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TECHNICAL NOTE

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Deuterium isotope effects in mechanistic studies of biotransformations of L-tyrosine and *p*-hydroxyphenylpyruvic acid catalyzed by the enzyme L-phenylalanine dehydrogenase

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Abstract. The mechanisms of the reversible oxidative deamination of L-tyrosine to *p*-hydroxyphenylpyruvic acid and reductive amination of phenylpyruvic acid to L-phenylalanine, both catalyzed by the enzyme L-phenylalanine dehydrogenase (PheDH, EC 1.4.1.20), were investigated using the kinetic isotope effect (KIE) and solvent isotope effect (SIE) methods. The values of deuterium kinetic effects in the 2-position of L-tyrosine and KIE in the (3*S*)-position of phenylpyruvic acid and solvent isotope effects for both reactions were determined using the non-competitive spectrophotometric method. Some mechanistic details of these biotransformations were discussed.

Keywords: Kinetic isotope effects • L-Phenylalanine • Phenylalanine dehydrogenase • *p*-Hydroxyphenylpyruvic acid • Solvent isotope effects • Tyrosine

Introduction

Type I phenylketonuria (PKU) [1–3] is possible and is caused by a mutation in the gene that encodes the enzyme L-phenylalanine hydroxylase (PAH). This enzyme is designed to convert L-phenylalanine (L-Phe) to L-tyrosine (L-Tyr). The accumulated L-phenylalanine is metabolized into dangerous phenylpyruvate, phenylacetate, and phenyl-L-lactate, which are excreted in the urine, see Fig. 1.

The excess of L-Phe also hinders the transport of other products within the cell. Used for the synthesis of neurotransmitters such as dopamine and melanin. Untreated type I PKU leads to mental retardation, brain damage, seizures, and skin discoloration.

Type I tyrosinemia [4], the most severe form of tyrosinemia, is an inborn genetic disorder, in which L-Tyr cannot be effectively broken down. It is accompanied by elevated levels of L-Tyr and its metabolites: *p*-hydroxyphenylpyruvate (*p*-OH PPA), *p*-hydroxyphenylacetate, and *p*-hydroxyphenyl-L-

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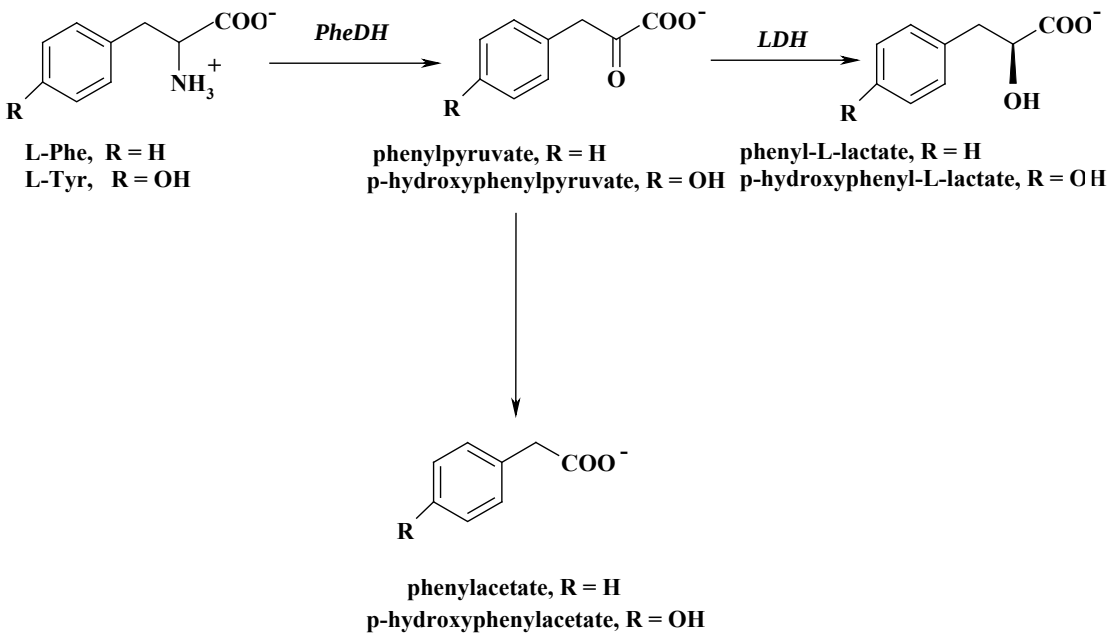

Fig. 1. Enforced metabolic pathways of accumulated L-Phe and L-Tyr in a person suffering from PKU and type I tyrosinemia diseases.

