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Dopamine β-hydroxylase from bovine adrenal medulla contains covalently-bound pyrroloquinoline quinone

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Treatment of homogeneous dopamine β-hydroxylase (DBH) preparations from bovine adrenals with the inhibitor phenylhydrazine (PH) changed the structureless absorption spectrum of DBH into spectra with a maximum at 350 nm. A product with this absorption spectrum could be detached with pronase, enabling its isolation. It appeared to be the C(5) hydrazone of pyrroloquinoline quinone (PQQ) and PH, as judged from its properties and the fact that it could be transformed into PQQ itself. From the yield obtained a ratio of 0.85 PQQ per enzyme subunit was calculated. In contrast to copper-quinoprotein amine oxidases (EC 1.4.3.6), hydrazone formation in DBH did not require saturation of the mixture with O_2. DBH is the first copper-quinoprotein hydroxylase found so far. The implications of this finding for the current views on mechanism of action and inhibition by hydrazines are discussed. The success of the recently developed 'hydrazine method' [(1987) FEBS Lett. 221, 299–304] for all different types of amine oxidoreductases, suggest that the method could also be applied to other enzymes for which hydrazines are inhibitors and where the identity of the cofactors has not been established or the presence of PQQ is suspected.

Dopamine β-hydroxylase; Pyrroloquinoline quinone; Hydrazine; Cofactor; Quinoprotein; Copper-containing enzyme

1. INTRODUCTION

Dopamine β-hydroxylase (DBH, EC 1.14.17.1) has been classified as a copper-protein monooxygenase which catalyzes the hydroxylation of dopamine to noradrenaline:

$$\text{dopamine} + O_2 + 2[H] \longrightarrow \text{noradrenaline} + H_2O$$

The kinetics of this enzyme have been intensively studied, leading to a scheme for the catalytic cycle in which the copper ions form part of the hydroxylating center [1]. However, the proposed mechanism is mainly based on this approach and the intermediates of the enzyme in the catalytic cycle have not been studied so well. For instance, the assumed reduction of Cu(II) to Cu(I) ions by ascorbic acid has been detected by ESR-spectroscopy but is not documented by changes in the absorption spectrum of the enzyme. Perhaps this one-sidedness has led to the general notion that the enzyme is a colorless protein (although the complete UV/VIS absorption spectrum has never been published) with copper ions as the only cofactor.

A similar situation exists with respect to the mechanism of inhibition of DBH: a lot of information has been obtained on the inhibitor specificity [2] but product analysis of the inhibited enzyme has scarcely been performed. For instance, the effect with hydrazines (irreversible inhibitors for DBH) has been ascribed to enzymic decomposition of these compounds, leading to the formation of a carbon-centered radical attacking the active site [2]. However, the putative product in the enzyme has not been characterized so that much uncertainty remains with respect to the proposed mechanism. In addition, although it is known that this type of inhibitor induces large spectral changes

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in inhibition-sensitive enzymes, this has not been documented for DBH.

During the past few years, a method has been developed to elucidate the nature of the covalently-bound organic cofactor in copper-containing amine oxidases (EC 1.4.3.6). The method is based on enzyme derivatization with suitable hydrazine inhibitors under specified conditions, detaching the product formed with protease, purifying the colored adduct, and comparing it with the corresponding model compound. Using this 'hydrazine method' the identity (and quantity) of the cofactor could be unambiguously established to be pyrroloquinoline quinone (PQQ) in several mammalian and microbial copper-containing amine oxidases [3–6], as well as in microbial methylamine dehydrogenase (EC 1.4.99.3) [7].

Since many other metal-containing enzymes have never been investigated for the presence of an organic cofactor (although participation of such a compound could explain anomalies in the proposed mechanisms in several cases) it could be reasoned that the presence of PQQ (at least 3 sites available for metal-ion binding) has been overlooked in these enzymes so that application of the hydrazine method should be tried. For these reasons and a possible relatedness to the copper-quinoprotein amine oxidases, DBH seemed an attractive candidate to test the suitability of the hydrazine method and to probe the versatility of PQQ as a cofactor.

