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(15) $k_{-2} = k_2 k_{123} K_f$ where k_{123} is the microscopic acid dissociation constant for the sulfhydryl group[9] and K_f the formation constant.[3]
(16) In the calculation of these rate constants, it was assumed that exchange by reaction 8 is negligible. If, however, the exchange at pH 2.12 is due solely to reaction 8, the rate constants obtained by the above procedure are $k_2 = 400 \pm 250\ sec^{-1}$, $k_{-2} = 3.4 \times 10^9\ M^{-1}\ sec^{-1}$, $k_4 = 2.5 \pm 1.5 \times 10^8\ M^{-1}\ sec^{-1}$, and $k_5 = 110\ M^{-1}\ sec^{-1}$.
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Spectral Studies of Copper(II) Carboxypeptidase A and Related Model Complexes

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Abstract: The near-infrared and visible electronic absorption spectrum of $Cu^{II}CPA$ exhibits a maximum at 12,580 cm^{-1} with a molar extinction coefficient of 124. Analysis of the frozen glass EPR spectrum of $Cu^{II}CPA$ in 1:1 ethylene glycol:buffer yields $g_\parallel = 2.327$, $g_\perp = 2.057$, $A_\parallel = 124$ G (13.5), $A_\perp = 15$ G (1.4 mK). Comparison of the electronic spectral and EPR properties of $Cu^{II}CPA$ with those of model Cu(II) complexes indicates that the coordination site is significantly distorted from square planar toward a tetrahedral geometry. Addition of the inhibitor sodium β-phenylpropionate (NaβPP) results in a shift of the principal peak in the electronic absorption spectrum to 11,400 cm^{-1} and an increase in molar extinction coefficient to 180, suggesting that the coordination geometry in $Cu^{II}CPA{\cdot}\beta PP$ is closer to tetrahedral than that in the unsubstituted derivative. A formation constant $K_1 = 1.44 \times 10^2\ M^{-1}$ was measured for the $Cu^{II}CPA{\cdot}\beta PP$ complex.

Carboxypeptidase A (CPA) is a zinc metalloenzyme that exhibits both peptidase and esterase activities.[1-3] An X-ray crystallographic study[4] has indicated that the zinc is probably in a distorted tetrahedral coordination environment at the active site of CPA. The probable donor-atom set in the resting enzyme is N_2O_2, comprised of the N(1)'s of His 69 and His 196, with oxygens furnished by Glu 72 and a water molecule.[4,5]

Numerous other dipositive metal ions have been substituted for the zinc in CPA with varying degrees of retention of peptidase and esterase activities.[6] Our electronic spectroscopic and magnetic susceptibility studies of two active derivatives, $Co^{II}CPA$ and $Ni^{II}CPA$, have demonstrated that the enzyme is flexible enough to accommodate both five- and six-coordinate active-site structures.[7] It appears, then, that as long as the metal center possesses a properly oriented, substitution-labile coordination position, a range of coordination numbers and geometries is possible for a peptidase-active derivative. In order to further examine the question of coordinative flexibility in the CPA system and the relationship of the coordination environment of the metal to enzymatic activity, study of an inactive metalloderivative appeared essential.

The $Cu^{II}CPA$ derivative is known to show neither peptidase nor esterase activity.[6a] Only sketchy information regarding its spectroscopic properties is available in the literature. The wavelength of maximum absorption of $Cu^{II}CPA$ has been mentioned,[8] but no details on the rest of the absorption spectrum appear to be available. A brief report stating that the EPR spectrum of a single crystal of $Cu^{II}CPA$ shows three principal g values and superhyperfine interaction from two equivalent nitrogen ligands has been published,[9] although no g values or hyperfine coupling constants were given. The only other EPR data for $Cu^{II}CPA$ are from a study of freeze-dried samples and pH 5.5 solutions.[10]

In this paper we report the results of our investigation of the electronic absorption and EPR spectra of $Cu^{II}CPA$. The spectroscopic data for $Cu^{II}CPA$ are compared with those obtained for a variety of cupric model complexes, with the aim of elucidating the coordination number and geometry of the metal center. We also report the electronic absorption spectrum and the formation constant of the complex of $Cu^{II}CPA$ with the inhibitor β-phenylpropionate (βPP).

