NUKLEONIKA 2013;58(2):255–259

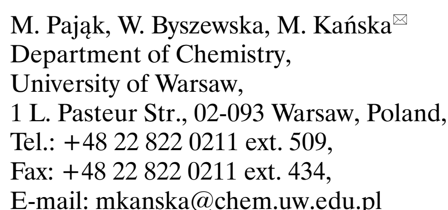
ORIGINAL PAPER

Deuterium isotope effects in oxidation of dopamine by tyramine oxidase

Małgorzata Pająk, Wioleta Byszewska, Marianna Kańska

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., [(1*R*)-^{2}H]-, and [(1*S*)-^{2}H]-DA were used. The numerical values of KIE's for the (1*R*)- and (1*S*)-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



M. Pająk, W. Byszewska, M. Kańska✉
Department of Chemistry,
University of Warsaw,
1 L. Pasteur Str., 02-093 Warsaw, Poland,
Tel.: +48 22 822 0211 ext. 509,
Fax: +48 22 822 0211 ext. 434,
E-mail: mkanska@chem.uw.edu.pl

Received: 28 November 2012
Accepted: 16 January 2013

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 quinines 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].

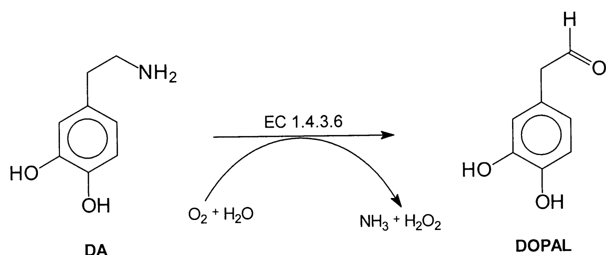


Fig. 1. The oxidation of DA to DOPAL catalyzed by the enzyme tyramine oxidase.

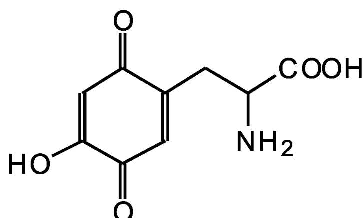


Fig. 2. Structure of topa quinone (TPQ).