2. MATERIALS AND METHODS

2.1. *Purification of DBH*

Since commercial DBH preparations appeared to be inhomogeneous, the following purification step was performed. 50 mg of lyophilized enzyme (Sigma) was dissolved in 2 ml of 0.1 M sodium phosphate, pH 7.0. The solution was injected (in portions) on an FPLC gel filtration column (Pharmacia Superose 12 HR 10/30) in 0.1 M sodium phosphate, pH 7.0, using a flow rate of 0.5 ml/min. The eluate was monitored with absorbance detection (Hewlett-Packard 1040A photodiode array detector) at 280 nm. Active fractions were pooled, and stored at −70°C.

Preparation of the copper-free enzyme was achieved as described [8].

2.2. *Adduct formation and characterization*

Derivatization of the cofactor in DBH with PH and isolation of the adduct was performed as described [7], with the following modifications: to a solution (2.5 ml) of DBH (4 mg) in 0.1 M sodium phosphate, pH 7.0, 2 μl of a 0.1 M solution of phenylhydrazine (PH) in conc. H_3PO_4/ethanol (1:1, v/v) [9] was added; the mixture was incubated at 40°C for 16 h; proteolysis was performed by addition of 2 mg of pronase E (Boehringer, Mannheim) and incubation at 40°C for 6 h. HPLC (on a 4 μm C_{18} RCM cartridge) of the adduct, its transformation into PQQ by dissolving in Me_2SO, the preparation of the model hydrazone from PQQ and PH, and the determination of PQQ concentrations were all performed as described [7].

2.3. *Enzyme assay*

A spectrophotometric assay, based on the tyramine-dependent oxidation of ferrocyanide to ferricyanide [10], was used to determine the enzymic activity of DBH under atmospheric conditions. Initial rates were derived from the increase in absorbance at 420 nm at 25°C. Reaction mixtures contained 10 mM 2-(*N*-morpholino)ethane sulfonic acid, 0.2 M sodium acetate, pH 6.0, 30 mM *N*-ethylmaleimide, 10 mM tyramine, and 1 mM $K_4Fe(CN)_6$. Activities were calculated using a molar extinction coefficient for $K_3Fe(CN)_6$ of 1000 $M^{-1} \cdot cm^{-1}$ [10]. The amount of purified DBH was calculated using the specific absorption coefficient determined by Skotland and Ljones [11] ($A_{280nm}^{1mg/ml} = 1.24$).

2.4. *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis in the absence and presence of SDS was performed on the Pharmacia Phastsystem equipment according to the instructions given by the manufacturer (Phastsystem separation technique file nos 120 and 110, respectively). Protein staining occurred with Phastgel Blue R (according to Phastsystem Development technique file no.200), while enzyme activity staining was carried out according to Ljones et al. [12].

3. RESULTS

Starting with a commercially available preparation, a single purification step was sufficient to provide an enzyme preparation with a specific activity of 5.4 μmol $[Fe(CN)_6]^{4-}$ oxidized $\cdot min^{-1} \cdot mg^{-1}$, comparable with the value (4.8 μmol $[Fe(CN)_6]^{4-}$ oxidized $\cdot min^{-1} \cdot mg^{-1}$) ascribed to a homogeneous DBH preparation by others [12]. The preparation appeared indeed to be homogeneous, as revealed by polyacrylamide gel electrophoresis in the absence and presence of SDS since no other bands were detectable. Therefore, this preparation was suited to investigate the presence of an organic cofactor.

