# Active Site of Dopamine β-Hydroxylase. Comparison of Enzyme Derivatives Containing Four and Eight Copper Atoms per Tetramer Using Potentiometry and EPR Spectroscopy†

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ABSTRACT: Potentiometric titrations, continuous wave EPR, and microwave power saturation measurements have been used to examine 8-Cu and 4-Cu forms of native dopamine β-hydroxylase and its azide derivative. The formation curve for the binding of $Cu^{2+}$ to the apoenzyme is best fit by assuming two independent binding sites per subunit, with p$K'$ values of 8.90 and 7.35 at pH 5.0. On the other hand, only minor differences are observed in either continuous wave EPR spectra or power saturation behavior of the 8- and 4-Cu forms of the native enzyme or of its azide derivative. The intensity of the EPR spectra of all derivatives integrates to >95% of the total copper, and the temperature dependence of $P_{1/2}$ shows no evidence for any $S = 1$ state of the copper ions in the enzyme. These results suggest a lower limit of ca. 7 Å for the separation between the two copper ions per subunit and thus rule out a type 3 site in the oxidized enzyme. The data are most consistent with Cu(II) sites consisting of two or three N (imidazole) and one or two O donor ligands in the coordination sphere. The similarity in EPR spectra and power saturation of 8- and 4-Cu derivatives suggests that the difference in Cu-binding constants may be due either to differences in the identity of an axial ligand or to solvation effects in the active site.

Dopamine β-hydroxylase (DBH) catalyzes the conversion of dopamine to noradrenalin in the sympathetic nervous system and thus plays an essential role in neurotransmitter biosynthesis (Villafranca 1981; Ljones & Skotland, 1984). In vitro, the enzyme has been shown to exhibit a broad substrate specificity that includes benzylic hydroxylation of aryl-substituted phenethylamines, ketonization of halophenethylamines (Miller & Klinman 1985; Mangold & Klinman, 1985; Bossard & Klinman, 1986), oxidation of phenylaminoalkyl sulfides and selenides to sulfoxides (May & Phillips, 1980; May et al., 1981) and selenoxides (May et al., 1987), epoxidation of olefins to alcohols (May et al., 1983; Padgette et al., 1985), and N-dealkylation of benzylic N-substituted analogues (Wimalasena & May, 1987). The reaction, which is of the monooxygenase type, consumes two electron equivalents, and consequently the enzyme has an absolute requirement for a reducing cofactor, which may be ascorbate, ferricyanide, or other one-electron reductant (Skotland & Ljones, 1980a; Skotland et al., 1980; Diliberto & Allen, 1981; Stewart & Klinman, 1987).

The broad substrate specificity has enabled detailed structure–reactivity correlations to be carried out on the hydroxylation step of the reaction. These studies in conjunction with the use of primary and secondary deuterium isotope effects (Miller & Klinman, 1985) have produced a remarkably detailed description of the transition state for substrate hydroxylation and thus of the mechanism of C–H bond activation. The latter has been shown to proceed via concerted homolysis of peroxide and C–H bonds, involving also an enzyme-bound proton. Indeed, formation of a radical species at the benzylic position is now believed to be a common feature of the reaction of the enzyme with a wide variety of substrates and mechanism-based inhibitors whose mode of action involves hydroxylation at carbon or a heteroatom at the benzylic position (Colombo et al., 1984; Fitzpatrick et al., 1985; Kruse et al., 1986; Goodhart et al., 1987; Wimalasena & May, 1987).

In contrast, the structure and coordination chemistry of the copper sites are less well understood. Evidence for imidazole coordination from histidine has been obtained from EXAFS spectroscopy, but the data were of insufficient quality to determine coordination numbers (Hasnain et al., 1984; Blackburn & Hasnain, 1986). Exogenous ligands such as azide and cyanide inhibit the enzyme via coordination to the copper centers (Blackburn et al., 1984), which leads to the displacement of a maximum of two bound water molecules (Obata et al., 1987). While it is known that reconstitution of apoenzyme with excess $Cu^{2+}$ followed by exhaustive dialysis against copper-free buffer results in binding of only four coppers per tetramer (one per subunit of 77 000 daltons) (Blackburn et al., 1984; Skotland & Ljones, 1979; Skotland et al., 1980), more recent work has established that the enzyme can bind additional copper and in fact requires a total of eight coppers per tetramer for maximal activity (Klinman et al., 1984; Ash et al., 1984; Colombo et al., 1984). The stoichiometry of two coppers per subunit would provide for the two-electron reduction of $O_2$ to form an enzyme-bound copper(II) hydroperoxide within a single active site, which is a feature of proposed mechanisms for the reaction (Miller & Klinman, 1985; May et al., 1981). The distance between the copper atoms in individual subunits is unknown, but no short-range magnetic interaction has ever been observed in native enzyme containing four coppers per tetramer (Walker et al., 1978; Skotland et al., 1980; Blackburn et al., 1984). There is unambiguous evidence that reduction to the Cu(I)

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form is a one-electron process (Diliberto & Allen, 1981) in which the reducing cofactor and amine substrate bind at separate sites on the protein (Stewart & Klinman, 1987). While these results argue against a binuclear site, detailed EPR spectral and power saturation data on the eight-copper form are required to establish the presence or absence of a copper–copper interaction at the active site.

In the present paper we report detailed EPR spectral comparisons of enzyme samples containing one and two coppers per subunit. Although small differences in binding constant are apparent between the two sites in the subunit, we have been unable to detect significant spectral differences or any evidence for short-range magnetic interactions between them. The results pose interesting questions as to the mode of copper binding and to the mechanism of electron transfer to oxygen, not least of which is the relatively weak binding of Cu(II) to the native enzyme.