Experimental Section

Materials. Crystalline carboxypeptidase A, isolated by the Cox procedure,[11] was obtained from Sigma Chemical Co. and used without further purification. The Cox method was chosen because it yields a relatively small amount of the undesirable CPA_γ form of the enzyme.[12] Samples were checked for peptidase activity[13] and metal content before and after metal replacement. Hippuryl-L-phenylalanine (Schwarz/Mann) was used as the substrate in all assays. $Cu^{II}CPA$ was prepared by the method of Coleman and Vallee.[14] Peptidase activity was found to be proportional to the zinc content of preparations containing mixtures of $Zn^{II}CPA$ and $Cu^{II}CPA$. The preparations of $Cu^{II}CPA$ used for the spectral studies were found to contain 1–3 mol % residual zinc and had a correspondingly low level activity. Extreme care was taken to prevent contamination of the CPA by adventitious metal ions.[15] Plastic lab ware was used, and all the Tris–HCl buffers were repeatedly extracted with dithizone in CCl_4 prior to use. Cupric ion solutions were made up by dissolving the pure metal in metal-free HCl. Minimum 99.9% pure Cu metal (J. T. Baker Co.) was used. D_2O was obtained from Columbia Organic Chemical Co. The D_2O

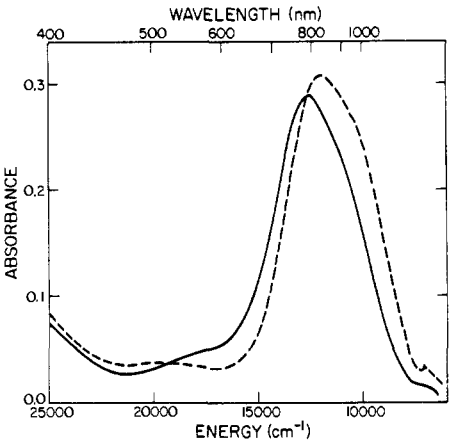

Figure 1. Absorption spectra of $Cu^{II}CPA$ in deuterated 1 *M* NaCl, 0.05 *M* (pH 7.8) Tris buffer (7–12°): (—) $[Cu^{2+}] = 4.66 \times 10^{-4}$ *M*; (- - - -) $[Cu^{2+}] = 4.25 \times 10^{-4}$. $[Na\beta PP] = 6.93 \times 10^{-3}$ *M*.

buffer was passed through a Chelex-100 column before use in the spectral experiments. Deuterated apoCPA was obtained by dialyzing apoCPA against a minimum of five changes of deuterated buffer. Sodium β-phenylpropionate (NaβPP) was prepared by neutralizing an alcoholic solution of hydrocinnamic acid (Eastern Chemical Co.) with sodium hydroxide. Samples of NaβPP were recrystallized from ethanol–hexane. The *N*-alkyl(aryl)salicylideneaminatocopper(II) complexes [abbreviated $Cu(N\text{-}R\text{-}Sal)_2$] were prepared by literature procedures[16] and recrystallized from chloroform–ethanol solution. Melting points agreed well with the published values.

Spectra. Near-infrared and visible absorption spectra were measured on a Cary 17I recording spectrometer which had been modified to run at constant slit width. This modification considerably reduces observed baseline variation on the 0.0–0.1 O.D. slidewire. A difference technique, which has been previously described,[7] was used to measure the spectra of $Cu^{II}CPA$ and its complex with β-phenylpropionate. Matched cells with a path length of 50 mm holding 1.95 ml of solution were obtained from Helma Cell, Inc. Spectra were measured between 7 and 12° in deuterated 1 *M* NaCl, 0.05 *M* (pH 7.8) Tris–HCl buffer. Corrections for dilution were applied to the spectral data. Enzyme concentrations were measured spectrophotometrically at 278 nm.[13]

The low temperature X-band EPR spectrum of $Cu^{II}CPA$ in a 1:1 buffer:ethylene glycol glass was measured on a Varian V4502 spectrometer equipped with a 9-in. Varian electromagnet and a Fieldial' fieldsweep control unit, and 100 kHz modulation was used. The magnetic field was calibrated in every experiment with a sample of solid DPPH placed in the rear compartment of the dual cavity. The DPPH signal was detected using a low frequency (20–400 Hz) modulation and detection system. The microwave frequency was measured by a wave meter attached to the microwave bridge. Low temperature measurements were made by passing a stream of nitrogen gas through a liquid helium heat exchanger and then through a quartz dewar containing the sample. A Varian V4540 temperature controller was used to monitor the gas flow. The computer simulation of the EPR spectrum of $Cu^{II}CPA$ was performed as described previously.[17]

