TEMPOH), 1.39 (s, 3.9, CH_2NCH_2 of TEMPOH), 1.44 (s, 6, $(CH_2)_3$), 2.67 (s, 4, CH_2NCH_2), 6.50-7.70 [br m, 4.2 (expected: 6), Ar H], 8.30-9.00 [br s, 1.3 (expected: 2), Ar H].

Addition of 1.5 equiv of TEMPOH (26 mg, 0.16 mmol) to a solution of **4** (30 mg, 0.11 mmol) in DMSO-d_6 (3 mL) afforded the following spectral characteristics: ^{1}H NMR ($(CD_3)_2SO$) δ 1.00 (s, 16.8, $(CH_2)_3$ of TEMPOH), 1.38 (s, 8.4, CH_2NCH_2 of TEMPOH), 1.44 (s, 6, $(CH_2)_3$), 2.67 (s, 4, CH_2NCH_2), 6.40-7.60 [br m, 4.6 (expected: 6), Ar H], 8.30-8.85 [br s, 1.1 (expected: 2), Ar H].

Addition of 4.0 equiv of TEMPOH (23 mg, 0.14 mmol) to a solution of **4** (10 mg, 0.036 mmol) in DMSO-d_6 (1 mL) afforded the following spectral characteristics: ^{1}H NMR ($(CD_3)_2SO$) δ 1.01 (s, 57, $(CH_2)_3$ of TEMPOH), 1.39 (s, 29, CH_2NCH_2 of TEMPOH), 1.45 (s, 6, $(CH_2)_3$), 2.69 (s, 4, CH_2NCH_2), 6.30-7.60 (br m, 6, Ar H), 8.30-8.95 (br s, 2, Ar H).

***N*-(9-Anthryl)piperidine (3). Anaerobic Conditions.** Anthrone (4.0 g, 21 mmol) and anhydrous *p*-toluenesulfonic acid (200 mg) were sealed under an argon atmosphere by a series of evacuation with a vacuum line, then filling with argon. Degassed toluene (20 mL) was added via syringe with stirring, giving a cream-yellow mixture. Degassed piperidine (8.3 mL, 84 mmol) was added via syringe producing a dark orange-red mixture. The solution was refluxed for 40 h, then cooled to room temperature, giving an orange precipitate. The precipitate was filtered in a drybox and washed with degassed toluene (10 mL) to yield an orange powder (**3**; 4.83 g, 88%): 164-169 °C; UV ($(CH_3)_2SO$) 328 nm (3550), 370 nm (1940), 396 nm (2050); ^{1}H NMR ($(CD_3)_2SO$) δ 1.45 (s, 6, $(CH_2)_3CH_2N$), 2.70 (d, 4, CH_2NCH_2), 7.27 (t, 2, Ar H), 7.40 (t, 2, Ar H), 7.71 (s, 1, Ar H), 7.85 (d, 2, Ar H), 8.48 (d, 2, Ar H); ^{13}C NMR ($(CD_3)_2SO$) δ 23.9 (t), 25.2 (t), 45.7 (t), 110.8 (d), 121.3 (d), 124.1 (d), 125.3 (d), 127.4 (d), 133.0 (2 overlapping singlets), 156.2 (s).

Anal. Calcd for $C_{19}H_{19}N\cdot H_2O$: C, 81.68; H, 7.58; N, 5.01. Found: C, 81.64; H, 7.45; N, 4.53.[15]

Aerobic Conditions. Anthrone (2.0 g, 10.5 mmol) and anhydrous *p*-toluenesulfonic acid (100 mg) were added to anhydrous toluene (10 mL) with stirring. No precautions were taken to exclude atmospheric oxygen. Upon addition of piperidine (4.2 mL, 42 mmol) the mixture changed from cream-yellow to orange-red. The mixture was heated to reflux, resulting in complete dissolution. Refluxing was continued for 22 h; then the solution was cooled to room temperature giving an orange precipitate in a dark red solution. Filtration and washing with toluene (15 mL) gave an orange powder (**3**; 2.32 g, 81%), identical by ^{1}H NMR with the material produced under anaerobic conditions.