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

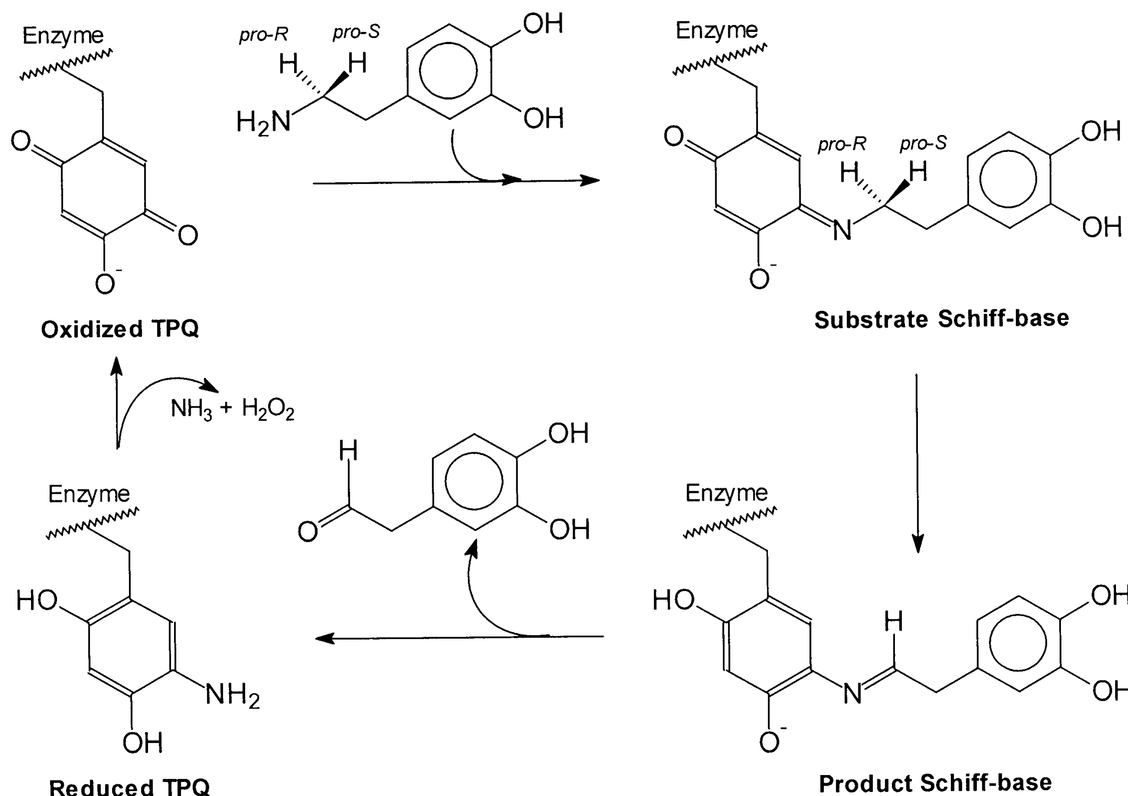


Fig. 3. Assumed mechanism of action of tyramine oxidase.

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/D_2O and 85% D_3PO_4/D_2O 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. Amberlite IRC-50 resin was obtained from Serva. Silica gel plates (silica gel 60 F_{254}) and aluminum oxide plates (aluminum oxide 60 F_{254}, neutral, type E) were obtained from Merck. 3-(N-ethyl-3-methylanilino)-2--hydroxypropanesulphonic acid sodium salt (TOOS), 4-aminoantipyrine (4-AA), L-DOPA, L-tyrosine, dopamine hydrochloride, and other chemicals needed for syntheses were from Sigma.

Methods

The extent of deuterium incorporation into 2-position of [2-^{2}H]-L-DOPA, the 1-positions of [(1*R*)-^{2}H]-, and [(1*S*)-^{2}H]-DA were determined from ^{1}H NMR spectra. The progress of all enzymatic reactions was monitored spectrophotometrically using Shimadzu UV-1202 spectrometer. Additionally, the presence of DA and L-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 acetonitryl: water (4:1, v/v) developing solvent were used. Visualization was obtained by UV lamp.

Synthesis

1. Synthesis of [(1*R*)-^{2}H]-DA. To encapped vial containing 20 mL of fully deuteriated 0.1 M phosphate buffer at pD 5.9 30 mg (0.15 mmol) of L-DOPA, 2.5 mL of 1 mM PLP/D_2O 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 by lyophilization to about 2 mL, and loaded on an Amberlite IRC-50 column (10 × 100 mm) previously equilibrated to pH 6.5 with 0.1 M KH_2PO_4. Unreacted L-DOPA was washed off with 0.1 M KH_2PO_4, pH 6.5, and the product was eluted with 0.5 M HCl. In each fraction the presence of deuteriated DA was checked by TLC. The fractions containing dopamine were combined and lyophilized. The residue was extracted with 15 mL of n-butanol, which in turn was lyophilized leaving 23 mg (0.12 mmol) [(1*R*)-^{2}H]-DA·HCl (80% yield). The extent of deuterium incorporation (near 100%) was shown by ^{1}H NMR spectrum (200 MHz, D_2O, TMS). The signals from α- and β-protons (δ 2.867 for 2βH, d and 3.192 for 1αH) are different from the signals from α- and β-protons of native DA (δ = 2.848 for 2βH, t and 3.199 for 2αH, t).
2. Synthesis of [(1*S*)-^{2}H]-DA. This isotopologue of dopamine was obtained as a result of three step reaction described earlier [20]. A 30 mg (0.16 mmol) sample of L-tyrosine, L-Tyr, was dissolved in fully deuteriated buffer and deuterium label was introduced at 2-position in the presence of enzyme tryptophanase (EC 4.1.99.1). The resulted [2-^{2}H]-L-Tyr was hydroxylated to [2-^{2}H]-L-DOPA using the activity of enzyme tyrosinase (EC 1.14.18.1). In the last step deuteriated [2-^{2}H]-L-DOPA was converted to [(1*S*)-^{2}H]-DA by the enzymatic decarboxylation according to procedure described in point 1 (Synthesis of [(1*R*)-^{2}H]-DA). In this case, L-DOPA decarboxylation was carried out in an authentic (not deuteriated) incubation medium. As a result, 8 mg (0.04 mmol) of (1*S*)-isotopologue was obtained. The overall chemical yield of this three step reaction was equal to ca. 25%. The incorporation of deuterium at 2-positions of intermediates: [2-^{2}H]--L-Tyr, [2-^{2}H]-L-DOPA, and at 1-position of the final product, [(1*S*)-^{2}H]-DA·HCl, was determined by the changes of signals in ^{1}H NMR spectra.

