Deuterium isotope effects in oxidation of dopamine by tyramine oxidase

Abstract. We report the studies on the mechanism of oxidation of dopamine (DA) to 3',4'-dihydroxyphenylacetaldehyde (DOPAL) by enzyme tyramine oxidase (EC 1.4.3.6) using kinetic isotope effects (KIE) and solvent isotope effects (SIE) methods. For kinetic studies, the selectively deuterium labelled isotopologues of dopamine, i.e., [(1R)-2H]-, and [(1S)-2H]-DA were used. The numerical values of KIE’s for the (1R)- and (1S)-isotopologues of dopamine in the enzymatic oxidation of DA to DOPAL were determined using the non competitive spectrophotometric method. Also, the SIE’s for this reaction carried out in heavy water were obtained spectrophotometrically. Some mechanistic details of enzymatic oxidation of DA to DOPAL were discussed.

Key words: deuterium • dopamine (DA) • isotope effects • oxidation • tyramine oxidase

Introduction

Dopamine a main endogenous catecholamine neurotransmitter in mammalian brain is produced by dopaminergic neurons specific for regions responsible for voluntary locomotion and behavioural processes such as mood, memory, learning, addiction, and stress [9, 17]. The dopaminergic neurons are also localized in peripheral tissues and are conjugated with cardiovascular, kidney, and hormonal systems [5]. In addition to the important role of dopamine in the vital physiological processes, its disturbed metabolism leads to a variety of disorders such as schizophrenia, Parkinson’s disease, drug addiction, and arterial hypertension [1, 3, 8, 23, 25].

The mechanism of action of DA as a neurotransmitter is quite well known, but the knowledge about causes of diseases associated with its abnormal metabolism is rather limited. It is understood that in the process of oxidation of DA catalyzed by the enzyme, monoamine oxidase (MAO) the toxic reactive oxygen species (ROS) such as hydroperoxide, oxygen radicals, and quinones are generated [5]. In the recent years DOPAL is suspected to contribute to apoptosis of the dopaminergic neurons in brain’s substantia nigra, and thus, to the appearance the symptoms of Parkinson’s disease [4, 13, 14, 16].
The enzymatic oxidation of DA to DOPAL (Fig. 1) catalyzed by the enzyme tyramine oxidase (EC 1.4.3.6) is not fully understood.

The particularly interesting issue in the mechanism of action of amine oxidases is their stereospecificity for proton removal. Series of experiments carried out for a number of amine oxidases isolated from animal tissues, plant and bacterial cells, allowed to divide these enzymes in three major groups. The first group includes enzymes that catalyze the proton elimination from the pro-S position of oxidized amine, such as an amine oxidase isolated from soybean seedling and pea seedling [7], rat aorta and porcine kidney [29], or from bacterial cells Arthrobacter globiformis [26]. To the second group belongs the enzymes MAO A and B isolated from different animal tissues [29] and amine oxidase isolated from porcine plasma [6] which catalyzes the elimination of proton from the pro-R position. The third group contains the enzymes catalyzing non specific elimination of proton, both from the pro-S and pro-R positions such as amine oxidase isolated from bovine plasma [24].

The aim of our studies is to elucidate the mechanism of oxidation of DA to DOPAL in the presence of tyramine oxidase (EC 1.4.3.6) isolated from Arthrobacter sp. This enzyme belongs to the class of copper-containing amine oxidases [28] and includes in its structure the carbonyl group-containing factor, which is probably topa quinone (3,4,6-trihydroxyphenylalanine quinone, TPQ, 6-hydroxydopa quinone) [2, 12, 15] (Fig. 2).

The proposed mechanism of action of tyramine oxidase is presented in Fig. 3.

Like other amino oxidases, tyramine oxidase catalyzes the elimination of proton from the 1-position of dopamine side chain, but the stereochemistry of this reaction has not been clearly established. To investigate some details of the above reaction we decided to use kinetic and solvent isotope effect methods to solve this issue. Determination of numerical values of KIE’s and SIE’s may shed a new light on the mechanism of enzyme-catalyzed oxidation of dopamine.