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Novel Inclusion of Bis(2-pyridylcarbinolato)copper(II) by Cyclodextrins

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Abstract: Formation of the inclusion complexes of bis(2-pyridylcarbinolato)copper(II) with cyclodextrins (CDs) has been studied extensively by electron spin resonance (ESR) and circular dichroism measurements. It has been found that the copper(II) complex forms a 1:1 inclusion complex with α-CD but a more stable 2:1 complex with γ-CD. The stability constants of these inclusion complexes have also been estimated by an ESR method. The 2:1 inclusion complex showed a triplet-state ESR spectrum characteristic of copper(II) dimers. A dimeric structure has been proposed based on computer simulation of the ESR spectrum. This inclusion system may be a new model for multifactorial biological systems for molecular recognition and for mimicking active transport or concentration of substances.

Cyclodextrins (CDs) are cyclic oligosaccharides with internal cavities capable of forming inclusion complexes with small organic and organometallic compounds in aqueous solutions.[1-5] They have been studied extensively as models for enzyme active sites with the intention of mimicking enzyme activity and understanding the mechanism of molecular recognition.[1-4] However, there are very few reports about the inclusion of small and ordinary metal complexes by CDs. The purpose of this paper is to report a novel inclusion phenomenon of bis(2-pyridylcarbinolato)copper(II) by CDs. The inclusion mode described here appears to be unique and suggestive in considering the mechanisms of molecular recognition and active transport or concentration of substances in biological systems.

Experimental Section

Materials. The α-CD, β-CD, γ-CD, and D-trehalose and 2-pyridylcarbinol were obtained from Nakarai and Aldrich, respectively, and were used as received. Bis(2-pyridylcarbinolato)copper(II) was prepared as a tetrahydrate by the following method. $CuCl_2\cdot 2H_2O$ (25 mmol) and 2-pyridylcarbinol (50 mmol) were dissolved in water (100 mL), the solution was adjusted to pH ~9, and the desired product was precipitated. The crude product was recrystallized from water at pH ~9. Anal. Calcd for $C_{12}H_{12}N_2O_2Cu\cdot 4H_2O$: C, 40.99; H, 5.73; N, 7.96. Found: C, 41.08; H, 5.66; N, 7.75.

Measurements. A JEOL JES-FE2XG ESR spectrometer (X-band) was used to measure ESR spectra of frozen aqueous solutions at 77 K; a Takeda Riken TR-5501 frequency counter and an ECHO Electronics EFM-2000 NMR field meter were used in measurements of the microwave frequency and magnetic fields, respectively. A computer simulation of the dimer ESR spectra of the above copper(II) complex was carried out at the Computer Center of Tohoku University with a program based on the point-dipole approximation.[6,7] Visible and UV spectra and cir-

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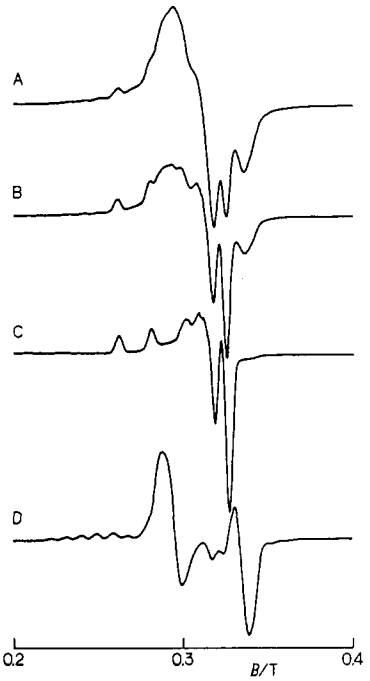

Figure 1. ESR spectra of Cu–PyC in aqueous solutions with the following additives (pH 6.5, ν = 9.054 GHz, [Cu–PyC] = 10.0 mM, I = 0.30 ($NaNO_3$), measured at 77 K): A, no additives; B, 40 mM D-trehalose; C, 10 mM α-CD; D, 10 mM γ-CD.

cular dichroism spectra were recorded at room temperature on a Shimadzu UV-240 spectrophotometer and a JASCO J-500 circular dichrograph with a J-DP 500 data processor, respectively.