## Materials and Methods

*Enzyme Isolation.* Dopamine $\beta$-hydroxylase was isolated from freshly dissected bovine adrenal medullae by using a modification of the method of Ljones et al. (1976). Medullae were frozen in liquid nitrogen and pulverized in a Waring blender. The cold powder was then suspended in 8 mM potassium phosphate buffer, pH 7.2, containing 25 $\mu$g/mL catalase (Sigma, 10000–20000 units/mg) in order to protect against enzyme inactivation by $H_2O_2$ produced as the result of autoxidation of ascorbate and catecholamines. A similar procedure has recently been described by Colombo and coworkers (1987), who have shown that addition of catalase is essential in order to protect against enzyme inactivation in the early stages of the isolation procedure. After poly(ethylene glycol) precipitation and washing as described in Ljones et al. (1976), the protein pellet was resuspended in 50 mM potassium phosphate, pH 7.5, containing 25 $\mu$g/mL catalase and passed rapidly through a column of DEAE-cellulose (Whatman, DE52) at a flow rate of 100 mL/h. Under these conditions the DBH activity passes through the column, but lipid and more acidic proteins are retained. The eluate was then adjusted to pH 6.5 and the ionic strength increased to 200 mM with solid sodium chloride. The enzyme solution was then applied to a column of Con-A–Sepharose (Pharmacia), washed with start buffer until $A_{280}$ of the washings dropped below 0.005, washed with 20 mM triethanolamine buffer, pH 7.3, and finally eluted at room temperature with triethanolamine buffer, pH 7.3, containing 10% methyl $\alpha$-mannoside. Fractions containing activity were pooled and passed directly onto a column of Q-Sepharose (Pharmacia) equilibrated to 20 mM triethanolamine chloride, pH 7.3. After washing to $A_{280}$ <0.005, the enzyme was eluted with a linear salt gradient of sodium chloride from 0 to 0.40 M. The broad band that eluted from the column between 50 and 150 mM sodium chloride was essentially homogeneous DBH as judged by PAGE. The enzyme was stored frozen in liquid nitrogen.

Enzyme activity was measured by monitoring oxygen consumption at 25.0 ± 0.3 °C using a polarographic $O_2$ electrode (Rank Brothers, Cambridge, U.K.). The constituents of the assay were as follows: sodium acetate buffer, pH 5.07, 200 mM (5.00 mL); catalase, 2500 units; $Cu(NO_3)_2$, 2 $\mu$M; tyramine, 20 mM; ascorbate, 10 mM; and enzyme. The reagents, excluding the ascorbate, were equilibrated at 25.0 °C under atmospheric oxygen (250 $\mu$M), and the reaction was initiated by addition of the ascorbate. Specific activities of the enzyme samples used in this study ranged from 11 to 14 $\mu$mol of $O_2$ consumed $min^{-1}$ (mg of enzyme)$^{-1}$ at 25 °C.

Apoenzyme was prepared via dialysis against 1 mM EDTA followed by exhaustive washing by ultrafiltration using a YM100 membrane in an Amicon 8200 ultrafiltration cell. The buffer used in the washing procedure was 100 mM acetate, pH 5.5, containing 1 mM $CaCl_2$. The apoenzyme was reconstituted by slow addition of ca. 10 molar equiv of $^{63}Cu(NO_3)_2$ in 100 mM acetate buffer, followed by dialysis against the same buffer containing 5 $\mu$M $^{63}Cu(NO_3)_2$. Copper analysis on the resulting preparation was performed by using a Perkin-Elmer atomic absorption spectrometer. Reconstituted enzyme prepared in this way typically contained 6.9–8.5 coppers per tetramer and is referred to as 8-Cu DBH in this paper. Enzyme with a lower copper content (ca. 3.5–4.2 Cu/tetramer) was routinely prepared by dialysis against 100 mM acetate buffer, pH 5.5, which had been passed through a small column of Chelex 100 (Sigma) equilibrated to the same ionic strength and pH. This enzyme preparation is referred to as 4-Cu DBH. Total protein was determined by using the method of Lowry et al. (1951).

*Measurement of Formation Constants.* The activity of free aqueous $Cu^{2+}$ was determined by using either a $Cu^{2+}$ Selectrode (V. A. Howe, London, U.K.) or an Orion $Cu^{2+}$-sensitive electrode. Both electrodes were found to give a linear response in $Cu^{2+}$ concentration in the range $[Cu^{2+}] = 10^{-2}$–$10^{-6}$ M for standard $Cu^{2+}$ solutions prepared by dilution of a stock $Cu(NO_3)_2$ solution (1 M, pH 1). The linearity of the calibration curve was extended to $[Cu^{2+}] = 10^{-10}$ M by use of $Cu^{2+}$NTA (NTA, nitrilotriacetic acid) metal buffers, pH 4.75, as described by Ruzicka and Lamm (1971). The slope of the calibration curve was 29.63 mV/pCu (pCu = −log $[Cu^{2+}]$, since $a_{Cu^{2+}} = [Cu^{2+}]$ in this concentration range), indicating Nernstian response.

