-$^{12}C/^{14}C$ kinetic isotope effect

The determined kinetic isotope effect values in position 3 of L-tyrosine are shown in Table 2. The average effect value is 1.05 ± 0.01, which was obtained from 4 measurements. The number of measurements was limited by the small amount of [3-^{14}C]-L-tyrosine available for this assay. Low number of measurements resulted in an increased value of experimental error.

The β-$^{12}C/^{14}C$ kinetic isotope effect is an usual value for primary effects of carbon.

Ring 1'-$^{12}C/^{14}C$ kinetic isotope effect

The obtained kinetic isotope effect values in position 1' of phenolic ring of L-tyrosine are presented in Table 3. The averaged value is 1.004 ± 0.007 from 3 measurements. Again, the low number of measurements resulted from difficulties in the synthesis of [1'-^{14}C]-L-tyrosine. The effect value is typical of secondary $^{12}C/^{14}C$ kinetic isotope effects.

Discussion

The mechanism of action of tyrosine phenol-lyase may be represented as shown in Scheme 2. E represents free holoenzyme TPL, S – tyrosine, ES – external adimine (Schiff base PLP – tyrosine) – complex with TPL, EQ – quinoid intermediate bound by the enzyme, PhOH – phenol, EC – complex of TPL with cyclohexadienone intermediate, EA – Schiff base of PLP and α-aminoacrylate in the active center, and finally P – ammonium pyruvate. Rate constants *k* are representatives for the elementary processes specified on the scheme. The last step of the catalysis is irreversible, since all of the formed pyruvate is rapidly trapped and converted to L-lactate by L-lactic dehydrogenase.

Table 3. Ring 1'-$^{12}C/^{14}C$ kinetic isotope effect values

f	α	*t* [ks]	PLP [μM]	TPL [nM]	S [μM]
0.130	1.009	14.40	48.4	31.7	984
0.158	1.001	14.28	49.8	32.7	1363
0.161	1.004	10.86	49.8	32.7	1363

Using the presented mechanism, the decrease of kinetic isotope effect in position 2 can be explained. Primary effects can be supposed on the α-deprotonation and reprotonation steps (described by k_2 and k_{-2} rate constants) and on final formation of pyruvate (k_5) exclusively, since these processes occur via bond formation or disruption on carbon 2. The whole process may be limited by the formation of ammonium pyruvate (k_5), which exhibits a large primary carbon isotope effect in position 2. Therefore, large effect may be observed for low conversion degrees. Assuming that α-$^{12}C/^{14}C$ kinetic isotope effect on reprotonation (k_{-2}) is significantly higher than on deprotonation (k_2), it is quite likely that the EA complex is continuously enriched with [2-^{14}C]-L-tyrosine, since EA is in dynamic equilibrium with the substrate, enzyme and all by-products. Therefore, a significant decrease of α-$^{12}C/^{14}C$ kinetic isotope effect to the low inverse values may be observed with the reaction extent.

The β-$^{12}C/^{14}C$ kinetic isotope effect in the reaction catalyzed by TPL is a large primary one. The value 1.05 is in good agreement with the results of Axelsson *et al.* [3], who determined $^{11}C/^{14}C$ effect in position 3 to be 1.08. They expected the value of $^{12}C/^{14}C$ effect of 1.04, which was proven by our results within the experimental error. The value of β-$^{12}C/^{14}C$ kinetic isotope effect confirms the importance of carbon atom 3 in the catalysis. Primary kinetic isotope effects in this position can be expected on phenol elimination (k_4) or addition (k_{-4}) and pyruvate formation (k_5). Since the latter seems to be the main rate-limiting step, the measured effect value probably reflects the kinetic isotope effect on k_5. It may also be concluded that β-$^{12}C/^{14}C$ effects on phenol elimination and addition are similar, since here no significant changes in observed kinetic isotope effect values with conversion were noticed, as it was the case with α-$^{12}C/^{14}C$ effect.

The ring 1'-$^{12}C/^{14}C$ kinetic isotope effect is 1.004, which is quite surprising, since the carbon atom 1' of the aromatic moiety is directly involved in the reaction course. Therefore, a large primary effect could have been expected. The significant effects can be anticipated on 1' atom protonation (k_3) and deprotonation (k_{-3}), as well as on phenol elimination (k_4) and addition (k_{-4}). However, the ring 1'-$^{12}C/^{14}C$ effect is not exhibited, which suggests that the mentioned elementary reactions are much quicker than the rate-limiting pyruvate formation step. These conclusions are consistent with the other elucidations resulting from $^{12}C/^{14}C$ kinetic isotope effects in positions α and β.

Our work on $^{12}C/^{14}C$ kinetic isotope effect in the reaction catalyzed by TPL allowed to elucidate that the ammonium pyruvate formation step is, at least partially, a rate-limiting step. Kinetic isotope effects allowed us to observe final steps of the reaction; such analysis was not accessible with spectral methods that were applied previously in studies on the mechanism of action of tyrosine phenol-lyase.

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Abbreviations

E – enzyme,
EA – complex of tyrosine phenol-lyase with Schiff base of α-acrylamide and pyridoxal 5'-phosphate,
EC – complex of tyrosine phenol-lyase with cyclohexadienone intermediate,
EQ – complex of tyrosine phenol-lyase with quinoid intermediate,
ES – complex of tyrosine phenol-lyase with Schiff base of tyrosine and pyridoxal 5'-phosphate,
f – conversion degree,
$k/{}^*k$ – kinetic isotope effect,
NADH – nicotinamide adenine dinucleotide, reduced form,
P – ammonium pyruvate,
PhOH – phenol,
PLP – pyridoxal 5'-phosphate,
R_0 – specific radioactivity of the starting substrate,
R_p – specific radioactivity of the product,
S – tyrosine,
TPL – tyrosine phenol-lyase

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