DOPAMINE β-HYDROXYLASE: EVIDENCE AGAINST A PING-PONG MECHANISM

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1. Introduction

The copper protein dopamine β-hydroxylase (dopamine β-mono-oxygenase; EC 1.14.17.1) catalyzes the hydroxylation of dopamine to noradrenaline and also the β-hydroxylation of similar substrates such as tyramine and phenylethylamine (for reviews see [1,2]). In this typical mono-oxygenase reaction ascorbate supports high rates of hydroxylation, but other electron donors can also function [3–5]. Based on experiments with stoichiometric amounts of enzyme and substrates, Friedman and Kaufman [4] proposed that each active site contains two copper atoms (Cu^{2+}) which accepts one electron each from ascorbate, followed by dissociation of dehydroascorbate before binding of O_2 and dopamine. Kinetic studies by Goldstein et al. [6] appeared to support this mechanism, as initial velocity patterns with variation of ascorbate concentrations at different levels of dopamine (or vice versa) were parallel in double-reciprocal plots and therefore consistent with a ping-pong mechanism. Similar results were later reported by Aunis et al. [7] with tyramine as the substrate. This ping-pong kinetic mechanism would seem to require that each active site accepts two electrons from the electron donor before binding of O_2.

Cleland [8] has pointed out that parallel kinetic patterns can also be consistent with certain types of sequential mechanisms, such as reactions involving large equilibrium constants. This possibility should be considered for dopamine β-hydroxylase catalysis, because many copper proteins have redox potentials that are much higher than for ascorbate [9]. The redox potential of the copper in dopamine β-hydroxylase has not been determined, but the present report shows that dopamine β-hydroxylase catalyzes high rates of hydroxylation of tyramine with $Fe(CN)_6^{4-}$ as electron donor. As the redox potential of the $Fe(CN)_6^{4-}$/$Fe(CN)_6^{3-}$ couple is approx. + 0.3 volt higher than the potential of the ascorbatedehydroascorbate couple at pH 7, the potential of the electron accepting group in this enzyme (presumably Cu^{2+}) is probably also higher than for ascorbate. Hence, the previously reported kinetic patterns may be consistent with a sequential mechanism as well as a ping-pong mechanism. In support of a sequential mechanism we report that kinetic patterns with $Fe(CN)_6^{4-}$ as electron donor are different from patterns with ascorbate and show variation in both ordinate intercepts and slopes of double-reciprocal plots. Mechanisms involving one copper atom per active site must therefore be considered possible.

2. Materials and methods

2.1. *Purification of dopamine β-hydroxylase from bovine adrenal glands*

Chromaffine granules were isolated from bovine adrenal medulla by the method of Helle et al. [10]. Dopamine β-hydroxylase was purified from the chromaffine granules by a method which was similar to the method of Foldes et al. [11]. The dialysis step was replaced with continuous ultrafiltration (diafiltration) through an Amicon Diaflow membrane XM-100. A linear salt gradient, 0–0.5 M NaCl, was used for chromatography on Whatman DE-52 DEAE-cellulose. The chromatography on Sephadex G-200 was omitted. After ion-exchange chromatography the dopamine β-hydroxylase preparation was concentrated by ultrafiltration with an Amicon PM-10 membrane and stored in liquid nitrogen.

2.2. *Analytical methods*

The rate of $Fe(CN)_6^{4-}$ oxidation was measured as an increase in absorbance at 420 nm with a Shimadzu model MPS-50L recording spectrophotometer. Rates were estimated from tangents to the initial parts of the curves. Concentration changes were calculated using the value 1000 M^{-1} cm^{-1} as the extinction coefficient of $Fe(CN)_6^{3-}$. Octopamine (*p*-hydroxyphenyl-ethanolamine) concentration was measured with the periodate oxidation assay as described by Wallace et al. [12], with octopamine from Sigma Chemical Co. as standard. With $Fe(CN)_6^{4-}$ as electron donor a correction for the absorbance of the oxidation product $Fe(CN)_6^{3-}$ at 330 nm was applied (assuming that 2 moles of $Fe(CN)_6^{3-}$ were formed per mole of octopamine, see Results). Protein was determined with the microbiuret method of Goa [13].