**Experimental**

**Materials**

Enzymes: tyrosinase (EC 1.14.18.1) from Neurospora crassa, tyrosine decarboxylase (EC 4.1.1.25) from Streptococcus faecalis, tryptophanase (EC 4.1.99.1) from...
E. coli, peroxidase, type II (EC 1.11.1.7) from horseradish, tyramine oxidase (EC 1.4.3.6) from Arthrobacter sp. were purchased from Sigma. Deuteriated 30% KOD/D2O and 85% D3PO4/D2O were obtained from POLATOM, Poland. Deuteriated water (99.9% D) and Amberlite IR-120 resin were from Aldrich. Aluminum oxide for column chromatography (activated) Brockmann Grade I was purchased from POCH, Poland. Deuteriated water (99.9% D) and [(1R)-2H]-L-DOPA, the 1-positions of [(1R)-2H]-, and [(1S)-2H]-DA were determined from 1H NMR spectra. The progress of all enzymatic reactions was monitored spectrophotometrically using Shimadzu UV-1202 spectrometer. Additionally, the presence of DA and t-DOPA in the course of reaction were checked qualitatively by thin-layer chromatography (TLC) using aluminum oxide plates and developing solvent: n-butanol:water:acetic acid (4:2:1, v/v/v). In the case of L-tyrosine for TLC silica gel plates the acetonitril: water (4:1, v/v) developing solvent were used. Visualization was obtained by UV lamp.

Methods

The extent of deuterium incorporation into 2-position of [2-2H]-t-DOPA, the 1-positions of [(1R)-2H]-, and [(1S)-2H]-DA were determined from 1H NMR spectra. The progress of all enzymatic reactions was monitored spectrophotometrically using Shimadzu UV-1202 spectrometer. Additionally, the presence of DA and t-DOPA in the course of reaction were checked qualitatively by thin-layer chromatography (TLC) using aluminum oxide plates and developing solvent: n-butanol:water:acetic acid (4:2:1, v/v/v). In the case of L-tyrosine for TLC silica gel plates the acetonitril: water (4:1, v/v) developing solvent were used. Visualization was obtained by UV lamp.

Synthesis

1. Synthesis of [(1R)-2H]-DA. To encapped vial containing 20 mL of fully deuteriated 0.1 M phosphate buffer at pH 6.5 (0.15 mmol) of t-DOPA, 2.5 mL of 1 mM PLP/D2O and 14 mg (5 U) of tyrosine decarboxylase (EC 4.1.1.25) from Streptococcus faecalis were added. The mixture was incubated at room temperature for 36 h. The enzyme was removed by centrifugation and the volume of post-reaction mixture was reduced to 0.5 mL (Table 1). The quantities of enzymes tyramine oxidase and peroxidase in each cuvette were 0.04 and 0.06 U respectively. To study the isotopic effects, the native DA and the previously synthesized isotopologues [(1R)-2H]- and [(1S)-2H]-DA were used. The reaction mixtures (prepared from the listed below buffered solutions) were placed in spectrometric cuvettes for monitoring the progress of oxidation.

2. Solutions “A” (substrates for coupled reaction forming colored dye). To 4.8 mL of Mc Ilvaine’s buffer, pH 5.6, a 5.5 mg (0.018 mmol) sample of 3-(N-ethyl-3-methylanilino)-2-hydroxypropanesulphonic acid sodium salt (TOOS), 4-aminoantipyrine (4-AA), t-DOPA, L-tyrosine, dopamine hydrochloride, and other chemicals needed for syntheses were from Sigma.

Kinetic assay

To study the isotopic effects, the native DA and the previously synthesized isotopologues [(1R)-2H]- and [(1S)-2H]-DA were used. The reaction mixtures (prepared from the listed below buffered solutions) were placed in spectrometric cuvettes for monitoring the progress of oxidation.