Determination of Stability Constants. The stability constants of the inclusion complexes formed in this work are calculated by assuming the following equilibriums

$$M_2{\cdot}\gamma\text{-CD} + 2\alpha\text{-CD} \overset{K}{\rightleftharpoons} \gamma\text{-CD} + 2M{\cdot}\alpha\text{-CD} \quad (1)$$

$$M + \alpha\text{-CD} \overset{K_\alpha}{\rightleftharpoons} M{\cdot}\alpha\text{-CD} \quad (2)$$

$$2M + \gamma\text{-CD} \overset{K_\gamma}{\rightleftharpoons} M_2{\cdot}\gamma\text{-CD} \quad (3)$$

where M, M_2, M·α-CD, and M_2·γ-CD denote the monomer and dimer of bis(2-pyridylcarbinolato)copper(II) and their inclusion complexes by α- and γ-CDs, respectively. K is defined by

$$K = \frac{[\alpha\text{-CD}][M{\cdot}\alpha\text{-CD}]^2}{[M_2{\cdot}\gamma\text{-CD}][\alpha\text{-CD}]^2} = \frac{K_\alpha^2}{K_\gamma} \quad (4)$$

where brackets indicate concentration. In the case of eq 1, two different ESR spectra superimposed on each other due to M_2·γ-CD and M·α-CD are simultaneously observed in varying intensity ratios according to the concentrations of α- and γ-CDs. In these spectra, however, two specific absorption lines characteristic of M_2·γ-CD and M·α-CD can be selected properly, and their peak heights are expressed by H_D and H_M, respectively. From the ESR spectra of M_2·γ-CD and M·α-CD in pure forms, we can evaluate $r_D = H_D/I_D$ and $r_M = H_M/I_M$, respectively, where I denotes the total spectral intensity obtained by computer double integration of an experimentally observed first-derivative ESR spectrum. R is defined by

$$R = \frac{H_D}{H_M} = \frac{2r_D}{r_M}\frac{[M_2{\cdot}\gamma\text{-CD}]}{[M{\cdot}\alpha\text{-CD}]} \quad (5)$$

In this study, $2r_D/r_M$ was evaluated to be 0.774 by selecting such two specific H_D and H_M lines as will be shown later (Figure 4). Now, we can calculate the K value of eq 4 for every ESR spectrum corresponding to eq 1, because K is expressed as a function of R and the initial concentrations of the copper(II) complex, α-CD, and γ-CD.

Results and Discussion

ESR Spectra. Figure 1 shows effects of various additives on the ESR spectra of bis(2-pyridylcarbinolato)copper(II) (hereafter abbreviated as Cu–PyC) in frozen aqueous solutions. The spectrum in Figure 1A is due to Cu–PyC in the solution containing no additives. This spectrum is composed of three types of components due to the monomers, dimers, and polymers of Cu–PyC at a constant concentration ratio. Figure 1B is obtained by the addition of D-trehalose (a nonreducing disaccharide) to the solution of Figure 1A in a 4-fold excess over the molar quantity of Cu–PyC. Figure 1B is in line shape different from Figure 1A but is essentially the same in composition, except for a decrease in the relative concentration of polymers. Similar spectra to Figure 1B were observed after the addition of small amounts of alcohols (methanol or ethanol) to the solution of Figure 1A. On the other hand, Figure 1C appears on addition of an equimolar amount of α-CD. This spectral line shape is similar to that obtained from mononuclear copper(II) complexes monomolecularly dispersed, suggesting that a 1:1 inclusion complex of Cu–PyC with α-CD is formed in the solution. The ESR parameters of this complex are as follows: $g_\parallel$ = 2.227 and $|A_\parallel|$ = 0.0212 cm^{-1}. Figure 1D, which was obtained by the addition of the equimolar amount of γ-CD, is quite different in line shape from the others. This line shape is typical of dimeric copper(II) complexes (see next paragraph).

