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Magnetic Resonance Studies on the Copper Site of Dopamine β-Monooxygenase in the Presence of Cyanide and Azide Anions

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ABSTRACT: In order to elucidate the coordination state of water molecules in the Cu(II) site of dopamine [(3,4-dihydroxyphenyl)ethylamine] β-monooxygenase, measurements of the paramagnetic ^1H nuclear magnetic relaxation rate of solvent water in the enzyme solution containing cyanide or azide as an exogenous ligand were carried out to obtain the values of intrinsic paramagnetic relaxation rate decrements R_p^1 and R_p^2 for the ligand–enzyme 1:1 and 2:1 complexes, respectively. R_p^1 (percent) values were 53 (pH 5.5) and 52 (pH 7.0) for cyanide and 38 (pH 5.5) and 32 (pH 7.0) for azide, while R_p^2 (percent) values were 98 (pH 5.5) and 96 (pH 7.0) for azide. Although no R_p^2 values for cyanide were obtained because of its reducing power at the Cu(II) site, the R_p^1 and R_p^2 values obtained above prove that the Cu(II) center has two coordinated water molecules that are exchangeable for exogenous ligands at either pH. Supporting evidence was provided by electron paramagnetic resonance (EPR) titration, in which the enzyme solution containing cyanide–enzyme (1:1) complex in an equal proportion to uncomplexed enzyme gave an observed paramagnetic relaxation rate decrement, R_p, of 23%. Another characteristic of the R_p^1 and R_p^2 values was their invariability with respect to pH, indicating that the three-dimensional structure of the Cu(II) site is pH-invariant within the range examined. Binding constants of ligand to enzyme K_b^1 and K_b^2 for 1:1 and 2:1 complex formation, respectively, were also determined through an analysis of the R_p values; it was found that K_b^1 was larger than K_b^2 irrespective of pH. On the basis of these results, together with the axial-symmetric EPR parameters of the 1:1 complexes, a possible coordination geometry of the two water molecules in the Cu(II) site of the enzyme is suggested.

Dopamine β-monooxygenase (EC 1.14.17.1), an indispensable copper enzyme in the human brain, regulates neurotransmitter levels by catalyzing the conversion of dopamine to norepinephrine (Rosenberg & Lovenberg, 1980). Although numerous studies have shown that the copper moiety in the enzyme is essential for the catalysis (Villafranca, 1981), the structure of the copper site has not yet been established. Indeed, even after a recent extended X-ray absorption fine

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structure (EXAFS) study (Hasnain et al., 1984), which has shown that the structure of the copper(II) site is best interpreted as being composed of four equatorially coordinated imidazole groups with one or two axial oxygen atoms (Musinu et al., 1983), it has remained in dispute whether the copper(II) site really has coordinated water molecules or other coordination ligands.

A recent electron paramagnetic resonance (EPR) study (Blackburn et al., 1984) has indicated that cyanide and azide anions, both of which are inhibitors of the enzyme, could function as inner-sphere coordination ligands for the copper(II) site, suggesting the presence of a solvent (water) accessible locus in the site. Therefore, if one could estimate the amount of water molecules left in the inner-sphere locus upon increasing binding of the anions to the copper(II) site, an answer to the problem of water molecule coordination in the copper(II) site would be expected. Indeed, useful information on coordinated water at the paramagnetic metal center of metal proteins or metal enzymes has been obtained by investigating the paramagnetic effects exerted by the paramagnetic center on the ^1H nuclear spin relaxation rates of solvent water (Koenig et al., 1985; Barker et al., 1979).

Encouraged by these successes, we carried out a series of measurements of the ^1H nuclear magnetic relaxation rate of solvent water in the enzyme solution in the presence of the anions, and the results were theoretically analyzed to evaluate the number of water molecules ligated to the copper(II) site. The relaxation studies also allowed us to probe the structure of the copper(II) site at two different pH values by estimating the distance between the ligated water molecules and copper(II) from the transverse and longitudinal relaxation times of the water protons.

An EPR experiment on the specimens used in the relaxation study was also carried out in parallel to examine the consistency of the results with the conclusion based on the relaxation studies and to investigate the nature of the copper(II) site anation complexes.

We report here on these magnetic resonance studies, which have allowed us to deduce the number of water molecules ligated to the copper(II) site of dopamine β-monooxygenase.

Experimental Procedures

Preparation and Analysis of Enzyme Samples. Dopamine β-monooxygenase was isolated from chromaffin granules of bovine adrenal medulla according to the reported procedure (Foldes et al., 1972), except that only the soluble form of the enzyme was collected by avoiding the Triton X-100 solubilization of the membrane-bound form (Aunis et al., 1977) and that a Bio-Gel A-1.5m agarose column was applied as the final purification step. Purified enzyme solution phosphate buffered at 20 mM was dialyzed in a collodion bag under reduced pressure at 0 °C to give a concentration of about 1 mg of protein/mL. Subsequently, the copper of the enzyme was substituted with ^{63}Cu by addition of a large excess of $^{63}CuCl_2$, prepared from ^{63}CuO, to the enzyme solution followed by incubation of the mixed solution for 5 min at 25 °C for quantitative substitution (Skotland & Flatmark, 1983). Free copper ions remaining in the solution were removed by the use of a Chelex 100 column. The eluent was reconcentrated to 10 mg of protein/mL in a collodion bag and used as a routine sample for magnetic resonance measurements.

