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INHIBITION OF DOPAMINE-β-HYDROXYLASE BY ALTERNATIVE ELECTRON DONORS

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Summary

The alternative electron donors ferrocyanide and hydroquinone have been shown to also act as inhibitors of dopamine-β-hydroxylase (3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1). Hydroquinone shows uncompetitive inhibition with respect to ascorbate and competitive inhibition with respect to tyramine. Ferrocyanide shows uncompetitive inhibition with respect to ascorbate and mixed type inhibition with respect to tyramine. Inhibition by ferrocyanide at concentrations at or above $2.5 \cdot 10^{-5}$ M was prevented by $2.5 \cdot 10^{-6}$ M cupric ion. These results indicate that the inhibitory action of these alternative electron donors is due to their interaction with a reduced enzyme species. The potency of inhibition of dopamine-β-hydroxylase by both ferrocyanide and hydroquinone is dependent on the degree of protonation of a group in the enzyme having a pK_a of 5.3.

Introduction

Dopamine-β-hydroxylase catalyzes the conversion of dopamine to norepinephrine in the catecholamine biosynthetic pathway. Molecular oxygen and an external electron donor are required for the catalytic benzylic hydroxylation to occur [1]. The stoichiometry of the dopamine-β-hydroxylase catalyzed reaction is such that the enzyme requires 2 mol electrons per mol hydroxylated product produced [1]. Ascorbate has been shown to be the most effective elec-

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tron donor to the enzyme in vitro [1]. The high concentration of ascorbate (of the order of millimolar) found in the chromaffin vesicles of the bovine adrenal medulla, where dopamine-β-hydroxylase is localized [2], has been taken as evidence that ascorbate is also the in vivo electron donor to the enzyme [3]. When the activity of dopamine-β-hydroxylase is measured in vitro, catalase is required in order for maximal enzymatic activity to be attained [4].

Although ascorbate is the best electron donor to dopamine-β-hydroxylase found to date, other reducing agents have been shown capable of serving as the electron donor to the enzyme in vitro. Among them are $Fe(CN)_6^{-4}$, dopamine, hydroquinone, dichlorophenolindophenol [1,4], and superoxide ion [5,6]. A preliminary study on the kinetic properties of ferrocyanide as an electron donor to dopamine-β-hydroxylase has been reported [7].

We have been interested in studying the effects of various inhibitors on dopamine-β-hydroxylase. In order to simplify such studies it would be convenient to use an electron donor to the enzyme that did not require the presence of catalase in the incubation mixture. This goal led us to a detailed investigation of the properties of some of the above mentioned alternative electron donors to the enzyme. Here we report our findings on the properties of ferrocyanide ($Fe(CN)_6^{-4}$) and hydroquinone as electron donors in the reaction catalyzed by dopamine-β-hydroxylase.

Experimental

Dopamine-β-hydroxylase was purified to homogeneity by either the procedure of Wallace [8] or that of Walker [9]. Equivalent results were obtained with enzyme obtained by either of these methods as well as with the fractions from the final purification steps having lower than maximal specific activities.

In the experiments where ferrocyanide was the sole electron donor present, the coupled phenylethanolamine-*N*-methyltransferase assay for dopamine-β-hydroxylase [10] was used. Neither ferrocyanide nor ferricyanide had any effect on the activity of phenylethanolamine-*N*-methyltransferase up to the highest concentrations tested (0.01 M). In all experiments multiple octopamine concentrations were used as internal standards to calibrate the phenylethanolamine-*N*-methyltransferase portion of the assay. Unless otherwise specified the reaction mixtures contained: Step 1: 0.08 mM tyramine-HCl; 0.01 M potassium fumarate; 0.05 M potassium phosphate (pH 6.0); step 2: 1.4 mM EDTA; *S*-adenosyl-l-methionine (20 Ci/M), 2.3 μM; 0.2 M potassium phosphate (pH 8.6); 0.005 unit/ml phenylethanolamine-*N*-methyltransferase. Each step of the assay was incubated for 20 min at 37°C in a shaking incubator with the tubes exposed to the atmosphere. With low concentrations of ferrocyanide the initial incubation was reduced to 15 min to insure linearity. Phenylethanolamine-*N*-methyltransferase was partially purified as previously described [11].

