NUKLEONIKA 2013;58(4):475–479

ORIGINAL PAPER

Deuterium isotope effects in oxidation of dopamine and norepinephrine catalyzed by horseradish peroxidase

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Abstract. Deuterium kinetic isotope effects (KIE's) in the reaction of oxidation of dopamine (DA) catalyzed by the enzyme horseradish peroxidase (HRP, EC 1.11.1.7) was determined using a non-competitive spectrophotometric method. For kinetic studies, the deuterium ring labelled isotopomer [2',5',6'-$^{2}H_3$]-dopamine was synthesized using acid catalyzed isotopic exchange between authentic dopamine and heavy water. Deuterium solvent isotope effects (SIE's) for dopamine and norepinephrine were determined separately for the enzymatic oxidation carried out in the presence or absence of hydrogen peroxide. Some mechanistic details of enzymatic oxidation of dopamine and norepinephrine to corresponding catecholchromes catalyzed by HRP were discussed.

Key words: deuterium • dopamine (DA) • norepinephrine • isotope effects • oxidation • peroxidase

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Received: 7 February 2013
Accepted: 19 September 2013

Introduction

Catecholamines, such as dopamine (DA) and norepinephrine (NE) found in the nervous system of mammals [14] are the major neurotransmitters and hormones. They are responsible for the regulation of physiological processes related with mood, memory or stress [6, 17]. Disturbance in the production and metabolism of DA and its derivatives leads to development of many pathologies, including neurological disorders, such as Alzheimer's or Parkinson's diseases [12, 16] and schizophrenia [8, 23]. Increased development of such diseases is observed in patients exposed to the prolonged oxidative stress. The oxidation reactions of catecholamines can be stimulated by both chemical or enzymatic agents [13] and lead to the formation of several toxic molecules, such as free radicals, reactive quinones or neurotoxic aminochromes. These species can react with proteins producing covalent modifications in their structure resulted in an impairment of protein physiological functions and disturbance in the normal redox state of cells [4].

The mechanisms of oxidation reactions of compounds with catechol moiety are complex and the knowledge of their early stages remains quite limited. When catalyzed by monoamine oxidase, MAO (EC 1.4.3.4) and aldehyde dehydrogenase, DH (EC 1.2.1.3) [5, 7, 10] they can lead to low or non-toxic metabolites, including aldehydes and carboxylic acids. On the other hand, it is possible that autooxidation processes of catecholamines form neuromelanins (NM) Fig. 1.

