Dopamine β-hydroxylase inactivation generates a protein-bound quinone derivative

Patrick Slama, Frédéric Jabre, Thierry Tron, Marius Réglie\*rt*

Chimie, Biologie et Radicaux libres, UMR-CNRS 6517, Faculté des Sciences et Techniques de St-Jérôme, case 432, Av. Escadrille Normandie-Nièmen, 13397 Marseille Cedex 20, France

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Abstract Bovine dopamine β-hydroxylase (DbH) was inactivated by hydrogen peroxide and ascorbate in the presence of dioxygen. Both inactivated forms of the enzyme were investigated. We could highlight the presence of a quinone derivative bound to the protein, assumed as being dopa-quinone, that is absent from active enzyme. Such results suggest that a tyrosinyl radical transiently forms during catalysis. Moreover we could show that addition of substrate tyramine to H\textsubscript{2}O\textsubscript{2} incubates is responsible for a partial protection of DbH against inactivation.

Key words: Dopamine β-hydroxylase; Tyrosine oxidation; Redox cycling staining; Dopa-quinone

1. Introduction

Dopamine β-hydroxylase (DbH) is the enzyme catalysing the conversion of dopamine to norepinephrine, two important neurotransmitters involved in the central nervous system [1]. This enzyme has been isolated from bovine chromaffin granules as a tetramer of 290 kDa [2], and exists as both membrane-associated and soluble forms. It is a copper protein [2] that contains two Type II copper atoms per active site, which both are Cu(II) in the resting state. No crystallographic data are available to date concerning DbH. However some indications about its structure have been given by EXAFS measurements [3], and by the examination of the recent publication of peptidyl glycine \(\alpha\)-amidating monoxygenase hydroxylating domain (PHM) structure [4]. PHM, which was shown to possess mechanistic similarities with DbH [5,6] and a sequence resembling that of DbH [7], contains two copper atoms 11 Å distant from each other, separated by a solvent pocket. In DbH, one copper (Cu\textsubscript{A}) is bound to three histidine and a water molecule, while the other (Cu\textsubscript{B}) is bound to two histidine residues and one additional methionine residue in the reduced form [3]. No magnetic coupling exists between the two copper ions, suggesting a distance more than 6 Å [8]. This would exclude the possibility of a transient Cu\textsubscript{B}-bis(\(\mu\)-oxo) active species, which is observed in various model compounds [9].

During catalysis, DbH copper atoms cycle between Cu(II) and Cu(I), as shown by EPR measurements [10]. A commonly used reducing cofactor is ascorbate, also present in chromaffin granules, and suggested to be a cofactor of DbH in vivo. Oxygen is the second cofactor for DbH, which accepts dopamine, tyramine or 2-phenyl-ethyamine as substrates. As early as 1961, Levin and Kaufman showed that incubation of the enzyme with either reducer ascorbate in the presence of air or oxidant H\textsubscript{2}O\textsubscript{2} was responsible for a loss of catalytic activity [11]. Kinetic measurements on the influence of inactivating species concentration onto activity have later been realised by Skotland and Ljones [12]. Up to now, no mechanistic study was able to provide an explanation for such losses of activity.

Reduction of the two copper atoms results in the activation of dioxygen by DbH and the generation of a peroxy-copper intermediate at Cu\textsubscript{B}, which undergoes a cleavage of the oxygen-oxygen bond [13]. As a next step, a hydrogen atom is abstracted from the substrate, before incorporation of the oxygen atom from the oxo-copper radical. The species responsible for this abstraction is not identified to date. Moreover it is not clear yet whether catalysis requires two copper atoms [14] or only one [15]. A tyrosinyl radical was proposed by Kliman and co-workers to form transiently during catalysis [13] (Scheme 1A). Therefore we decided to investigate the degradation of a tyrosine residue following inactivation by ascorbate/oxygen or H\textsubscript{2}O\textsubscript{2}.

In this article, we characterise the inactivated form of the enzyme produced by reaction with H\textsubscript{2}O\textsubscript{2} or ascorbate/oxygen. We show that inactivation of DbH by hydrogen peroxide is partially prevented by substrate tyramine. Using visible spectroscopy and the quinone staining method by Paz et al. [16], we prove that inactivated DbH contains a protein-bound quinone derivative.