1. Mc Ilvaine’s buffers [22]. 25.25 mL of 0.2 M Na2HPO4 were mixed with 14.75 mL of 0.1 M citric acid. The desired pH 5.6 was adjusted by adding 0.2 M Na2HPO4 and 0.1 M citric acid. In parallel, fully deuteriated Mc Ilvaine’s buffer was prepared by dissolving appropriate quantities of Na2HPO4 and citric acid in heavy water and adjusted to pH 6 using 0.2 M D3PO4/D2O and 0.1 M citric acid/D3O solutions.

2. Solutions “A” (substrates for coupled reaction forming colored dye). To 4.8 mL of Mc Ilvaine’s buffer, pH 5.6, a 5.5 mg (0.018 mmol) sample of 3-(N-ethyl-3-methylanilino)-2-hydroxypropanesulphonic acid sodium salt (TOOS), 100 μL solution of 4-aminoantipyrine, 4-AA, 3.8 mg 4-AA/mL), and 100 μL (13 U) solution of enzyme peroxidase (EC 1.11.1.7) were added. TOOS, 4-AA, and enzyme were dissolved in Mc Ilvaine’s buffer. The same quantities of substrates in deuteriated Mc Ilvaine’s buffer were used to prepare parallel fully deuteriated solution “A”, pH 6.

3. Solutions “B”. 1 mM solution of native dopamine, [(1R)-2H]-, and [(1S)-2H]-DA in Mc Ilvaine’s protonated or fully deuteriated buffers, pH 5.6 and pH 6, respectively, used to appropicates kinetics runs.

4. Solutions of enzyme tyramine oxidase (5 U/mL) in Mc Ilvaine’s protonated or fully deuteriated buffers, pH 5.6 and pH 6, respectively.

Each kinetic run consists of six measurements carried out in disposable 550 μL plastic spectrosopic cuvettes containing solutions with different concentration of dopamine (from 0.066 to 0.232 mM range). To these cuvettes, the exact volumes of buffered solutions (listed in points 1 to 4) were added to reach the 0.5 mL final volume (Table 1). The quantities of enzymes tyramine oxidase and peroxidase in each cuvette were 0.04 and 0.05 U (units), respectively. The progress of DA oxidation was registered spectrophotometrically by measuring the
increase in absorbance of quinoneimine dye formed at $\lambda = 570$ nm at 1 min intervals for 60 min.

**Result and discussion**

**Synthesis**

The isotopologues [$(1R)$-2H]- and [$(1S)$-2H]-DA labelled with deuterium, synthesized by slightly modified procedures described earlier [19, 20] were used for study the enzymatic oxidation of DA presented in Fig. 1. [$(1R)$-2H]-DA was obtained by enzymatic decarboxylation of L-DOPA in fully deuteriated incubation medium catalyzed by enzyme tyrosine decarboxylase (EC 4.1.1.25) while the $(1S)$-isotopologue was prepared by enzymatic decarboxylation of [2-2H]-L-DOPA in authentic (protonated) incubation medium. Strongly documented previous studies proved that the decarboxylation of $\alpha$-L-amino acids occurs with retention of configuration at the $\alpha$-carbon atom [10, 27]. Thus, enzymatic decarboxylation of the native L-DOPA carried out in fully deuteriated incubation medium leads to incorporation of deuterium from solvent entirely in the $(1R)$-position. According to this rule, the decarboxylation of [2-2H]-L-DOPA in protonated medium yields [$(1S)$-2H]-DA as deuterium at $C_6$ retains the configuration in the resulting dopamine. The intermediate [2-2H]-L-DOPA [20] needed for synthesis of [$(1S)$-2H]-DA was prepared according to the scheme presented in Fig. 4.

**Kinetic assays**

The non competitive spectroscopic method was used to study the H/D kinetic and solvent isotope effects in the enzymatic oxidation of DA to DOPAL (Fig. 1). The kinetic assays were carried out in Mc Ilvaine’s buffers (protonated and deuteriated, pH 5.6 and pD 6, respectively). Deuteriated Mc Ilvain’s buffer was prepared as described in point 1 (calculated fraction of $H^+$/D+ ions was equal to 0.0025) and was adjusted to pD 6 due to higher pK(D2O) [11].