A small portion of sample solution was used for enzyme assay; it was electrophoretically pure, showing a single band in polyacrylamide gel electrophoresis. The specific activity was determined spectrophotometrically, by converting octopamine, produced enzymatically from tyramine, to *p*-hydroxybenzaldehyde in the presence of periodate as an oxidant, to be 40 μmol/(mg·min). This is comparable with the highest values obtained previously (Blackburn et al., 1980). Protein concentration was determined from the absorbance of the protein itself at 280 nm with $E^{1\%} = 12.4$ (Skotland & Ljones, 1977). The concentration thus obtained was confirmed by colorimetry (Bradford, 1976) of Coomassie Brilliant Blue complexes of the enzyme and of bovine serum albumin. Total copper was assayed on a Japan Jarell-Ash atomic absorption spectrometer. Most of the enzyme samples were found to contain copper in the range of four to six atoms per tetramer of enzyme (M_r 290 000). The copper contents were in good accordance with those determined spectrophotometrically by means of bathocuproinedisulfonate chelation to monovalent copper (Skotland & Ljones, 1979) after complete reduction of divalent copper to monovalent copper with an excess of ascorbate. The bathocuproine assay method could also be utilized to estimate the population of monovalent copper involved in the native form of the enzyme and formed in the course of its titration with CN^- or N_3^-. Native enzyme samples always included monovalent copper amounting to less than 10% of divalent copper.

Magnetic Resonance Measurements. Nuclear magnetic relaxation of water protons was observed at 100 and 270 MHz, by using JEOL PFT-100 and GX-270 spectrometers, respectively. Spin–lattice relaxation times (T_1) were determined by the inversion recovery technique and spin–spin relaxation times (T_2) by the Meiboom–Gill modification (Meiboom & Gill, 1958) of the Curr–Purcell technique. The contributions of paramagnetic relaxation rates ($1/T_{1p}$ and $1/T_{2p}$) were derived by subtracting, from the observed relaxation rates, the diamagnetic contributions ($1/T_{1d}$ and $1/T_{2d}$), which were determined with the enzyme sample of monovalent copper obtained by ascorbate reduction.

EPR experiments were carried out on a JEOL FE-3XG spectrometer driven at 100-kHz field modulation in the X-band. The copper spectra were usually recorded at 0.5 mT modulation amplitude and at 10 mW microwave power, where no extra line broadening was detectable.

NMR and EPR titration experiments were performed by adding 1-μL aliquots of concentrated NaCN or NaN_3 solution successively to 100 μL of the enzyme solution in a quartz tube (3-mm ID), with a Drummond micropipet. The concentrations of the constituents in the enzyme solution were redetermined at every step of the above procedures. For nuclear relaxation measurements, the quartz tube was inserted in a Pyrex tube (5-mm ID), with acetone-d_6 being placed between two tubes for field locking.

Reagents and Materials. Chelex 100, agarose A-1.5m, and Coomassie Brilliant Blue were purchased from Bio-Rad Laboratories and collodion bags (cutoff M_r 75 000) from Sartorius Co. All other reagents were of analytical grade. Inorganic inhibitors (NaCN and NaN_3) and buffer reagents [KH_2PO_4, K_2HPO_4, and 2-(*N*-morpholino)ethanesulfonic acid (MES)] were freed from heavy metal ions by treatment with Chelex 100. Deionized water (10^8 Ω/cm) was used for every step of sample preparation.

Theory

A paramagnetic site in water produces an increase of the nuclear spin relaxation rate of the water protons when a rapid coordination exchange of the water molecules occurs at the paramagnetic site. This paramagnetic effect is reduced in the case that the site is occupied by an ligand other than water. Thus, we may define this reduction of the paramagnetic effect, R_p, as

$$R_p = (1/T_{1p}^f - 1/T_{1p})/(1/T_{1p}^f) \quad (1)$$

Here T_{1p} is the longitudinal nuclear spin relaxation time of the protons of water at the paramagnetic centers in the presence of the ligands, and T_{1p}^f is T_{1p} in the absence of the ligands.

Considering the binding equilibrium between a paramagnetic site (M) and a ligand (L), the binding constants K_b^1, K_b^2, ... may be written

$$K_b^1 = [LM]/[L][M]$$

$$K_b^2 = [L_2M]/[L][LM] \quad (2)$$

Thus, the paramagnetic reduction R_p may be formulated as eq 3 by introducing R_p^1, R_p^2, ..., the intrinsic paramagnetic

$$R_p = R_p^1([LM]/[M]_0) + R_p^2([L_2M]/[M]_0) + \ldots \quad (3)$$

reductions for the binding complexes LM, L_2M, ..., respectively. $[M]_0$ is the total concentration of the paramagnetic site, M. If one restricts the binding only to the formation of LM, its concentration is given strictly by

$$[LM] = (1/2)[[L]_0 + [M]_0 + (1/\alpha K_b^1) - \sqrt{\{[L]_0 + [M]_0 + (1/\alpha K_b^1)\}^2 - 4[L]_0[M]_0}] \quad (4)$$

where $[L]_0$ is the total concentration of the ligand L, including the concentration of the conjugate acid H^+L with dissociation degree α, which is dependent on the pH of the medium. Thus, R_p can be evaluated from the first term of the right-hand side in eq 3 to give the optimum values of R_p^1 and K_b^1.