2. Materials and methods

2.1. Materials

Tyramine hydrochloride, nitroblue tetrazolium chloride (NBT) and phosphate potassium salts were from Aldrich. Sodium ascorbate was from Fluka, hydrogen peroxide from Normapur\textsuperscript{9}. Bovine liver catalase and bovine plasma amine oxidase (Cu-AO) were purchased from Sigma.

2.2. Protein purification

Bovine adrenal medullae DbH was purified according to the method by Ljones [17], except for the ConA-Sepharose that was replaced by a phenyl-Sepharose affinity column (1×10 cm, Pharmacia, Uppsala, Sweden).

2.3. Enzyme activity assay

DbH activity was measured following the decrease in oxygen con-
centration in the enzyme containing buffer. Inactivation by hydrogen peroxide was performed by mixing 200 μl of DbH (51 μg) with 0.5 mM H$_2$O$_2$ and null or 4 mM tyramine in 50 mM phosphate buffer, pH 6.0, and stirring at 25°C. At different times, 50 μl aliquots were removed, mixed with catalase (800 U in 50 mM phosphate buffer, pH 6.0) and stirred for 12 more min. The aliquot was then added to a 50 mM phosphate buffer, pH 6.0 solution, containing 10 mM tyramine and 5 mM sodium ascorbate. Dissolved oxygen consumption was followed using a Strathkelvin Instruments (Glasgow, UK) Oxygen Monitor, model 781, at a fixed temperature of 25°C. Inactivation experiments were carried out on two different enzyme preparations, producing three similar sets of data for each experiment.

When measuring inactivation by ascorbate and dioxygen, aliquots were directly taken out of a mixture containing an identical amount of DbH as that used with H$_2$O$_2$ and 0.25 mM ascorbate in 50 mM phosphate buffer, pH 6.0, and tested for activity in the same conditions as described above.

2.4. Preparation of Apo-DbH

Active DbH was diluted in a 50 mM phosphate buffer at pH 6.0, containing EDTA at 1 mM and incubated for 5 min at 4°C. EDTA was then removed by ultrafiltration on a 10 kDa membrane in a microconcentrator (microcon$^\text{0}$, Amicon, Inc., Beverly, MA, USA) and washing twice with phosphate buffer, once with a 0.5 M NaCl solution, and twice more with phosphate buffer.

2.5. Quinone staining

Protein samples of either active or inactivated DbH were submitted to SDS-PAGE and transferred onto a nitrocellulose membrane (Protran$^\text{0}$, Schleicher and Schuell). The membrane was then revealed for quinoproteins, using the method previously described by Paz et al. [16]. Bovine monoamine oxidase was used as a positive reference, 12 μg being used per lane. Active DbH was introduced in identical amounts as treated enzyme. Inactivated DbH samples were prepared as following.

2.5.1. Inactivation by hydrogen peroxide. DbH (7.7 μg) was incubated with 1.6 mM H$_2$O$_2$ at pH 6.0 for 17 min at 20°C. Catalase (900 U) was added, and the solution was stirred for 10 more minutes.

2.5.2. Inactivation by ascorbate. DbH (16 μg) was incubated for 20 min at 20°C in a 50 mM phosphate solution buffered at pH 6.0, in the presence of 0.5 mM ascorbate. Nitrocellulose membranes were incubated once or twice in an NBT-glycinate buffer at pH 10, depending on the intensity of the formazan stains.

2.6. UV–visible spectroscopy

DbH (300 μl, 0.3 mg) was incubated for 10 min with 0.5 mM ascorbate at 25°C. Inactivated enzyme was then denatured by adding 2 μl dithiothreitol at 1 M and heating at 65°C for 90 s. Near UV–visible spectrum was recorded using a Uvikon 943 Spectrophotometer (Kontron Instruments, St-Quentin-en-Yvelines, France). Active enzyme was similarly denatured right after introduction of ascorbate, and both spectra were compared.