Inhibition of dopamine-β-hydroxylase by the alternative electron donors in the presence of ascorbate was assayed by the spectrophotometric procedure of Nagatsu and Udenfriend [12]. Unless otherwise specified the following conditions were employed: 0.02 M tyramine-HCl; 0.01 M ascorbic acid; 0.01 M sodium fumarate; 2000 units/ml catalase; 0.2 M sodium acetate (pH 5.5). Incubations were for 20 min at 37°C as described above. In the experiments

investigating the pH dependence of the inhibition of dopamine-β-hydroxylase by ferrocyanide or hydroquinone, constant ionic strength sodium acetate buffers, $I = 0.2$, were used.

In all experiments initial velocities were calculated from the amount of octopamine produced in a fixed time. Extreme care was taken to insure that octopamine production was linear with time under the conditions of length of incubation, substrate, and enzyme concentrations employed. All experiments were done in triplicate. Lineweaver-Burk and Dixon plots were obtained from least-squares analysis of the data.

All chemicals were of the highest grade commercially available. Tyramine-HCl was recrystalized from absolute ethanol. Catalase was obtained from Calbiochem. Both $Na_4Fe(CN)_6 \cdot 10\ H_2O$ and $K_4Fe(CN)_6 \cdot 3\ H_2O$ gave identical results.

Results

In studying $Fe(CN)_6^{-4}$ as an electron donor for dopamine-β-hydroxylase several differences between it and ascorbate were noted. Catalase, which is required when ascorbate is used as the electron donor to the enzyme, had no effect on the activity of the enzyme when ferrocyanide is used. The lack of activating effect of added catalase was observed at both high (1 mM) and low (10 μM) concentrations of ferrocyanide. As a result of this finding catalase was omitted from the reaction mixture in all experiments that did not also involve ascorbate.

A further difference between ferrocyanide and ascorbate as electron donors for dopamine-β-hydroxylase is in the apparent pH optimum for the dopamine-β-hydroxylase-catalyzed reaction. Ljones and Flatmark [7] have reported that with ferrocyanide the pH optimum is around pH 6.0, which is significantly higher than the value of between pH 5.3 and 5.5 found when ascorbate is the electron donor. Our results confirm the findings of these workers as regards the pH optimum for the ferrocyanide supported hydroxylation reaction catalyzed by the enzyme, as we also find the pH optimum to be 6.0 when ferrocyanide is employed as the sole electron donor. Unless otherwise specifed all experiments with ferrocyanide as the sole electron donor for dopamine-β-hydroxylase were carried out at the optimum pH of 6.0.

In examining the properties of ferrocyanide as an electron donor to dopamine-β-hydroxylase, we noticed that cupric ion appeared to substantially activate the enzyme when it is added to the assay mixture, as shown in Fig. 1. Other divalent metal ions such as Zn^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, Cd^{2+}, had no effect (as the chloride or sulfate salts). Preincubation of the enzyme with Cu^{2+}, even under assay conditions (in the absence of the substrate tyramine) followed by dialysis or chromatography on Sephadex G-25 did not result in any retention of activation. Addition of comparable concentrations of cupric ion when ascorbate was used instead of ferrocyanide also did not produce any activation, which indicates that a phenomenon associated with ferrocyanide was involved.

When the dependence of the initial rate of the hydroxylation reaction on the concentration of ferrocyanide was investigated, the explanation for the activating effect of Cu^{2+} become clear. Fig. 2 shows the concentration depend-

