Biochimica et Biophysica Acta, 482 (1977) 309—322

BBA 68158

AN INVESTIGATION OF THE COPPER SITE(S) OF DOPAMINE-β-HYDROXYLASE BY ELECTRON PARAMAGNETIC RESONANCE

GUSTAVUS A. WALKER *, HIDEO KON and WALTER LOVENBERG

Section on Biochemical Pharmacology, National Heart, Lung and Blood Institute, National Institutes of Health, and Laboratory of Chemical Physics, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)

(Received July 28th, 1976)
(Revised manuscript received November 15th, 1976)

Summary

1. Dopamine-β-hydroxylase (3,4-dihydroxyphenylethylamine, ascorbate: oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1) contains approximately four copper atoms per tetramer. These can be fully reduced by ascorbate and fully oxidized by potassium ferricyanide or by oxygen in the presence of fumarate, tyramine or octopamine.

2. Upon the addition of tyramine (substrate) or octopamine (product) to dopamine-β-hydroxylase, several superhyperfine lines with a splitting of about 15 G are detected at the g_1 region of the EPR spectrum of the enzyme. The involvement of nitrogen ligands in the binding of the enzyme copper is suggested. The absence of superhyperfine lines upon the addition of the substrate, phenethylamine, implicates the involvement of the 4-hydroxyl group on tyramine and octopamine in the appearance of these lines.

3. A decrease in the amplitude of the EPR signal of dopamine-β-hydroxylase upon the deoxygenation of the enzyme was consistently observed and could suggest that oxygen is reversibly bound in a protein-copper-oxygen intermediate complex.

4. By the use of EPR as a probe for the extent of copper oxidation, the midpoint potential of dopamine-β-hydroxylase is estimated to be +310 mV.

* Present address: School of Medicine, St. Louis University, St. Louis, Mo. 63104, U.S.A.
The abbreviations used are: dopamine, 3,4-dihydroxyphenylethylamine; tyramine, 4-hydroxyphenyletheylamine; octopamine, 4-hydroxyphenylethanolamine; dopamine-β-hydroxylase (DBH), 3,4-dihydroxyphenylethylamine-β-hydroxylase; EPR, electron paramagnetic resonance; bathocuproine, disulfonated sodium salt of 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline.

Introduction

Dopamine-β-hydroxylase (3,4-dihydroxyphenylethylamine, ascorbate: oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1) a copper-containing monooxygenase, catalyzes the conversion of dopamine to norepinephrine according to this reaction [1]:

$$\text{Dopamine} + \text{ascorbate} + O_2 \rightarrow \text{norepinephrine} + \text{dehydroascorbate} + H_2O$$

Many derivatives of phenethylamine such as tyramine and epinine serve as substrates for this enzyme [2]. Studies on the stoichiometry of this reaction show that for each mole of hydroxylated product formed, one mole each of oxygen and ascorbate are consumed [1.]

Fumarate and other dicarboxylic acids or salts have been shown to stimulate dopamine-β-hydroxylase activity by nonspecific anion activation, which is thought to occur near the copper center [3]. EPR studies showed that fumarate would increase the intensity of cupric signal of the enzyme [4]. Later kinetic studies showed that fumarate facilitated the interaction of the reduced enzyme with oxygen and lowered the K_m for the substrate [5].

Fully-active dopamine-β-hydroxylase contains four copper atoms per tetramer [6]. The copper in the enzyme has been shown by chemical methods and EPR analysis to undergo valence changes during enzyme-catalyzed hydroxylation reactions [4,7]. Oxygen must react with the reduced copper to form a postulated active intermediate complex which, in turn, hydroxylates the substrate to yield product, water, and an oxidized copper enzyme.

In this paper we have studied the effect of substrate analogues, fumarate, and oxygen removal on the EPR spectrum of dopamine-β-hydroxylase, and we report new observations. We have also estimated the midpoint potential of the enzyme using the EPR signal to probe the oxidation state of the copper center.

Materials and Methods

Protein purification

Dopamine-β-hydroxylase was isolated as a pure protein from bovine adrenal medullae by a modification of the method of Friedman and Kaufman [3]. After the 60% ammonium sulfate fractionation and extensive dialysis, the protein was adsorbed on a DEAE-cellulose column and eluted with a linear salt concentration gradient (0—0.8 M NaCl in 10 mM potassium phosphate buffer, pH 7.2). The most active fractions were pooled, concentrated, and twice applied to two BioGel A-0.5 m agarose columns (2.5 × 80 cm) in series which had been equilibrated with 10 mM potassium phosphate buffer containing 0.1 M NaCl, pH 7.2. The most active fractions had a specific activity between 25 and 31 μmol octopamine produced per min per mg of protein, using a spectrophotometric assay in which octopamine formed from tyramine is converted to *p*-hydroxybenzaldehyde [6]. The protein was judged to be pure, as only one band was detected after disc gel electrophoresis. Enzyme that was to be used for EPR experiments was additionally dialyzed against 50 mM potassium phosphate buffer, pH 7.0.

