Copper–Cytochrome *c*

Meredith C. Findlay, L. Charles Dickinson, and James C. W. Chien*

Contribution from the Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003. *Received January 24, 1977*

Abstract: Copper cytochrome *c* (^{Cu}cyt-*c*) has been synthesized. It has the same electrophoretic and ion-exchange mobilities as the native enzyme. EPR and electronic spectra of the molecule from pH 4–11 showed it to be six-coordinated, exemplifying the effect of protein as a ligand which determines the unusual coordination chemistry of the metal ion since copper porphyrins do not usually complex with two axial Lewis base ligands. The EPR parameters are: $g_\perp = 2.050$, $g_\parallel = 2.216$, $^{Cu}A_\perp = 19.0$ G, $^{Cu}A_\parallel = 183$ G, $^{N}A_\perp = 14.5$ G, and $^{N}A_\parallel = 11.5$ G. The unpaired electron densities are: N(2s) = 2.4%, N(2p) = 4.8%, Cu($d_{x^2-y^2}$) = 76%, Cu(4s) = 4%. The bonding orbital is primarily the $\psi(b_{1g}) = \beta_1 d_{x^2-y^2} - \beta_1(\sigma_1 - \sigma_2 + \sigma_3 - \sigma_4)$. The energy differences $E(B_{1g}) - E(B_{2g})$ and $E(B_{1g}) - E(E_g)$ are estimated to be 23 000 cm^{-1} and 25 750 cm^{-1}, respectively. Thus $e_g < b_{2g} < a_{1g} < b_{1g}$ in energy for ^{Cu}cyt-*c*. ^{Cu}cyt-*c* undergoes conformational transitions at p*K* of 4 and 12. At extreme pHs the molecule dimerizes, but it probably still retains one axial ligand. A side product was always obtained in the insertion reaction of Cu(II) ion with metal-free porphyrin cytochrome *c*. This fraction has reduced electrophoretic mobility and is appreciably dimerized.

Recently there has been a great deal of interest in the synthesis of metal-substituted enzymes and in the study of their physical, chemical, and biochemical properties.[1] Because the heme in hemoglobin and myoglobin is held to the globin only by noncovalent forces and can be readily removed by acidification,[2,3] and because hemoglobin is a widely accepted model for allosteric enzymes, metal substituted derivatives of myoglobins and hemoglobins have been extensively studied.[4-7] The work on the cobalt myoglobin and hemoglobins alone was able to show the structure of the metal–dioxygen bond,[8,9] to refine the triggering mechanism for the allosteric transition,[4,5] to understand the role of the metal in determining the protein conformation,[10] and to evaluate the influence of globin on the thermodynamics of ligand binding.[11] Electron transfer between hemoglobin molecules can also be studied.[12]

Metal substitution has also been accomplished for cytochrome *c* (cyt-*c*)[13-15] even though the heme is covalently linked to the protein moiety. The cobalt derivative (^{Co}cyt-*c*) has been shown to have Met-80 and His-18 for axial ligands as in the native species.[16,17] This contrasts with the coordination chemistry of cobalt porphyrins which have been shown to form 1:1 and 1:2 complexes with nitrogenous bases[18-20] and 1:1 complexes with thio compounds,[20] but do not form complexes with both types of ligands in the axial positions.[20] ^{Co}cyt-*c* is capable of reducing cytochrome oxidase, but ^{Co}cyt-*c*$^+$ cannot be reduced by cytochrome reductases.[16,21] The surface lysyl residues are found to play an important role in the biochemical electron transfer.[16,17] The study also showed that the channel of aromatic residues proposed by Dickerson and co-workers[22] for these processes is probably not a valid mechanism.

Other metal-substituted cytochromes *c* have been synthesized in our laboratories to further our understanding of the role of metal ion in electron-transfer enzymes and the effect of the protein environment on the coordination chemistry of the metal ion. In this paper we report the preparation and properties of copper–cytochrome *c* (^{Cu}cyt-*c*).

Experimental Section

Preparation of ^{Cu}cyt-c. The procedure for the preparation of metal-free porphyrin cytochrome *c* (^{0}cyt-*c*) from the reaction of ^{Fe}cyt-*c* (Sigma Type VI) with anhydrous HF has been described previously.[16] The method developed for cobalt insertion is not used here because of the far greater affinity of porphyrin for cupric ion. Instead copper insertion is accomplished simply through dialysis at 4 °C of a freshly prepared ^{0}cyt-*c* solution (0.2 mM) against 1.25 mM cupric acetate (Fisher) solution. The reaction is complete in 4 h as evidenced by the disappearance of the four characteristic absorption bands of ^{0}cyt-*c* in the visible wavelength region. Excess cupric acetate is removed by a Sephadex G-25 column or by dialysis. Amberlite CG-50 was used to separate the copper insertion products using a 0.0 to 0.5 M NaCl gradient in 0.02 M, pH 8.0 phosphate buffer.[16] The concentration of NaCl solution was monitored by its refractive index.

Methods. Electronic spectra were obtained with a Cary-14 spectrometer. Atomic absorption and dry weight methods were both used in the determination of extinction coefficients. Circular dichroism spectra were obtained with a Cary-60 spectrometer.