-lactate in body fluids (Fig. 1). This disease is caused by a deficiency of the enzyme fumarylacetoacetate hydrolase (EC 3.7.1.2). The high concentration of these metabolites inhibits the activity of other enzymes. Type I tyrosinemia causes liver, spleen, and kidney disturbances and mental retardation.

Similarly, type II tyrosinemia is caused by a deficiency of the enzyme tyrosine aminotransferase (EC 2.6.1.5). In this case, the accumulation of L-Tyr forces the alternative path of its decomposition, leading to *N*-acetylotyrosine and tyramine (Fig. 2). This form of disease can affect the eyes, skin, and mental development.

The key stage of the disease – PKU – is the transformation of L-phenylalanine into phenylpyruvic acid. This is oxidative deamination [5, 6] which is a reversible process, catalyzed by the enzyme L-phenylalanine dehydrogenase, Fig. 1. In an alkaline environment, PheDH catalyzes the oxidative deamination of L-α-amino acids into the corresponding 2-oxo acids [7, 8] showing maximum activity at pH 10.7. The reversible process is reductive amination (conversion of 2-oxo acids into L-α-amino acids) [9–11] and occurs under milder conditions, reaching the highest rate at pH 8.5.

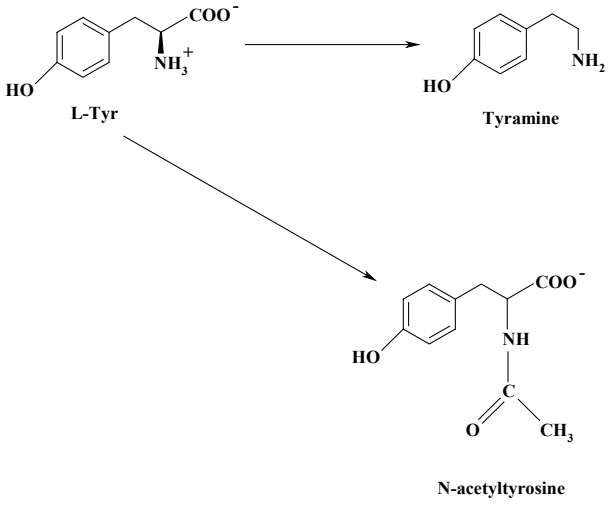

Fig. 2. Alternative metabolic pathways of L-Tyr in a person suffering from type II tyrosinemia disease.

The mechanisms of L-Phe and L-Tyr metabolism have not been fully understood, despite many reports in the literature. Therefore, we decided to investigate and learn more about the details of the reactions catalyzed by PheDH presented in Fig. 1, i.e., the reversible reductive amination of PPA to L-Phe and the oxidative deamination of L-Tyr to *p*-OH PPA by using methods of kinetic isotope effect (KIE) [12] and solvent isotope effect (SIE) [13].

The determined numerical values for isotope effects allowed us to elucidate the internal details of the mechanism and helped to distinguish between alternative mechanisms. Studies of the above enzymatic reactions required the use of isotopically labeled PPA and L-Tyr isotopomers. For this purpose, we use deuterium labeled in the (3*S*)-position isotopomer [(3*S*)-^{2}H]-PPA and deuterium labeled in the 2-position isotopomer [2-^{2}H]-L-Tyr. These compounds were obtained according to the earlier developed methods [14–16].

Experimental

Materials

Enzyme PheDH (L-Phenylalanine dehydrogenase, EC 1.4.1.20) from *Sporosacrina* sp. and cofactor – NADH disodium salt – were purchased from Sigma. Deuterated water (99.9% D) and deuterated 30% KOD/D_2O (99.9% D) were purchased from Polatom, Poland. L-Phenylalanine, L-tyrosine, sodium phenylpyruvate, *p*-hydroxyphenylpyruvic

acid, and other chemicals needed for trial synthesis were purchased from Aldrich. The compounds for kinetic assays, i.e., PPA, selectively labeled with deuterium in the 3*S*-position ([(3*S*)-^{2}H]-PPA) and L-Tyr, selectively labeled with deuterium in the 2-position ([2-^{2}H]-L-Tyr), were obtained by the methods described earlier.