EPR spectral measurements

The EPR spectra, taken at 77 K, were recorded with a Varian E-9 spectrometer operating at about 9 GHz with a 100 KHz modulation frequency. The first derivative of the absorption curve was plotted as a function of the applied magnetic field using an XY recorder. The g values near 2.0 were measured by comparison with the free radical standard 2,2-diphenyl-1-picrylhydrazyl.

Copper determinations

The amount of copper in dopamine-β-hydroxylase was determined chemically by using the bathocuproine reagent [8] and spectrally by manual integration of the EPR signal intensities of the fully oxidized species after addition of excess ferricyanide or tyramine. The area under the integrated spectra was determined by cutting the shapes out with scissors, weighing them and comparing the values with the integrated spectra of standard copper similarly treated. Copper sulfate (0.1 mM) dissolved in dilute ammonium hydroxide (7.2 mM) was used to make a standard solution of copper.

Protein determination

Protein was determined by the procedure of Lowry et al. [9] used with crystalline bovine serum albumin (Sigma) as a standard. The concentration of dopamine-β-hydroxylase could also be estimated spectrophotometrically from its absorbance at 280 mμ. An absorbance of 1.16 for 1 mg of protein per ml and a 1-cm lightpath was obtained by calibration against the Lowry method.

Anaerobic technique

For most experiments, dopamine-β-hydroxylase was initially deoxygenated. Protein was placed in a 4-ml serum vial which was capped with a rubber stopper. The vial was connected by syringe to a vacuum pump line through a manifold system of glass and butyl rubber tubing. The sample was gently agitated during 10 cycles of evacuation and flushing with argon that had twice been passed through acidic vanadyl sulfate solution to remove traces of oxygen in the gas. If strict anaerobic conditions were to be maintained, all solutions, reagents, and containers were similarly degassed and flushed with argon. Solutions were transferred anaerobically with a syringe and mixed at room temperature under argon in a rubber capped serum vial. Samples were then transferred to rubber capped quartz EPR tubes, previously degassed and flushed with argon, and promptly frozen by immersion in liquid nitrogen. Control samples or those where substrate or product were to be added were handled similarly but under aerobic conditions.

Estimation of midpoint potential

Hydroquinone in 10 mM sodium acetate, pH 5.0 or potassium ferrocyanide was mixed anaerobically with protein that had been thoroughly degassed and flushed with argon. After five minutes (equilibration time) the mixture was transferred by syringe to rubber capped EPR tubes that had been flushed with argon. The samples were immediately frozen in liquid nitrogen. The oxidation level of the protein was proportional to signal height at $g_{\perp}$. Maximum signal height (100%) oxidized protein) was determined by treating the protein with a

15-fold excess of potassium ferricyanide and by subtracting any contribution to the signal produced by the protein when in the presence of ascorbate. The EPR spectra were recorded at 8.919 GHz at 77 K with a modulation amplitude of 32 G, modulation frequency of 100 KHz, a 5 min scan time and microwave power of 6 mW.

In determining the E_H of the system at pH 7.0, values of +275 mV and +430 mV were used respectively for the midpoint potential of the hydroquinone/quinone couple and the potassium ferricyanide/potassium ferrocyanide couple [10].

Chemicals

The chemicals used were of the highest commercial quality available. Ascorbic acid was obtained from Sigma; hydroquinone, from Fisher Scientific; tyramine hydrochloride, D,L-octopamine hydrochloride, dopamine hydrochloride and bathocuproinedisulfonic acid, from Aldrich; and potassium ferricyanide, from Baker and Adamson. The concentration of potassium ferricyanide was determined spectrophotometrically using a molar extinction coefficient of 1000 $M^{-1} \cdot cm^{-1}$ at 420 nm. An examination of the spectra of hydroquinone in 10 mM NaAc buffer, pH 5.0 indicated that less than 1% quinone was present under anaerobic conditions.

All solutions were prepared from distilled water that had been filtered and passed through a set of mixed bed ion exchange columns designed by Hydro Service and Supplies, Inc. Water which had been treated in this manner had a specific resistance of 2.5 MΩ per cm^3.

Results

The EPR spectrum of dopamine-β-hydroxylase, as first reported by Blumberg et al. [11], showed magnetic parameters which suggested that the protein-bound copper was in rhombic symmetry. Friedman and Kaufman [4] subsequently published a spectrum of the enzyme, of greater purity, which showed a signal more typical of copper in a complex of tetragonal symmetry. We have isolated a highly purified preparation of dopamine-β-hydroxylase with the highest specific activity yet published, and its EPR spectrum is shown in Fig. 1, top. The magnetic parameters are very similar to those published by Friedman and Kaufman. In this spectrum of the enzyme in the resting state (as isolated), the signal height at $g_\perp$ is about 66% of that found in the spectrum of the fully oxidized enzyme. After the addition of excess ascorbate the signal virtually disappears (Fig. 1, bottom).