Electron paramagnetic spectra (EPR) were obtained with a Varian E9 X-band spectrometer using a sample of diphenylpicrylhydrazyl in a dual cavity as the reference. Simulation of the EPR spectra was performed with a program based on that by Toy et al.[23] and kindly furnished by Dr. Phil Aisen of the Albert Einstein College of Medicine.

The electrophoretic mobility of ^{Cu}cyt-*c* was measured on cellulose acetate strips (Sepraphore III, Gelman) in pH 7.0, 0.05 M phosphate buffer at 200 mV and 2 mA. A sample of native ferric cytochrome *c* (^{Fe}cyt *c*$^+$) was always measured simultaneously. Direct comparison gave the R_f values.

Results

The solution of ^{Cu}cyt-*c* of a typical preparation has two Soret bands at 403 and 421 nm, indicating the formation of two products. After removal of excess cupric ion, the two products were separated on an Amberlite CG-50 column with a NaCl gradient (Figure 1). The first fraction, henceforth designated as ^{Cu}cyt-*c* A, elutes at 0.15 M NaCl. The second fraction, ^{Cu}cyt-*c* B was eluted at 0.27 M. The electrophoretic mobility of ^{Cu}cyt-*c* A is identical with that of ^{Fe}cyt-*c*$^+$, i.e., $R_f = 1$. On the other hand, ^{Cu}cyt-*c* B has much lower mobility than the other two proteins having a R_f value of 0.8. Systematic variations in the synthetic procedure showed that the relative yields of the two fractions depend primarily on the starting material ^{Fe}cyt-*c*$^+$, being independent of reaction time, reaction temperature, and $Cu(OAc)_2$ concentration. All preparations using the same batch of ^{Fe}cyt-*c*$^+$ yielded the same A/B product ratio. Different batches of ^{Fe}cyt-*c*$^+$ from Sigma gave different ratios of A/B. Because ^{Cu}cyt-*c* A has the same electrophoretic mobility as the native protein, it is more thoroughly characterized than the B fraction.

Both atomic absorption and dry weight methods were used to determine the copper content for the proteins. The two methods gave 1.1 ± 0.05 Cu(II) ions per molecule.

The electronic spectra of copper–cytochromes *c* are shown in Figure 2; the values of λ_{max} and extinction coefficients are given in Table I. All the absorptions of ^{Cu}cyt-*c* A lie at longer wavelengths than the corresponding bands for ^{Cu}cyt-*c* B.

The EPR spectra of copper–cytochrome *c* are shown in Figure 3.

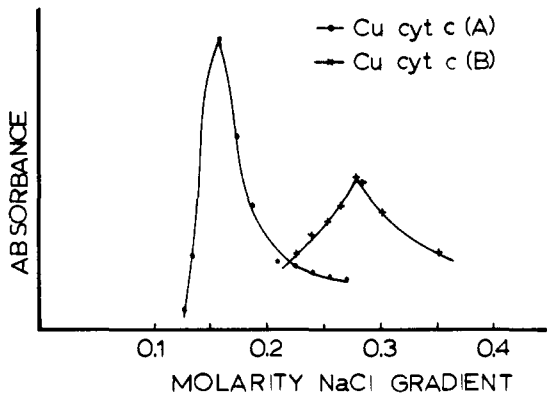

Figure 1. Elution pattern of copper-cytochrome *c*.

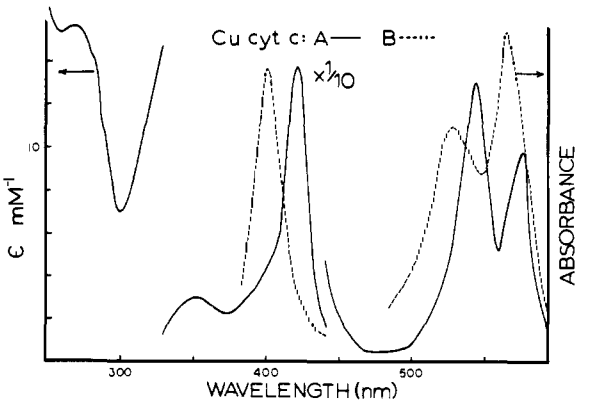

Figure 2. Electronic spectra of ^{Cu}cyt-*c* A and B at pH 7 in 0.1 M Na-phos.

Both fractions A and B of copper-cytochrome *c* show pH-dependent conformational changes. The p*K* of transitions for the native-like ^{Cu}cyt-*c* A are found to be 4.5 and 12.0. The two conformations at extreme pHs have electronic spectra characteristic of metalloporphyrins (Figure 4), indicating that there is no dissociation of the copper ion from the porphyrin moiety. At pH 1.5 the α and β bands appear at 568 and 532 nm, respectively, with peak ratio of $\alpha/\beta \approx 1.71$. The Soret band appears at 401 nm. This spectrum is very similar to that of ^{Cu}cyt-*c* B at neutral pH (Figure 2b). The two species are, however, different. When the pH of a solution of ^{Cu}cyt-*c* A is lowered to 1.5 and then brought back up to pH 7.0, the electronic spectrum of the A species (Figure 2a) was obtained. If we designate the low pH conformation as state I and the one between pH 4 and 11 as state II, then the transformation between