3. Results

3.1. Kinetics of the inactivation by hydrogen peroxide and protection by substrate

Enzyme activity was monitored as dissolved oxygen consumption. Incubation with H$_2$O$_2$ was responsible for a loss of activity (Fig. 1). These results are similar to those obtained by Skotland and Ljones in different buffer conditions [12].

Addition to the incubation buffer of substrate tyramine at 4 mM partly protected the enzyme from inactivation (Fig. 1). Plots of remaining activity vs incubation time revealed an exponential dependence on incubation time over a time period of about 30 min. In the presence of 4 mM tyramine, inactivation was 54% of that observed with 0.5 mM H$_2$O$_2$ only.

Incubation of DbH with 250 μM ascorbate at pH 6.0 led to an even faster decrease in catalytic activity than that observed with 500 μM H$_2$O$_2$ (Fig. 1).

![Scheme 1. Proposed mechanisms for DbH at copper B. Substrate transformation (A) and enzyme inactivation (B).](image-url)

Fig. 1. DbH residual activity after incubation with 0.5 mM H$_2$O$_2$ (open circles), 0.5 mM H$_2$O$_2$ plus 4 mM tyramine (full circles), 0.25 mM ascorbate (full squares). Reference is the activity of the enzyme sample prior to any incubation.
3.2. Formation of a quinone derivative by inactivation

3.2.1. Ascorbate. The method described by Paz et al. for the detection of the topa-quinone (TPQ) cofactor in amine oxidase [16] was applied to DbH. After incubation in the presence of ascorbate on native and demetallated DbH, protein solutions were submitted to SDS-PAGE. Ascorbate-inactivated DbH loaded on the gel showed 10% residual activity as compared to the enzyme prior to incubation (data not shown). Apo-enzyme showed no detectable activity. NBT colouring showed a formazan stain for inactivated DbH, that was absent from non-inactivated DbH and Apo-DbH samples (Fig. 2). Monoamine oxidase was used as a positive reference during NBT colouration. The experiment was repeated five times, producing identical results.

3.2.2. Hydrogen peroxide. We submitted H$_2$O$_2$-inactivated DbH samples to the quinone-staining method. A formazan stain was present for DbH (Fig. 3). The membrane also showed a formazan stain for catalase in all experiments. This stain was attributed to a modification of iron tyrosine ligand after inactivation by H$_2$O$_2$. A blank experiment with catalase only incubated with 1.6 mM H$_2$O$_2$ for 10 min was performed, where this stain was still observed. Formazan stains for DbH were obtained in a reproducible way (experiments repeated five times).

3.3. UV–visible spectroscopy

Near UV–visible spectra of ascorbate-inactivated and active DbH were measured, inactivated enzyme showing about 15% of its initial activity. We were able to observe a peak centred at about 408 nm. This peak was absent from the spectrum of active DbH (Fig. 4). The presence of this absorption peak could reflect the presence of an oxidised form of a tyrosine residue.