In the case that the binding also involves the formation of L_2M, we can assume that an excess of ligand over the paramagnetic site exists ($[L]_0 \gg [M]_0$), so that [LM] and $[L_2M]$ are given simply by

$$[LM] = \alpha K_b^1[L]_0[M]_0/(1 + \alpha K_b^1[L]_0 + \alpha K_b^1 \alpha K_b^2[L]_0^2)$$

$$[L_2M] = \alpha K_b^1[L]_0 \alpha K_b^2[L]_0[M]_0/(1 + \alpha K_b^1[L]_0 + \alpha K_b^1 \alpha K_b^2[L]_0^2)$$

and hence R_p (eq 3) is given by eq 5, which contains four

$$\frac{1}{R_p} = \left(\frac{1}{\alpha K_b^1(R_p^1 + R_p^2 \alpha K_b^2[L]_0)}\right)\left(\frac{1}{[L]_0}\right) + \frac{1 + \alpha K_b^2[L]_0}{R_p^1 + R_p^2 \alpha K_b^2[L]_0} \quad (5)$$

parameters, R_p^1, R_p^2, K_b^1, and K_b^2, to be determined. Note that, when only LM is formed, eq 5 reduces to give a linear relation between $1/R_p$ and $1/[L]_0$, from which K_b^1 and R_p^1 can be explicitly determined.

Next, let us consider a competitive binding equilibrium, i.e., binding in the presence of two kinds of ligand, L^A and L^B. Suppose that a large excess of L^B is added to M containing a minor proportion of L^AM. In this case, $L^A{}_2M$ is scarcely formed and the concentration of L^AL^BM also remains low relative to those of L^AM, L^BM and $L^B{}_2M$. Thus, the paramagnetic reduction R_p can be formulated:

$$R_p = R_p^{1A}([L^AM]/[M]_0) + R_p^{1B}([L^BM]/[M]_0) + R_p^{2B}([L^B{}_2M]/[M]_0) \quad (6)$$

where R_p^{1A}, R_p^{1B}, and R_p^{2B} are the intrinsic paramagnetic reductions for L^AM, L^BM, and $L^B{}_2M$, respectively. Denoting the dissociation degrees of the conjugate acids of L^A and L^B as α^A and α^B, respectively, and utilizing the condition $[L^B]_0 \gg [M]_0$, we obtain equations for $[L^AM]$, $[L^BM]$, and $[L^B{}_2M]$:

$$[L^AM] = \alpha^a K_b^{1A}[L^A]_0[M]/(1 + \alpha^A K_b^{1A}[M])$$

$$[L^BM] = \alpha^B K_b^{1B}[L^B]_0[M]$$

$$[L^B{}_2M] = \alpha^B K_b^{1B} \alpha^B K_b^{2B}[L^B]_0^2[M]$$

where K_b^{1A}, K_b^{1B}, and K_b^{2B} are the binding constants in the formation of L^AM and L^BM from M and $L^B{}_2M$ from L^BM, respectively. Note that, in the derivation of $[L^BM]$, the concentration arising from the possible process $L^AM + L^B = L^BM + L^A$ is neglected for the same reason that L^AL^BM is considered negligible. Reduction of [M], which is necessary for numerical evaluation of R_p, can be done by solving a quadratic equation[1] on [M], which is derived by inserting the above expressions for $[L^AM]$, $[L^BM]$, and $[L^B{}_2M]$ into the formula defining $[M]_0$.

Note that throughout the analysis M refers to the Cu(II) in the enzyme, L refers to CN^- or N_3^- and L^A and L^B refer to CN^- and N_3^-, respectively, in this study. Further, the intrinsic binding constants K_b, which are independent of pH, always appear in the form multiplied by the dissociation degree α. The term αK_b is thus an apparent binding constant, depending on pH.

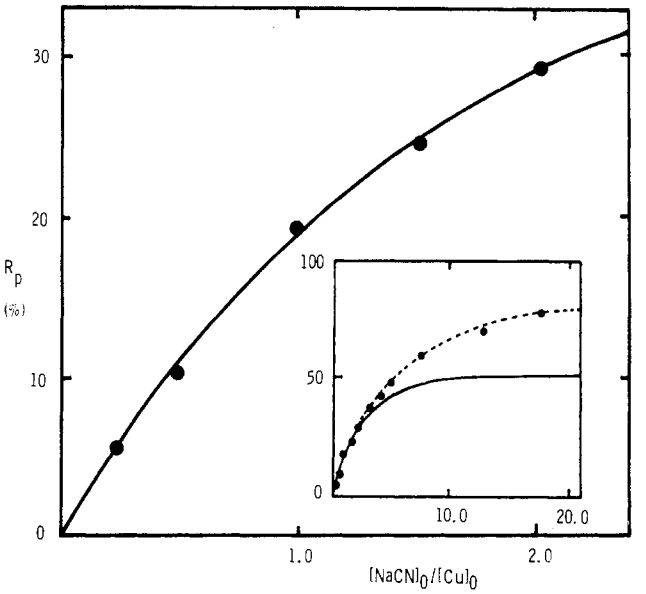

FIGURE 1: Correlation between paramagnetic reduction, R_p, and the ratio $[NaCN]_0/[Cu]_0$ in the ^1H nuclear spin–lattice relaxation of the enzyme solution containing NaCN. The inset shows the correlation extended to a higher range of $[NaCN]_0/[Cu]_0$ (abscissa). Closed circles, experimental points; solid line, theoretical curve based on eq 4. Enzyme solution: protein concentration 4.55×10^{-2} mM, $[Cu]_0$ 2.07×10^{-1} mM, and MES buffer 200 mM, pH 5.5. Titrations with NaCN were performed as described under Experimental Procedures.