Formation constants for copper binding to apo-DBH were obtained by titration of the apoenzyme with $Cu^{2+}$. The method used employed a combination of equilibrium dialysis and $Cu^{2+}$ potentiometry as follows. Aliquots (1.00 mL) of apo-DBH (10.86 $\mu$M in enzyme, 1.25 $\mu$M in residual copper, 12.50 specific activity in the presence of excess $Cu^{2+}$ at 25 °C) were dialyzed for 17 h against 250-mL volumes of 100 mM acetate buffer, pH 5.00, containing 68 $\mu$M NTA and a concentration of $Cu^{2+}$ that was varied from 0 to 120 $\mu$M. The presence of the copper-binding ligand NTA served to buffer the free copper concentration at the appropriate levels for attainment of low and intermediate copper to protein equilibria while maintaining a sufficiently high total copper concentration for accurate determination by atomic absorption. It was not possible to obtain reliable results without the use of such a metal buffering system because of the effects of adsorption of $Cu^{2+}$ on the walls of the titration vessel and dialysis tubing and complexation with $OH^-$ and/or acetate buffer ions at pCu values above 6. A concern with the use of a copper buffering ligand such as NTA is electrostatic binding of the free anionic NTA ligand to the protein surface, as has been demonstrated for EDTA (Colombo et al., 1987). However, such effects are expected to be minimized in the present study by the large excess of acetate over NTA (0.068 and 100 mM, respectively). The temperature was maintained at 25.0 ± 0.3 °C. When dialysis was complete, the uncomplexed $[Cu^{2+}]$ ($Cu_F$) in the dialysate (in equilibrium with the $Cu^{2+}$ bound to the enzyme) was measured by using the $Cu^{2+}$-sensitive electrode. Total copper was determined both inside the dialysis bag ($Cu_{T'}$) and in the dialysate ($Cu_{T''}$) by atomic absorption spectrophotometry. If $Cu_B$ represents the concentration of $Cu^{2+}$ bound to the enzyme, and $E_T$ the total concentration of enzyme, then the degree of formation, $F$, can be expressed as

$$F = Cu_B/E_T \quad (1)$$

or

$$F = (Cu_{T'} - Cu_{T''})/E_T \quad (2)$$

A plot of $F$ versus pCu gives the experimental formation curve for the system.

*Calculation of Theoretical Formation Curves.* Binding of $Cu^{2+}$ to apo-DBH may be considered in terms of the equilibria

$$E + Cu^{2+} = E\text{–}Cu \quad \text{formation constant } K_1 \quad (3)$$

$$ECu + Cu^{2+} = E\text{–}Cu_2 \quad \text{formation constant } K_2 \quad (4)$$

$$ECu_{n-1} + Cu^{2+} = E\text{–}Cu_n \quad \text{formation constant } K_n \quad (5)$$

The formation constants $K_1$ to $K_n$ are defined by

$$K_n = [E\text{–}Cu_n]/[E\text{–}Cu_{n-1}][Cu^{2+}] \quad (6)$$

in the approximation that activity is equal to concentration, which is valid in the present study since measurements were carried out in the presence of a constant (0.100 M) concentration of acetate, pH 5.00, and the total concentrations of enzyme and copper species never exceeded $10^{-4}$ M. The degree of formation, $F$, can be derived from expressions for total protein concentration, $E_T$, and formation constants $K_1$ to $K_n$ as follows.

If $F$ = average number of bound $Cu^{2+}$ ions per enzyme molecule, then

$$F = \frac{([E\text{–}Cu] + 2[E\text{–}Cu_2] + \ldots + n[E\text{–}Cu_n])}{[E] + [E\text{–}Cu] + [E\text{–}Cu_2] + \ldots + [E\text{–}Cu_n]} \quad (7)$$

Substitution of mass action expressions for the $K_n$ leads to

$$F = \frac{(K_1[Cu] + 2K_1K_2[Cu]^2 + \ldots + nK_1\ldots K_n[Cu]^n)}{(1 + K_1[Cu] + K_1K_2[Cu]^2 + \ldots + K_1\ldots K_n[Cu]^n)} \quad (8)$$

The above equation relates the degree of formation to the uncomplexed copper concentration (pCu) and the formation constants for the system. Theoretical formation curves were calculated by using the expression and inputing a set of formation constants, $K_j$, and calculating $F$ as a function of pCu in the range pCu = 5–10. The $K_j$ were varied until the best fit to the experimental formation curve was obtained.

*Relationship between Constants.* In the present study a number of different models were examined with respect to relationships between constants.

(i) Two identical binding sites per subunit (or eight identical binding sites per tetramer): In this case $K_1$–$K_8$ must be related by statistical considerations. Binding of the first $Cu^{2+}$ can occur at any of the eight sites, but dissociation can only occur from a single site; on the other hand, only a single site is available for binding of the eighth $Cu^{2+}$, while dissociation can occur from any of eight sites. Applying these arguments leads to the relationship $K_n = K_1/n$ for statistical binding to eight identical sites.

(ii) Two nonidentical binding sites per subunit: This model leads to two independent constants, $K_1$ and $K_2$, with statistical binding among each set of four sites per tetramer.

(iii) Two nonidentical sites per subunit with positive or negative cooperativity: In this case, no prior constraints can be imposed on the magnitude of the eight constants. This situation leads to too many variable parameters in the fit to the experimental data such that a unique solution could not be obtained.

*EPR Measurements.* EPR spectra were recorded on a Varian E112 spectrometer operating either at X-band (ca. 9.25 GHz) or Q-band (35.0 GHz) for samples contained in narrow-bore silica tubes as described by Garner et al. (1972). Relative values of the microwave power were read directly from the attenuation meter on the Varian E102 microwave bridge. Sample cooling was achieved at X-band by using an Oxford Instruments ESR9 continuous flow cryostat, Harwell DT temperature controller, and VC flow controller with cooling effected by liquid nitrogen (for temperatures from 300 to 77 K) or liquid helium (77 to 3.6 K). At Q-band a sample temperature of 150 K was achieved by immersing the cavity tuning rod in a narrow-necked Dewar containing liquid nitrogen. All the enzyme samples were reconstituted with pure isotope of copper ($^{63}Cu$) in order to eliminate the extra line broadening due to the different nuclear magnetic moments of the natural abundance $^{63}Cu$ and $^{65}Cu$ and facilitate spectral simulation.

Spectral simulation and saturation analysis were performed on the Amdahl 470/V7-CDC7600 computer system at the University of Manchester Regional Computer Centre, using programs that have been described elsewhere (Collision & Mabbs, 1982; Gahan & Mabbs, 1983).