$$\underset{\text{pH 7}}{^{Cu}\text{cyt-}c\ \text{AII}} \underset{}{\overset{H^+}{\rightleftharpoons}} \underset{\text{pH 1.5}}{^{Cu}\text{cyt-}c\ \text{AI}} \qquad (1)$$

is reversible and does not involve ^{Cu}cyt-*c* B as an intermediate, because spectroscopically it can be shown that

$$\underset{\text{pH 7}}{^{Cu}\text{cyt-}c\ \text{B}} \overset{H^+}{\rightleftharpoons} \underset{\text{pH 1.5}}{^{Cu}\text{cyt-}c\ \text{BI}} \qquad (2)$$

The electronic spectrum of the B fraction at low pH is shown in Figure 4c. There is only one Soret band which is blue shifted by about 15 nm from the band at neutral pH. The shifts for the α and β bands are less than 1 nm for the two spectra (Figures 2b and 4c).

New species are formed in strongly alkaline media which are designated as state III. For ^{Cu}cyt-*c* AIII, there are two Soret bands at 400 and 390 nm. The electronic spectra of ^{Cu}cyt-*c* B at acidic and basic pHs are indistinguishable. However, they do have different EPR spectra (vide infra).

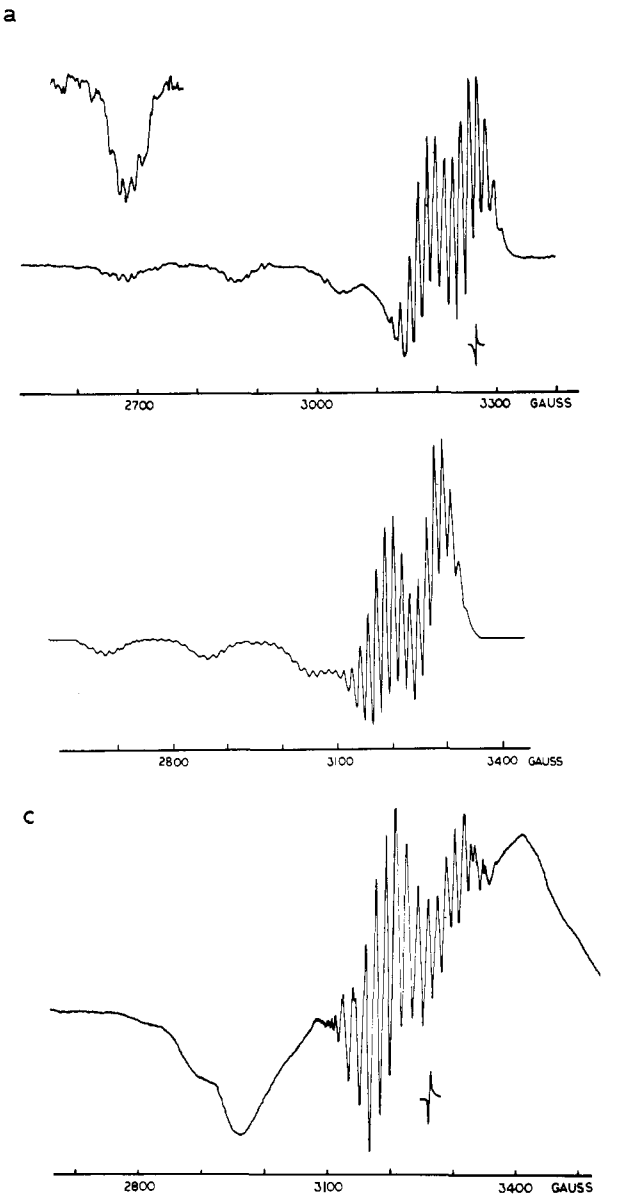

Figure 3. EPR spectra of ^{Cu}cyt-*c* at 77 K: (a) 2 mM of ^{Cu}cyt-*c* AII; (b) computer simulated spectrum; (c) ~1 mM ^{Cu}cyt-*c* BII.

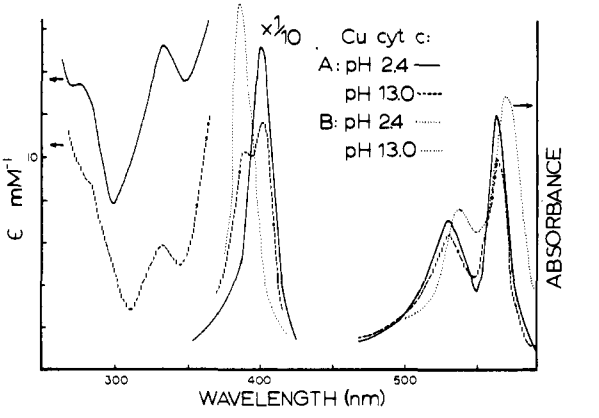

Figure 4. Electronic spectra of ^{Cu}cyt-*c* at extreme pHs.

The pH-dependent EPR spectra of ^{Cu}cyt-*c* are shown in Figure 5. A model compound study was also made on copper protoporphyrin IX dimethyl ester (^{Cu}PPIX DME). EPR spectra are given in Figure 6.