3. Results

3.1. *Hexacyanoferrate(II) ion as electron donor in the hydroxylation of tyramine*

Friedman and Kaufman [4] reported that $Fe(CN)_6^{4-}$ can function as electron donor in the dopamine β-hydroxylase catalyzed hydroxylation of tyramine, but they gave no values for reaction rates. We therefore compared rates of octopamine formation with $Fe(CN)_6^{4-}$ and with ascorbate as electron donors. Reaction mixtures contained in a volume of 1.0 ml: 10 mM tyramine, 10 mM fumarate, 50 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), 0.1 mg/ml catalase (Sigma Chemical Co., from beef liver, twice crystallized) and either 1 mM ascorbate or 1 mM $K_4Fe(CN)_6$; pH was 6.0 and temperature 25°C. The reactions were started by addition of purified dopamine β-hydroxylase (4.5 μg protein) and stopped with 0.2 ml concentrated aqueous ammonia after incubation for different times on a shaking water-bath. The specific activities were calculated from the linear parts of the time-courses. The values found were, in units of μmoles octopamine formed per min per mg protein, with 1 mM ascorbate: 1.13 and with 1 mM $K_4Fe(CN)_6$: 1.54.

The conditions used were not optimal for ascorbate supported hydroxylation, and for comparison, the specific activity of the dopamine β-hydroxylase preparation was determined with the assay conditions described by Wallace et al. [12] i.e. 20 mM tyramine, 10 mM fumarate, 200 mM acetate, 10 mM ascorbate, pH 5.0, and 37°C. We found a specific activity of 12.6 μmoles · min^{-1} · mg^{-1}, compared with 14–16.5 μmoles · min^{-1} · mg^{-1} reported by Wallace et al.

3.2. *Stoichiometry between oxidation of $Fe(CN)_6^{4-}$ and hydroxylation of tyramine*

In most mono-oxygenase reactions there is a 1:1 stoichiometry between the consumption of electron donor (expressed as two-electron equivalents) and the formation of hydroxylated product, but for some mono-oxygenases this ratio becomes higher than 1 with some non-physiological substrates [14,15]. $Fe(CN)_6^{4-}$ is certainly not a physiological substrate, but the stoichiometry is nevertheless 1:1, as three different experiments with conditions as described in the previous section gave the values 0.98; 0.95; and 1.03 for the ratio of half the number of moles of $Fe(CN)_6^{4-}$ oxidized to the number of moles octopamine formed.

3.3. *Initial velocity studies*

In order to establish whether the points of addition of $Fe(CN)_6^{4-}$ and tyramine are reversibly connected in the dopamine β-hydroxylase reaction, we measured initial velocities at different levels of these two substrates (for theory, see [8]). Apparent maximal velocities *(V)* and Michaelis constants *(K)* were estimated with the method of direct linear plots [16]. According to the recommendation in [16] the results were plotted as $\frac{s}{v}$ versus s (s = substrate concentration and v = initial velocity; slopes in fig. 1 are equal to $\frac{1}{V}$ and ordinate intercepts are equal to $\frac{K}{V}$). Table 1 lists the kinetic parameters when $Fe(CN)_6^{4-}$ is considered the changing fixed substrate [8], and table 2 lists kinetic parameters from the same data, but with tyramine considered as changing fixed substrate.

The requirement for a ping-pong mechanism is that $\frac{K}{V}$ (equal to slopes of double-reciprocal plots used by Cleland [8]) is independent of the concentration of the changing fixed substrate. Tables 1 and 2 show that $\frac{K}{V}$ decreases with increasing concentrations of changing fixed substrate, and similar results have been obtained in a number of different experiments. Thus, it is clear that the dopamine β-hydroxylase reaction with $Fe(CN)_6^{4-}$ as electron donor is sequential, as opposed to ping-pong, between the points of addition of electron donor and substrate to be hydroxylated.