4. Discussion

NBT redox cycling staining was performed on ascorbate or hydrogen peroxide-inactivated DbH. This method proved that a protein-bound quinone derivative is present in inactivated enzyme. Our study and that describing this method [16] do not reveal any quinonoid component bound to active DbH. Therefore, the quinone derivative is generated during inactivation of the enzyme. Moreover, the absence of any NBT reduction by Apo-DbH demonstrates that copper is essential to the formation of this derivative. The presence of an oxidised phenolic side-chain is a proof for an oxidising attack on a tyrosine residue during DbH inactivation, thus suggesting the formation of a tyrosinyl radical during catalysis. Tyr residues have been shown to intervene as radical intermediates in other metalloenzymes, such as RNR [18]. The nature of this oxidised species deserves discussion. Indeed, oxidation of a tyrosine residue can either generate a dopa-quinone (DPQ) or a TPQ, after insertion of one or two oxygen atoms on its side-chain. A protein-bound quinone has already been observed in other copper containing proteins. In copper monoamine oxidase, a carbonyl cofactor was evidenced, which was first proposed as a pyrroquinolinequinone [19] and later identified as a TPQ [20]. It was later established that this essential cofactor was generated through a copper involving redox process [21]. The presence of this modified residue can be let out both by visible absorption spectroscopy and using the NBT redox cycling test [16]. In that paper, it is proved that the intensity of the blue colouration depends on the reducing quinone compound tested. For instance, DPQ has to be about 10 times as concentrated as TPQ to reduce identical amounts of NBT to formazan. In our redox cycling assays, we always
obtained less intense formazan stains for DbH than for Cu-AO, despite using comparable amounts of proteins (220 vs 140 pmol of monomers). Such results suggest that the protein-bound quinone in inactivated DbH is not a TPQ but a DPQ. On the other hand, direct spectroscopic detection of the oxidised tyrosine residue in inactivated DbH discloses an absorption band centred at 408 nm, as opposed to the broad 474 nm band from MAO TPQ [22]. Our value is rather close to that observed for free quinones, such as that produced by the oxidation of di-tert-butyl-catechol [23]. On grounds of both redox cycling and visible spectroscopy results, we therefore propose that inactivation of DbH by ascorbate/oxygen or hydrogen peroxide generates a DPQ species that would be located nearby the active site copper responsible for dioxygen binding.

We propose two different pathways for the production of this species: either that described by Dooley [24], where the phenol ring is first activated by binding of its oxygen to copper, or one where a copper-peroxo species would directly react with an active site tyrosine residue (Scheme 1B). This second pathway would exhibit differences between inactivation by ascorbate and inactivation by H2O2. Generation of a copper-peroxo species by ascorbate would start with the reduction of Cu(II) to Cu(I) by ascorbate, this species then reacting with oxygen. On the other hand, production of such an adduct caused by H2O2 would imply direct reaction between peroxide and the active site copper. We believe that the less intense formazan stains obtained with H2O2 could either prove that DPQ formation occurs through various pathways, or that H2O2 is the cause for further degradations of the quinone, for example to muconic acid [25].

DbH activity was followed by oxymetry, and we showed that tyramine was responsible for a partial protection from H2O2-mediated inactivation. Since a peroxo-copper species could form, samples of DbH incubated with H2O2 and tyramine were probed for activity by HPLC, but octopamine could only be detected in very small amounts under such conditions, and its formation could not be correlated to H2O2 and tyramine concentrations (data not shown). The substrate role in the protection of the enzyme can be explained in various ways: first, it could be thought as a purely physical role. Indeed, if H2O2 and substrate bind the enzyme at a same site, assumed as being CuA, they could compete for this site. Introduction of tyramine to the inactivation incubate should thus cause hydrogen peroxide to be partly excluded from this site, and therefore decrease its inactivating capacity. Second, recognition of substrate tyramine by the enzyme could induce structural modifications in its tertiary structure that would prevent any attack by hydrogen peroxide. Protection of catalytic activity by substrate tyramine, at about a 50% rate, gives us some hints about the way H2O2 induces inactivation of the protein. Indeed, substrate tyramine is only recognised by CuA, and H2O2 is not specific regarding copper. Therefore we believe that introduction of tyramine in the incubation buffer prior to activity measurement protects CuA from an attack by hydrogen peroxide, while CuA or any residue in its environment still is available for oxidation, thus producing an enzyme that exhibits partial activity. In the presence of hydrogen peroxide only, some residues in the CuB environment are also likely to be modified under the action of H2O2. For instance, it was observed using model copper complexes that a copper-bound thioether group could be oxidised into a sulfoxide after introduction of H2O2 [26]. The methionine residue located near CuB could undergo such a modification in our inactivation experiments. Thus, inactivation by H2O2 could occur through various mechanisms, while inactivation by ascorbate would only lead to DPQ formation.

We showed that substrate tyramine is able to protect DbH from H2O2-mediated inactivation. DbH inactivation by either H2O2 or ascorbate/dioxygen produced a protein-bound quinone derivative, which formation was also evidenced on ascorbate-inactivated DbH by UV-visible spectroscopy. This derivative is proposed to be DPQ. Its formation suggests the involvement of a tyrosine residue during catalysis.

References


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