The amount of Cu^{2+} present in the isolated enzyme, as judged by signal intensity at $g_\perp$, was found to vary from preparation to preparation. In Table I the percentage of maximum signal height for total Cu^{2+} varied from 55% to 84%. Freezing and thawing tended to increase the intensity of Cu^{2+} signal. If however, the protein solution was thoroughly degassed of air and flushed with argon, an appreciable decrease in the signal intensity resulted. Differences in signal amplitude as high as 31% or as low as 10% were found. No other changes in the spectrum were observed. The activity of the degassed samples was determined subsequent to each EPR experiment and found to be identical to that of

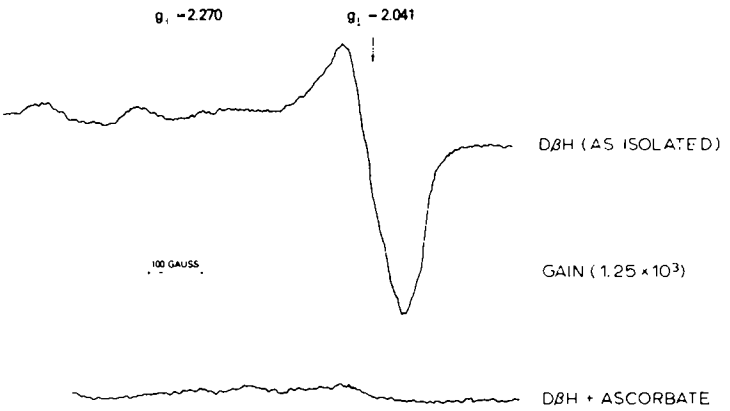

Fig. 1. EPR spectra of dopamine-β-hydroxylase. Top, native enzyme (as isolated), 1.7 mg of protein, and 10 μmol of potassium phosphate buffer, pH 7.0, in 0.22 ml. Bottom, same as above after the anaerobic addition of 88 nmol of ascorbate to the protein solution which had been previously degassed and flushed with argon. In these spectra and all others the magnetic field, H_0, is increasing to the right. Modulation amplitude, 20 G; modulation frequency, 100 KHz; scan time, 2.5 min; temperature, 77 K; microwave frequency, 8.879 GHz; microwave power, 3.5 mW. For further information see Materials and Methods.

the undegassed samples. This indicates that no change in concentration of active enzyme had occurred during the degassing process and by implication, no change in copper content of the enzyme.

Upon the anaerobic addition of excess potassium ferricyanide to dopamine-β-hydroxylase, the EPR signal height was maximized (Fig. 2, top). By quantitative evaluation of the EPR spectrum by manual integration, the observed signal accounted for 4.03 gatoms of copper per mol of protein, or 100% of the chemically determined copper. When the substrate, tyramine, was added to the previously degassed enzyme in the presence of oxygen, the EPR signal increased from 55% to full intensity (Fig. 2, bottom). Several superhyperfine lines were detected at the $g_\perp$ region of the spectrum with a splitting of about 15 g. Some line narrowing was also apparent, especially at the three hyperfine lines at low field.

Fig. 3 compares the EPR spectra of dopamine-β-hydroxylase in the presence of octopamine, phenethylamine and tyramine. Interestingly, octopamine,

TABLE I

EFFECT OF DEGASSING ON Cu^{2+} EPR SIGNAL HEIGHT OF DOPAMINE-β-HYDROXYLASE

Experimental conditions were the same as in Fig. 1 except anaerobic technique was employed. "Degassing" refers to deoxygenating a sample by evacuation followed by flushing with argon (see Materials and Methods). Each tube contained 1.7 mg of protein, and 10 μmol of potassium phosphate buffer, pH 7.0, in 0.22 ml.

	% Maximum signal height at $g_\perp$	
	As isolated	After degassing
Experiment 1	84 ± 1	53 ± 1
Experiment 2	65 ± 2	55 ± 2
Experiment 3	83	70
Experiment 4	55	34

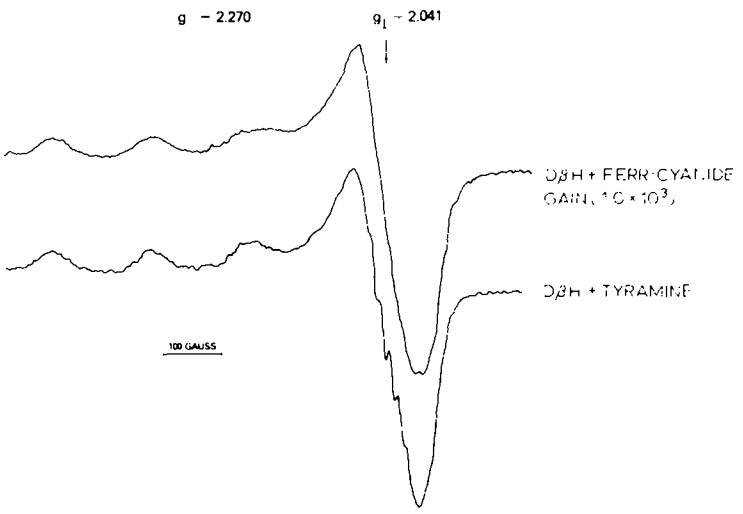

Fig. 2. EPR spectra of dopamine-β-hydroxylase in the presence of ferricyanide or tyramine. Each tube contained 1.7 mg of protein and 10 μmol of potassium phosphate buffer, pH 7.0, in 0.22 ml. Top, after the anaerobic addition of 88 nmol of deoxygenated potassium ferricyanide to the protein solution which had been previously degassed and flushed with argon. Bottom, after the addition of 4 μmol of tyramine hydrochloride. In this instance, only the protein was previously deoxygenated. Other experimental details were the same as in Fig. 1.