RESULTS

^1H Nuclear Magnetic Relaxation Studies. R_p values obtained according to eq 1 by using T_{1p}'s that were determined for enzyme solutions containing NaCN were plotted against the ratio of total ligand concentration to total metal concen-

[1] Coefficients a, b, and c in the quadratic equation $a[M]^2 + b[M] + c = 0$ are

$$a = \alpha^A K_b^{1A} + \alpha^A K_b^{1A} \alpha^B K_b^{1B}[L^B]_0 + \alpha^A K_b^{1A} \alpha^B K_b^{1B} \alpha^B K_b^{2B}[L^B]_0^2$$

$$b = 1 + \alpha^A K_b^{1A}[L^A]_0 + \alpha^B K_b^{1B}[L^B]_0 + \alpha^B K_b^{1B} \alpha^B K_b^{2B}[L^B]_0^2 - \alpha^A K_b^{1A}[M]_0$$

$$c = -[M]_0$$

Table I: Binding Constants (K_b^1 and K_b^2) and Intrinsic Reduction of the Paramagnetic Relaxation Rates (R_p^1 and R_p^2) in the Binding of CN^- or N_3^- to the Cu(II) Site of Dopamine β-Monooxygenase

		pH 5.5	pH 7.0
CN^-–Cu(II)	K_b^1 (M^{-1})	8.67×10^6	7.49×10^5
	R_p^1 (%)	53	52
N_3^-–Cu(II)	K_b^1 (M^{-1})	5.55×10^2	1.78×10^2
	K_b^2 (M^{-1})	8.1	3.1
	R_p^1 (%)	38	32
	R_p^2 (%)	98	96

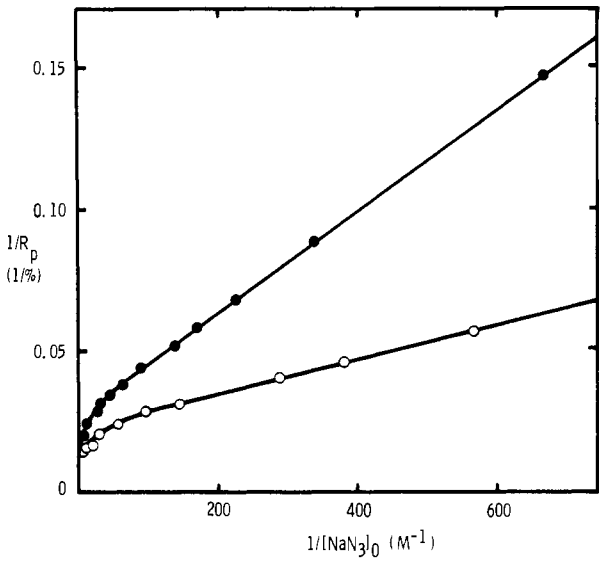

FIGURE 2: Correlation between the reciprocal of paramagnetic reduction, $1/R_p$, and the reciprocal of $[NaN_3]_0$ in the ^1H nuclear spin–lattice relaxation of the enzyme solution containing NaN_3. Closed circles, experimental points at pH 7.0 (200 mM potassium phosphate buffer); open circles, experimental points at pH 5.5 (200 mM MES buffer); solid lines, simulated curves based on eq 5. Enzyme solution: protein concentration 4.14×10^{-2} mM and $[Cu]_0$ 1.64×10^{-1} mM. Titrations with NaN_3 were performed as described under Experimental Procedures.

tration, i.e., $[NaCN]_0/[Cu]_0$, as indicated by closed circles in Figure 1 for the case of enzyme solution at pH 5.5. Care must be taken for analyzing these data by using eq 4 or eq 5, since it is well-known that an excess of cyanide in the enzyme solution causes a substantial reduction of Cu(II) to Cu(I) (Blackburn et al., 1984) to give a reduced R_p owing to the deparamagnetization of the site. The reduction of Cu(II) found by the determination of Cu(I) with bathocuproine in the enzyme solution containing NaCN at pH 5.5 reached 87% at $[NaCN]_0/[Cu]_0 = 17.5$ but was <5% at $[NaCN]_0/[Cu]_0 = 2$. Thus, the analysis using eq 4 was applied to the data within the ratio 2 to give the best fit as shown by the solid line in Figure 1; the line at higher ligand concentrations is drawn in the inset, showing an apparent deviation from the experimental points. A similar plot of R_p's vs. the ligand-to-metal ratios was also obtained at pH 7.0, and an analogous fitting procedure gave R_p^1 and K_b^1. The results are listed in Table I, together with those obtained at pH 5.5.

In the case of N_3^- ligand, no reduction of Cu(II) was observed over the whole range of ligand concentration, i.e., $10 \lesssim [NaN_3]_0/[Cu]_0 \lesssim 750$, so that the R_p values were determined by using eq 5 to give simulation curves (solid lines) that well described the experimental points (closed circles), as shown in Figure 2. Thus, the four constants R_p^1, R_p^2, K_b^1, and K_b^2 were determined at pH 5.5 and 7.0 as summarized in Table I.