## RESULTS

*Formation Constants for Copper Binding to DBH.* Titration of nitrilotriacetic acid with Cu(II) nitrate at pH 5.00 using the Orion $Cu^{2+}$-sensitive electrode gave results that followed the theoretical formation curve for the $Cu^{2+}$NTA system at this pH, indicating that the electrode behaves satisfactorily and gives Nernstian response. Some small deviations from Nernstian response were observed at $F$ values less than 0.1, and the early part of the formation curve is therefore expected to be less reliable in the enzyme titrations. (A formation curve for the $Cu^{2+}$NTA system documenting the electrode response is available as supplementary material.) Figure 1 shows experimental versus theoretical formation curves for copper binding to DBH, obtained by using the equilibrium dialysis method described above. The end point that best fits the data is calculated to be 8.3 coppers per tetramer or 2.08 per subunit. The theoretical curve shown in Figure 1a has been calculated by assuming a stoichiometry equivalent to two nonidentical sites per subunit ($pK_1 = 8.9 \pm 0.1$, $pK_2 = 7.35 \pm 0.1$) with copper binding in a statistical fashion among the two sets of four sites. In Figure 1b the theoretical fits have assumed two identical binding sites per subunit with statistical binding of the coppers among eight identical sites per tetramer, and a family of such curves with $pK_1$ values of 8.9, 8.4, 7.9, and 7.4 have been plotted. It is clear that the "one-site" model is unsatisfactory and that the results support strongly the model which assumes nonidentical copper-binding sites, with $pK$ values of 8.9 and 7.35, respectively. The difference in binding constants, however, is small and could arise either from a small difference in ligand environment or from differences in solvation at or near the copper centers. Nevertheless, these results suggest the presence of two nonidentical coppers per active unit and raise the possibility that the two coppers have different roles in the catalytic event. It should be noted, however, that an alternative interpretation is that two subunits of the tetramer have a higher affinity for copper than the other two, thereby fully loading two subunits before the other two.

Syvertsen and co-workers (1986) have reported results on the copper-binding equilibria in the DBH system that do not agree with the present work. They have found a single end point equivalent to 4 coppers per tetramer, with a formation constant which is dependent on the nature of the buffer ion present. It is worth noting that the method used in the present study, combining equilibrium dialysis and the use of a copper-sensitive electrode, eliminates any effects due to complex

Table I: $g$-Values and Hyperfine Splitting Constants Derived from Simulation of EPR Spectra of Various Forms of $^{63}$Cu-Substituted Dopamine Hydroxylase

| enzyme form | $g_z$ | $g_x$ | $g_y$ | $A_z$ (mT) | $A_x$ (mT) | $A_y$ (mT) |
|---|---|---|---|---|---|---|
| 8-Cu native X-band[a] | 2.277 | 2.051 | 2.037 | 15.5 | 1.20 | 0.90 |
| 8-Cu native Q-band[b] | 2.277 | 2.057 | 2.043 | 15.5 | 1.20 | 0.90 |
| 8-Cu azide X-band[a] | 2.246 | 2.049 | 2.032 | 15.5 | 2.30 | 0.90 |
| 8-Cu azide Q-band[b] | 2.246 | 2.053 | 2.041 | 15.5 | 2.30 | 0.90 |
| 4-Cu native X-band[a] | 2.276 | 2.046 | 2.034 | 15.7 | 1.05 | 1.15 |
| 4-Cu native Q-band[b] | 2.276 | 2.055 | 2.047 | 15.7 | 1.05 | 1.15 |
| 4-Cu azide X-band[a] | 2.241 | 2.042 | 2.036 | 16.0 | 2.20 | 0.80 |
| 4-Cu azide Q-band[b] | 2.241 | 2.049 | 2.036 | 16.0 | 2.20 | 0.80 |
| errors[c] | 0.003 | 0.005 | 0.005 | 0.3 | 0.5 | 0.5 |

[a] Measured at 77 K. [b] Measured at 150 K. [c] Values of any one of the parameters outside the limits quoted with the other parameters fixed at the values in the table give a simulated spectrum visibly different from that observed.

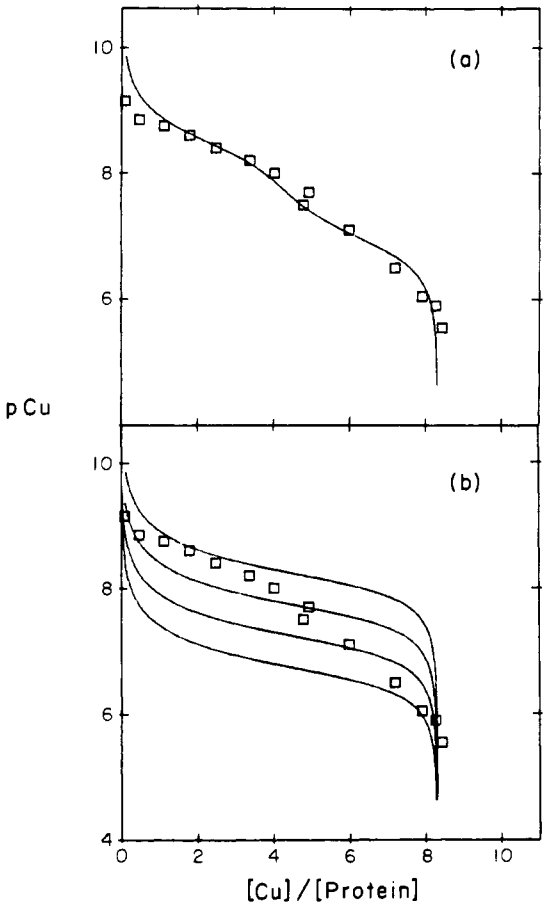


FIGURE 1: (a) Experimental (open squares) and theoretical (solid lines) formation curves for copper binding to dopamine $\beta$-hydroxylase. The theoretical curve was calculated by assuming two independent copper-binding sites per subunit ($pK_1 = 8.9$, $pK_2 = 7.35$) with statistical binding of copper among the two sets of four identical sites per tetramer. (b) Theoretical fits to the experimental formation curve assuming two identical binding sites per subunit with statistical binding among the eight identical sites per tetramer: (a) $pK_1 = 8.9$; (b) $pK_1 = 8.4$; (c) $pK_1 = 7.9$; (d) $pK_1 = 7.4$.