which is formed by the enzyme from tyramine, not only caused the appearance of superhyperfine lines at high field but also increased the signal amplitude to the same extent as the substrate, tyramine. Phenethylamine, a substrate which is about 15% as effective as tyramine [2] and which lacks the 4-hydroxyl group

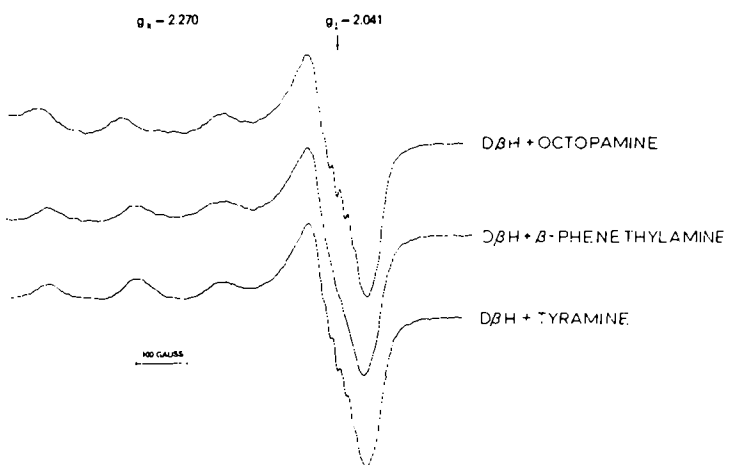

Fig. 3. Comparison of the EPR spectra of dopamine-β-hydroxylase in the presence of octopamine, phenethylamine, or tyramine. Each tube contained 1.6 mg of protein (initially degassed) and 10.5 μmol of potassium phosphate buffer, pH 7.0, in 0.22 ml. All additions were made in the presence of air. Top, after the addition of 4 μmol of octopamine hydrochloride. Middle, after the addition of 4 μmol phenethylamine. Bottom, after the addition of 4 μmol of tyramine hydrochloride. Incubation time 15 min at room temperature. Modulation amplitude 25 G microwave power, 6.0 mW. Other experimental details were the same as in Fig. 1.

on the ring, increased the signal amplitude but did not effect superhyperfine splitting.

Table II summarizes the relative intensities of the signals and the presence of detectable superhyperfine splitting after the addition of various derivatives of phenethylamine. The effects of ferricyanide and fumarate are added for comparison. Note that *N*-methyl phenethylamine, a substrate analogue which possesses little or no activity, neither increases the signal strength nor causes superhyperfine splitting. The addition of dopamine to the enzyme caused a decrease in the signal intensity since, under the conditions used, it can serve as an electron donor forming dopamine quinone with concomitant enzyme reduction [3]. Because of the small intensity of the signal and the influence of background noise, it was difficult to determine whether superhyperfine lines appeared after the addition of dopamine to the enzyme.

Since superhyperfine splitting was observed after the addition of either substrate (tyramine) or product, (octopamine), it was necessary to determine whether both compounds or only the product could induce this effect. Conceivably, the octopamine formed from the added tyramine by dopamine-β-hdyroxylase could remain bound to the substrate site until subsequent reduction of the enzyme. We were able to distinguish between these possibilities by first oxidizing the degassed enzyme anerobically with potassium ferricyanide, then adding tyramine or octopamine. As indicated in Table II, superhyperfine splitting still occurs after the addition of tyramine. Evidently, either compound can effect this change without a concomitant oxidation of enzyme copper by oxygen. It is also important to note that the subsequent addition of either tyramine or octopamine to the enzyme oxidized by potassium ferricyanide did

TABLE II

SPECIFICITY OF SUBSTRATE-INDUCED EPR SIGNAL CHANGE OF DOPAMINE-β-HYDROXYLASE

The experimental procedure was the same as in Fig. 3. Each tube contained 1.6 mg of protein and 10.5 μmol of potassium phosphate buffer, pH 7.0, in 0.22 ml. To the various samples, 4 μmol of tyramine hydrochloride, octopamine hydrochloride, dopamine hydrochloride, β-phenethylamine or *N*-methylphenethylamine were added where indicated. When potassium ferricyanide was used, 33 nmol were added anaerobically to degassed protein solution, and after 5 min incubation, tyramine or octopamine were added where indicated. To the final sample of 5 μmol of fumarate were added.