Methods

The extent of deuterium incorporation into the (3*S*)--positions of [(3*S*)-^{2}H]-PPA and 2-positions of [2-^{2}H]-L-Tyr was determined from ^{1}H NMR spectra recorded on a Varian Unity + 500 MHz spectrometer (TMS, DMSO-d_6). The progress of enzymatic reductive amination and oxidative deamination was determined indirectly by measuring the concentration of NADH spectrophotometrically at λ_{max} = 340 nm using a Shimadzu UV-102-CE-LV spectrophotometer.

Synthesis

Synthesis of [(3S)-^{2}H]-PPA

1) **Synthesis of [(3*S*)-^{2}H]-L-Phe.** This intermediate for the synthesis of [(3*S*)-^{2}H]-PPA was obtained by adding ammonia to (E)-cinnamic acid (45 mg, 0.3 mmol) catalyzed by the enzyme Phenylalanine Ammonia Lyase (PAL, EC 4.3.1.24, 2 U) using the fully deuterated instead of tritiated incubation medium. The details of this enzymatic synthesis were described earlier [14]. As a result, a 17.4 mg (0.106 mmol) sample of [(3*S*)-^{2}H]-L-Phe was obtained with 35.7% yield.
2) **Synthesis of [(3*S*)-^{2}H]-PPA.** The oxidative deamination of the sample of [(3*S*)-^{2}H]-L-Phe (17.4 mg, 0.106 mmol) converted to [(3*S*)-^{2}H]--PPA catalyzed by enzyme PheDH (2.8 U) was carried out in glycine buffer as described earlier [15]. As a result, 8.1 mg (0.049 mmol) of [(3*S*)-^{2}H]-PPA was collected (46% yield). The position and extent of deuterium incorporation was verified by ^{1}H NMR (100% D incorporation in the (3*S*)-position) [15], i.e., ^{1}H NMR (500 MHz, DMSO-d_6): δ2.71 [1H, (3*R*)-H, s), δ6.91 (1H, ArH, m), δ6.98 (1H, ArH, m), δ7.15 (1H, ArH, m), δ7.20 (1H, ArH, m), δ7.31 (H, ArH, m). Signal δ2.59 (s) from (3*S*) hydrogen disappeared.

Synthesis [2-^{2}H]-L-Tyr

The reductive amination of *p*-OH PPA to [2-^{2}H]--L-Tyr was carried out as described earlier [16]. A sample of 20 mg (0.11 mmol) of *p*-OH PPA was converted to 9 mg (0.05 mmol) of [2-^{2}H]-L-Tyr (45% chem. yield) using fully deuterated 0.7 M ammonium buffer (pD 8.9) containing 100 mg (0.14 mmol) of disodium NADH salt and 4.5 U of enzyme PheDH (EC 1.4.1.20). The ^{1}H NMR spectrum has shown near 100% incorporation of deuterium into the 2-position of deuterated L-Tyr, i.e., ^{1}H NMR (500 MHz, DMSO-d_6): δ3.03 (1H, β-H, d), δ3.18 (1H, β-H, d), δ6.89 (2H, ArH, d), δ7.18 (2H, ArH, d). Signal from α-proton, i.e., δ 3.93 (1H, α-H, dd), disappeared.

Kinetic assays

Determination of deuterium isotope effects of reductive amination of PPA catalyzed by PheDH

1) **SIE's for reductive amination of PPA to L-Phe.** The kinetic experiments for determining SIE's of reductive amination of PPA were carried out using the subsequent buffered solutions:

(a) 0.7 M ammonium buffer, pH 8.5; pH was adjusted with 3.0 M NH_3. The fully deuterated buffer medium was corrected to pD 8.9 [17] with 30% KO^{2}H/^2H_2O,
(b) 5.4 mM solution of PPA,
(c) 1.2 mM solution of NADH,
(d) L-PheDH solution (5.4 U mL^{-1}).