Kinetic assay

To study the isotopic effects, the native DA and the previously synthesized isotopologues [(1*R*)-^{2}H]-and [(1*S*)-^{2}H]-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 Na_2HPO_4 were mixed with 14.75 mL of 0.1 M citric acid. The desired pH 5.6 was adjusted by adding 0.2 M Na_2HPO_4 and 0.1 M citric acid. In parallel, fully deuteriated Mc Ilvaine's buffer was prepared by dissolving appropriate quantities of Na_2HPO_4 and citric acid in heavy water and adjusted to pD 6 using 0.2 M D_3PO_4/D_2O and 0.1 M citric acid/D_2O 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", pD 6.
3. Solutions "B". 1 mM solution of native dopamine, [(1*R*)-^{2}H]-, and [(1*S*)-^{2}H]-DA in Mc Ilvaine's protonated or fully deuteriated buffers, pH 5.6 and pD 6, respectively, used to appropriates kinetics runs.
4. Solutions of enzyme tyramine oxidase (5 U/mL) in Mc Ilvaine's protonated or fully deuteriated buffers, pH 5.6 and pD 6, respectively.

Each kinetic run consists of six measurements carried out in disposable 550 μL plastic spectroscopic 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 0.3 U (units), respectively. The progress of DA oxidation was registered spectrophotometrically by measuring the

Table 1. The composition of reaction mixtures in separate cuvettes

Cuvette's no.	1	2	3	4	5	6
Solution "A" (μL)	133	133	133	133	133	133
Solution "B" (μL)	33	50	66	83	100	116
Mc Ilvaine's buffer (μL)	326	309	293	276	259	243
Tyramine oxidase (μL)	8	8	8	8	8	8

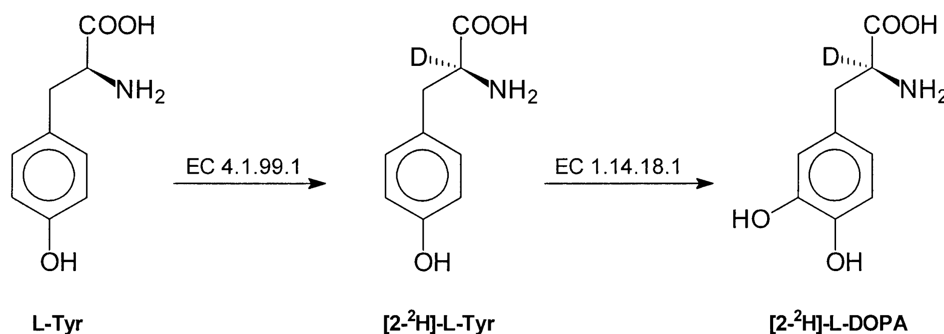


Fig. 4. Two step enzymatic synthesis of [2-^{2}H]-L-DOPA.

increase in absorbance of quinoneimine dye formed at λ = 570 nm at 1 min intervals for 60 min.