Let us first consider the intrinsic R_p values, R_p^1 and R_p^2, the decrements of the paramagnetic effect for the LM and L_2M complexes, respectively. The R_p^1's, found to have approximately equal values at both pH's, indicated that CN^- bound singly to the Cu(II) site eliminated half of the total paramagnetic effect of the free Cu(II) site, whereas N_3^- bound singly to the Cu(II) site left about two-thirds of the total effect, reflecting a difference in the coordination strength of the respective ligands. In the case of CN^-, hampered by its reducing ability, we could not estimate the R_p^2 values. However, R_p^2's in N_3^- binding, found to have equal values at both pH's, showed that the Cu(II) site doubly ligated by N_3^- lost almost all of its paramagnetic influence, implying the presence in the Cu(II) site of two coordinated water molecules that are exchangeable for exogenous ligands.

Table II: Estimation of Cu(II)–H and Cu(II)–O Distances in the H_2O Coordination to the Cu(II) Site of Dopamine β-Monooxygenase[a]

	pH 5.5	pH 7.0
$1/T_{1M}$ (s^{-1})	3.82×10^4	4.36×10^4
$1/T_{2M}$ (s^{-1})	2.24×10^5	2.03×10^5
τ_c (s)	1.55×10^{-9}	1.35×10^{-9}
R_{Cu-H}^{av} (Å)	2.69	2.68
R_{Cu-O}^{av} (Å)	2.00	1.98

[a] The terms $1/T_{1M}$, $1/T_{2M}$, τ_c, R_{Cu-H}^{av}, and R_{Cu-O}^{av} are defined under Results.

Let us turn next to the binding constants, K_b's. In the case of CN^- binding, K_b^1 at pH 5.5 was larger by a factor of about 10 than K_b^1 at pH 7.0. A similar tendency was also seen in the case of N_3^- binding; that is, K_b^1 at pH 5.5 was larger by a factor of about 3 than K_b^1 at pH 7.0, and interestingly, the K_b^2's behave similarly, with approximately the same magnification factor, 3. It should be noted, however, that K_b^2's were always smaller than K_b^1's by the factor of about $^1/_{60}$ at either pH, indicating the difficulty of substituting the second water molecule of the two ligated to the Cu(II) site.

The remarkable pH effect observed with the K_b's stimulated us to examine the coordination distance of the water molecules to the Cu(II) site. Measurements of transverse nuclear spin relaxation rates of the water protons in enzyme solutions at pH 5.5 and pH 7.0 enabled us to estimate the distances, in combination with the longitudinal relaxation times, by utilizing the Solomon–Bloembergen equations (Solomon, 1955; Solomon & Bloembergen, 1956). Table II shows the results; the Cu(II)–H distances R_{Cu-H}^{av} were calculated on the assumption of an equivalence of the two water molecules ligated to the Cu(II) site, and the Cu(II)–O distances R_{Cu-O}^{av} were derived from R_{Cu-H}^{av}, by considering that the O atoms of the ligated water molecules were at the nearest allowable position to the Cu(II). As can be seen from the table, the pH dependences of the magnitudes of $1/T_{2M}$ and $1/T_{1M}$ (see footnote 2) are opposite, thus giving nearly the same rotational correlation time τ_c and hence almost the same distance at either pH. Thus, it can be concluded that pH effect in the K_b's does not arise from a change in the distance between Cu(II) and the coordinated water molecules. Rather, the immutability of the distances explains well the pH independence of R_p^1 and R_p^2.

EPR Studies. To confirm the inference obtained from the relaxation studies, EPR spectra of enzyme solutions containing various concentrations of the ligands were recorded. As was already observed, the EPR spectrum of the enzyme solution containing CN^- showed a new signal reflecting the formation

[2] T_{1M} and T_{2M} are the spin–lattice and spin–spin relaxation times per one Cu(II) atom, respectively, and are given by the relation $1/T_{1M} = 1/T_p(m_H/nm_{Cu})$, where m_H and m_{Cu} are the number of moles of water protons and that of Cu(II) in the specimen, respectively, and n is the number of water protons coordinated to the Cu(II) site.

Table III: EPR Parameters[a] of ^{63}Cu–Dopamine β-Monooxygenase Solution Alone (Native) and Containing NaCN (Native–NaCN) or NaN_3 (Native–NaN_3)

	$g_\parallel$	$g_\perp$	$A_\parallel$ (mT)
native	2.273	2.062	16.6
native–NaCN ($[L]_0/[Cu]_0$ = 1.25)			
component I	2.273	2.060	16.1
component II	2.218	2.025	17.0
native–NaN_3 ($[L]_0/[Cu]_0$ = 10)	2.249	2.056	16.4
native–NaN_3 ($[L]_0/[Cu]_0$ = 100)	2.242	2.056	15.8
native–NaN_3 ($[L]_0/[Cu]_0$ = 750)	2.241	2.053	15.2

[a] The parameters were determined on the basis of spectra shown in Figure 3.

of the complex with CN^- bound at the Cu(II) site (Blackburn et al., 1984). Therefore, in the case of CN^-, our attention was focused on the development of this new signal as $[NaCN]_0/[Cu]_0$ was increased. Figure 3A shows a series of these spectral changes of specimens buffered at pH 7.0. The nature of the spectral change was similar to that observed by Blackburn et al. at pH 5.5, and the changes were reproducible in spectra simulated by assuming proper combinations of two components I and II both with axially symmetric $g_\parallel$ and $A_\parallel$ tensors as given in Table III. Of these simulated spectra, the spectrum composed of equal proportions of component I and component II is shown by the dashed line in Figure 3A. It appears that the spectrum corresponding to equal proportions of the two components was obtained at an $[NaCN]_0/[Cu]_0$ ratio of 1.25, when R_p was 23.5%, indicating a loss of about one-fourth of the water molecules ligated to the Cu(II) site. This finding led naturally to the conclusion, in good accordance with the result of the relaxation study in the case of N_3^- binding, that component I represents the Cu(II) site with two water molecules and component II represents the Cu(II) site that has lost one water molecule as the result of ligation of one CN^-.