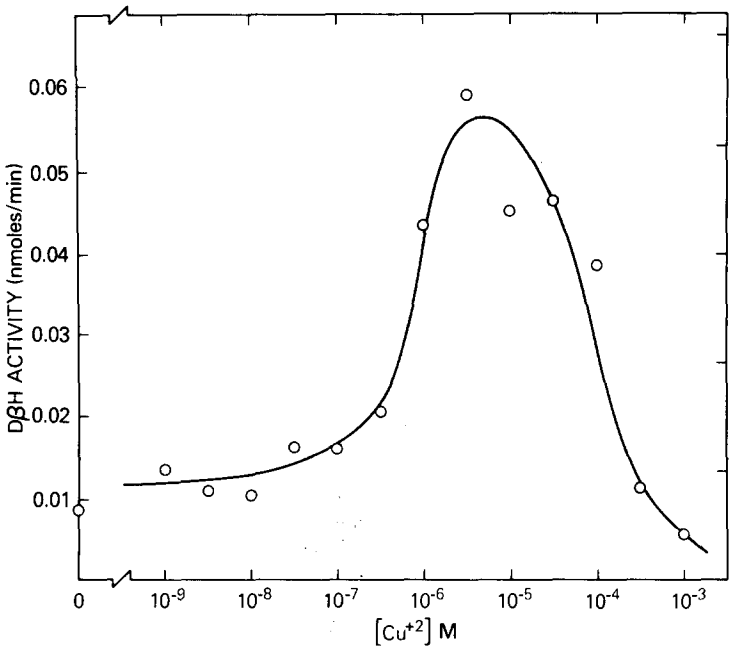


Fig. 1. Effect of added Cu^{2+} ($CuSO_4 \cdot 5\,H_2O$) on the activity of dopamine-β-hydroxylase when ferrocyanide was the electron donor. Reaction conditions and assay procedure were as described in Methods, except tyramine-HCl was 0.4 mM, and 0.1 mM $Na_4Fe(CN)_6 \cdot 10\,H_2O$.

ence of the initial rate of the hydroxylation reaction on ferrocyanide with and without exogenous Cu^{2+}. The shape of the curve with no added Cu^{2+} indicates substrate inhibition by ferrocyanide. Addition of Cu^{2+} appears to have a dual effect on the system. At high ferrocyanide concentrations Cu^{2+} appears to

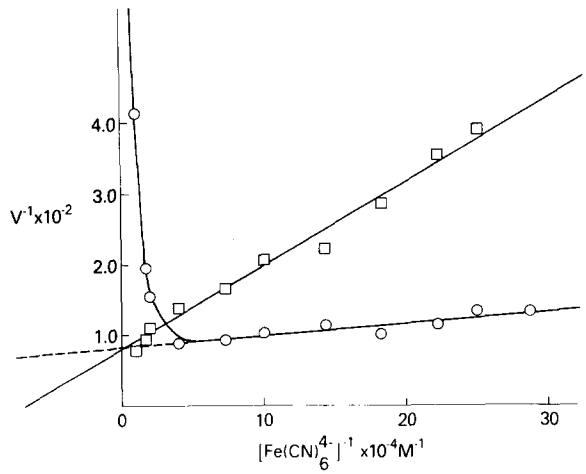


Fig. 2. V^{-1} vs. S^{-1} plot of data with sodium ferrocyanide as the varied substrate with and without added Cu^{2+} ($CuSO_4 \cdot 5\,H_2O$). Conditions and assay procedures were as described in Methods except tyramine-HCl was 0.125 mM. Velocity is in units of nmol per min. ○, no Cu^{2+}; □, $2.5 \cdot 10^{-6}$ M Cu^{2+}.

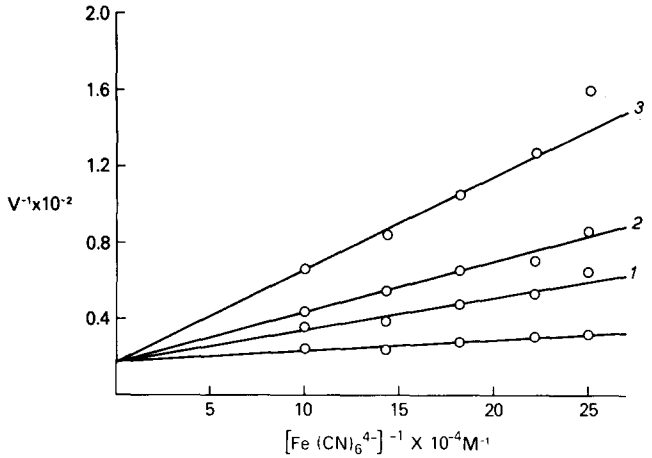


Fig. 3. Inhibition of dopamine-β-hydroxylase by Cu^{2+} ($CuSO_4 \cdot 5\ H_2O$) with sodium ferrocyanide as the sole electron donor. Conditions and assay procedures were as described in Methods. Velocity is in units of nmol per min. *1*, $1 \cdot 10^{-6}$ M Cu^{2+}; *2*, $2 \cdot 10^{-6}$ M Cu^{2+}; and *3*, $3 \cdot 10^{-6}$ M Cu^{2+}.

reverse the inhibitory effects of ferrocyanide, while Cu^{2+} itself appears to be inhibitory.