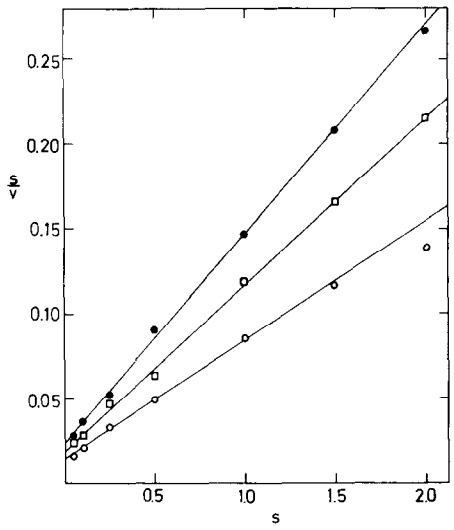

Fig. 1. $\frac{s}{v}$ versus s plot of data from kinetic experiment with $K_4Fe(CN)_6$ and tyramine as variable substrates, s is the concentration of tyramine in mM and v is the rate of $Fe(CN)_6^{4-}$ oxidation in nmoles · min^{-1}. Slopes and intercepts indicate the kinetic parameters that were estimated with the method of direct linear plots. Reaction mixtures contained 10 mM fumarate, 50 mM 2(*N*-morpholino)-ethanesulfonic acid, tyramine and $K_4Fe(CN)_6$ as indicated, and 4 μg/ml dopamine β-hydroxylase. Total volume was 1.0 ml, temperature 25°C, and pH 6.0. (○–○–○) 0.5 mM $K_4Fe(CN)_6$; (□–□–□) 0.2 mM $K_4FE(CN)_6$; (●–●–●) 0.1 mM $K_4Fe(CN)_6$.

Table 1
Kinetic parameters from data in fig. 1 with tyramine considered variable substrate and $Fe(CN)_6^{4-}$ considered changing fixed substrate

$K_4Fe(CN)_6$ mM	K mM	V nmoles/min	$\frac{K}{V}$
0.5	0.22	14.4	0.0153
0.2	0.19	10.2	0.0186
0.1	0.19	8.1	0.0234

4. Discussion

The results in the present study require new interpretations of previously published kinetic studies on dopamine β-hydroxylase. It seems probable that the redox potential of the copper in dopamine β-hydroxylase is considerably more positive than for ascorbate, which means that the initial reduction step is almost

Table 2
Same as table 1, but with $Fe(CN)_6^{4-}$ as variable substrate and tyramine as changing fixed substrate

Tyramine mM	K mM	V nmoles/min	$\frac{K}{V}$
2.0	0.12	16.6	0.0072
1.5	0.12	15.9	0.0075
1.0	0.11	14.3	0.0077
0.5	0.13	12.9	0.0101
0.25	0.08	8.8	0.0091
0.10	0.11	5.8	0.0190
0.05	0.115	3.8	0.0303

irreversible. Hence, $\frac{K}{V}$ could be independent of changing fixed substrate when ascorbate is electron donor, without the need to postulate a ping-pong mechanism. We report here that the mechanism is indeed sequential with $Fe(CN)_6^{4-}$ as electron donor, and similar mechanisms, with provisions for the difference between one-electron and two-electron donors, seem plausible for other electron donors as well.

From the stoichiometry of the reaction it is well established that all of the oxygen incorporated into the substrate is derived from molecular oxygen [17] and that two electrons must be utilized for the activation of O_2 [3]. Although electron paramagnetic resonance (EPR) spectra of dopamine β-hydroxylase have suggested an electron transferring function of the protein bound copper [18,19], the mechanism of the O_2 activation is not yet known. The EPR spectrum of the enzyme in the resting state has been interpreted as the oxygenated form of cupric dopamine β-hydroxylase [19], and evidence has been presented that oxygen activation follows the reduction of Cu(II) to Cu(I) [4]. Friedman and Kaufman [4] have proposed that O_2 interacts simultaneously with two copper atoms, thus explaining how this one-electron acceptor overcomes the difficulties of a two-electron transfer to oxygen. As pointed out by Vänngård [20], this mechanism is somewhat difficult to reconcile with the EPR spectra of the oxidized species showing no indications of interactions between neighboring copper atoms. Furthermore, as the present kinetic study support a sequential rather than a ping-pong mechanism, the involvement of only one copper atom per catalytic center must be considered equally acceptable. Report-

ed values of the copper content vary, but highly purified dopamine β-hydroxylase was recently found to contain 4 copper atoms per molecule [12] and to be a tetramer of four subunits [12,21]. It is therefore possible that there is one active site with one copper atom on each subunit.

For a complete understanding of the steady-state kinetic mechanism of dopamine β-hydroxylase, initial velocity studies with variation of the concentrations of all three substrates and the activator fumarate must be carried out. Inhibition studies are also necessary to further corroborate proposed mechanisms. There is, nevertheless, reason to believe that the mechanism is of the ter bi sequential type (for 2-electron donors), where the sequence of addition of substrates is electron donor, O_2 and substrate to be hydroxylated (RH). This proposal is based on our conclusion that the electron donor and RH add sequentially, and on the kinetic study of Goldstein et al. [6]. They reported that kinetic patterns with O_2 as variable substrate at different levels of dopamine showed variation in both $\frac{K}{V}$ and $\frac{1}{V}$, whereas patterns with dopamine as variable substrate at different levels of oxygen showed variation in $\frac{K}{V}$, but not in $\frac{1}{V}$. This suggests that the mechanism is equilibrium ordered [8] for O_2 and RH with O_2 adding first.

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