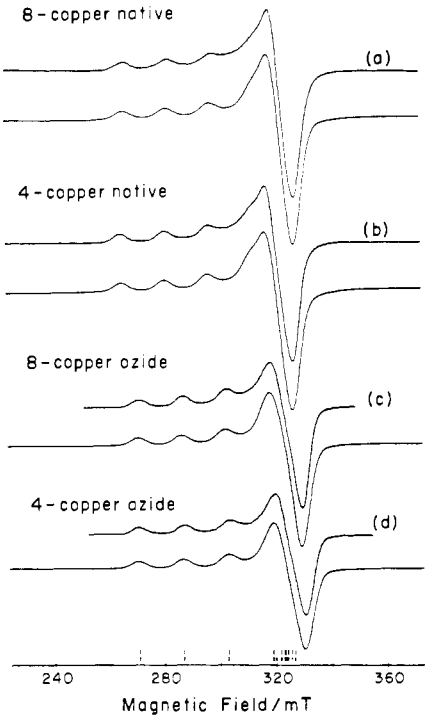


FIGURE 2: 9-GHz experimental and simulated EPR spectra of 8- and 4-$^{63}$Cu native dopamine $\beta$-hydroxylase and their derivatives treated with sodium azide. The simulated spectra are shown under each experimental spectrum: (a) 8-Cu native, 1.25 mM in total copper; (b) 4-Cu native, 0.921 mM in total copper; (c) 8-Cu azide, 1.25 mM in total copper; (d) 4-Cu azide, 0.921 mM in total copper. (Microwave frequency, 9.252 GHz; modulation amplitude, 1 mT; microwave power, 20mW; $T$, 77 K.)

formation between copper and the buffer ion, since the concentrations of $Cu^{2+}$ low molecular weight complexes are the same inside and outside the dialysis bag. While the results presented in the present work are in broad agreement with activity–stoichiometry relationships reported by Klinman et al. (1984) and Villafranca and co-workers (Ash et al., 1984; Colombo et al., 1984), the lack of agreement with Syvertsen et al. (1986) must remain unresolved.

*Electron Paramagnetic Resonance (EPR).* EPR studies were carried out on the 8-copper and 4-copper forms of DBH at both X-band (ca. 9.3) and Q-band (35.0 GHz) in order to probe further any structural and/or electronic differences between the individual copper atoms in the active unit. The frozen solution EPR spectra of the native enzyme and the azido derivative are shown in Figure 2 together with the results of spectral simulation. The integrated intensities of the X-band spectra of both the 8-Cu and 4-Cu forms shown in Figure 2 were 95 and 97%, respectively, indicating the absence of strong magnetic coupling between the two coppers of the subunit. The parameters necessary to reproduce the experimental spectra are given in Table I. The simulations were performed on the assumption of coincidence between the principal axes of the $g$- and $A$-tensors. The line-width components were computed by using a formalism that permitted a fractional ratio of Gaussian to Lorentzian line-shape functions. The values of the line widths and the line-shape ratios are available as supplementary material. Adequate simulations of all the spectra were achieved by using the criterion of "best fit by eye".

It has proved possible to obtain adequate simulations of all spectra on the basis that each contains only a single EPR-active species. Although comparison of equivalent X- and Q-band

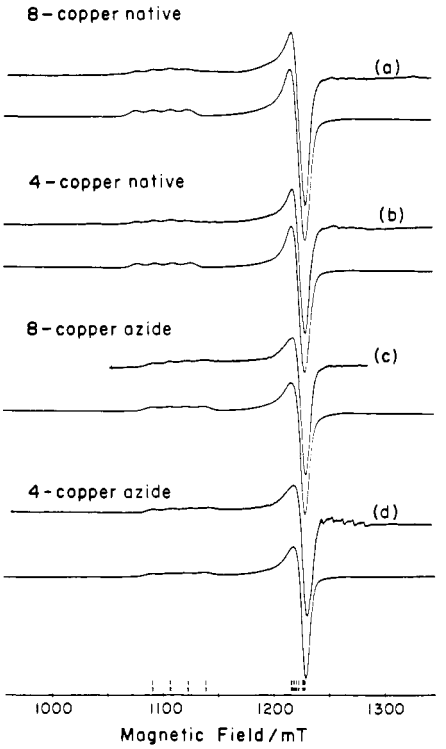


FIGURE 3: 35-GHz experimental and simulated EPR spectra of 8- and 4-$^{63}$Cu native dopamine β-hydroxylase and their derivatives treated with sodium azide. The simulated spectra are shown under each experimental spectrum: (a) 8-Cu native, 1.25 mM in total copper; (b) 4-Cu native, 0.921 mM in total copper; (c) 8-Cu azide, 1.25 mM in total copper; (d) 4-Cu azide, 0.921 mM in total copper. (Microwave frequency, 9.252 GHz; modulation amplitude, 1 mT; microwave power, 20mW; $T$, 77 K.)

spectra shows that in all cases the copper is not grossly distorted from axial symmetry, it has not been possible to obtain satisfactory simulations of the spectrum of any of the species without incorporating a small rhombic splitting of the $x$ and $y$ components of the $g$- and $A$-tensors. Whereas in all cases the values of the $z$ parameters are well-defined, there is a strong correlation between line width, line shape, and hyperfine splitting constants for the largely unresolved band envelopes in the $x$ and $y$ directions.