According to the measurement being performed, all reagents necessary for kinetic assays were dissolved in protonated or fully deuterated ammonium buffer solutions. Every kinetic experiment, which included six runs with varied concentrations of PPA, was carried out in 3 mL quartz spectroscopic cuvettes filled with precise volumes of the buffered solution to reach the final concentrations of reactant ranging from 0.036 mM to 0.22 mM with 0.04 mM intervals. Exact volumes of NADH and PheDH solutions were added to obtain 0.08 mM and 0.2 U mL^{-1} concentrations, respectively. At last, each cuvette was filled with ammonium buffer (protonated or deuterated) up to 3 mL. The progress of reduction in room temperature was monitored spectrophotometrically by measuring the decreasing absorbance of the reduced form of cofactor NADH at λ_{max} = 340 nm for 20 min with 1-min intervals.

2) **KIE's for reductive amination of PPA to L-Phe.** The kinetic experiments for determining KIE's of reductive amination of PPA and [(3*S*)-^{2}H]-PPA were carried out as described above using 0.7 M ammonium buffer, pH 8.5 mM and 5.4 mM solutions of PPA, and [(3*S*)-^{2}H]-PPA.

Determination of deuterium isotope effects of oxidative deamination of L-Tyr catalyzed by PheDH

1) **SIE's for oxidative deamination of L-Tyr to *p*-OH PPA.** The kinetic experiments for studying SIE's of oxidative deamination of L-Tyr were performed using the following buffered solutions:

(a) 0.1 M glycine buffer, pH 10.7; pH was adjusted with 2.0 M KOH. The fully deuterated buffer medium was prepared and corrected to pD 11.1 with 30% KO^{2}H/^2H_2O,
(b) 3.0 mM solution of L-Tyr,
(c) 30 mM solution of NAD^+,
(d) L-PheDH solution (6.7 U mL^{-1}).

All reagents necessary for kinetic assays were dissolved in protonated or fully deuterated glycine buffer solution depending on the measurement being registered. Every kinetic experiment included six runs with varied concentrations of L-Tyr. 3 mL

Fig. 3. Reversible conversion of L-Phe and L-Tyr into corresponding oxoacids catalyzed by the enzyme PheDH.

quartz spectroscopic cuvettes were filled with precise volumes of the buffered solution to reach the final reactant concentrations ranging from 6 mM to 12 mM with 1 mM intervals. Exact volumes of NAD^+ and PheDH solutions were added to obtain 0.2 mM and 0.2 U mL^{-1} concentrations, respectively. Finally, each cuvette was filled with glycine buffer (protonated or deuterated) up to 3 mL. The progress of oxidation was monitored spectrophotometrically by measuring the increasing absorbance related to the reduced form of cofactor NADH at λ_{max} = 340 nm for 45 min with 30 s intervals.

2) **KIE's for oxidative deamination of L-Tyr to *p*-OH PPA**. The kinetic experiments for studying KIE's of oxidative deamination of L-Tyr and [2-^{2}H]-L-Tyr were performed using the following buffered solutions: 0.1 M glycine buffer, pH 10.7; 3.0 mM solution of L-Tyr and [2-^{2}H]-L-Tyr. The kinetic experiments were carried out the same way as described earlier [18].

Results and discussion

The purpose of this study was to investigate deuterium KIE and SIE in the reactions of reductive amination of PPA to L-Phe and oxidative deamination of L-Tyr to *p*-OH PPA catalyzed by the NADH-dependent enzyme PheDH (Fig. 3).

The kinetics of the above-presented (Fig. 3) reactions at room temperature were determined using the non-competitive spectrophotometric method [19]. Because the products and substrates of these reactions do not adsorb UV-VIS light in the spectrometric region, the loss of PPA or L-Tyr is determined indirectly by the increase (in case of oxidative amination of L-Tyr) or decrease (in case of reductive amination of PPA) of absorbance of NADH involved in the course of these reactions.