Table I. Electronic Spectra of Copper-Cytochrome *c* and Model Complexes

Compound	Solvent	Absorption bands: α λ_{max}, nm	α ϵ_{max}, $(mM\ cm)^{-1}$	α $\Delta\nu$, cm^{-1}	β λ_{max}, nm	β ϵ_{max}, $(mM\ cm)^{-1}$	β $\Delta\nu$ cm^{-1}	Soret λ_{max}, nm	Soret ϵ_{max}, $(mM\ cm)^{-1}$	Soret $\Delta\nu$, cm^{-1}
Cu-OEP[a]	Vapor	566			531			387.5		
Cu-PPIXDME · py[b]	Toluene/py	573		−216	536		−175	408		−1296
Cu-PPIXDME dimer, polymer	Toluene	573		−216	536		−175	408		−1296
Cu-cyt-*c* AII	pH 7	577	9.61	−337	545	12.9	−483	422	138	−2109
Cu-cyt-*c* BII	pH 7	566		0	530		+36	403		−992
Cu-cyt-*c* AI	pH 1	568	12.0	−62	532	7.07	−35	403	154.6	−992
Cu-cyt-*c* BI	pH 1	568		−62	533		−70	340		−165
Cu-cyt-*c* AIII	pH 14	568	9.98	−62	532	6.31	−35	390		−165
								403		−992
Cu-cyt-*c* BIII	pH 14	568		−62	533		−70	390		−165

[a] Copper octaethylporphyrin, L. Edwards, D. H. Dolphin, and M. Gouterman, *J. Mol. Spectrosc.*, **35**, 90 (1970). [b] Copper protoporphyrin IX dimethyl ester, py = pyridine.