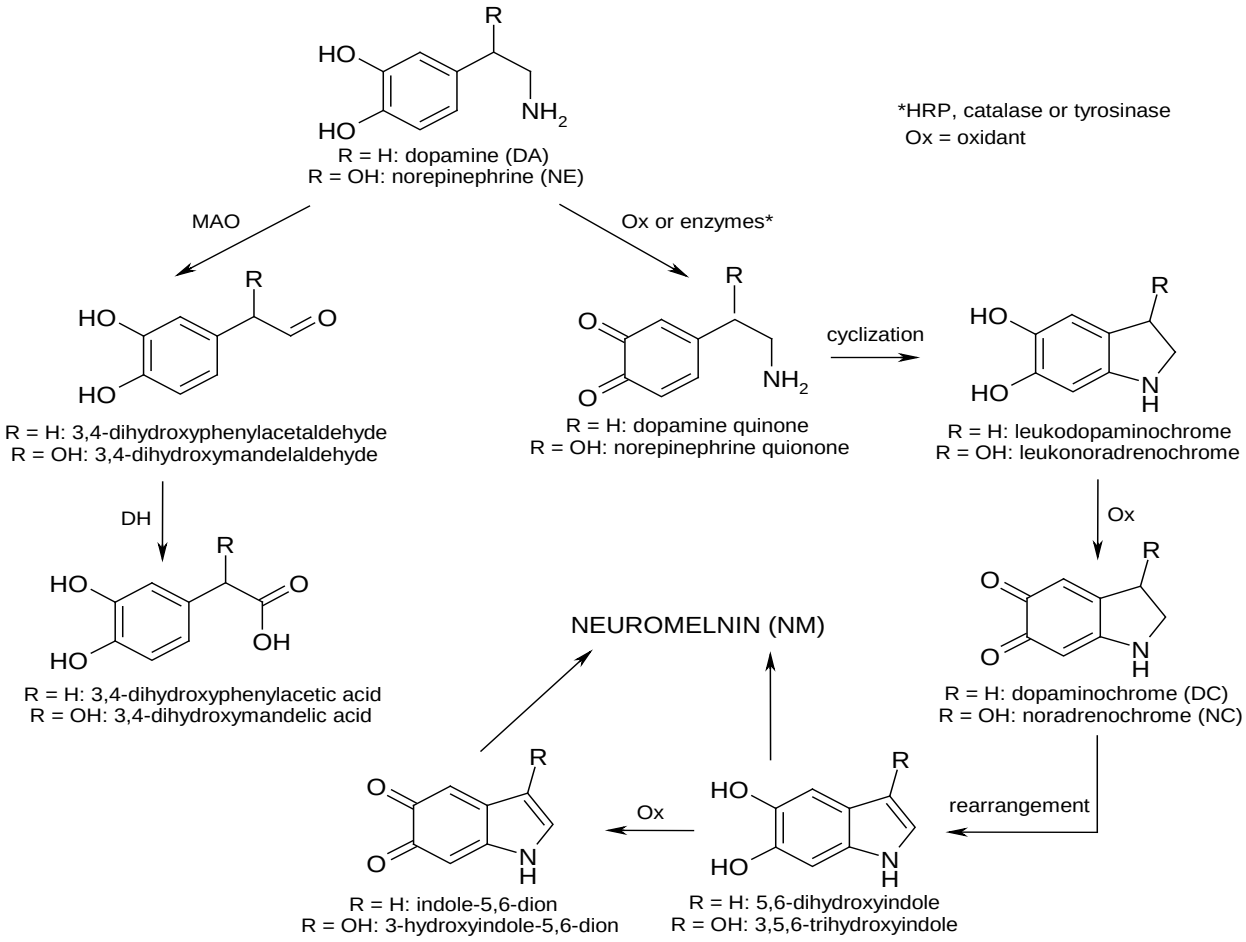

Fig. 1. Oxidation processes of dopamine and norepinephrine.

It is supposed that autooxidation involves the formation of intermediate quinones and subsequent their cyclization to aminochromes followed by formation of unstable leucoaminochromes and polymerization to NM [4, 13]. In the cyclization step of these following reactions, the 6-position of the quinone ring is attacked by the nitrogen atom via 1,4-intramolecular Michael's addition reaction [3, 14].

According to one of the hypotheses, the increased level of adrenochrome enhances the symptoms of schizophrenia [8, 9], and the decreased DA level in brain is a potential cause of Parkinson's and Alzheimer's diseases [4]. Therefore, it is important to understand the kinetics of stages of catecholamines oxidation leading to formation of aminochromes.

The aim of our studies was the kinetic analysis of oxidation of DA and NE to dopaminochrome (DC) and noradrenochrome (NC) catalyzed by enzyme peroxidase isolated from a root of horseradish plant (HRP, EC 1.11.1.7). This enzyme belongs to the class iron-containing oxidases with heme as a prosthetic group at its active site. The activity of HRP is based on the formation of complex with hydrogen peroxide, effective species for oxidizing several substances as phenols or nitrates [2] but the mechanisms of these actions have not been clearly established yet.

To investigate the above reactions the kinetic (KIE) and solvent isotope effect (SIE) methods were used [19, 22]. Determination of numerical values of SIE's and KIE's can provide relevant information about the mechanism of enzyme-catalyzed oxidation of the catecholamines studied.

Experimental

Materials

Solutions of 37% DCl/D_2O, 30% NaOD/D_2O and 83% D_3PO_4/D_2O were obtained from POLATOM, Poland. Deuteriated water (99.9% D) was from Aldrich. Aluminum oxide plates (aluminum oxide 60 F_{254}, neutral, type E) was from Merck. DL-Norepinephrine hydrochloride was purchased from Aldrich. Dopamine hydrochloride, enzyme peroxidase, type II (EC 1.11.1.7) from horseradish, HRP, and other chemicals needed for syntheses and trial experiments were from Sigma.

Methods

The ^1H NMR spectra were recorded in D_2O using tetramethylsilane (TMS) as internal standard on a Varian 500 MHz Unity-Plus spectrometer. The kinetic assays were performed using a UV/VIS Lambda 25 spectrometer (Perkin Elmer Precisely).