We have investigated the inhibition of dopamine-β-hydroxylase by copper ion in more detail at low (non-inhibitory concentrations of ferrocyanide). As the data in Fig. 3 indicate copper appears to be a competitive inhibitor with respect to ferrocyanide at the concentrations studied. Analysis of the data by the method of Dixon showed that the inhibition of the enzyme by Cu^{2+} is not

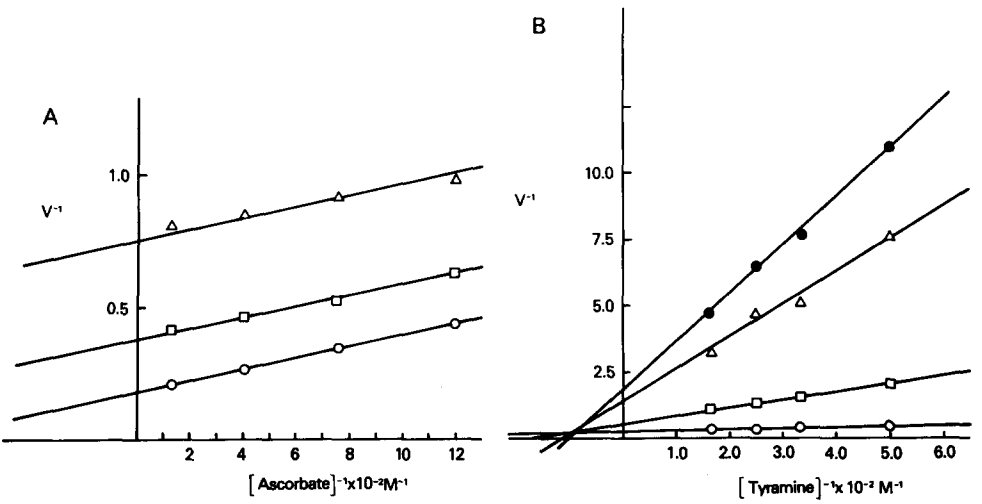


Fig. 4. Inhibition of dopamine-β-hydroxylase by sodium ferrocyanide with ascorbate as the electron donor. (A) Ascorbate as the varied substrate. Ferrocyanide concentrations are: ○, zero; □, $1.5 \cdot 10^{-5}$ M; and △, $3.0 \cdot 10^{-5}$ M. (B) Tyramine as the varied substrate. Ferrocyanide concentrations are: ○, zero; □, $5.0 \cdot 10^{-5}$ M; △, $10.0 \cdot 10^{-5}$ M; ●, $12.5 \cdot 10^{-5}$ M. Conditions and methods were as described in Methods. Velocity is in units of nmol per min.

simple intersecting linear in character. Inhibition of purified dopamine-β-hydroxylase by Cu^{2+} with ascorbate as the electron donor has been reported [14,15] but the inhibition was not characterized kinetically.

In order to confirm that ferrocyanide can inhibit dopamine-β-hydroxylase we have examined the effects of ferrocyanide on the hydroxylation reaction with ascorbate present. The results of these experiments are summarized in Fig. 4. Ferrocyanide shows uncompetitive inhibition with respect to ascorbate and mixed type inhibition with respect to tyramine. Since ferrocyanide is a highly charged anion, and anions such as fumarate have been shown to activate the enzyme [1,16], we also examined the effects of ferrocyanide with respect to fumarate. Mixed type inhibition was observed when fumarate was treated as the varied substrate (data not shown). The observation of different types of inhibition patterns when ascorbate and tyramine were the varied substrates clearly indicates that the inhibition of dopamine-β-hydroxylase by ferrocyanide is not being mediated by catalase, since in that case we would have expected to see only a V effect with either substrate.