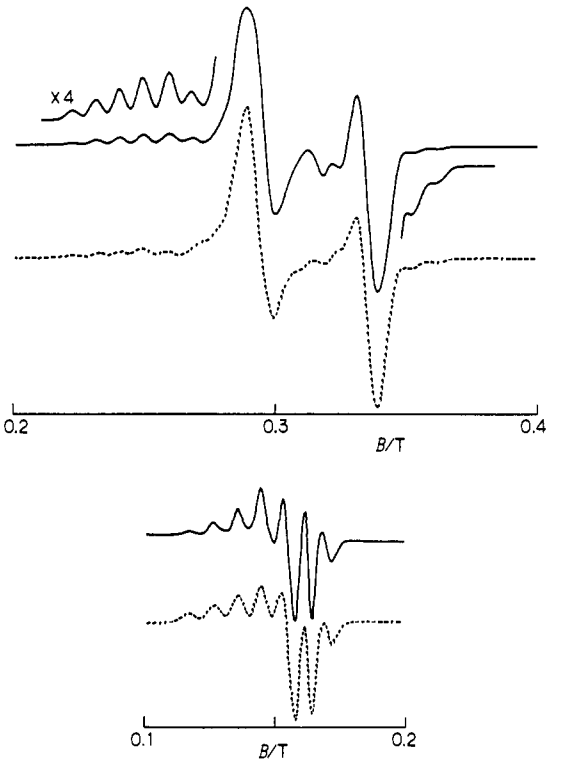

Figure 2. ESR spectra of Cu–PyC in an aqueous solution containing an equimolar γ-CD: (—), observed under the same conditions as in Figure 1D; (- - -), simulated with the parameter values written in the text.

Dimeric Structure of Cu–PyC in the Presence of γ-CD. Figure 2 shows an expanded spectrum of Figure 1D, together with the half-field spectrum simultaneously observed. Since Cu–PyC is a stable planar complex molecule, its dimer must be of a parallel-planar type. Therefore, Figure 2 has been analyzed in full detail by a computer simulation method in which the coordinate of the dimer is of a parallel-planar type with the structural parameters of r and ξ (r, the Cu–Cu distance; ξ, the angle between the Cu–Cu direction and the normal to the molecular plane) and in which a point-dipole approximation is used.[6,7] The best fit simulation spectrum calculated with the parameters of $g_\parallel$ = 2.240, $g_\perp$ = 2.050, $A_\parallel'$ = 0.0090 cm^{-1}, $A_\perp'$ = 0.0010 cm^{-1}, r = 4.1 Å, ξ = 15°, ΔH_1 = 25 G, and ΔH_2 = 19 G is shown as a dotted line in Figure 2, where $A_\parallel' = |A_\parallel|/2$, $A_\perp' = |A_\perp|/2$, and ΔH_1 and ΔH_2 are the half-widths of Gaussian line shape in the ΔM = 1 and 2 spectra, respectively (1 G = 10^{-4} T). From these structural parameter values, the structure of the present dimer can be visualized as follows: The interplanar distance of this parallel-planar dimer

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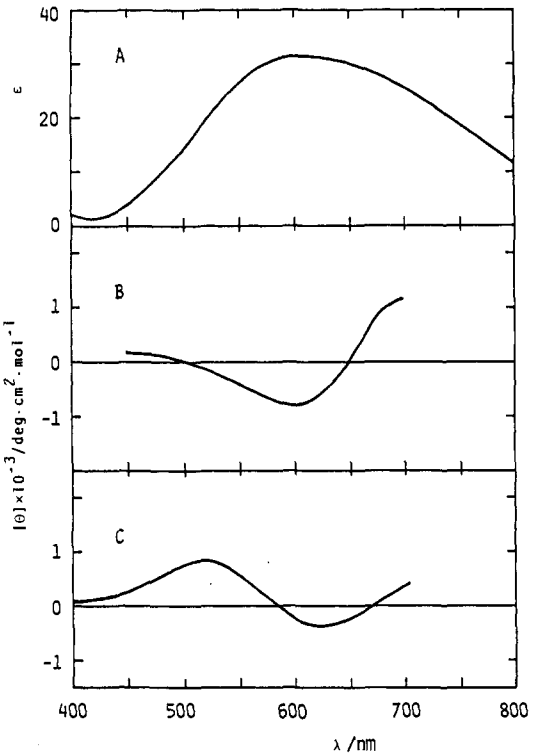