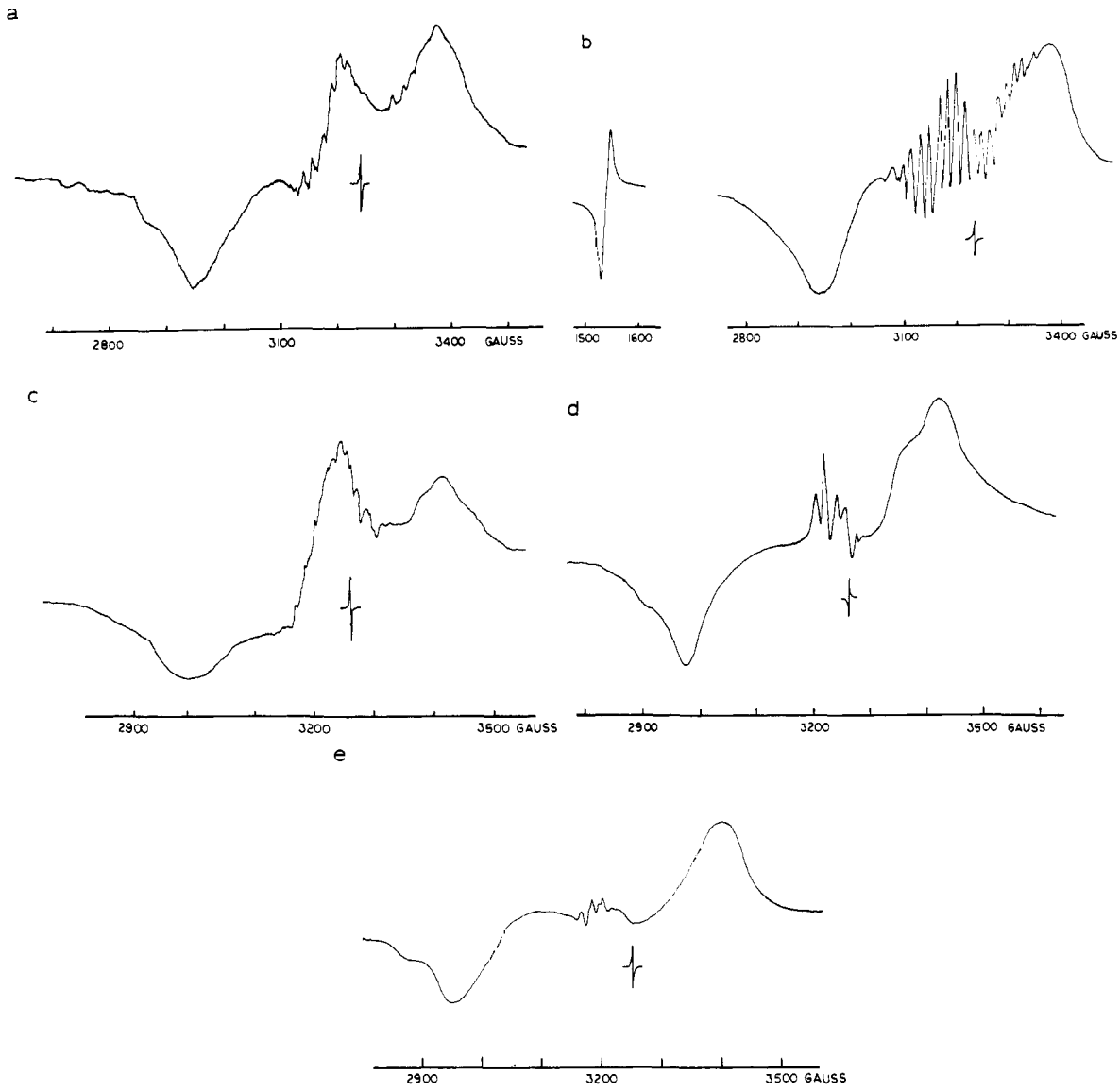

Figure 5. EPR spectra of ^{Cu}cyt-*c* at extreme pHs and 77 K: (a) ^{Cu}cyt-*c* AI pH 1.5; (b) ^{Cu}cyt-*c* AIII pH 14.0; (c) ^{Cu}cyt-*c* BI pH 1.3; (d) ^{Cu}cyt-*c* BIII pH 12; (e) ^{Cu}cyt-*c* A in saturated guanidine hydrochloride solution.

The ultraviolet circular dichroism (CD) spectra of ^{Cu}cyt-*c* A are shown in Figure 7 as a function of pH along with those of the native enzymes at neutral pH.

Attempts to reduce or oxidize ^{Cu}cyt-*c* met with no success. The EPR spectrum was unchanged with the addition of an excess of either dithionite or ferricyanide. ^{Cu}cyt-*c* has no pro-

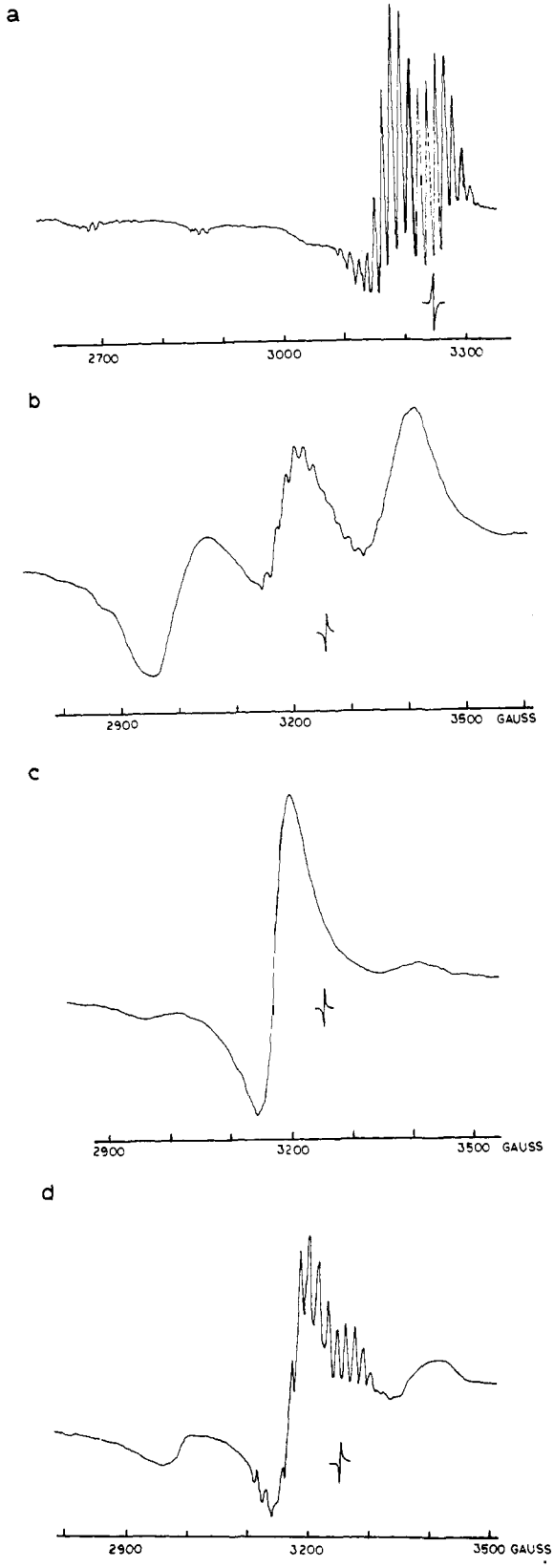

Figure 6. EPR spectrum of copper protoporphyrin IX dimethyl ester at 77 K: (a) 1.6 mM in 1:1 toluene–pyridine; (b) dimer in toluene; (c) polymer in toluene; (d) in 1:1:2 pyridine–methylthioethanol–toluene.

pensity to bind CO or NO; neither caused any change in the UV–vis or the EPR spectra.

Discussion

The electrophoretic mobility of ^{Cu}cyt-*c* A is identical with that of the native enzyme, indicating the two species have the same charges at the surfaces of the proteins. However, differences could still exist for the charged surface groups either in their distribution or local structure. For instance, in the cobalt-substituted cytochromes *c*, proton magnetic resonance showed that several of the surface lysyl residues are more free to rotate in ^{Co}cyt-*c* than for ^{Fe}cyt-*c*. In fact, copper substitution does significantly alter the conformation of the protein. Ultraviolet CD spectra showed ^{Cu}cyt-*c* to have only three/fourths of the helicity as the native protein. The conformational difference probably is the consequence of the repulsion of the axial ligands by the antibonding d electrons in ^{Cu}cyt-*c*, causing displacement of peptide residues. There are no antibonding d electrons in the low-spin ^{Fe}cyt-*c*. These conformational differences are clearly discernible in the near ultraviolet and visible CD spectra which will be reported elsewhere.

