Biochimica et Biophysica Acta 1784 (2008) 1687–1693

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Oxidative metabolism of dopamine: A colour reaction from human midbrain analysed by mass spectrometry

Angela De Iuliis [a,*], Giorgio Arrigoni [b], Liselotte Andersson [b], Pamela Zambenedetti [c], Alessandro Burlina [d], Peter James [b], Paola Arslan [a], Fabio Vianello [e]

[a] Department of Medical Sciences, University of Padua, Padova, Italy
[b] Department of Protein Technology, University of Lund, Lund, Sweden
[c] Pathology Division and Brain Bank, General Hospital of Dolo-Venice, Italy
[d] Neurological Unit, San Bassiano Hospital, Bassano del Grappa, Italy
[e] Department of Biological Chemistry, University of Padua, Padova, Italy

ARTICLE INFO

Article history:
Received 14 March 2008
Received in revised form 2 July 2008
Accepted 3 July 2008
Available online 15 July 2008

Keywords:
Dopamine
Dopaminochrome
Dopamine peroxidizing activity
Human brain
Parkinson's disease
Mass spectrometry

ABSTRACT

In order to identify the protein responsible for a dopamine peroxidizing activity, previously described in human normal and parkinsonian substantia nigra by our group, we developed non-denaturing polyacrylamide gel electrophoresis conditions, mimicking the characteristic colour *in vitro* reaction, resulting from cyclic oxidation of dopamine (DA). After separating protein mixtures from human normal midbrain homogenates on two sets of identical native gels, one gel set was subjected to specific activity staining by using DA and hydrogen peroxide. An activity red/orange band appeared in midbrain tissue lanes, similarly to the lane where commercial horseradish peroxidase (HRP) was present as control of peroxidative activity. The second set of gels, stained with Coomassie Blue, showed other, not enzymatically active protein bands. Mass spectrometry analysis of the bands containing the activity and the corresponding Coomassie Blue bands revealed the presence of proteins that may play a role in neurodegenerative disease, highlighting a possible functional link among dopamine/dopaminochrome redox cycle and protein metabolism.

1. Introduction

Over the past years, the role of dopamine metabolites has been focus of neurodegeneration studies in Parkinson's disease (PD). A characteristic autoptic finding of this disease is a depigmented substantia nigra with loss of dopaminergic neuromelanin-containing neurons and post-mortem studies have consistently indicated oxidative damage in PD pathogenesis [1,2]. The primary cause of oxidative stress has not been clarified but the leading candidate is the metabolism of DA itself, since it gives rise to various toxic species. Reactive products of partially reduced oxygen, such as superoxide, hydrogen peroxide, and hydroxyl radical are generated from monoamine oxidase (MAO)-deamination, during normal catabolism of DA, and also from catechol oxidation, during oxidative pathways in which DA can serve as substrate for neuromelanin synthesis [3]. The pathway of the reactions converting dopamine to neuromelanin is not yet well understood, but it is thought to proceed through DA oxidation to dopamine *o*-quinone, cyclization of dopamine *o*-quinone to dopaminochrome, leading to the formation of leukoaminochrome, oxidation of leukoaminochrome to dopaminochrome and polymerization of dopaminochrome to neuromelanin (Fig. 1) [4,5]. The DA oxidation can occur spontaneously [3], is accelerated by transition metal ions (Mn^{2+} or Fe^{2+}) [6], or can be catalyzed by a number of different enzymes [7–11]. The quinone consequently produced has an electron-deficient ring, which readily forms covalent bonds with available nucleophiles [12], especially the cellular important sulphydryl groups, such as L-cysteine, glutathione, and proteins containing cysteinyl residues [13]. Several *in vitro* studies have demonstrated the binding of quinone formed by DA to protein sulphydryl groups, leading to inactivation of enzymes of vital importance for the cell function, and such reactions have been implicated in the neurodegeneration process of catecholaminergic neurons [14–17]. The *in vivo* occurrence of dopamine oxidation has been demonstrated by the recovery of dopamine adducts in human and in other mammalian brain extracts [18–22]. However, in the absence of competing nucleophiles, the amine side chain of DA is readily available for 1,4-intramolecular ring closure and oxidation, forming relatively more stable dopaminochrome (2,3-1H-indole-5,6-quinone), the potential precursor of neuromelanin [12]. Dopaminochrome is a member of the family of red to violet coloured indoline-5,6-quinones, known as aminochromes, which are readily obtained on oxidation of the corresponding catecholamines [23]. Occurrence in the brain of dopaminochrome comes from the fact that this metabolite was identified as part of neuromelanin, along with 5,6-dihydroxyindole, a rearrangement product of dopaminochrome itself