As mentioned above, hydroquinone and catechol are also able to serve as the electron donor in the dopamine-β-hydroxylase catalyzed reaction. We have found that both of these compounds appear to act as inhibitors of the enzyme when they are included in the ascorbate assay system. Both hydroquinone and catechol (data not shown) show uncompetitive inhibition with respect to ascorbate and competitive inhibition with respect to tyramine (Fig. 5). Their affinities for the enzyme appear to be comparable. Substrate inhibition analogous to that found for ferrocyanide was not observed when hydroquinone was used as the sole electron donor for the enzyme, as the double reciprocal plots were linear at fixed levels of tyramine. However when both tyramine and hydroquinone concentrations were varied simultaneously a family of skewed lines, neither parallel nor intersecting at one point were obtained (data not shown), indicating that hydroquinone can interact with more than one form of the enzyme [17].

In the course of our studies on the inhibition of dopamine-β-hydroxylase by ferrocyanide, it was noted that the potency of the inhibition appeared to depend on pH. A similar, although less pronounced effect was also observed with hydroquinone. For hydroquinone, analysis of the data by the method of Dixon showed a 3-fold increase in K_i for hydroquinone as the pH decreased from 5.9 to 4.9 as is shown in Fig. 6. Although such treatment of the data was not possible in the case of ferrocyanide, it was found that at a constant ferrocyanide concentration the percentage of inhibition relative to controls containing no ferrocyanide also increased as the pH of the solution was lowered from 5.9 to 4.7. These results are shown in Fig. 7. A similar pH dependence was obtained in identical experiments with hydroquinone instead of ferrocyanide (data not shown). These studies suggest that the degree of protonation of a group having a pK_a of between 5.2 and 5.3 appears to effect the potency of the inhibition of dopamine-β-hydroxylase by both of these species. As neither hydroquinone nor ferrocyanide has a pK_a in this region, it would appear that protonation of a group on the enzyme increases the potency of inhibition by these compounds.