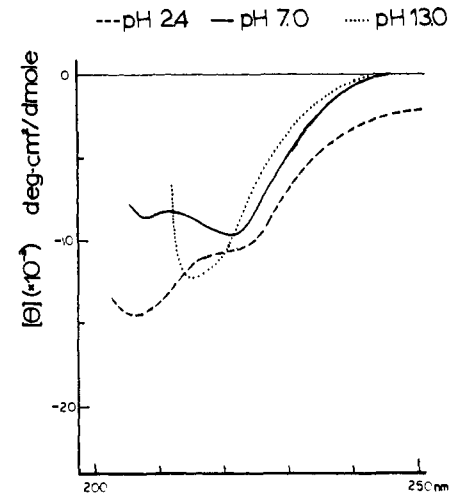

Figure 7. Mean residue ultraviolet circular dichroism spectra of ^{Cu}cyt-*c* A: low, neutral, and high pH.

The coordination chemistry of copper in ^{Cu}cyt-*c* is of interest. Copper porphyrins are known to bind one but not two nitrogenous ligands.[24,25] The ^{0}cyt-*c* may be thought of as a macromolecular ligand which offers two residues for axial coordination with the metal ion. That both Met-80 and His-18 are axial ligands for ^{Fe}cyt-*c* and ^{Co}cyt-*c* have been well documented.[16,17,26-29] The results for ^{Cu}cyt-*c* suggest that copper also binds two axial ligands. Even though we favor His-18 and Met-80 as the fifth and sixth ligand for ^{Cu}cyt-*c* as in the iron and cobalt species, positive evidence is lacking. High-resolution proton NMR spectroscopic studies are underway for ^{Cu}cyt-*c* but we are encountering difficulty from paramagnetic line broadening. Accordingly, the identification of the axial ligands is uncertain at present.

The position of the Soret band of proteins with metalloporphyrin prosthetic group is sensitive to axial ligation due to stereoelectronic interaction between the axial ligands and π-electron system of the porphyrin,[24,25,30] to cause red shift of the Soret band. This effect is quite regular for Co(II), Ni(II), and Cu(II) porphyrins. Compared with the Soret bands of metalloporphyrins in the vapor phase,[31] the absorption maximum is red shifted by 1000 to 1400 cm^{-1} for 1:1 complexes with nitrogenous bases. The Soret red shift is about twice as much (2000–2800 cm^{-1}) with double axial coordination. Table I, column 11, shows that the Soret band of ^{Cu}cyt-*c* (A) is shifted -2109 cm^{-1} from ^{Cu}OEP in vapor phase, thus establishing that Cu is six coordinated in this metalloprotein.

The EPR spectra of Cu(II) have been actively investigated. Depending upon whether Jahn-Teller distortion stabilizes the $d_{x^2-y^2}$ or the d_{z^2} orbital, the unpaired electron will be found in the other orbital. The fact that $g_{\parallel} \gg 2$ and that the hyperfine interaction with the in-plane nitrogen atoms of the porphyrin is large favors a $(d_{x^2-y^2})^1$ configuration.

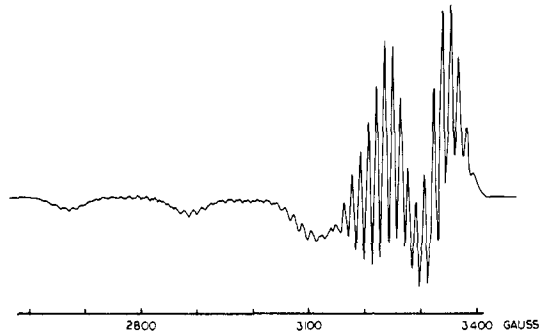

Figure 8. Computer simulated EPR spectrum for copper tetraphenylporphyrin in CH_3Cl_3 glass. Parameters taken from ref 38 and spectrum to be compared with Figure 5a of that reference.

From the Cu hyperfine parameters, the unpaired electron density can be estimated. The hyperfine tensor has the form $|T_\parallel, T_\perp, T_\perp| = |A_\parallel - a, A_\perp - a, A_\perp - a|$, where the A's are the observed parameters $^{Cu}A_\parallel = 183$ G and $^{Cu}A_\perp = 19$ G and a is the isotropic value and is equal to $\frac{1}{3}(^{Cu}A_\parallel + 2^{Cu}A_\perp) = 73.7$ G. For an electron in the $d_{x^2-y^2}$ orbital,[32] $T_\parallel = -\frac{4}{7}P$ and $T_\perp = +\frac{2}{7}P$, where $P = g_e\beta_e g_N\beta_N/\langle r^3\rangle d_{x^2-y^2}$. The fraction of electron in the $d_{x^2-y^2}$ orbital, $^{Cu}f_d$, is found to be about 76% taking the principal value of the anisotropic tensor $^{Cu}A_{zz} = -250$ G.[32] The fractional electron density in the 4s orbital, $^{Cu}f_s$, is given by

$$^{Cu}f_s = 3a(8\pi g_N\beta_n|\psi(4s)|^2)^{-1} \quad (3)$$

Substituting $|\psi(4s)|^2 = 2.82 \times 10^{25}$ cm^{-1} and the weighted average of g_n (^{63}Cu) = 1.4804 and g_n (^{65}Cu) = 1.586[32] into eq 3 yields a value of about 4% for $^{Cu}f_s$.