Synthesis

Synthesis of [2',5',6'-$^2H_3$]-DA·DCl. A sample of 40 mg (0.22 mmol) dopamine hydrochloride dissolved in a 4 mL solution of 6 M DCl/D_2O was placed in a glass ampoule, frozen with liquid nitrogen, outgased, and sealed. The exchanging mixture was kept at 50°C for 24 h. After this time, the solvent was lyophilized to dryness. The residue was dissolved in 1 mL of water, loaded on an Amberlite IRC 50 H^+ column (100 × 10 mm), and washed with water to remove deuterium atoms incorporated into labile positions (-OH and -NH_2 groups) of dopamine. Next, the product was eluted with 2 M DCl/D_2O and evaporated to dryness under reduced pressure. As a result, 39.5 mg (0.215 mmol) of [2',5',6'-$^2H_3$]-DA·DCl was obtained (near 100% chemical yield). The purity of product was checked by TLC (developing solvent – n-butanol:acetic acid:water; 4:1:2; v/v/v). The extent of deuterium incorporation close to 100% in the 2',5' and 6'-ring positions of dopamine hydrochloride obtained was determined by ^1H NMR spectrum. ^1H-Chemical shifts for DA; relative to TMS in D_2O, 500 MHz: δ (ppm): 2.859 ($2H_\beta$), 3.209 ($1H_\alpha$) and 6.739, 6.829, 6.886 ($3H_{ring}$). The peaks for three protons in the aromatic ring in DA disappeared after deuterium exchange [20].

Kinetic

To study isotopic effects in the enzymatic oxidation the native DA and NE, as well as, isotopomer [2',5',6'-$^2H_3$]-DA·DCl were used. The kinetic experiments were carried out using the following solutions:

a) 50 mM phosphate buffer, pH 7.0;
b) 30 mM solutions of catecholamines in distilled water;
c) 9.8 M hydrogen peroxide;
d) peroxidase (type II (EC 1.11.1.7) from horseradish, HRP) solution in 50 mM phosphate buffer (200 U/mL activity).

For studying kinetics in deuteriated incubation media, the fully deuteriated 50 mM phosphate buffer corrected to pD 7.4 [11] or heavy water, were used to solubilize all reagents and enzymes.

Each kinetic run was consisted of six measurements carried out in 1 mL quartz spectroscopic cuvettes containing solutions with six different concentrations of catecholamines. To these cuvettes exact volumes of solutions listed in points (a–d) were added to reach the concentrations of each catecholamine in the range between 0.09–0.39 mM with 0.06 mM intervals. In each cuvette, the concentration of enzyme and H_2O_2 were constant (0.05 U/mL and 9.8 mM, respectively). The progress of oxidation reaction was registered spectrophotometrically by measuring the increase of absorbance of aminochromes formed at λ = 480 nm, 1 min intervals for 20 min at 37°C.

Result and discussion

Synthesis

The deuterium ring labelled isotopomer [2',5',6'-$^2H_3$]-DA·DCl, was obtained using slightly modified acid-catalyzed isotopic exchange between DA and heavy water described earlier, Fig. 2, [20].

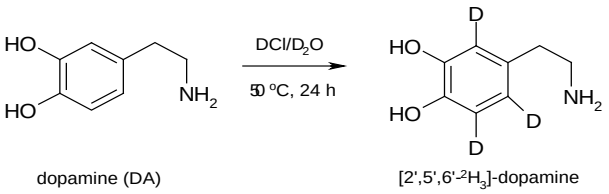

Fig. 2. Isotopic H/D exchange into dopamine ring.

The synthesis was carried out in heavy water acidified with deuterochloric acid under anaerobic conditions at 50°C for 24 h. The degree of deuterium incorporation in the three positions in the ring was determined by ^1H NMR spectroscopy and was close to 100% after 22.5 h. The incorporation of deuterium takes place only into the aromatic positions of DA and the rates of H/D exchange were practically the same for three ring protons.

Kinetic assays

The non-competitive spectrophotometric method was used to determine the KIE for [2',5',6'-$^2H_3$]-DA and SIE's separately for DA or NE in the oxidation of catecholamines to DC and NC catalyzed by horseradish peroxidase, HRP (Fig. 3).

The kinetics assays were carried out in phosphate buffers (protonated and deuteriated, pH 7 and pD 7.4, respectively) at 37°C for 20 min.