In the case of N_3^- binding buffered at pH 7.0, as shown in Figure 3B, no signal separation was observed, as was also the case at pH 5.5 (Blackburn et al., 1984). Thus, the spectra were simulated with a single component with axial-symmetric parameters, as summarized in Table III. If one assumes that the parameter changes do reflect the formation of an N_3^--bound complex at the Cu(II) site, an interesting difference in the parameters between the Cu(II)–CN^- and Cu(II)–N_3^- complexes appears. That is, the $A_\parallel$ value was larger in the former than in the native site, but smaller in the latter, although the $g_\parallel$ values were smaller in both complexes than in the native site. This tendency was the same for the parameters obtained at pH 5.5. These results suggest that there is a structural and/or electronic difference between the two complexes.

Thus, it may be important to discriminate whether these complexes are formed by binding of each ligand to the same locus or to different loci in the native Cu(II) site. To resolve this problem, titration of the Cu(II)–CN^- complex with N_3^- ligand by using the EPR spectra as a probe was carried out. As can be clearly seen from Figure 4, further addition of NaN_3 to the specimen with equal concentrations of components I and II resulted in spectra with a decreased amount of component II and with an increased amount of a lower magnetic field component III due to the formation of the Cu(II)–N_3^- complex. Qualitatively, this feature can be understood to indicate that the N_3^- ligand reversibly competes with the CN^- ligand to occupy the same coordination locus of the Cu(II) site.

Quantitative treatment of this competitive process was attempted by utilizing R_p values determined for specimens corresponding to various steps of the titration to give a hyperbolic trace of $1/R_p$ values against $1/[NaN_3]_0$ as shown by closed circles in Figure 5. In the figure, the solid line was obtained by using eq 6 with the K_b^{1A}, K_b^{1B}, K_b^{2B}, R_p^{1A}, R_p^{1B}, and