The pH dependence of the inhibition of dopamine-β-hydroxylase by ferro-

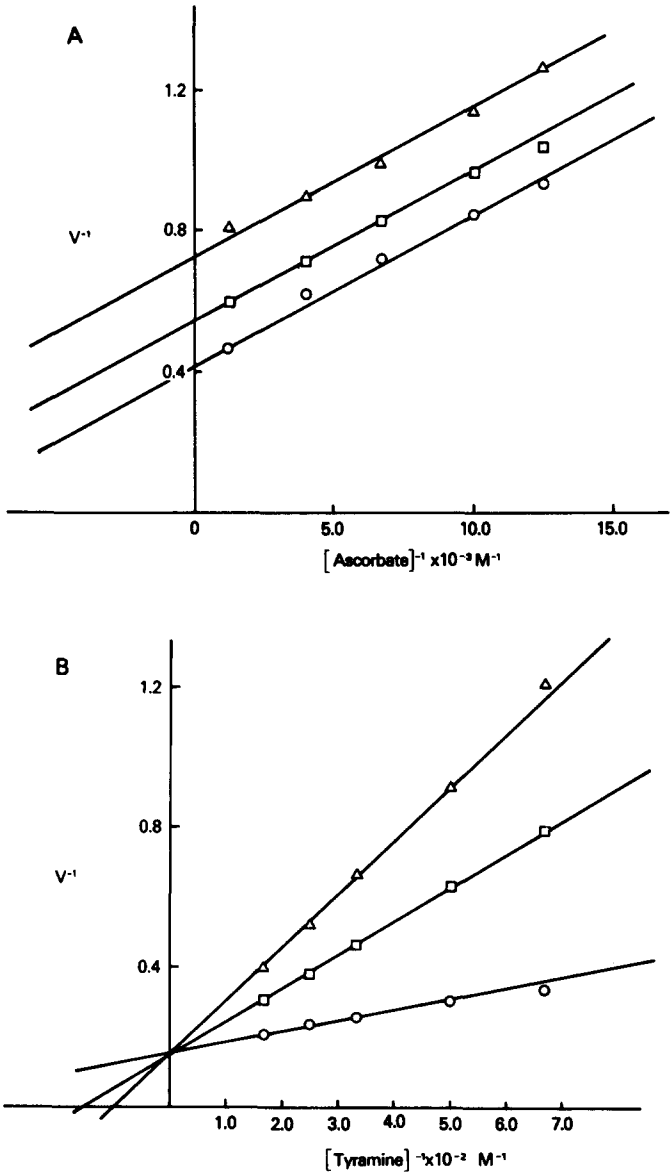


Fig. 5. Inhibition of dopamine-β-hydroxylase by hydroquinone with ascorbate as the electron donor. (A) Ascorbate as the varied substrate. Hydroquinone concentrations are: ○, zero; □, $2.5 \cdot 10^{-3}$ M; and △, $7.5 \cdot 10^{-3}$ M. (B) Tyramine as the varied substrate. Hydroquinone concentrations are: ○, zero; □, $1 \cdot 10^{-3}$ M; and △, $2 \cdot 10^{-3}$ M. Conditions and assay procedures were as described in Methods. Velocity is in units of nmol per min.

cyanide might be the basis of the shift in the pH optimum from 5.4 to 6.0 observed when ferrocyanide replaces ascorbate as the electron donor for the enzyme. Since ferrocyanide, as shown above, is a more potent inhibitor of the enzyme at lower pH values, the differential sensitivity of the enzyme to ferro-