The unpaired electron density for the Cu orbitals can be estimated also from the principal molecular orbital for the interaction between metal and porphyrin:

$$\psi(b_{1g}) = \beta_1 d_{x^2-y^2} - \beta_1'(\sigma_1 - \sigma_2 + \sigma_3 - \sigma_4) \quad (4)$$

The normalization condition for this orbital is

$$\beta_1^2 + \beta_1'^2 - 2\beta_1\beta_1' S = 1 \quad (5)$$

where S is the overlap integral:

$$S = 2\langle d_{x^2-y^2}|\sigma\rangle \quad (6)$$

and has been found to have a value of 0.092 for ^{Cu}TPP.[33] The values of β_1 and β_1' can be obtained from the observed nitrogen hyperfine splittings as follows:[33,34]

$$^NA_\parallel = (\beta_1'/2)^2 g_n\beta_n(8\pi|\psi_0|^2\tfrac{2}{3} + 8\langle r^{-3}\rangle_{2p}/15) \quad (7)$$

$$^NA_\perp = (\beta_1'/2)^2 g_n\beta_n(8\pi|\psi_0|^2\tfrac{2}{3} - 4\langle r^{-3}\rangle_{2p}/15) \quad (8)$$

where $\langle r^{-3}\rangle_{2p} = 21.1 \times 10^{24}$ cm^{-1}.[35] From the value of β_1 we find that the unpaired electron has 77% $d_{x^2-y^2}$ character in agreement with the value estimated above.

To estimate the unpaired electron densities on the nitrogen 2s and 2p orbitals, ^Nf_s and ^Nf_p, we first correct the observed ^{14}N hyperfine parameters, $^NA_\parallel = 11.5$ G and $^NA_\perp = 14.5$ G for the dipolar interactions, d:

$$^NA'_\parallel = {}^NA_\parallel - d,\ {}^NA'_\perp = {}^NA'_\perp + 2d \quad (9)$$

where $^NA'_\parallel$ and $^NA'_\perp$ are the values after dipolar corrections, and

$$d = g_e\beta_e g_N\beta_N/R^3 = 0.2 \times 10^{-4}\ \text{cm}^{-1} \quad (10)$$

taking the Cu–N bond distance R to be 2.0 Å. The electron densities can be calculated from

$$^Nf_s = \frac{{}^NA'_\perp + 2{}^NA'_\parallel}{(8\pi/hc)g_e\beta_e g_N\beta_N|\psi(2s)|^2} \quad (11)$$

and

$$^Nf_p = \frac{{}^NA'_\perp - {}^NA'_\parallel}{(2/5hc)g_e g_N\beta_N\langle 1/r^3\rangle_{2p}} \quad (12)$$

where the A's are in the unit of cm^{-1} and c is the velocity of light. The values of $|\psi(2s)|^2$ and $\langle 1/r^3\rangle_{2p}$ for ^{14}N have been given[36] to be 5.6 and 3.6 a.u., respectively. Using eq 11 and 12, we estimated $^Nf_s = 2.4\%$ and $^Nf_p = 4.8\%$. The ratio of ^Nf_p to ^Nf_s is consistent with a sp^2 hybridization for the pyrrole nitrogen atoms and suggests that there is no significant π bonding between the Cu atom and the porphyrin.

The values of unpaired electron density at Cu and N found for ^{Cu}cyt-c are comparable with those obtained for ^{Cu}TPP.[37] Also the value of ^Nf_s estimated for ^{Cu}cyt-c is nearly the same as that reported for hemin[38] which is 2.7%. It suggests that the σ-type bonding with the porphyrin is largely independent of the metal. The sum of the unpaired electron densities in ^{Cu}cyt-c is $4(0.024 + 0.048) + 0.75 + 0.04 = 1.08$, which accounts for all the densities.

The g values and unpaired electron densities permit evaluation of the relative energies of the d orbitals. The approximate expression for the principal g values, neglecting π bonding, are:

$$g_\parallel = 2.0023 - \frac{8\lambda\beta_1^2\beta_2^2}{E(B_{1g}) - E(B_{2g})}[1 - (\beta_1'/\beta_1)S] = 2.216 \quad (13)$$

$$g_\perp = 2.0023 - \frac{2\lambda\beta_1^2\epsilon^2}{E(B_{1g}) - E(E_g)}[1 - (\beta_1'/\beta_1)S] = 2.050 \quad (14)$$

where β_2^2 and ϵ^2 are bonding coefficients in the $\psi(b_{2g})$ and $\psi(e_g)$ molecular orbitals, respectively, and can be taken as unity in the absence of any π bonding;[37] λ is the spin–orbit coupling constant for the Cu(II) ion and is -830 cm^{-1}. Substitution into eq 13 and 14 give $E(B_{1g}) - E(B_{2g}) = 23\,000$ cm^{-1} and $E(B_{1g}) - E(E_g) = 25\,750$ cm^{-1}. These values lead to the following order of d orbitals in ^{Cu}cyt-c: $e_g < b_{2g} < a_{1g} < b_{1g}$.