As shown in fig.1, DBH is not a 'colorless protein' since substantial absorbance is observed above 300 nm, resulting in an, albeit structureless, absorption spectrum. Obviously, the copper ion is not the only species responsible for this since the copper-depleted enzyme still shows absorbance above 300 nm. Derivatization with PH led to a

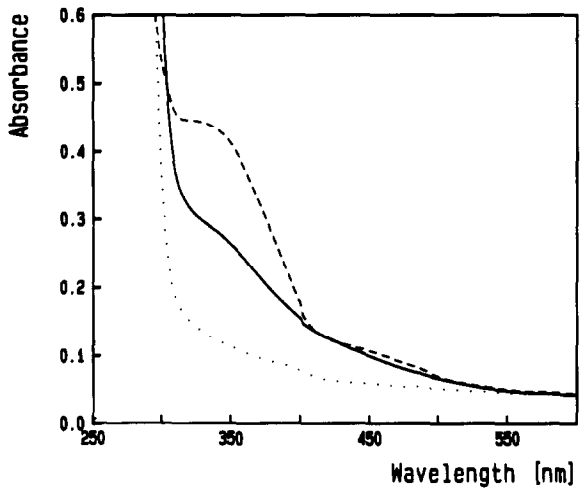


Fig.1. Absorption spectra of dopamine β-hydroxylase (1.6 mg/ml in 0.1 M sodium phosphate, pH 7.0) before (——) and after (– – –) derivatization with PH; and of the copper-depleted enzyme (···).

pronounced spectral change, inducing a maximum at 350 nm. The product having this spectrum was not released on acidification to pH 2.0, but it required prolonged incubation with pronase before adsorption took place to the Seppak cartridge (prior dissolution of the disulphide bonds appeared unnecessary). Comparison of the chromatographic and spectral properties of the adduct and the model compound (fig.2) showed that the adduct is the C(5) hydrazone of PQQ and PH. This was confirmed by conversion experiments in Me_2SO, showing that the product had a retention time and absorption spectrum identical to that of PQQ. Quantitative determinations of the PQQ formed indicated that the cofactor adduct was obtained in 85% yield (assuming that the transformation into PQQ occurred quantitatively and that DBH contains 1 PQQ per enzyme subunit). This is comparable to the finding of Fitzpatrick and Villafranca [13] who observed binding of 0.94 ^{14}C-labeled PH per subunit.