Sample	% Maximum signal height at $g_\perp$	Presence of superhyperfine splitting at $g_\perp$
Dopamine-β-hydroxylase only	77	—
Dopamine-β-hydroxylase + tyramine	100	+
Dopamine-β-hydroxylase + octopamine	98	+
Dopamine-β-hydroxylase + β-phenethylamine	92	—
Dopamine-β-hydroxylase + *N*-methylphenethylamine	78	—
Dopamine-β-hydroxylase + dopamine	16	?
Dopamine-β-hydroxylase + ferricyanide	100	—
Dopamine-β-hydroxylase + ferricyanide + tyramine	100	+
Dopamine-β-hydroxylase + ferricyanide + octopamine	100	+
Dopamine-β-hydroxylase + fumarate	93	—

not result in any further increase in signal amplitude or any major change in signal shape. Therefore, the increase in amplitude seen upon the addition of tyramine or octopamine can be qualitatively ascribed to an apparent oxidation of the copper rather than a change in the signal shape.

Friedman and Kaufman [4], having reported that the addition of fumarate increased the amplitude of the EPR signal of isolated dopamine-β-hydroxylase, speculated that the absence of oxygen such an increase should not occur. We tested this by degassing the protein solution and flushing with argon before fumarate was added either in the presence or absence of air. As can be seen by Table III, a 5-min incubation of fumarate in the presence or absence of air produced no increases in the signal relative to the control. Only when tyramine was added in the presence of air was there an intensification of the signal. However, when the incubation period was extended to fifteen minutes with a second protein preparation which was initially more oxidized, the addition of fumarate in the presence of air increased the amount of Cu^{2+} signal to 95% of maximum intensity. If the degassed enzyme was exposed to air or fumarate separately, little change in the signal amplitude resulted. Also, the addition of fumarate to the enzyme in the presence of excess ascorbate did not restore the signal, nor did its addition further increase the signal if tyramine was added first. The enzyme used in the second experiment was recycled; that is, after having been used for the first experiment, it was reused following dialysis, concentration and freezing. This fact may also affect the accessibility of the enzyme copper to oxygen, especially in the presence of fumarate.

An examination of the EPR spectrum of high specific activity (31 μmol/min mg) dopamine-β-hydroxylase (Fig. 2) showed that upon integration 4.03 gatoms of copper per tetramer were associated with the fully oxidized enzyme

TABLE III

EFFECT OF FUMARATE AND SUBSTRATE ON Cu^{2+} EPR SIGNAL HEIGHT OF DOPAMINE-β-HYDROXYLASE

The experimental procedure was similar to that in Fig. 1. Anaerobic technique was employed except where indicated that the sample was exposed to air (see Materials and Methods). Samples contained 1.7 mg of protein, 10 μmol of potassium phosphate buffer, pH 7.0, and where indicated, 5 μmol of fumarate, 88 nmol of ascorbate, 88 nmol of potassium ferricyanide and 4 μmol of tyramine in 0.22 ml.

Sample	% Maximum signal height at $g_\perp$	
	Experiment 1 5 min incubation	Experiment 2 15 min incubation
Dopamine-β-hydroxylase * only	56	70
Dopamine-β-hydroxylase + ascorbate	0	0
Dopamine-β-hydroxylase + ferricyanide	100	100
Dopamine-β-hydroxylase + air	56	74
Dopamine-β-hydroxylase + fumarate	54	75
Dopamine-β-hydroxylase + fumarate + air	54	95
Dopamine-β-hydroxylase + fumarate + ascorbate	—	0
Dopamine-β-hydroxylase + tyramine + air	100	95
Dopamine-β-hydroxylase + tyramine + fumarate + air	70	93

* All Dopamine-β-hydroxylase samples were initially degassed

signal. A similar value of 3.96 gatoms of copper was obtained by chemical determination using the copper reagent bathocuproine. Since all the enzyme copper can be fully reduced by ascorbate and fully oxidized by potassium ferricyanide or by oxygen in the presence of substrate, product or fumarate, it is suggested that a four electron transfer is involved when dopamine-β-hydroxylase goes from a fully reduced state to the fully oxidized state. A direct titration of the enzyme with potassium ferricyanide should confirm this. Because dopamine-β-hydroxylase had no visible absorption, changes in the EPR signal height at $g_{\perp}$ were used to measure the extent of Cu^{2+} oxidation. During the course of the titration, no new signals nor the change in the signal shape appeared. As shown in Fig. 4, we obtained a linear titration curve, starting with the isolated form of the enzyme with 34% of the maximum signal intensity and going to an oxidized form of the enzyme with 82% of the maximum signal intensity. This difference in signal intensity was used to calculate the number of electrons which could be removed from fully reduced enzyme. Since potassium ferricyanide is a one-electron acceptor, it was determined that approximately 6 electrons could be removed from fully reduced enzyme that had only 3.2 gatoms of copper per mol. This preparation of dopamine-β-hydroxylase had about 77% of the activity and about 80% of the copper of our most active enzyme preparation. Since it gave a single band upon disc gel electrophoresis, it appears that apoprotein was present. When the enzyme was first reduced with ascorbate (Fig. 4, insert) and then titrated with potassium ferricyanide to about