The measured absorbance values were used to calculate deuterium KIE’s and SIE’s on $V_{max}$ and $V_{max}/K_M$. Since the DA and the resulting DOPAL show the same absorbance in the UV-VIS region ($\lambda = 280$ nm), thus the reaction progress was measured indirectly by registering the growth of absorbance of quinoneimine dye at $\lambda = 570$ nm. For this purpose, the coupled reaction (Fig. 5) leading to the formation of red dye was used. The resulting H2O2, formed during oxidation of DA reacts with 3-(N-ethyl-3-methylanilino)-2-hydroxypropanesulfonic acid (TOOS) and 4-aminoantipyrine (4-AA) forming a color dye absorbing light at $\lambda = 570$ nm. This reaction is catalyzed by the enzyme peroxidase, type II from horseradish (EC 1.11.1.7), Fig. 5, [22].

The kinetic reaction parameters were calculated from the measured spectrophotometric data, which were then optimized to the Michaelis-Menten equation using the program Enzfitter 1.05. The values of KIE on $V_{max}$ and KIE on $V_{max}/K_M$ for enzymatic oxidation of DA are presented in Table 2. The experimental error

### Table 1. The composition of reaction mixtures in separate cuvettes

<table>
<thead>
<tr>
<th>Cuvette’s no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution “A” (μL)</td>
<td>133</td>
<td>133</td>
<td>133</td>
<td>133</td>
<td>133</td>
<td>133</td>
</tr>
<tr>
<td>Solution “B” (μL)</td>
<td>33</td>
<td>50</td>
<td>66</td>
<td>83</td>
<td>100</td>
<td>116</td>
</tr>
<tr>
<td>Mc Ilvaine’s buffer (μL)</td>
<td>326</td>
<td>309</td>
<td>293</td>
<td>276</td>
<td>259</td>
<td>243</td>
</tr>
<tr>
<td>Tyramine oxidase (μL)</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Fig. 4. Two step enzymatic synthesis of [2-2H]-L-DOPA.

Fig. 5. The coupled reaction used to spectrophotometric monitoring the progress of oxidation of DA to DOPAL.

### Table 2. KIE’s in enzymatic oxidation of DA to DOPAL

<table>
<thead>
<tr>
<th>Isotopologue</th>
<th>KIE on $V_{max}$</th>
<th>KIE on $V_{max}/K_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>[$(1S)$-2H]-dopamine</td>
<td>5.80 ± 0.40</td>
<td>5.66 ± 0.70</td>
</tr>
<tr>
<td>[$(1R)$-2H]-dopamine</td>
<td>1.35 ± 0.08</td>
<td>1.75 ± 0.27</td>
</tr>
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</table>
was calculated with Student’s t-distribution for 95% confidence interval.

SIE’s were calculated using the kinetic data obtained as a result of enzymatic oxidation of DA to DOPAL in protonated and fully deuteriated Mc Ilvaine’s buffers separately (Table 3). The kinetic experiments were carried out in the same way as when determining the KIE’s for this reaction.

### Conclusion

The developed method allowed to determine for the first time the deuterium KIE’s and SIE’s in the oxidation reaction of dopamine to 3',4'-dihydroxyphenylacetaldehyde, catalyzed by the enzyme tyramine oxidase from *Arthrobacter* sp. The comparison of the measured KIE on V max and KIE on V max/K M for [(1S)-H]-DA and for [(1R)-H]-DA isotopologues (Table 2), indicate that the enzyme catalyzes the stereospecific proton abstraction from the pro-S position at the α-carbon atom of dopamine, and that this proton separation process occurs in the reaction rate determining step [18]. The values of deuterium KIE on V max = 1.35 and KIE on V max/K M = 1.75 for the pro-1R position are typical for secondary isotope effects, whereas the small values of SIE’s indicate that the solvent has little effect into the conversion of “enzyme-substrate” complex into the “enzyme-product” complex [21].

### Acknowledgment

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### References