*Comparison of 8-Cu and 4-Cu DBH at X-band: Native Enzyme.* Direct visual comparison of 8-Cu and 4-Cu forms (Figure 2a,b) indicates that any distinction lies in the lowest field line, where there is an apparent low-field shoulder for the 8-Cu form that is absent in the 4-Cu form. This low-field feature is more clearly resolved at 35 GHz (see below). We find this to be a reproducible phenomenon between different preparations of the enzyme with EPR-detectable copper concentrations ranging from 6.5 to 8.1. Although it is possible that the low-field shoulder arises from the presence of a small amount of adventitious copper, this is thought to be unlikely since it is present at stoichiometries less than 8 per tetramer. In order to elucidate further the nature of this species, separate samples of 4-Cu and 8-Cu DBH were titrated with potassium iodide to a final concentration of 12 molar equiv of iodide per $Cu^{2+}$. This treatment failed to remove the low-field shoulder in 8-Cu DBH and in fact produced no change in any region of the spectrum of either form. The addition of iodide to adventitious $Cu^{2+}$ should cause reduction to EPR-nondetectable $Cu^{+}$. Apart from this low-field feature, only minor differences can be detected in the simulation parameters listed in Table I.

*Azide Derivatives.* Treatment of 8-Cu and 4-Cu forms with azide (Figure 2c,d) leads in both cases to a decrease in $g_z$ and

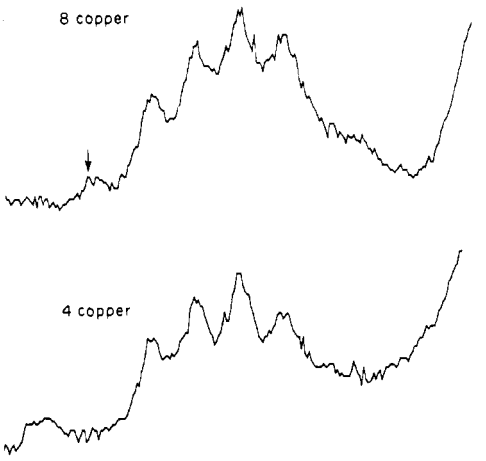


FIGURE 4: Expansion of the parallel region of the 35-GHz EPR spectra of 8- and 4-$^{63}$Cu native dopamine β-hydroxylase. Conditions as for (a) and (b) of Figure 3.

to an increase in the degree of rhombicity as indicated by the $x$ and $y$ parameters in Table I. No significant differences exist between the simulated $g$-values of either azide form, although a small increase in $A_z$ of 4-Cu azide is observed relative to the 8-Cu derivative.

*Comparison of 8-Cu and 4-Cu Forms at 35 GHz: Native Enzyme.* Spectra measured at 35 GHz are not as well resolved as their 9-GHz counterparts, as indicated by the increase in the line widths deduced from the simulations. Furthermore, this increase in line width appears to be anisotropic, being much more pronounced in the parallel than in the perpendicular region (Figure 3). This is probably due to $g$-strain since the parallel region is reported to be most sensitive to this effect (Cannistraro & Guigliarelli, 1986; Guigliarelli & Cannistraro, 1985; Hearshen et al., 1986).

Figure 4 shows a blow up of the parallel region of the 35-GHz spectra of the 8- and 4-copper forms of the native protein. A low-field feature (marked with an arrow) appears to be present in the 8-copper form but absent in the 4-copper form. As discussed above, this feature could arise from binding of adventitious copper to the protein surface, but in view of the measured difference in copper-binding constants, it is tempting to speculate that it arises from some structural or electronic difference between the copper atoms of the subunit. (The broad feature at lower field in the 4-copper spectrum is probably due to base-line drift since it is not observed at X-band.) However, the expanded spectra are of poor signal to noise ratio, and we would not wish to overinterpret these minor spectral differences.

A small shift in the values of $g_x$ and $g_y$ occurs from those obtained at X-band, which has been shown from X-band studies at 77 and 300 K to arise from a temperature dependence of the $g$-value on raising the temperature of measurement from 77 to 150 K. A similar dependence of $g$-value on temperature has been observed in laccase (Morpurgo et al., 1985). The addition of azide (Figure 3c,d) appears to increase the rhombicity of the spectrum. No significant differences exist in the simulation parameters of 8-Cu versus 4-Cu forms of either native or azido enzyme.

*Power Saturation Studies.* The power dependence of the EPR spectra of 4- and 8-Cu DBH, both native and azide treated, were measured at 3.6, 4.2, 9, 15, 27, 40, and 77 K at X-band frequency. Measurements were confined to the lowest field copper hyperfine line and the high-field perpendicular line of each spectrum. Since the band shape in each case changed little with incident power at any particular temperature, the signal intensity was taken as being propor-

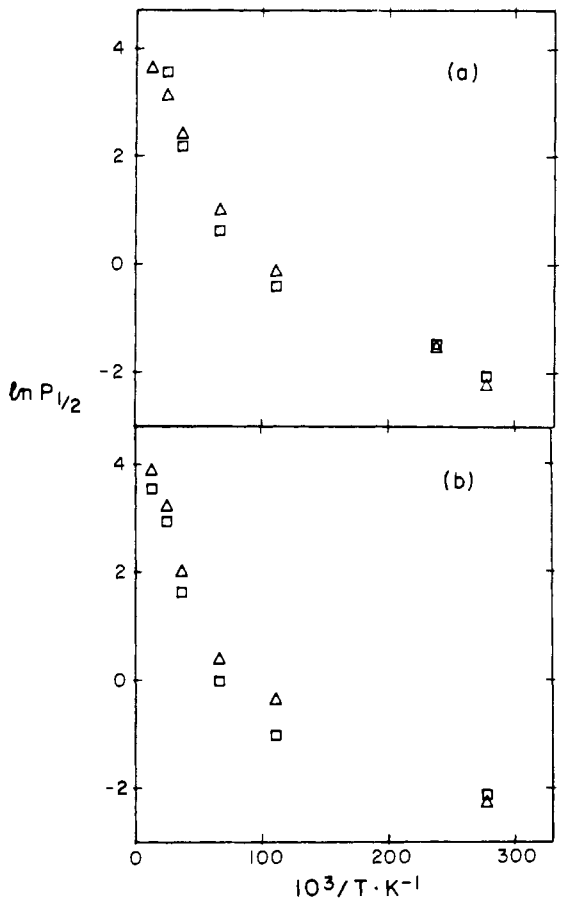


FIGURE 5: Temperature dependence of $P_{1/2}$ for (a) native dopamine $\beta$-hydroxylase and (b) the azide-treated derivatives. Squares represents data for 8-Cu enzyme; triangles represent data for 4-Cu enzyme. Saturation measurements were made at 3.6, 4.2, 9, 15, 27, 40, and 77 K.