Result and discussion

Synthesis

The isotopologues [(1*R*)-^{2}H]-, and [(1*S*)-^{2}H]-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. [(1*R*)-^{2}H]-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 (1*S*)-isotopologue was prepared by enzymatic decarboxylation of [2-^{2}H]-L-DOPA in authentic (protonated) incubation medium. Strongly documented previous studies proved that the decarboxylation of α-L-amino acids occurs with retention of configuration at the α-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 (1*R*)-position. According to this rule, the decarboxylation of [2-^{2}H]-L-DOPA in protonated medium yields [(1*S*)-^{2}H]-DA as deuterium at C_α retains the configuration in the resulting dopamine. The intermediate [2-^{2}H]-L-DOPA [20] needed for synthesis of [(1*S*)-^{2}H]-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 $pK(D_2O)$ [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 (λ = 280 nm), thus the reaction progress was measured indirectly by registering the growth of absorbance of quinoneimine dye at λ = 570 nm. For this purpose, the coupled reaction (Fig. 5) leading to the formation of red dye was used. The resulting H_2O_2, 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 λ = 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

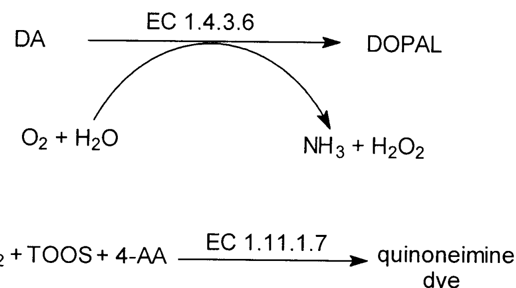


Fig. 5. The coupled reaction used to spectrophotometric monitoring the progress of oxidation of DA to DOPAL.

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

Isotopologue	KIE on V_{max}	KIE on V_{max}/K_M
[(1*S*)-^{2}H]-dopamine	5.80 ± 0.40	5.66 ± 0.70
[(1*R*)-^{2}H]-dopamine	1.35 ± 0.08	1.75 ± 0.27

Table 3. SIE's in enzymatic oxidation of DA to DOPAL

Isotopologue	SIE on V_{max}	SIE on V_{max}/K_M
Dopamine	1.7 ± 0.03	1.26 ± 0.16

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 [(1*S*)-^{2}H]-DA and for [(1*R*)-^{2}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*-1*R* position are typical for secondary isotope effects, whereas the small values of SIE's indicate that the solvent has little effect on the conversion of "enzyme-substrate" complex into the "enzyme-product" complex [21].

Acknowledgment. This work was supported by grant BST 501/86-DSM-100100.