* Corresponding author. Tel.: +39 49 827 6143; fax: +39 49 807 3310.
E-mail address: angela.deiuliis@unipd.it (A. De Iuliis).

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doi:10.1016/j.bbapap.2008.07.002

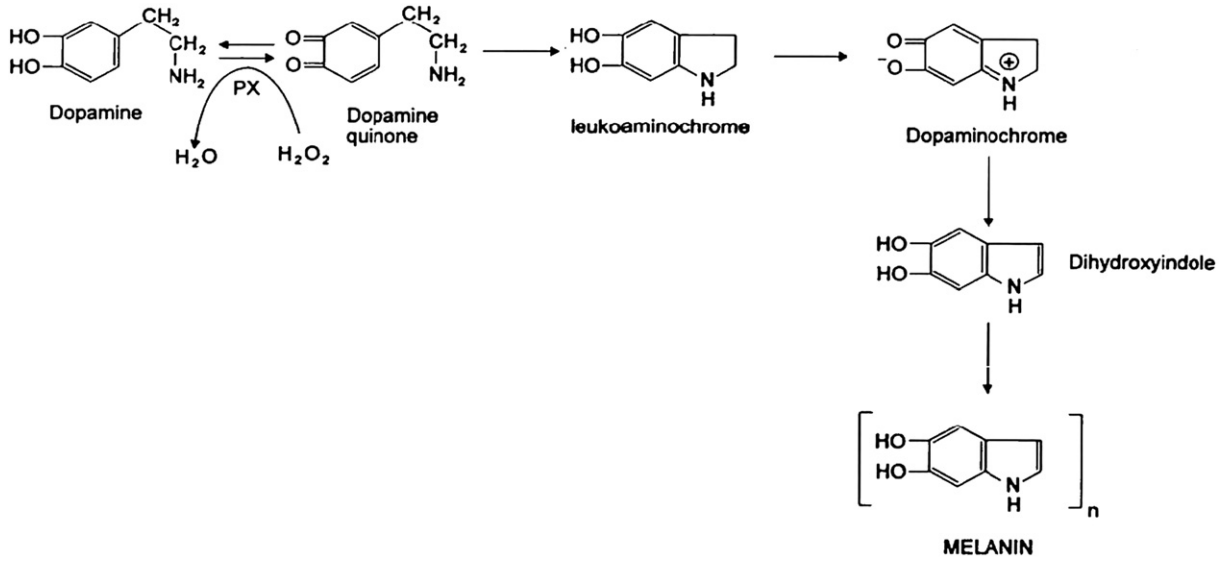

Fig. 1. Neuromelanin formation. The reaction pathway converting dopamine to neuromelanin is a normal process in the substantia nigra, which it is thought to involve several steps: dopamine oxidation to dopamine *o*-quinone catalyzed by metals, oxygen, peroxynitrite or peroxidative activity of several enzymes (PX), such as prostaglandin H synthase, cytochrome P450 1A2, xantine oxidase, cyclization of dopamine *o*-quinone to dopaminochrome via an addition, at physiological pH values, leading to the formation of unstable leukoaminochrome and oxidation of leukoaminochrome to dopaminochrome and polymerization of dopaminochrome to neuromelanin, via dihydroxyindole formation (see the text).