Figure 3. Visible absorption and circular dichroism spectra of Cu–PyC in aqueous solutions (pH 7.15, measured at room temperature): A, visible absorption spectrum (almost invariant, whether any CD is present or not); B and C, circular dichroism spectra in the presence of α-CD and γ-CD, respectively (the solutions of B and C correspond to parts C and D of Figure 1, respectively).

selected is ~3.8 Å, and the copper atom of one monomeric half is located nearly ±1 Å from the position where the copper atoms are right above each other. The same half-field spectra as shown in Figure 2 were also observed for the solutions of Figure 1A,B. This fact indicates that the presence of γ-CD does not affect the dimeric structure.

Circular Dichroism. As has been described above, ESR studies of Cu–PyC in the presence of CDs have suggested the possibility that Cu–PyC may form 1:1 and 2:1 inclusion complexes with α- and γ-CDs, respectively. However, direct evidence as to whether these inclusion complexes are actually formed or not cannot be obtained by the ESR method. We have tried to obtain such direct evidence by measuring induced circular dichroism for the above systems. Figure 3 shows their visible absorption and circular dichroism spectra observed at room temperature. The fact that circular dichroism is induced at the absorption bands of the achiral copper(II) complex in the presence of CDs clearly indicates that the copper(II) complex is included in the cavities of chiral CD host molecules. On the basis of the literature,[8] it seems that the spectral patterns of Figure 3B,C are characteristic of the 1:1 and 2:1 inclusion complexes, respectively. However, detailed analyses of these circular dichroism spectra were not attempted in this work, because of some extra unknown and complicated factors on the d–d spectral bands of Cu–PyC.

Stereochemical examination by the Corey–Pauling–Koltun (CPK) molecular model demonstrates that the monomer and dimer of Cu–PyC are well fitted into the cavities of α- and γ-CDs, respectively; α-CD is not capable of including the dimer.

Stability of Inclusion Complexes. The stabilities of the inclusion complexes of M_2·γ-CD and M·α-CD are discussed according to eqs 1–3. Figure 4 shows the ESR spectra of 0.010 M Cu–PyC

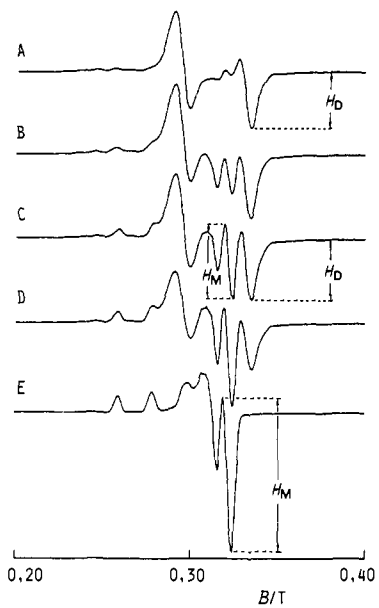

Figure 4. ESR spectra of Cu–PyC in aqueous solutions with the following additives (pH 7.0, [Cu–PyC] = 10.0 mM, I = 0.30 ($NaNO_3$), measured at 77 K): A, 10.0 mM γ-CD; B, 5.0 mM γ-CD; C, 10.0 mM γ-CD and 18.7 mM α-CD; D, 10.0 mM γ-CD and 37.5 mM α-CD; E, 100 mM α-CD.

Table I. K and R Values for Inclusion Complex Formation of Cu–PyC in the Presence of Both α- and γ-CDs in Various Concentration Ratios[a]

[α-CD]/M	R	K[b]
0.0187	0.853	0.0763
0.0375	0.470	0.0497
0.0750	0.162	0.0621

[a] [Cu–PyC] = 0.0100 M; [γ-CD] = 0.0100 M. [b] Average 0.0627 ± 0.0052.