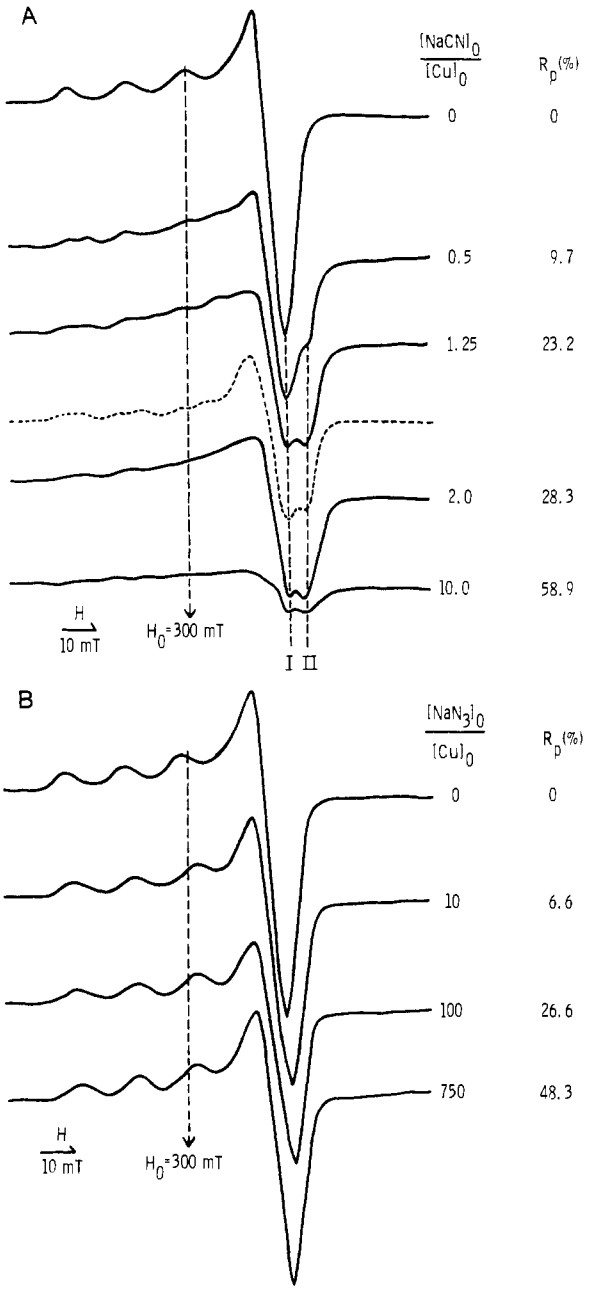

FIGURE 3: (A) Change of EPR spectra of ^{63}Cu–dopamine β-monooxygenase solution with increase of the $[NaCN]_0/[Cu]_0$ ratio. Enzyme solution: protein concentration 2.86×10^{-2} mM, $[^{63}Cu]_0$ 1.54×10^{-1} mM, and potassium phosphate buffer 200 mM, pH 7.0. Titrations with NaCN were performed as described under Experimental Procedures. The dashed line is the spectrum simulated on the basis that the solution consisted of equal proportions of component I and component II by using the parameters in Table III. EPR spectra were recorded at −150 °C as described under Experimental Procedures, and the parallel nuclear relaxation rate measurements were also carried out according to the same procedure as in Figure 1 to give the R_p values shown in the figure, along with the $[NaCN]_0/[Cu]_0$ ratio. (B) Change of EPR spectra of ^{63}Cu–dopamine β-monooxygenase solution with increase of the $[NaN_3]_0/[Cu]_0$ ratio. Enzyme solution: protein concentration 2.86×10^{-2} mM, $[^{63}Cu]_0$ 1.54×10^{-1} mM, and potassium phosphate buffer 200 mM, pH 7.0. Titrations with NaN_3 were performed as described under Experimental Procedures. EPR spectra were recorded at −150 °C as described under Experimental Procedures and the parallel nuclear relaxation rate measurements were also carried out according to the same procedure as in Figure 2 to give the R_p values shown in the figure, along with the $[NaN_3]_0/[Cu]_0$ ratio.

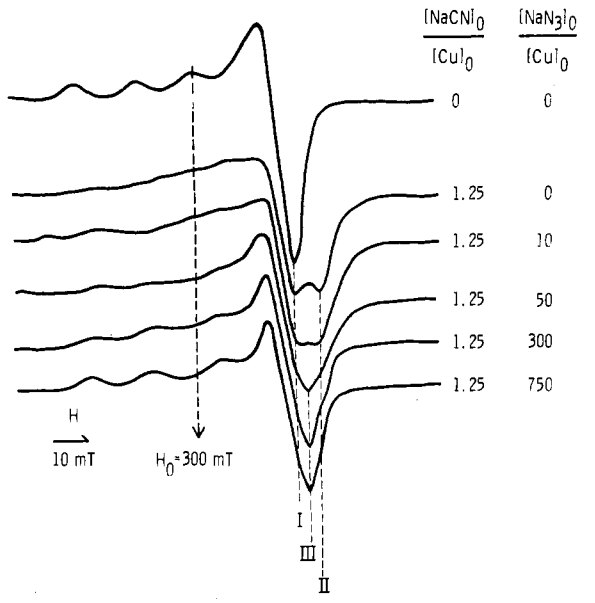

FIGURE 4: EPR titration of ^{63}Cu–dopamine β-monooxygenase–CN^- (1:1) complex with NaN_3. Enzyme solution: protein concentration 4.17×10^{-2} mM, $[^{63}Cu]_0$ 1.83×10^{-1} mM, and potassium phosphate buffer 200 mM, pH 7.0. Titration with NaN_3 was performed as described under Experimental Procedures except for the use of enzyme solution containing NaCN in the ratio of 1.25 for $[NaCN]_0/[Cu]_0$. EPR spectra were recorded at −150 °C as described under Experiment Procedures.

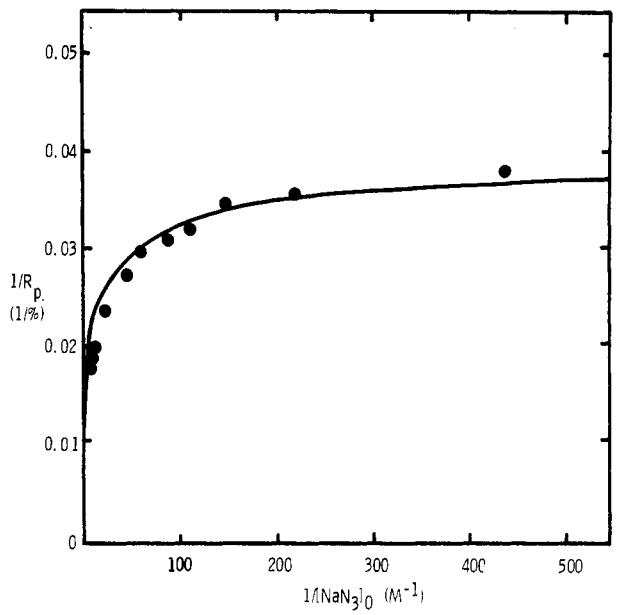

FIGURE 5: Correlation between the reciprocal of paramagnetic reduction, $1/R_p$, and the reciprocal of $[NaN_3]_0$ in the titration of ^{63}Cu–dopamine β-monooxygenase solution containing NaCN with NaN_3. Closed circles, experimental points; solid line, theoretical curve based on eq 6. Enzyme solution: protein concentration 4.86×10^{-2} mM, $[^{63}Cu]_0$ 2.29×10^{-1} mM, $[NaCN]_0/[Cu]_0 = 1.0$, and potassium phosphate buffer 200 mM, pH 7.0. Titration with NaN_3 were performed as described under Experimental Procedures except for the use of enzyme solution containing NaCN in the above ratio to $[^{63}Cu]_0$.

R_p^{2B} values already determined in the individual bindings (Table III). The solid line is in satisfactory agreement with the experimental hyperbolic trace, indicating that the competitive process was substantially governed by the parameters of the individual bindings. This further supports the view that the exogenous ligands bind at the same coordination locus of the Cu(II) site.