and noradrenochrome [24–26]. Hastings [8] described dopaminochrome *in vitro* formation from Prostaglandin H synthase (PHS)-mediated dopamine oxidation, while Mattamal showed the *in vivo* reaction, by a mass spectrometry study [9]. Among possible other candidates able to oxidize DA to dopaminochrome, cytochrome P450 1A2 [10], xanthine oxidase [11] and peroxynitrite [27,28] were reported, but to date, none of them has been identified as specifically responsible for such reaction in nigral dopaminergic neurons.

We have previously provided spectrophotometric evidence of the presence of an enzymatic activity in human substantia nigra [29]. This activity, catalyzing the formation of dopaminochrome from dopamine and hydrogen peroxide, was first demonstrated in rat brain fraction [30]. We subsequently observed increased dopamine peroxidation in the midbrain and basal ganglia of Parkinsonian brain, obtained at autopsy [31].

The colour reactions resulting from the cyclic oxidation of catecholamines formed the basis of qualitative and quantitative assay procedures [23]. In an attempt to identify the protein responsible for dopamine peroxidizing activity in substantia nigra, we have analysed the characteristic, coloured reaction forming the dopaminochrome by using a proteomic approach.

We report here the specific staining procedure, developed for in gel-detection of human DA peroxidizing activity, after electrophoresis of midbrain tissue homogenates, and the mass spectrometry analysis, which revealed the presence of proteins in the activity bands.

2. Materials and methods

2.1. Chemicals and instruments

Dopamine, hydrogen peroxide, reagents used for homogenate buffer, and horseradish peroxidase (TYPE VI-A, P6783) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Acrylamide, TEMED, ammonium persulfate, Coomassie Brilliant Blue G-250 as well as all the reagents and apparatus for gel electrophoresis were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

Spectrophometric measurements were performed at 25 °C, with a Beckman DU-640 recording instruments equipped an Epson FX-850 recorder. Brain homogenates were obtained by a Beckman, J2-21 model fixed angle rotor, centrifuge.

Mass spectra were acquired with a Q-TOF Ultima mass spectrometer (Waters Manchester, UK).

2.2. Sample preparation

Human normal midbrain tissues from four different specimens were obtained from the Brain Bank of the General Hospital of Dolo (Venice, Italy). Each specimen was accompanied by a histopathological report, certifying they were not affected by neurodegenerative diseases, neither brain vasculopathy involving mesencephalic regions (sex: 3 males, 1 female, aged 75±5 years). The autopsies were performed within 24 h of death. Midbrain has been excised from thawed autoptic specimens, using ceramic tools to avoid metal contamination of the samples. When present, blood clots were removed before sample processing.

Brain specimens (1–3 g) were homogenized and the supernatant was spectrophotometrically tested for the enzymatic activity, as described [31]. Protein content was estimated by Bio-Rad assay.

2.3. Gel electrophoresis

Two identical sets of three non-denaturing polyacrylamide gel (3,5% acrylamide stacking gel and 7% separating gel) were performed in Bio-Rad Mini Protean II cell (1 mm thickness, 10 cm × 10 cm gel size). Ten microliters of homogenates from midbrain tissues, corresponding to 10 μg of total proteins, was loaded onto each gel, along with 2 ml (2 μg) of horseradish peroxidase (HRP), used, without further purification, as control of peroxidative activity.

Electrophoresis was carried out with a current of 10 mA/gel at room temperature, one extra hour after the dye front had migrated to the end of the gel.

2.4. Peroxidative activity and protein staining

After electrophoresis, one gel set was soaked in substrate solution (0. 2 M sodium phosphate buffer, pH 7.4, containing 2 mM dopamine and 30 mM hydrogen peroxide), with gentle shaking at room temperature, until an orange activity band appeared.