in the presence and/or absence of α- and γ-CDs in varying concentrations. We selected two specific absorption lines characteristic of M_2·γ-CD and M·α-CD with the peak heights of H_D and H_M, respectively, in such a way as shown in Figure 4. Table I summarizes the R and K values obtained from three different spectra and leads to the conclusion that $K \cong 0.063$. From the experimental datum of R = 1.87 in Figure 4 in the absence of α-CD, the K_γ value was evaluated to be 1.8×10^6 in a similar way. Accordingly, $K_\alpha = 3.4 \times 10^2$ was calculated by eq 4. K = 0.063 means that 96% of Cu–PyC is included by γ-CD as dimers for the solution containing 10 mM Cu–PyC, α-CD, and γ-CD. After all, it is safely said that M_2·γ-CD is more stable than M·α-CD. However, it is worth mentioning that these stability constants were estimated in frozen aqueous solutions; the observation was made in a frozen solution state in which aqueous solutions were quickly frozen in liquid nitrogen with use of quartz sample tubes of the same size. Therefore, the stability constants described above are considered to be pertinent for a temperature somewhat lower than 0 °C.[7] The discussion given above about the stability of M_2·γ-CD and M·α-CD also seems reasonable at room temperature in a relative sense.

On the other hand, the β-CD, which is intermediate in the size of cavity between α- and γ-CDs, simultaneously formed both 1:1 and 1:2 inclusion complexes with Cu–PyC. Accordingly, β-CD is reasonably considered to be intermediate between α- and γ-CDs in the inclusion behavior too.

Concluding Remarks

In this work, we have found that γ- and α-CDs form 1:2 and 1:1 inclusion complexes with Cu–PyC, respectively, and that the former inclusion complex is much more stable than the latter. These facts are of great interest in connection with molecular recognition and active transport or concentration of substances in biological systems. One should note that γ-CD can sensitively

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recognize Cu-PyC in the dimeric form. It is well-known that the dimeric structure and dimer formability of a copper(II) complex are quite dependent upon its structure and nature.[7,9,10] Therefore, multiple factors such as dimer formability, dimeric structure, and inclusion by γ-CD must be important in determining sensitivity in this kind of molecular recognition. The present inclusion system can be regarded as a new and multifactorial model for the biological systems of molecular recognition.

Another interesting point of the present inclusion phenomena is concerned with the active transport or concentration of substances. Cu-PyC can form a 1:1 inclusion complex with α-CD but a more stable 2:1 one with γ-CD. This fact gives us an idea about a chemical substance transport system in which Cu-PyC migrates to an adjacent area of α-CD and then to the next area of γ-CD. Furthermore, there may exist some unknown host compound capable of forming a much more stable inclusion complex with some higher polymeric species of Cu-PyC. A certain system composed of the CDs and the above unknown host compound is considered as a new model for the active transport or concentration of substances in biological systems. This model is also based on high sensitivity in molecular recognition, as mentioned above.

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Ligand Spin Densities in Blue Copper Proteins by Q-Band ^1H and ^{14}N ENDOR Spectroscopy

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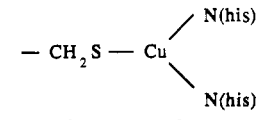
Abstract: The type 1 or blue copper centers of poplar plastocyanin (*Populus nigra var italica*), azurin (*Pseudomonas aeruginosa*), stellacyanin (*Rhus vernicifera*), and type 2 reduced fungal (*Polyporous versicolor*) and tree (*R. vernicifera*) laccase have been studied by Q-band (35 GHz) ENDOR spectroscopy. At this microwave frequency the ^1H and ^{14}N resonances occur in completely distinct radio-frequency ranges, and this has enabled us to study them individually for the first time. Each protein exhibits strongly coupled methylene protons of cysteine with isotropic hyperfine splittings in the range 16–31 MHz. The measurements indicate that the geometry of the Cu-cys linkage as measured by the H^{β}-C^{β}-S-Cu dihedral angles is remarkably similar in all these proteins, $-58° \lesssim \theta(H^{\beta 2}) \lesssim -50°$. With one exception, all the proteins have a similar, large total spin density on sulfur; fungal laccase appears to have a larger value but rather may differ slightly in structure. The Cu-bound nitrogens of the two histidine ligands of plastocyanin give a single ^{14}N resonance with isotropic coupling ($A^N \sim 22$ MHz) and thus the Cu-N bonds appear effectively equivalent although they differ metrically. In contrast, azurin, stellacyanin, and fungal laccase exhibit ^{14}N signals with isotropic hyperfine interactions from two inequivalent histidyl nitrogen ligands. We estimate the sum of the spin densities on N to be $\gtrsim 0.1$ and the overall spin density on ligands to be over 50%. The ^{14}N ENDOR of the similar site of tree laccase requires that it be unlike any of the other type 1 centers studied, with at least one ^{14}N ligand whose hyperfine tensor is highly anisotropic. Together, the ^1H and ^{14}N data suggest that the single-site proteins and the laccases fall into different subclasses. The advantages of the Q-Band ENDOR technique over alternate methods of determining ligand superhyperfine couplings also are discussed.