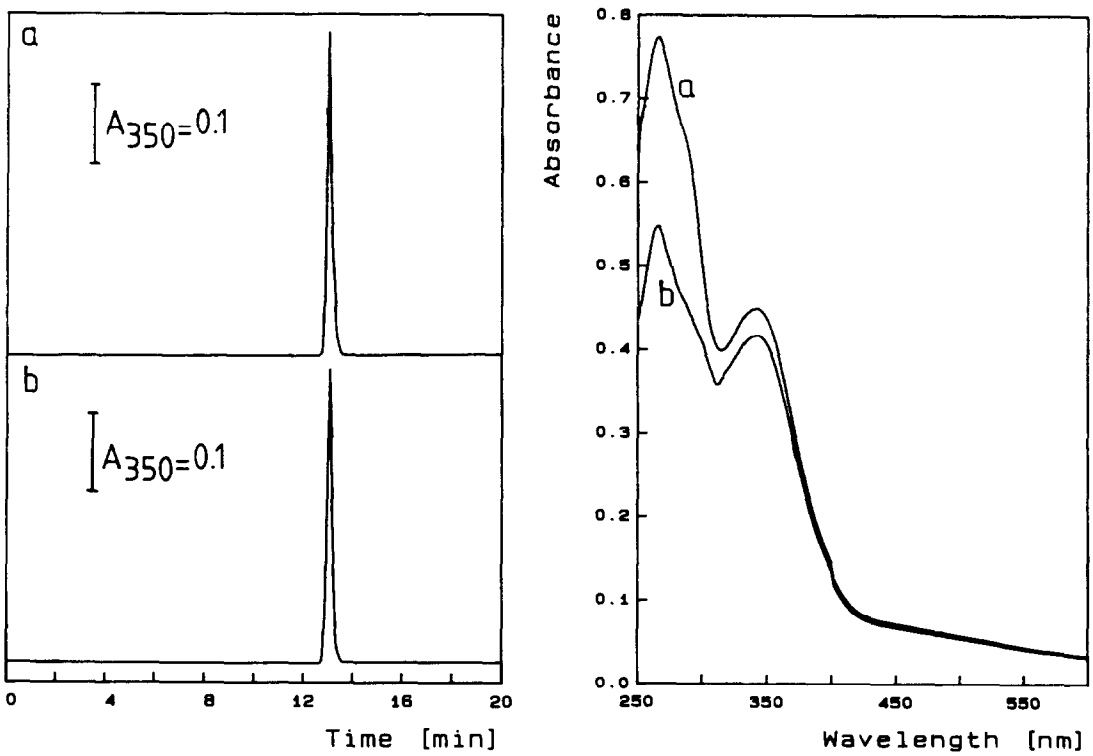


Fig.2. Chromatograms (left) and absorption spectra (right) of: a, adduct isolated from enzyme treated with PH for 16 h at 40°C; b, C(5) hydrazone of PQQ and PH. HPLC and photodiode array detection were performed as described in the text. Spectra were taken at the top of the peaks.

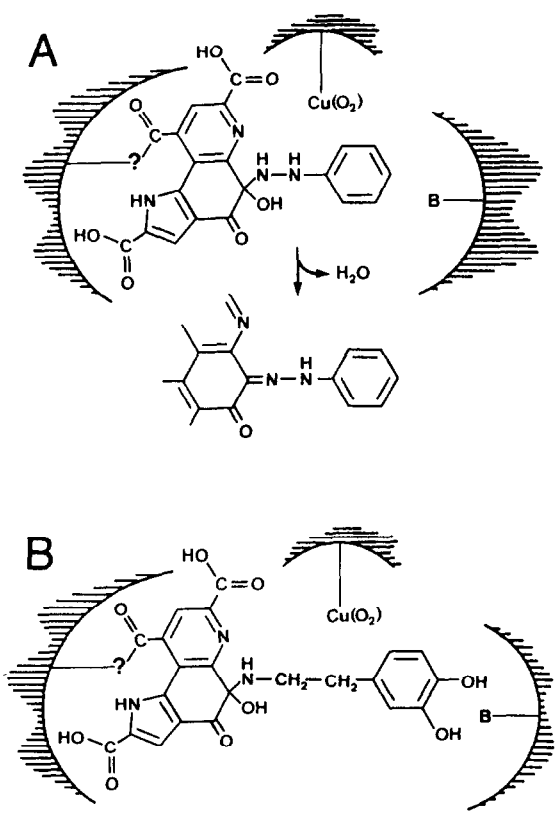
Although the enzyme became immediately inhibited after PH addition, derivatization of the cofactor to the hydrazone required long incubation. The hydrazone formation proceeded under atmospheric conditions and even under (semi) anaerobic conditions, this in contrast to copper-quinoprotein amine oxidases where azo adduct is formed at the very first moment and hydrazone adduct formation requires high O_2 concentrations. The presence of electron donor (ascorbate or ferrocyanide) prevented hydrazone formation in the enzyme but not in the model system. Derivatization of copper-depleted enzyme led to the hydrazone.

4. DISCUSSION

Derivatization of DBH with PH led to product formation, as is evident from the change in the absorption spectrum (fig.1). The product is the C(5) hydrazone of PQQ and PH and not the modified amino acid assumed by Fitzpatrick and Villafranca [13]. Therefore, the proposed mechanism for PH inhibition [2,13] should be reconsidered. Since product release required incubation with pronase, the hydrazone is most probably bound to the protein via a peptide bond, as in the case of copper-quinoprotein amine oxidases ([3–6]; Van der Meer, R.A., unpublished). Inhibition of DBH with PH has been reported by several other groups so that it is highly unlikely that the PQQ in this enzyme preparation was fortuitously bound. Although a role as allosteric modulator cannot be excluded at the moment, most probably PQQ functions as a cofactor in DBH, in accordance with the following observations: the inhibition of enzyme with PH and the nearly stoichiometric amounts (with respect to subunits) of hydrazone formed; the prevention of derivatization by the presence of electron donor; the unimpaired inhibition and derivatization with PH of copper-depleted enzyme.