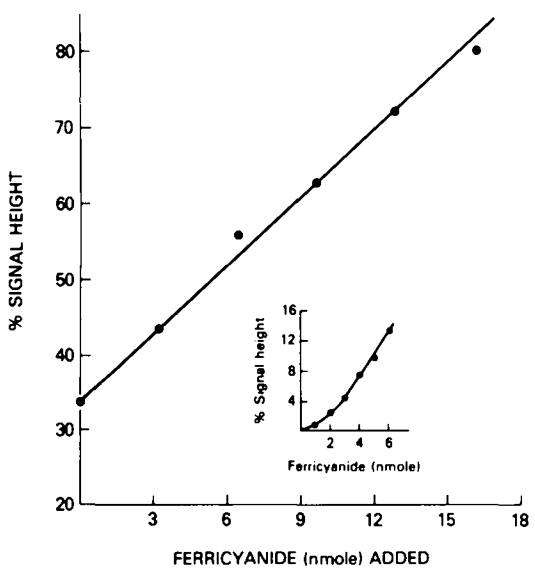

Fig. 4. Anaerobic titration of dopamine-β-hydroxylase with potassium ferricyanide. Each tube contained 1.6 mg of protein and 10 μmol of potassium phosphate buffer, pH 7.0, in 0.22 ml, previously degassed and flushed with argon. Aliquots of deoxygenated potassium ferricyanide were added as indicated. Maximum signal height at $g_{\perp}$ was determined using a 10-fold excess of potassium ferricyanide and subtracting any contribution to the signal when the protein was in the presence of ascorbate. Insert, each tube contained 1.7 mg of protein and 10.5 μmol of potassium phosphate buffer, pH 7.0, in 0.22 ml. The protein was initially reduced with ascorbate prior to titration with potassium ferricyanide. Modulation amplitude, 20 G; sweep time, 2 min; microwave power, 6 mW. Other experimental details were the same as in Fig. 1.

13% of the maximum signal intensity, a value of 5.7 electrons per mole of enzyme was calculated using the linear portion of the curve.

It was of interest to determine whether octopamine could be formed when tyramine alone was added to isolated enzyme that was partially oxidized and, if so, whether the amount of octopamine formed would be stoichiometric. Ideally, one should expect one mole of product formed for every two Cu^{2+} oxidized to Cu^{2+} [7]. However, when this type of experiment was performed, we consistently found more octopamine produced than could be accounted for by reduced enzyme copper present. This observation is consistent with the excess number of electrons found by titrating the reducing enzyme with potassium ferricyanide.

An important aspect in understanding the mechanism of the dopamine-β-hydroxylase catalyzed reaction is the determination of the number of reducible sites on the enzyme and the potential at which electrons are transferred from these sites. Much of this information must await direct potentiometric titrations of the enzyme. However, an estimation of the midpoint potential can be made by using the EPR Cu^{2+} signal as a probe to monitor the extent of copper oxidation after equilibrium has been established between the enzyme copper and a reducing mediator. Hydroquinone, which we found to be about 3% as efficient as ascorbate in the standard assay for dopamine-β-hydroxylase, was

TABLE IV

ESTIMATION OF MIDPOINT POTENTIAL OF DOPAMINE-β-HYDROXYLASE

Anaerobic technique was employed for these experiments (see Materials and Methods). Each sample contained 1.7 mg of protein and 10 μmol of potassium buffer, pH 7.0, in 0.22 ml. "As isolated" in this instance refers to native protein which has been degassed and flushed with argon. Additional experimental detail is contained in Materials and Methods.

Electron donor: dopamine-β-hydroxylase *		% Oxidation of dopamine-β-hydroxylase		E_H of system ** (mV)		E'_0 of dopamine-β-hydroxylase *** (mV)	
		As isolated	After equilibrium	A	B	A	B
Hydroquinone							
1	1 : 1	49	22	+273	+290	+307	+324
2	1 : 1	4	2	+229	+238	+325	+334
3	1 : 1	29	9	+265	+280	+326	+341
4	2 : 1	29	7	+255	+265	+321	+332
5	3 : 1	29	7	+249	+259	+318	+328
6	21 : 1	59	20	+243	+248	+279	+284
Ferrocyanide							
1	100 : 1	48	39	+283	+296	+295	+308
						310 + 16 †	321 + 18 †

* Based on tetramer molecular weight of $2.9 \cdot 10^5$.

** Extent of donor oxidation calculated from electrons accepted by dopamine-β-hydroxylase, based on (A) copper content, or (B) electrons titrated with ferricyanide.

*** Assuming that $n = 1$.

† Average ± S.D. ($n = 7$).

added anaerobically to the enzyme, with hydroquinone to enzyme ratios varying from 1 to 1 to 21 to 1 (Table IV). The reduction was performed using dopamine-β-hydroxylase at different initial levels of oxidation. Potassium ferrocyanide, added anaerobically to dopamine-β-hydroxylase at a ratio of 100 : 1, was used as an electron donor in one instance.