Introduction

The type 1 sites of blue copper proteins have unusual optical and magnetic properties which include intense (ϵ = 3500–6000 M^{-1} cm^{-1}) absorption bands in the visible region of the spectrum (λ = 600–625 nm), an approximately axial EPR spectrum with very low hyperfine splitting constant ($A_{\parallel} \approx 0.006$ cm^{-1}), and relatively high reduction potentials.[1] Despite the great effort made toward understanding the spectral features and the associated electronic structure of these sites, they continue to be of interest because they are so different from ordinary tetrahedral or square-planar Cu^{2+} complexes. Crystal structures[2] have been reported for the two single-site blue copper electron-transfer proteins, plastocyanin[2a] and azurin.[2b] In both cases, the Cu^{2+} ion has three strongly bonded ligands, the thiolate sulfur of a cysteine and the imidazole nitrogens of two histidines. The primary coordination geometry of Cu^{2+} in the two proteins is very similar, with unequal Cu-N1 and Cu-N2 bond distances and unequal S-Cu-N1 and S-Cu-N2 bond angles,[3] and can be schematically represented as follows



The structure of azurin from *Pseudomonas aeruginosa* (2.7 Å) has not yet been fully refined, but present coordinates[2c] are not inconsistent with the results for azurin from *Azotobacter denitrificans*.

Electron-nuclear double resonance (ENDOR) spectroscopy provides detailed information about the coordination sphere of

(1) (a) Ainscough, E. W.; Bingham, A. G.; Brodie, A. M.; Ellis, W. R.; Gray, H. C.; Loehr, T. M.; Plowman, J. E.; Norris, G. E.; Baker, E. N. *Biochemistry* **1987**, *26*, 71–82. (b) Gray, H. B.; Solomon, E. I. In *Copper Proteins*; Spiro, T. G., Ed.; Wiley: New York, 1981; Vol. 3, pp 1–39.

(2) (a) Guss, J. M.; Freeman, H. C. *J. Mol. Biol* **1983**, *169*, 526–563. (b) Norris, G. E.; Anderson, B. F.; Baker, E. N. *J. Am. Chem. Soc.* **1986**, *108*, 2784–2785. (c) Adman, E. T.; Sieker, L. C.; Jensen, L. H.: coordinates deposited (1980) with Brookhaven Protein Data Bank, Brookhaven National Laboratory, Upton, New York 11973.

(3) (a) The most recent crystallographic coordinates of plastocyanin are the result of a 1.33-Å refinement (Guss, J. M.; Freeman, H. C., personal communication). They give a short, 1.91 Å, Cu-N(1) bond length and a S(cys)-Cu-N(1) bond angle of 132°. The other coordinated nitrogen has a relatively long bond, 2.07 Å, with a S(cys)-Cu-N(2) bond angle of 126°. The Cu-ligand bond distances and bond angles obtained from the high-resolution structure of *A. denitrificans* azurin are quite similar: Cu-N(1), 1.96 Å; Cu-N(2), 2.06 Å; N(1)-Cu-S, 137°; N(2)-Cu-S, 119° (ref 2b). (b) The Cu^{2+} ion of plastocyanin has in addition a weaker interaction with methionine sulfur; that of the azurin has a methionine sulfur and a main chain carbonyl oxygen (from glycine) that form significantly weaker interactions in axial positions, completing an axially elongated trigonal bipyramid.

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