The mechanism of action of DBH has been the subject of intensive research by a number of groups [1,10,13–15]. As a result of these efforts, considerable progress has been made in understanding the kinetic features of the enzyme-catalyzed reaction. In particular, compelling evidence has been presented for the participation of a substrate-derived benzylic radical intermediate. However, the nature of dioxygen activation by enzyme-bound copper is still unclear. In this respect, the requirement of two copper atoms per subunit of fully active enzyme has not been convincingly accommodated in any of the catalytic schemes presented so far. The present finding of PQQ as an integral part of DBH does not seem to relieve this ambiguity. This may also form an explanation for the fact that the presence of PQQ has been overlooked in the past.

On the basis of current knowledge it is not feasible to ascribe an unequivocal role to PQQ in the catalytic cycle of DBH. However, from the fact that PQQ derivatization by PH leads to irreversible inactivation of the enzyme, the conclusion may be drawn that PQQ is (at least partly) exposed in the active site. Although the enzyme shows a rather broad substrate specificity, the requirement for



Scheme 1. Binding of inhibitor and substrate in the active site. A possible binding mode of PH to PQQ, leading in a slow reaction to the hydrazone adduct, is depicted in A. An analogous arrangement is proposed for the substrate (B). The present data give no information on the possible participation of Cu(II) in the anchoring of substrate or inhibitor in the active site.

substrates containing a phenyl group seems absolute. Provided that this requirement is innate to a hydrophobic pocket in which either substrates or PH will be held in similar positions, it appears likely that the physiological, catecholamine, substrate(s) are oriented with the amino group in close proximity to the PQQ ortho-quinone moiety. Recalling the (in vitro) tendency of PQQ to form adducts with suitable nucleophiles at the 5-position [16], a redox-silent role for PQQ as a mere 'anchoring' point should be considered. A schematic topology for the active site with respect to this view is given in scheme 1.

However, the observation made by us and others that reduced enzyme does not incorporate PH, may well indicate that PQQ is redox-linked to the copper centers. Coupling of a copper center with an organic redox center on the enzyme has at one time been proposed to account for the properties of DBH, then thought to contain only one copper per enzyme subunit [15]. We feel that the discovery of PQQ in DBH warrants a reconsideration of this proposal. A possible cycle is depicted in scheme 2.

As a third possibility, present knowledge might be adequately represented by invoking two copper atoms spaced and redox-connected by a PQQ molecule. Experiments in which $PQQH_2$ and Cu(II) were mixed anaerobically showed the rapid oxidation of $PQQH_2$ to PQQ, indicating that an enzyme form with a Cu(I)/PQQ cluster might exist (Jongejan, J.A., unpublished). Such a situation might well account for the observed (indistinguishable) redox properties of the two copper atoms in each subunit, thus obviating the conceptual need for a closely spaced binuclear copper site.

Discrimination between the three mechanisms is not clearly possible at the moment and we are well aware of the fact that the proposed mechanisms are not mutually exclusive. DBH is the first copper-quinoprotein hydroxylase found so far, illustrating the versatility of PQQ. It is therefore a matter of course to apply the 'hydrazine method' to other enzymes for which the presence of PQQ is suspected.

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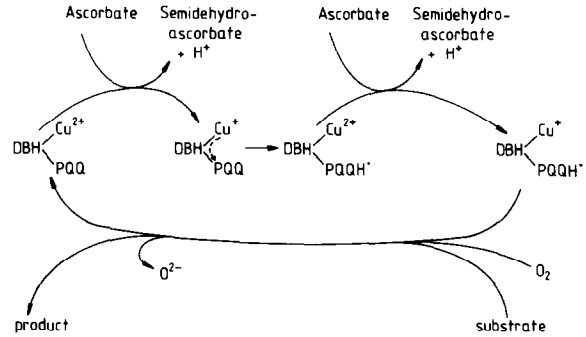

Scheme 2. The catalytic cycle of dopamine β-hydroxylase. In the mechanism proposed, enzyme is reduced by ascorbate in two steps (a generally accepted reduction mechanism for this enzyme) via bound Cu(II), leading to an enzyme form containing Cu(I) and $PQQH^{\cdot}$. This form reacts with O_2 and substrate, as indicated.

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