The KIE and SIE in enzymatic reductive amination of ([(3*S*)-^{2}H]-PPA) and oxidative deamination of [2-^{2}H]-L-Tyr) were calculated using the method described earlier [20]. The K_M and V_{max} in Michaelis–Menten Eq. (1) were calculated using Enzifitter™ software from experimentally measured reaction rates (v) of given concentrations of PPA and L-Tyr.

$$K_M = [S]\left[\frac{V_{max}}{v} - 1\right] \quad (1)$$

The values of isotope effects were obtained from the initial rate (v) for corresponding isotopomers of PPA and L-Tyr using Eq. (2)

Table 1. Solvent isotope effects (SIE) and kinetic isotope effects (KIE) in the oxidative deamination of L-Tyr and reductive amination of PPA catalyzed by enzyme PheDH

Reactant	SIE	
	on V_{max}	on V_{max}/K_M
L-Tyr	1.43 ± 0.09	1.48 ± 0.12
PPA	1.44 ± 0.08	1.58 ± 0.14
	KIE	
	on V_{max}	on V_{max}/K_M
L-Tyr/[2-^{2}H]-L-Tyr	2.26 ± 0.10	2.87 ± 0.11
PPA/[(3*S*)-^{2}H]-PPA	1.55 ± 0.06	1.53 ± 0.16

$$v = \frac{[S]}{\left(\frac{1}{V_{max}K_M} + \frac{[S]}{V_{max}}\right)} \quad (2)$$

where K_M is a Michaelis constant; [S] is a concentration of reactant ($S \gg$ concentration of enzyme); v is the initial rate; and V_{max} is the maximum reaction rate.

The KIE values, presented in Table 1, were obtained by dividing the parameters for the reactions carried out with PPA and L-Tyr by parameters for the reactions with ([(3*S*)-^{2}H]-PPA) and ([2-^{2}H]-L--Tyr), respectively.

The SIE values, presented in Table 1, were calculated by dividing the parameters for PPA and L-Tyr carried out in protonated reaction media by parameters obtained in fully deuterated buffers. The determined KIE values (KIE on V_{max} = 1.55 and KIE on V_{max}/K_M = 1.53) for the 3*S*-position in the reaction of reductive amination of [(3*S*)-^{2}H]-PPA to L-Phe catalyzed by enzyme PheDH indicate that replacing the protium with deuterium slightly affects the reaction rate. In the case of oxidative deamination of [2-^{2}H]-L-Tyr deuterium KIE obtained for the 2-position of tyrosine (KIE on V_{max} = 2.26 and KIE on V_{max}/K_M = 2.87) shows that the cleavage of C–D bond in this place is probably the reaction rate-determining step.

The SIE values for the above reactions (SIE on V_{max} = 1.44 and KIE on V_{max}/K_M = 1.58 for reductive amination of PPA to L-Phe, and SIE on V_{max} = 1.43 and KIE on V_{max}/K_M = 1.48 for oxidative deamination of L-Tyr to *p*-OH PPA) indicate that the solvent has little effect on the conversion of the enzyme--substrate complex to the enzyme-product complex.

The commonly distributed enzymes that belong to the amino-acid dehydrogenase group, including the PheDH, catalyze a reversible oxidative deami-

nation of keto acids (Fig. 1). For many years, the PheDH enzyme was a scope of comprehensive mechanistic studies [21] and has found an application as a biosensor to screen the PKU [22, 23]. It is also useful for industrial-scale manufacturing of L-Phe [24] as a component for a sweetener aspartame. Although the comprehensive description of the structure, kinetics, and proposed mechanism of action of reactions catalyzed by the PheDH has been previously described [6, 25], we have not determined the rate-limiting reaction step. Thus, the values of KIE and SIE for the deuterium, which were determined in this work, can be used for a complete understanding of the mechanism of action of this enzyme.

Conclusion

Despite numerous literature reports, the mechanism of this reaction is not fully known. In this manuscript, we present our research aiming at a deeper understanding of the mechanism of action of L-phenylalanine dehydrogenase (PheDH) by studying isotope effects. The values of KIE and SIE of deuterium have been determined. Based on the SIE values, it has been found that the solvent has a negligible effect on the reaction rate. The KIE data indicate that the substitution of deuterium for protium in the (3*S*)-position has a small effect on the rate of reductive amination of PPA to L-Phe. The KIE value for deuterium in the 2-position of L-Tyr indicates that cleavage of a C–D bond significantly influences the rate of oxidative deamination of L-Tyr to *p*-OH PPA. Together, the obtained values of KIE and SIE of deuterium indicate that the proton transfer effect in the tested reactions is partially masked and does not fully determine the rate of the tested reactions. Further research, which is in progress, will allow us to explain in more detail the mechanism of action of L-phenylalanine dehydrogenase.

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