The measured absorbance values of dopaminochrome and noradrenochrome were used to calculate the reaction rates using the Michaelis-Menten equation [18]

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

The maximal reaction rates, V_{max}, and Michaelis-Menton constants, K_m are optimized by the Lineweaver-Burk plot (or double reciprocal plot) [15].

$$\frac{1}{V} = \frac{K_m + [S]}{V_{max}[S]} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \quad (2)$$

where V is the reaction rate, K_m is the Michaelis-Menten constant, V_{max} is the maximum reaction rate, and S is the substrate concentration.

The values of deuterium KIE's for [2',5',6'-$^2H_3$]-DA were calculated from the ratio of the V_{max} or V_{max}/K_m using the kinetic data obtained as a result of enzymatic oxidation of labelled and unlabelled DA separately to DC in protonated or fully deuteriated buffers. SIE's for DA and NE were calculated in a similar way as the KIE's, using the ratio of the V_{max} and V_{max}/K_m obtained

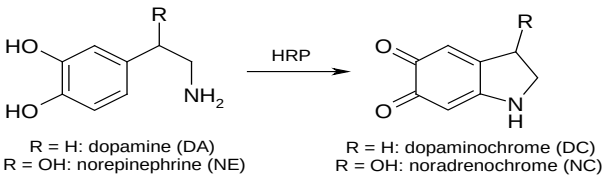

Fig. 3. Oxidation of dopamine and norepinephrine catalyzed by HRP.

Table 1. Deuterium KIE's and SIE's for oxidation of dopamine and norepinephrine catalyzed by HRP

Isotopologe	KIE on V_{max}	KIE on V_{max}/K_m	SIE on V_{max}	SIE on V_{max}/K_m
[2',5',6'-$^2H_3$]-DA in protonated buffer	1.30 ± 0.2	1.11 ± 0.07	–	–
[2',5',6'-$^2H_3$]-DA in deuteriated buffer	1.15 ± 0.05	1.05 ± 0.15	–	–
[2',5',6'-$^2H_3$]-DA	–	–	2.70 ± 0.2	3.20 ± 0.1
DA	–	–	3.10 ± 0.3	3.40 ± 0.4
NE with H_2O_2	–	–	2.03 ± 0.26	1.38 ± 0.05
NE without H_2O_2	–	–	3.90 ± 0.5	3.80 ± 0.3

from the kinetic parameters for oxidation of unlabelled catecholamines carried out in fully deuteriated or protonated buffers. The values of SIE's and KIE's for enzymatic oxidation of formation of DA and NE are presented in Table 1.

According to earlier reports, the oxidation reactions of compounds which catechol moiety catalyzed by HRP can be carried out without the addition of hydrogen peroxide to incubation media [1, 13]. Unlike NE, the progress of the enzymatic reaction in the absence of hydrogen peroxide for DA was not observed. Therefore, the kinetic assays for DA were carried out only with H_2O_2 present in incubation medium and the reactions for NE were studied both in the presence or absence of H_2O_2. The formation of NC under strong oxidizing conditions using hydrogen peroxide and HRP, proceeds much more dramatically than using only HRP. Nevertheless, the numerical values of SIE determined in medium not containing H_2O_2 are higher than for the reaction carried out with the addition of hydrogen peroxide to the incubation media. Thus, it was proposed that during the oxidation of the catechol moiety hydrogen peroxide is generated endogenously and used as a catalyst for HRP.

The method allows the determination for the first time of the deuterium SIE's and KIE's in the oxidation of DA and NE catalyzed by HRP and provides information about the mechanism of this reactions.

The values we observed of SIE's on $V_{max} = 3.9 \pm 0.5$ and on $V_{max}/K_m = 3.8 \pm 0.3$ for the oxidation reaction of NE without H_2O_2 in the incubation medium suggest that the protons used to the formation of endogenous hydrogen peroxide were derived from the medium. On the other hand, in the presence of exogenous H_2O_2, the lower observed values of SIE's (2.03 ± 0.26 and 1.38 ± 0.05, respectively) are due to the competition between endo- or exogenous peroxide randomly used by HRP. Furthermore, the calculated values of SIE's for both DA and NE indicate that the solvent plays an important role during the conversion of "enzyme-substrate" complex to "enzyme-product" complex [22].

The values of deuterium KIE's on V_{max} and V_{max}/K_m close to unity (no isotope effect) in the studied reaction for [2',5',6'-$^2H_3$]-DA are typical of secondary isotope effects and indicate that the deuterium substitution at the 2'-, 5'- and 6'-ring positions of DA only slightly affects the conversion of "enzyme-substrate" complex to "enzyme-product" complex [21].

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

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