The EPR spectral parameters used in the above calculations were obtained by the best computer fit. The simulated spectrum (Figure 3b) is in good agreement with the experimental spectrum (Figure 3a). These parameters are, however, significantly different from those reported for ^{Cu}TPP.[37] This may be due to the presence of the axial ligands and the substituent groups of the porphyrin ring. On the other hand, simulation of the EPR spectrum of ^{Cu}TTP in chloroform glass using the reported parameters[37] gave a spectrum very different from the experimental spectrum (compare our Figure 8 with Figure 5a of ref 37). It is not clear to us how these workers obtained the EPR parameters. The discrepancy remains to be resolved.

^{Cu}cyt-c (A) undergoes pH-dependent conformational transitions. At pH 14 there are two Soret bands, indicating the presence of two species. The bands are red shifted with respect to ^{Cu}OEP by only -165 and -992 cm^{-1} (Table I). The former we attribute to monomeric copper-cytochrome c which has either lost its axial ligands, or their bonds to Cu are greatly weakened. The band at 403 nm is attributed to a dimeric Cu_2(II) triplet species. Cu(II) complexes dimerize readily; dimers have been reported for dialkyldithiocarbamates,[39] various amino acids,[40] dimethylglyoxime,[41] protoporphyrin IX,[42] and uroporphyrin III.[43] It is worthwhile to note that copper uroporphyrin III also forms a dimer at alkaline conditions.

For an axial system the triplet spin Hamiltonian is

$$\mathcal{H} = \beta(gH_zS_z + g(H_xS_x + H_yS_y)] + D[S_x^2 - S(S+1)/3] + AS_zI_z + B(S_xI_x + S_yI_y) \quad (15)$$

At midfield the parallel lines are split by $2D$ and the perpendicular lines by D, where D is the zero-field splitting parameter. In addition, there may be observable a $\Delta m = 2$ transition at half-field, H_{min}:

$$H_{min} = [\omega_o^2 - 4/3D^2]^{1/2}(2g\beta)^{-1} \quad (16)$$

where ω_o is the microwave frequency.

The EPR spectrum of ^{Cu}cyt-*c* (A) at pH 14 showed clearly the presence of a dimeric triplet species with $g = 4.251$ and $D = 0.0437$ cm^{-1}. Superimposed on it at midfield is the signal from the monomeric species.

At acidic pH, the visible spectrum of ^{Cu}cyt-*c* (AI) has only one Soret band due to the dimers. Its EPR spectrum at midfield is very similar to that at alkaline pH except the monomer contribution is smaller. The $\Delta m = 2$ signal was not observable at pH 1.5.

It is interesting to compare the EPR spectrum of ^{Cu}cyt-*c* (A) in guanidine hydrochloride (Figure 5e) with those obtained at extreme pHs (Figure 5a and b). There is virtually no monomeric species in guanidine hydrochloride.

^{Cu}cyt-*c* (B) is of interest because it is obtained in amounts which apparently depend upon the starting material ^{Fe}cyt-*c*. If that is true then the best Sigma cytochrome *c* may be heterogeneous, at least with regard to protein conformation responses to copper ion substitution. At neutral pH, ^{Cu}cyt-*c* (BII) is apparently largely dimeric as judged by its EPR spectrum (Figure 3c) and its Soret band (Table I). At extreme pHs, the extent of dimerization seems to increase. The zero-field splitting at pH 1.3 is, however, smaller than at pH 12.

^{Cu}cyt-*c* differs in one respect from copper uroporphyrin III. The latter at low pHs forms polymers.[43]

Boas et al.[42] had reported EPR spectra of ^{Cu}PPIX in dimethylformamide, trimethyl phosphate, and 1-chloronaphthelene. We have obtained spectra for the dimethyl ester of ^{Cu}PPIX in other media. The molecule is monomeric in 1:1 toluene–pyridine (Figure 6a). In neat toluene the EPR spectrum (Figure 6b) suggests an admixture of dimers and polymers. Upon standing for several days polymerization seems to be largely completed as judged by spectrum 6c, which resembles the spectrum of polymeric copper uroporphyrin in HOAc.[43] In pyridine–methylthioethanol solvent ^{Cu}PPIX DME exists as both monomers and dimers (Figure 6d). These results suggest that polymerization occurs only when both axial coordination positions are free and dimerization can occur when the Cu is five coordinated. It is recalled that the corresponding conformational state of ^{Co}cyt-*c* retains His-18 as the axial ligand at alkaline pH and it retains Met-80 as the axial ligand in acidic pH.[17] Finally, the fact that all the divalent transition metal substituted cytochromes *c* have the same two p*K*s of transition[44] suggest that they result from ionization of the peptide residues.

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