tional to peak height per unit gain. The results obtained for native and azide-treated enzymes were analyzed from plots of log $(S/P^{1/2})$ versus log $P$, where $S$ is the signal amplitude and $P$ is the incident power. The analytical expression for the observables $S$ and $P$ (Rupp et al., 1978) is given in eq 9, where

$$S = CP^{1/2}(1 + P/P_{1/2})^{b/2} \quad (9)$$

$b$ is the inhomogeneity parameter, $P_{1/2}$ is the power at half-saturation, and $C$ is a proportionality constant.

Theoretical fitting of the saturation data of 8- and 4-Cu forms of DBH and their azide derivatives was achieved by using a nonlinear least-squares minimization fit to the experimental data for the adjustable parameters $P_{1/2}$ and $b$. The temperature dependence of the saturation parameter $P_{1/2}$ is presented in Figure 5, plotted as log $P_{1/2}$ versus $1/T$ (Makinnen et al., 1985). The results show that it is difficult to distinguish 4- and 8-Cu forms of the native enzyme on the basis of their relaxation behavior. On the other hand, the data suggest that the 4- and 8-Cu forms of the azide derivative may be distinct. Although the data are limited, there is no indication of linearity in the 5–30 K temperature regime; linearity would be expected if two Cu(II) sites exhibited a measurable degree of interaction, where the slope of the linear portion of the curve is proportional to the zero field splitting of the triplet-state level (Makinnen et al., 1985). We may infer that the copper centers are likely to be separated by a distance in excess of ca. 7 Å. (The fits to the experimental data obtained by using the curve-fitting procedure and the values of $P_{1/2}$ and $b$ derived therefrom for each enzyme derivative measured at the six different temperatures between 3.6 and 77 K are available as supplementary material.)

## Discussion

The results of quantitative studies of copper binding to DBH indicate that the two copper atoms of the subunit appear to be inequivalent, although the difference in binding constants is small. Furthermore, the magnitudes of both $pK_1$ and $pK_2$ are themselves small and imply relatively weak binding of $Cu^{2+}$ to the native enzyme. The weak binding poses interesting questions regarding the role of copper in the enzyme and the degree of copper loading to the enzyme in the intact chromaffin vesicle. Reference to the formation curves shown in Figure 1 indicates that a free copper concentration of at least $10^{-6}$ M is required to saturate the enzyme at pH 5 with its full complement of bound $Cu^{2+}$. However, the chromaffin vesicles are known to contain concentrations of ascorbate equivalent to ca. 20 mM (Ingebretson et al., 1980). It is well-known that free aqueous $Cu^{2+}$ ions catalyze the autoxidation of ascorbate, producing hydrogen peroxide (Zuberbuhler, 1981), which is itself a powerful irreversible inhibitor of DBH (Skotland & Ljones, 1980b). Thus, either some mechanism must exist to protect the enzyme from inactivation (high levels of catalase) or a separate system for active reconstitution of DBH must be present. It is interesting in this connection to mention the discovery of a small acidic non-blue protein, neurocuproin, that appears to be present in catecholamine storage vesicles of both adrenal medullae and adrenergic neurons (Grigoryan et al., 1981; Sharoyan et al., 1977), which may be a possible candidate for copper transfer to DBH. Of particular significance is the finding that aponeurocuproin is able to inhibit DBH activity, presumably by a mechanism related to copper chelation, and regulation of DBH activity has been suggested as a possible physiological role for the holoneurocuproin molecule (Markossian et al., 1986).

It is of interest to compare our measured $pK$ values for copper binding to DBH with literature values for copper binding in complexes of known structure. Histidine coordination has been implicated from EXAFS data (Hasnain et al., 1984), but while a satisfactory fit to the data could be obtained by using four coordinated imidazoles, the data were of insufficient quality to distinguish this fit from one involving mixed imidazole and oxygen donor ligands. Since the $pK$ values for the enzyme have been determined at only a single pH, they represent composite constants that include the $pK_a$s of the ligands which bind Cu in the active site. The true formation constant, $pK_M$, is related to the apparent constant $pK_M'$ measured at fixed pH, as shown in eq 10. Thus, com-

$$pK_M = pK_M' - \log (1 + K_1[H] + K_1K_{12}[H]^2 + \ldots + K_1\ldots K_{1n}[H]^n) \quad (10)$$

parison with literature $pK$ values requires evaluation of eq 10, which itself requires a knowledge of the $pK_a$ values of the copper-binding ligands. This information is unavailable at the present time, but the $pK_a$ values of histidine in proteins are known from NMR titrations to range from 4 to 8 depending on the degree of hydrogen bonding and hydrophobicity within the site (Markley, 1975). However, a simple calculation based on literature values of $\beta_1$–$\beta_4$ for imidazole binding to copper (Sillen & Martell, 1964, 1971), using an average $pK_a$ of 6.5 for histidine (Markley, 1975), shows that our values are certainly not inconsistent with a coordination environment containing histidine together with a number of additional O donor ligands. A more detailed discussion of these points will emerge from measurements of the pH dependence of the formation constants for copper binding to DBH, which are under way in our laboratory, but the values reported here are

entirely relevant to the catalytically competent copper site since they have been determined at the pH optimum for substrate hydroxylation.