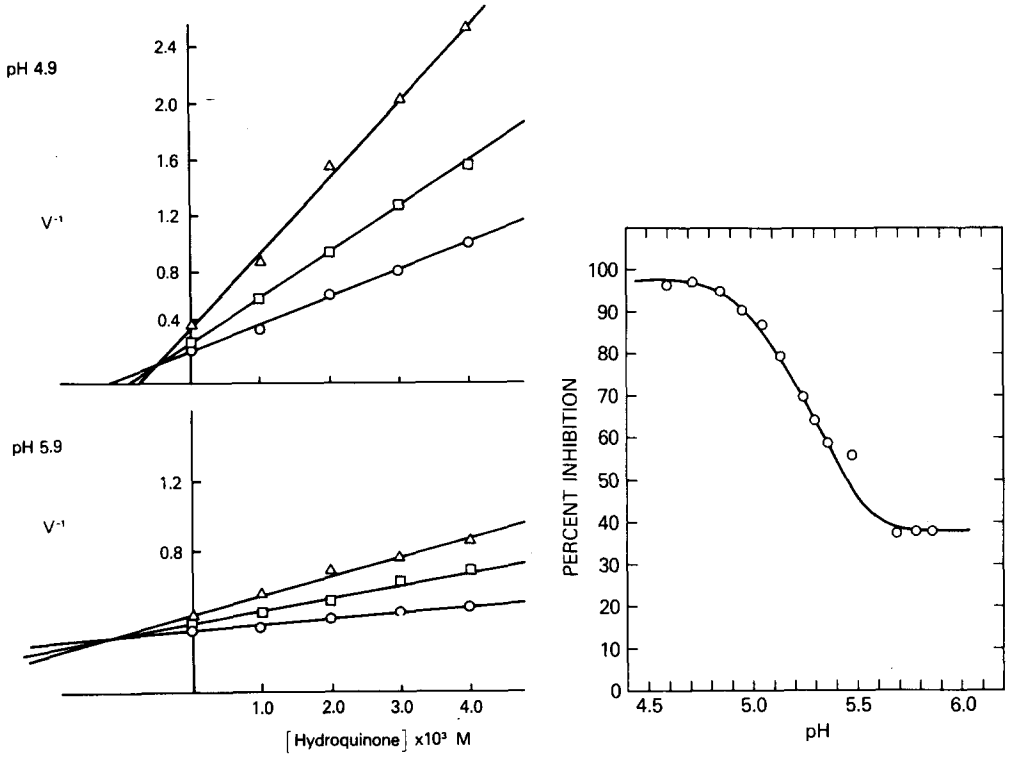


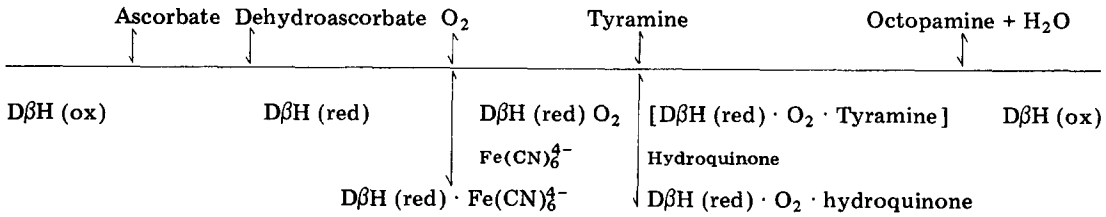
Fig. 6. pH dependence of the inhibition of dopamine-β-hydroxylase by hydroquinone with ascorbate as the electron donor plotted by the method of Dixon. At pH 4.9 K_i for hydroquinone is $0.37 \pm 0.04 \cdot 10^{-3}$ M, while at pH 5.9 $K_i = 1.05 \pm 0.05 \cdot 10^{-3}$ M. Conditions and assay procedures were as described in Methods. Velocity is in units of nmol per min. Tyramine concentrations were: ○, $6.0 \cdot 10^{-3}$ M; □, $3.5 \cdot 10^{-3}$ M; and △, $2.5 \cdot 10^{-3}$ M.

Fig. 7. pH dependence of inhibition of dopamine-β-hydroxylase by $5 \cdot 10^{-5}$ M sodium ferrocyanide with ascorbate as the electron donor. Percent inhibition at each pH is relative to dopamine-β-hydroxylase activity under identical conditions with no sodium ferrocyanide present. Conditions and assay procedures are as described in the Methods.

cyanide inhibition could skew a pH activity profile so that the apparent pH optimum occurs at higher pH when ferrocyanide is the electron donor.

Discussion

In their preliminary study of ferrocyanide as an electron donor for dopamine-β-hydroxylase, Ljones et al. [7] reported linear intersecting kinetic plots. We have not been able to confirm these particular findings. On the contrary we find that substrate inhibition by ferrocyanide is apparent at or above ferrocyanide concentrations of $2.5 \cdot 10^{-5}$ M, under conditions where the enzyme is not saturated with tyramine (Fig. 2). Inhibition of dopamine-β-hydroxylase by ferrocyanide was further confirmed by our studies with both ascorbate and ferrocyanide present (Fig. 4). This behavior has been consistently observed with enzyme from independent preparations. At this point we are at a loss to explain the discrepancies in our results and those of Ljones, et al. [7].



DβH = dopamine-β-hydroxylase

Scheme I. Kinetic scheme for dopamine-β-hydroxylase catalyzed reaction showing possible sites of inhibition by ferrocyanide and hydroquinone.

Although different assay techniques were used in these two studies, we do not feel that the observed inconsistencies can be ascribed to this factor, since we have observed the inhibition of dopamine-β-hydroxylase by ferrocyanide using two different assay procedures.

One possible explanation of the linear plots observed by Ljones et al. [7] is suggested by the results of our experiments on the effect of added Cu^{2+} on the inhibition of dopamine-β-hydroxylase by ferrocyanide. As shown in Fig. 2, Cu^{2+} at concentrations between 10^{-6} and 10^{-5} M appears to give a linear double reciprocal plot when ferrocyanide is the varied substrate and sole electron donor present. Thus adventitious copper at the 0.1—1.0 ppm level can overcome the effects of ferrocyanide substrate inhibition and could possibly have given rise to the type of behavior observed by Ljones et al. [7].

The data shown in Figs. 4 and 5 indicate that neither ferrocyanide nor hydroquinone bind to the same form of dopamine-β-hydroxylase as does ascorbate. Since both electron donors show uncompetitive inhibition with respect to asorbate it is likely that they bind to enzymatic species that are formed subsequent to the interaction of ascorbate with the enzyme. One possible kinetic scheme that is consistent with all of the observed inhibition patterns, as well as with previous [18] kinetic studies of the enzyme is shown in Scheme I. According to this scheme, in the presence of both ascorbate and an inhibitory alternative electron donor, the order of addition of the phenylethylamine and molecular oxygen substrates is ordered, with molecular oxygen adding first. If the additions of oxygen and tyramine form an ordered rapid equilibrium segment, as suggested by the data of Goldstein et al. [18], our inhibition data imply that the reduced enzyme-ferrocyanide complex is capable of binding tyramine.