Discussion

First of all, it should be noted that our analysis assumes the equivalence of the Cu(II) sites. In fact, it has been shown that the Cu(II) sites can be regarded as equivalent up to eight copper atoms per tetramer of enzyme, as evaluated in terms of the paramagnetic relaxation rates of bulk water (Villafranca et al., 1984). We have confirmed that result with our enzyme samples and further confirmed that the R_p values on ligand binding to the Cu(II) sites are not affected by the number of copper atoms per tetramer of enzyme. Thus, no nonequivalence of the Cu(II) sites was detectable in our relaxation studies, in accordance with the assumption underlying our theoretical treatment.

As was shown by the analysis of the competitive binding data, CN^- and N_3^- compete for the same coordination position of the Cu(II) site, in a manner that is compatible with the parameters in an individual binding. This self-consistency of the parameters supports the reliability of the values. The results represent further evidence for the hypothesis that the Cu(II) site in dopamine β-monooxygenase ligates two water molecules that are exchangeable for exogenous ligands as indicated by the results of the ^1H nuclear relaxation and EPR studies.

Thus, the next question that arises is how the two water molecules are located in the Cu(II) site. In order to approach this problem, we need to consider the pH dependences of the parameters obtained by the relaxation experiments.

The R_p^1 and R_p^2 values, which can be regarded as intrinsic structural parameters of the ligand-bound Cu(II) site (vide ante), were not altered by a change of pH, indicating that the local structure of the Cu(II) site was preserved through the pH change. This conclusion is supported by the constancy of the Cu(II)–H_2O distances at both pH's. However, the K_b values were always larger at pH 5.5 than at pH 7.0 for both ligands, and this might be interpreted in terms of conformational change in the broader environment of the Cu(II) site, not at the site itself. If our conclusion concerning the invariability of the local structure is correct, the fact that K_b^1 was always very much larger than K_b^2 in N_3^- binding at either pH has important implications in considering the location of the water molecules.

The characteristics of the N_3^- binding constants for the present Cu(II) site resemble those of the F^- binding constants for the tetragonal-pyramidal Cu(II) site of galactose oxidase, where the first anation occurs 500 times more readily in the basal plane than at the apical position, in terms of the binding constant ratio (Kosman, 1984). Further, we found that the $g_\parallel$ values in the EPR parameters showed an apparent reduction in both complexes compared with the native Cu(II) site. A substantial reduction in the $g_\parallel$ value on basal ligation of CN^- or N_3^- has been observed in the square-pyramidal Cu(II) site of galactose oxidase (Giordano et al., 1974) and pig plasma amine oxidase (Marwedel et al., 1981).

Therefore, our experimental data seem to favor placing one of the two water molecules at an equatorial position and the other at an apical vertex in the axially symmetric field of the Cu(II) site. However, a final conclusion on the location of the water molecules may be premature, since, insofar as we have examined the site structure only through the use of products with exogenous ligands, we cannot rule out the possibility of rearrangement of the native coordination ligands in the presence of exogenous ligand, as has been considered in the Cu(II) site of superoxide dismutase (Bertini et al., 1983).

Although the structure of the Cu(II) site has not been proven, confirmation of the existence of two water molecules coordinated to the structure of the Cu(II) site is important

not only in relation to the Cu(II) site but also in connection with the catalytic mechanism of dopamine β-monooxygenase, which has yet to be determined in detail.

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Registry No. Cu, 7440-50-8; CN^-, 57-12-5; N_3^-, 14343-69-2; dopamine β-monooxygenase, 9013-38-1; water, 7732-18-5.

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Characterization of Tryptophan Environments in Glutamate Dehydrogenases from Temperature-Dependent Phosphorescence

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ABSTRACT: Tryptophan room temperature phosphorescence in solution was detected in glutamic dehydrogenase from bovine liver and *Escherichia coli* with lifetimes of 1.2 and 0.65 s, respectively. Although these enzymes possess three and five tryptophanyl residues per polypeptide chain, respectively, the temperature dependence of the phosphorescence quantum yield estimates that the room temperature emission is due, in either case, to a single residue. Long triplet-state lifetimes and very small rates of O_2 quenching indicate that these tryptophanyl side chains are embedded in a highly inflexible internal region of the macromolecule. Aided by sequence homology with dehydrogenases of known structure and theoretical predictions of secondary structure [Wootton, J. C. (1974) *Nature (London) 252*, 542–546; Brett, M., Chambers, G. K., Holder, A. A., Fincham, J. R. S., & Wootton, J. C. (1976) *J. Mol. Biol. 106*, 1–22], the phosphorescing tryptophans have been tentatively placed in the catalytic coenzyme binding domain of each enzyme. The particular sensitivity of the triplet-state lifetime in probing local changes in conformation provides a strong indication that within the time window of phosphorescence measurements the six subunits in the hexameric enzymes are equivalent. Furthermore, while in the bovine enzyme this parameter is markedly affected by the interaction with ligands which have a funtional role, the constancy of the phosphorescence lifetime at various degrees of polymerization suggests that the association process is not accompanied by important conformational changes in the macromolecule.

It has recently been shown that the phosphorescence lifetime and quantum yield of tryptophan are strongly affected by the fluidity of the surrounding matrix (Strambini & Gonnelli, 1985). As a result of this dependence, phosphorescence is not detectable in fluid solutions. In globular proteins, this emission has been rarely reported in aqueous solution at room temperature, being confined invariably to tryptophan residues

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