After the addition of hydroquinone or potassium ferrocyanide, differences in the signal height in the perpendicular region of the EPR spectrum were used to calculate the extent of electron donor oxidation as well as enzyme reduction. The Nernst equation [10] was used to calculate the E_H of the system and E_o' (midpoint potential of enzyme at pH 7.0). Since there was some discrepancy between the amount of copper present in dopamine-β-hydroxylase and the number of electrons available for titration, calculations for estimations of E_H and E_o' were based on (a) copper content, or (b) electrons titrated with potassium ferricyanide. For example, in experiment 1 with hydroquinone or potassium ferrocyanide as the electron donor, the copper content was 3.6 gatoms of copper per mol of enzyme; in experiment 2 thru 5, 3.2 gatoms of copper; in experiment 6, 4.3 gatoms of copper. A value of 6 electrons per mol of enzyme was used when calculations of E_H and E_o' were based on titratable electrons. The average values for the midpoint potentials obtained by the two different methods of calculation differ only by 11 mV.

Discussion

In this paper it was shown that valence changes in enzyme copper can occur under a variety of conditions without concomitant hydroxylation of a substrate. It was also shown that the changes which occur in the EPR spectrum of dopamine-β-hydroxylase can be related to the structural differences of substrate analogues. In addition, we have monitored the EPR signal of the enzyme after addition of reductant to estimate the potential at which electrons are transferred from the copper center.

All the copper in dopamine-β-hydroxylase which can be determined chemically can be accounted for by integration of the fully oxidized EPR signal of the enzyme. In our most active preparations each tetramer of enzyme contains about 4 gatoms of copper, which can be fully reduced by ascorbate and fully oxidized by potassium ferricyanide or by oxygen in the presence of fumarate, substrate or product. It would seem likely then that the ligand environment of all the copper atoms in dopamine-β-hydroxylase is equivalent.

The appearance of several superhyperfine lines with a splitting of about 15 G at the perpendicular region of the EPR spectrum of dopamine-β-hydroxylase upon the addition of tyramine or octopamine suggests the involvement of some nitrogen ligands in the binding of the copper [12]. Some of the lines could be due to the hyperfine interaction with the copper nucleus. However, since only one copper nucleus is apparently associated with the paramagnetic center (as is evident from the four hyperfine lines in the low field parallel absorption), other magnetic nuclei, such as ^{14}N, must be involved to account for the observed 6—8 hyperfine lines in the perpendicular absorption. The nitrogen nucleus ^{14}N can show a hyperfine splittings of similar magnitude as the ones due to copper [13]. Either the nitrogen from the substrate or product amine group or nitro-

gen from amino acid residues (e.g. imidazole group of histidine) could give rise to the observed superhyperfine lines. However, data from nuclear magnetic resonance experiments in this laboratory and others [11] show that tyramine does not affect the proton relaxation rate enhancement of solvent by protein-bound copper.

A more likely explanation for the substrate and product induced EPR spectral changes would be a change in protein ligand conformation around the copper center(s). In addition, superhyperfine splitting seen with the addition of tyramine and octopamine but not with the substrate, phenethylamine, implicates the involvement of the 4-hydroxyl group on tyramine and octopamine as playing an indirect but essential role in the appearance of these lines.

Craine et al. [3] have presented a scheme for the mechanism of dopamine-β-hydroxylase catalyzed hydroxylation, which shows molecular oxygen in equilibrium with an intermediate which is a reduced enzyme-oxygen complex. According to this scheme, a resonance exists between limiting forms consisting of one in which the electrons are apparently localized on the bound oxygen to form an enzyme copper peroxide complex. No comment was made about the predicted EPR properties of these intermediates although the second species was depicted with copper in the cupric state. We were therefore curious as to whether deoxygenation of dopamine-β-hydroxylase would affect the intensity or shape of the copper signal. As can be seen in Table I, degassing (deoxygenating) the enzyme consistently resulted in an appreciable decrease in the signal amplitude. Although it is beyond the scope of this paper to extract from this simple experiment any information about the nature of the proposed reduced enzyme-oxygen complex, it does not appear that oxygen is reversibly bound in an enzyme-oxygen complex. Presumably, deoxygenation of the enzyme shifted the equilibrium towards an EPR undetectable form.

There are, of course, many basic questions which must be answered about the proposed enzyme-copper-oxygen complex put forth by Craine et al. [3]. There is no evidence to show that oxygen is bound to the enzyme copper in an intermediate complex. As to the paramagnetic nature of such a complex it is probable that any cupric ion bound to oxygen would be either EPR undetectable or would give rise to an EPR signal of a different line shape.

How then is a decrease in signal amplitude upon deoxygenation of dopamine-β-hydroxylase to be explained? A possibility is that some of the cupric ions may be in such close proximity that spin coupling is likely. We must assume then that there may be more copper present than we can measure in our chemical determinations. This would mean that the EPR may not be totally quantitative because of spin-spin coupling. It can be postulated that oxygen binding may change the conformation of the protein sufficiently to eliminate spin-spin coupling of two cupric atoms and that when oxygen is removed, the copper atoms assume a position to allow spin-spin coupling and the disappearance of the EPR signal.