Although ferrocyanide and hydroquinone apparently have their inhibitory interaction with different forms of the enzyme in the catalytic cycle, the similarities in the pH dependence of the inhibition of dopamine-β-hydroxylase by these two species raises the possibility that the sites of interaction may be physically close together, and could reflect the influence of a single amino acid residue in close proximity to both binding sites. Clearly, based on kinetic data alone, we can not eliminate the possibility that the agreement of the pK_a values for inhibition is fortuitious and due to similar but widely separated residues. It is interesting however that a similar pK_a has been observed for the pH depend-

ence of the apparent K_m for the phenylethylamine substrate, tyramine [19,20]. Since hydroquinone is a competitive inhibitor with respect to tyramine it would appear that the affinity of the enzyme for this inhibitor and substrate may be influenced by the degree of protonation of the same group in the enzyme.

Our results at low concentrations of ferrocyanide confirm that ferrocyanide can function as an electron donor to dopamine-β-hydroxylase in the catalytic process. Ferrocyanide can serve only as a one electron donor, whereas both ascorbate and hydroquinone can function as either one or two electron donors since both the monodehydroascorbate radical [21] and the semiquinone radical [22] have been identified and studied. It has been generally assumed in the literature, except by Blumberg et al. [23], that ascorbate functions as a two electron donor to the enzyme. However such an assumption is not required in order to explain the available kinetic and spectroscopic data. The disproportionation of the monodehydroascorbate radical is very rapid at the relatively acid pH values where the enzyme shows optimum activity [24]. The reduced form of dopamine-β-hydroxylase could be formed by a series of two ping-pong additions of ascorbate and releases of the monodehydroascorbate radical. Disproportionation of the monodehydroascorbate radicals would then give the observed one to one stoichiometry of dehydroascorbate to hydroxylated phenylethylamine. Blumberg et al. [23] have reported evidence for the existence of a free radical under turnover conditions in the dopamine-β-hydroxylase catalyzed reaction, which they assigned to the monodehydroascorbate free radical. Evidence for the monodehydroascorbate free radical being an intermediate in the reduction of the copper-containing enzyme ascorbate oxidase has also been reported [25], where a similar one electron reduction mechanism was proposed.

Available data on the reduction potentials of the various species involved in the dopamine-β-hydroxylase catalyzed reaction also indicate that the one electron transfer mechanism we have proposed is reasonable. Walker et al. [9] have found that at pH 7.0 the midpoint potential of the copper in dopamine-β-hydroxylase is +0.31 V. Ljones et al. [26] have also reported preliminary results of a redox titration of the enzyme in which they found the midpoint potential to be around +0.37 V at pH 7.0. As with the studies reported by Walker et al. [9], Ljones et al. [26] also found that their data could best be explained by assuming that the EPR detectable redox site in the enzyme is a one electron acceptor/donor. Although the values of the midpoint potentials for dopamine-β-hydroxylase reported in these two studies appear to differ significantly, both values are considerably more positive than the value of the ascorbate/dehydroascorbate couple, which has a midpoint potential of +0.06 V at pH 7.0 [27]. The large differences in potentials for dopamine-β-hydroxylase and its presumed natural electron donor led Ljones et al. [7,26] to conclude that reduction of the enzyme by ascorbate is essentially irreversible and that it is this irreversible step that gives rise to the apparent ping-pong type steady state kinetics.

In contrast to the low potential of the ascorbate/dehydroascorbate couple, Weiss [28] has found that the ascorbate/monodehydroascorbate couple has a considerably more positive midpoint potential of +0.32 V at pH 7.0, which is

much closer to that of the EPR detectable redox site in dopamine-β-hydroxylase. So if ascorbate functions as a one electron donor to the enzyme, the reduction step is not necessarily irreversible in a thermodynamic sense, as suggested by Ljones et al. [7,26]. Thus the apparent ping-pong type kinetics may reflect the true mechanism of the dopamine-β-hydroxylase catalyzed reaction. Although the fully reduced enzyme is capable of stoichiometric substrate hydroxylation in the presence of molecular oxygen [14], the kinetic competence of the fully reduced enzyme has been recently questioned [29].

Although the data cited above are consistent with ascorbate acting as a one electron donor to dopamine-β-hydroxylase, it should be noted that both of these midpoint potential determinations as well as the turnover experiments of Skotland et al. [29] were done at pH 7.0, which is considerably more basic than the natural environment of the enzyme in the chromaffin vesicle [30,31], and the pH values where the enzyme shows optimal activity in vitro. Unfortunately no information regarding the pH dependence of any of these midpoint potentials is currently available.

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