References

1. Asico L, Zhang X, Jiang J *et al.* (2011) Lack of renal dopamine D_5 receptors promotes hypertension. J Am Soc Nephrol 22:82–89
2. Brown DE, McGuirl MA, Dooley DM, Janes SM, Mu D, Klinman JP (1991) The organic functional group in copper--containing amine oxidase. J Biol Chem 266:4049–4051
3. Burke WJ, Li SW, Chung HD *et al.* (2004) Neurotoxicity of MAO metabolites of catecholamine neurotransmitters: role in neurodegenerative diseases. Neurotoxicology 25:101–115
4. Burke WJ, Li SW, Williams EA, Nonneman R, Zahm DS (2003) 3,4-Dihydroxyphenylacetaldehyde is the toxic dopamine metabolite in vivo: implications for Parkinson's disease pathogenesis. Brain Res 989:205–213
5. Chinta SJ, Andersen JK (2005) Dopaminergic neurons. Int J Biochem Cell Biol 37:942–946
6. Coleman AA, Hindsgaul O, Palcic MM (1989) Stereochemistry of copper amine oxidase reactions. J Biol Chem 264:19500–19505
7. Coleman AA, Scaman CH, Kang YJ, Palcic MM (1991) Stereochemical trends in copper amine oxidase reaction. J Biol Chem 266:6795–6800
8. De Biasi M, Dani JA (2011) Reward, addiction, withdrawal to nicotine. Annu Rev Neurosci 34:105–130
9. Drożak J, Bryła J (2005) Dopamine: not just a neurotransmitter. Postępy Higieny i Medycyny Doświadczalnej 59:405–420 (in Polish)
10. Dunathan HC (1971) Stereochemical aspects of pyridoxal phosphate catalysis. Adv Enzymol Relat Areas Mol Biol 35:79–134
11. Gary R, Bates RG, Robinson RA (1964) Second dissociation constant of deuteriophosphoric acid in deuterium oxide from 5 to 50°. Standardization of a pD scale. J Phys Chem 68:3806–3809
12. Klinman JP (1996) New quinocofactors in eukaryotes. J Biol Chem 271:27189–27192
13. Lamensdorf I, Eisenhofer G, Harvey-White J, Nechustan A, Kirk K, Kopin IJ (2000) 3,4-Dihydroxyphenylacetaldehyde potentiates the toxic effects of metabolic stress in PC12 cells. Brain Res 868:191–201
14. Li SW, Lin TS, Minteer S, Burke WJ (2001) 3,4-Dihydroxyphenylacetaldehyde and hydrogen peroxide generate a hydroxyl radical: possible role in Parkinson's disease pathogenesis. Mol Brain Res 93:1–7
15. Matsuzaki R, Suzuki S, Yamaguchi K, Fukui T, Tanizawa K (1995) Spectroscopic studies on the mechanism of the topa quinine generation in bacterial monoamine oxidase. Biochemistry 34:4524–4530
16. Mattammal MB, Haring JH, Chung HD, Raghu G, Strong R (1995) An endogenous dopaminergic neurotoxin: implication for Parkinson's disease. Neurodegeneration 4:271–281
17. Medina MA, Urdiales JL, Rodriguez-Caso C, Ramirez FJ, Sanchez-Jimenez F (2003) Biogenic amines and polyamines: similar biochemistry for different physiological missions and biomedical applications. Crit Rev Biochem Mol Biol 38:23–59
18. Northrop DB (1975) Steady-state analysis of kinetic isotope effects in enzymic reactions. Biochemistry 14:2644–2651
19. Pająk M, Kańska M (2006) Synthesis of isotopomers of dopamine labeled with deuterium or tritium. J Labelled Compd Radiopharm 49:1061–1067
20. Pająk M, Kańska M (2009) Synthesis of isotopomers of L-DOPA and dopamine labeled with hydrogen isotopes in the side chain. J Radioanal Nucl Chem 281:365–370
21. Schowen RL (1972) Mechanistic deductions from solvent isotope effects. Progr Phys Org Chem 9:275–332
22. Shimizu H, Taniguchi K, Sugiyama M, Kanno T (1990) Rapid enzymatic analysis of plasma for tyrosine. Clin Chem 36:32–35
23. Siever LJ, Davis KL (2004) The pathophysiology of schizophrenia disorders: perspectives from the spectrum. Am J Psych 161:398–413
24. Summers MC, Markovic R, Klinman JP (1979) Stereochemistry and kinetic isotope effects in bovine plasma amine oxidase catalyzed oxidation of dopamine. Biochemistry 18:1969–1979
25. Thomas B, Beal MF (2007) Parkinson's disease. Hum Mol Genet 16:R183–R194
26. Uchida M, Ohtani A, Kohyama N, Okajima T, Tanizawa K, Yamamoto Y (2003) Stereochemistry of 2-phenylethylamine oxidation catalyzed by bacterial copper amine oxidase. Biosci Biotechnol Biochem 67:2664–2667
27. Vederas JC, Reingold ID, Sellers HW (1979) Stereospecificity of sodium borohydride reduction of tyrosine decarboxylase from *Streptococcus faecalis*. J Biol Chem 254:5053–5057
28. Wouters J, Perpete P, Hayen P, Anceau N, Durant F (1994) Kinetic characterization of tyramine oxidase of Arthrobacter species. Biochem Mol Biol Int 32:737–743
29. Yu PH (1988) Three types of stereospecificity and the kinetic deuterium isotope effect in the oxidative deamination of dopamine as catalyzed by different amine oxidases. Biochem Cell Biol 66:853–861