The data from the titration of dopamine-β-hydroxylase with potassium ferricyanide also seems to present more questions than it readily answers, yet there is a thread of consistency which may reveal some insights into the enzyme mechanism. The problem, as previously mentioned, is that although the enzyme titrates in an apparently linear fashion, it appears to donate more

electrons than it has copper atoms. There is no evidence yet that dopamine-β-hydroxylase contains other prosthetic groups or that oxidation of sulfhydryl groups by potassium ferricyanide is occurring; if this were the case, the titration would be expected to deviate from linearity. Whatever the source of the extra electrons, they must feed into the copper at the same potential at which electrons are transferred from copper. Again, an explanation which is consistent with the decrease in EPR signal upon deoxygenation of the enzyme is the possibility that the extra electrons are localized on copper atoms, which in the cupric form are spin coupled. However, it would be unusual for such copper to be titrated at the same potential as the EPR detectable copper; one would expect a deviation from linearity in the titration with ferricyanide either at the onset or towards the end of the titration.

An alternative explanation of finding excess electrons over copper is, of course, the possibility that dopamine-β-hydroxylase contains a second prosthetic group other than copper. If this were the case, we would also expect an excess of product over apparent reduced enzyme copper. While we have found no spectral evidence to substantiate the presence of a second prosthetic group, studies are currently underway to explore further this possibility.

In contrast to studies with the blue copper oxidases, little work has been done in determining the potential of the copper centers in Type 2 copper enzymes, of which dopamine-β-hydroxylase is apparently one. Reinhammar and Vänngård [14] estimated that the type 2 copper ion in laccase from *Rhus vernicifera*, has a potential of +390 mV at pH 7.5. Anaerobic titration of the EPR intensity of this copper with quinol was used to estimate the potential, since type 2 copper has no resolvable absorption band in the visible spectrum. Using similar techniques with hydroquinone, we have estimated the potential of dopamine-β-hydroxylase to be about +310 mV. Unlike the copper contained in laccase, all the copper atoms in dopamine-β-hydroxylase appear to be equivalent. Besides the type 2 copper, laccase contains a type 1 copper with a potential of +420 mV and a cupric-cupric pair with a potential of +460 mV at pH 7.5 [14]. Conceivable, the potential of the type 2 copper in laccase could be influenced in the positive direction by close association with the higher potential copper centers. Additionally, the ligand environment of enzyme bound metal atoms can affect their oxidation reduction potential. For example, the 2 iron-2 sulfur centers of putidaredoxin, adrenodoxin, and plant ferredoxins have potentials varying from −240 to −432 mV [15]. We feel that our estimated value for the potential of dopamine-β-hydroxylase falls in a reasonable range and that our assumption of $n = 1$ in the Nernst equation is warranted since this is a general characteristic of enzyme metallo-prosthetic groups [14,16]. However, we hope to be able soon to directly titrate the enzyme potentiometrically in order to determine absolute potential values.

Acknowledgements

The authors wish to thank Dr. Margaret N. Walker for her helpful discussions. The senior author (G.A.W.) was supported by a postdoctoral fellowship from the National Institutes of Health (F22HL0172001). Some of this work

was presented at the American Society of Biological Chemists Meeting in San Francisco, California, June 1976.

References

1 Levin, E.Y., Levenberg, B. and Kaufman, S. (1960), J. Biol. Chem. 235, 2080—2086
2 Creveling, C.R., Daley, J.W., Witkop, B. and Udenfriend, S. (1962) Biochim. Biophys. Acta 64, 125—134
3 Craine, J.E., Daniels, G.H. and Kaufman, S. (1973) J. Biol. Chem. 248, 7838—7844
4 Friedman, S. and Kaufman, S. (1966) J. Biol. Chem. 241, 2256—2259
5 Goldstein, M., Joh, T.H. and Garvey, III, T.Q. (1968) Biochemistry 7, 2724—2730
6 Wallace, E.F., Krantz, M.J. and Lovenberg, W. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2253—2255
7 Friedman, S. and Kaufman, S. (1965) J. Biol. Chem. 240, 4763—4773
8 Poillon, W.N. and Dawson, C.R. (1963) Biochim. Biophys. Acta 77, 27—36
9 Lowry, O.H. Rosebrough, N.J. Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
10 Clark, W.M. (1960) Oxidation-Reduction Potentials of Organic Systems, Chapters 1 and 14, Williams and Wilkins Co., Baltimore
11 Blumberg, W.E., Goldstein, M., Lauber, E. and Peisach, J. (1965) Biochim. Biophys. Acta 99, 187—190
12 Gersmann, H.R. and Swalen, J.D. (1962) J. Chem. Phys. 36, 3221—3233
13 Kuska, H.A. and Rogers, M.T. (1965) J. Chem. Phys. 43, 1744— 1747
14 Reinhammar, R.M. and Vänngård, T.I. (1971) Eur. J. Biochem. 18, 463—468
15 Gunsalus, I.C. and Lipscomb, J.D., (1973) In Iron-Sulfur Proteins, Vol. I (Lovenberg, W., ed.), pp. 151—171, Academic Press, New York, N.Y.
16 Eisenstein, K.K. and Wang, J.H. (1969) J. Biol. Chem. 244, 1720—1728