The integrated intensity of 8-Cu native DBH has been found to be 95%. This result, together with the temperature dependence of $P_{1/2}$, effectively rules out any magnetic coupling between the two $Cu^{2+}$ ions of the subunit. The $g$- and $A$-values determined from simulation of the EPR spectra of the native enzyme are in the range expected for 2N–2O or 3N–1O according to the correlation diagrams of Peisach and Blumberg (1976). Furthermore, binding of azide results in a decrease in $g_{\parallel}$ and a small increase in $A_{\parallel}$ consistent with the substitution of an O donor by N from azide. Taken together, these considerations suggest a $Cu^{2+}$ polyhedron comprising two or three histidines with one or two O donor ligands also present in the basal plane of a coordination site of slightly lower than axial symmetry. This description is also entirely consistent with the recent water proton relaxation measurements of Obata and co-workers (1987), which have provided good evidence that the copper sites of oxidized native DBH are ligated to two water molecules, at least one of which is coordinated in an equatorial position.

The one spectral feature that distinguishes 8-Cu DBH from the 4-Cu enzyme would seem to be the low-field copper hyperfine feature present in the 8-Cu EPR spectrum but absent in the 4-Cu spectrum. The small increase in the $g_z$ or $A_z$ (or both) parameters required to produce such a feature would be consistent with the presence of a second Cu species containing one additional oxygen donor ligand (Peisach & Blumberg, 1976). However, any such assignment must be made with extreme caution, because despite the measured differences in p$K$s for $Cu^{2+}$ binding, the power saturation studies have failed to detect any important differences between the copper sites of the native enzyme. This may suggest that the differences in binding constants arise from differences in solvation and/or H-bonding within the active-site locus rather than from differences in the identity or site symmetry of coordinated ligands. Alternatively, the sites might be distinguished by the identity of a weakly binding axial ligand, which would have only a small effect on the EPR parameters. Miller and Klinman (1985) have concluded from deuterium isotope studies that an essential amino acid residue in the active-site cavity protonates the putative $Cu^{2+}$–$O_2^{2-}$ intermediate to release water, which emphasizes the mechanistic importance of solvent access to the copper site involved in oxygen atom transfer.

There is general consensus from a large number of studies relating to novel monooxygenase activities of DBH, including sulfoxidation and selenoxidation of phenylaminoalkyl sulfides and selenides (May & Phillips, 1980; Padgette et al., 1985), oxidative ketonization of halophenylethylamines (Klinman & Krueger, 1982; Mangold & Klinman, 1984), N-dealkylation of benzylic N-substituted analogues, and reaction inactivation caused by $p$-cresol (Goodhart et al., 1987), that a reactive Cu–oxygen intermediate attacks the benzylic position of all these substrates or suicide inhibitors in a regiospecific fashion to produce a cation radical which then partitions between reaction to products and enzyme inactivation, depending on the reactivity of the particular radical species. Kruse and co-workers (1986) have also demonstrated enhanced binding to the enzyme of bifunctional inhibitors that incorporate a Cu(I)-binding donor atom in addition to the substituted phenylalkyl structural entity common to the majority of substrates and suicide inhibitors. These studies argue forcibly for juxtapositioning of the Cu(I)–oxygen binding site and phenylalkyl substrate. On the other hand, the present work suggests that the two copper atoms in the active center are separated by a distance greater than 7 Å. Thus, unless a major conformational change occurs upon reduction, it is unlikely that both copper atoms are involved in the oxygen binding and substrate hydroxylation event, and we must consider a separate functional role for the second copper. The nature of this role has yet to be determined, but an electron-transfer center remains an attractive possibility, especially as binding of the reducing cofactor and substrate hydroxylation are known to occur at different sites within the enzyme (Stewart & Klinman, 1987). Further work is under way to gain insights into these aspects of DBH catalysis.

## Supplementary Material Available

Tables and figures examining the formation curve for the $Cu^{2+}$NTA system, the values of line widths and line-shape ratios, and the values of $P_{1/2}$ and $b$ (11 pages). Ordering information is given on any current masthead page.

**Registry No.** Cu, 7440-50-8; dopamine β-hydroxylase, 9013-38-1.

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# Fidelity of DNA Synthesis by the *Thermus aquaticus* DNA Polymerase

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ABSTRACT: We have determined the fidelity of in vitro DNA synthesis catalyzed at high temperature by the DNA polymerase from the thermophilic bacterium *Thermus aquaticus*. Using a DNA substrate that contains a 3'-OH terminal mismatch, we demonstrate that the purified polymerase lacks detectable exonucleolytic proofreading activity. The fidelity of the *Taq* polymerase was measured by two assays which score errors produced during in vitro DNA synthesis of the *lacZα* complementation gene in M13mp2 DNA. In both assays, the *Taq* polymerase produces single-base substitution errors at a rate of 1 for each 9000 nucleotides polymerized. Frameshift errors are also produced, at a frequency of 1/41 000. These results are discussed in relation to the effects of high temperature on fidelity and the use of the *Taq* DNA polymerase as a reagent for the in vitro amplification of DNA by the polymerase chain reaction.

Studies on the fidelity of DNA synthesis by purified DNA polymerases have provided useful insights into the factors that influence mutation rates. In addition, DNA polymerases are valuable reagents for a variety of molecular techniques which require in vitro DNA synthesis. Depending on the anticipated use of synthesized DNA, the fidelity of polymerization may be a significant factor in the choice of polymerase. The fidelity of DNA synthesis, at least for base substitution errors at single codons, has been described for several DNA polymerases useful in molecular biology, including *Escherichia coli* DNA polymerase I, T4 DNA polymerase, and reverse transcriptase from avian myeloblastosis virus (AMV)[1] [for review, see Loeb

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