The X-band, frozen-glass EPR spectra of the $Cu(N\text{-}R\text{-}Sal)_2$ complexes were measured on a Varian V-4502 EPR spectrometer equipped with a 12-in. Varian electromagnet and a Varian Mark II Fieldial field sweep control unit. A Varian V4532 rectangular cavity fitted with a quartz liquid nitrogen dewar was used. The magnetic field was calibrated with a solid sample of DPPH and the microwave frequency was measured by a wave meter attached to the microwave bridge. Spectra at low temperature (77°K) were obtained by immersing a 1:1 toluene–methylcyclohexane solution of the sample in liquid nitrogen. The parallel region of each of the spectra was analyzed according to eq 1. As $|A_\perp|$ is usually about

$$H(0,M_I) = H_\parallel^0 - |A_\parallel| M_I \quad (M_I = {}^3/_2, {}^1/_2, -{}^1/_2, -{}^3/_2) \quad (1)$$

an order of magnitude smaller than $|A_\parallel|$ in most monomeric copper(II) complexes, the second-order contribution to the spectrum in the parallel region was neglected. The uncertainty of the $|A_\parallel|$ values so determined is estimated to be ±0.4 mK.

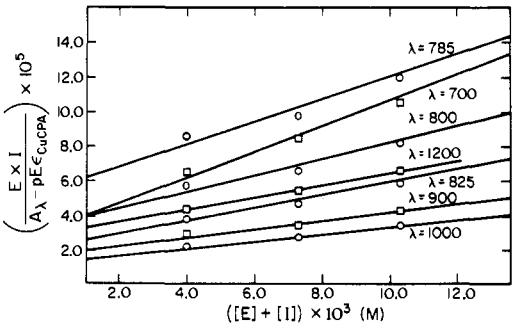

Figure 2. Plots of $EI/(A_\lambda - pE\epsilon_{Cu^{II}CPA})$ vs. $E + I$ for the reaction of $Cu^{II}CPA$ with NaβPP in deuterated 1 *M* NaCl, 0.05 *M* (pH 7.8) Tris buffer (7–12°).

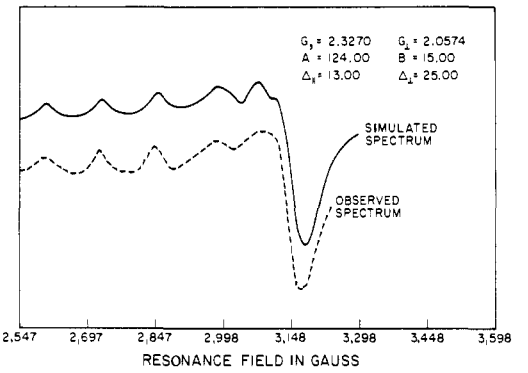

Figure 3. Observed (85°K, 1:1 buffer–ethylene glycol glass) and simulated EPR spectra of $Cu^{II}CPA$.

Metal Analyses. Metal analyses were done by atomic absorption spectroscopy using a Varian Techtron Model AA-5 unit equipped with a Jarrell-Ash Model 82-000 monochromater and Varian Techtron element specific, hollow cathode lamps. Copper(II) and zinc(II) standards containing the metal ions in Tris–HCl–NaCl buffer were made up from 1000 ppm standard solutions obtained from Varian Techtron.

Results

The electronic absorption spectrum of $Cu^{II}CPA$ in D_2O buffer is shown in Figure 1. The principal absorption maximum occurs at 795 nm (12,580 cm^{-1}) and has a molar extinction coefficient, ϵ_m, of 124, in substantial agreement with the values mentioned by Vallee.[8] There is also a distinct shoulder on the low energy side of the absorption envelope in the region of 920 nm (10,800 cm^{-1}). Upon addition of a 16-fold excess of the inhibitor β-phenylpropionate, the band maximum shifts to lower energy and the molar extinction coefficient increases (Figure 1). The low energy shoulder also becomes more pronounced. Using the procedure of Furman and Garner,[18] the formation constant K_I of $Cu^{II}CPA{\cdot}\beta PP$ was found to be $(1.44 \pm 0.24) \times 10^2$. Representative plots of $EI/(A_\lambda - pE\epsilon_{CuCPA})$ vs. $E + I$ are shown in Figure 2 (E and I are the total enzyme and inhibitor concentrations, respectively, A_λ is the observed absorbance at wavelength λ, and p is the path length of the cell in centimeters). The data also indicate that an isosbestic point occurs somewhere between 750 and 770 nm, suggesting that only two species are involved in the equilibrium. Calculation of the spectrum of the $Cu^{II}CPA{\cdot}\beta PP$ complex by a standard procedure[18] yields an absorption maximum at about 875 nm (11,400 cm^{-1}), with $\epsilon_m \sim 180$.

Experimental and the best computer simulated EPR spectra of a frozen sample of $Cu^{II}CPA$ are shown in Figure 3. In the simulation, different line widths (Δ) were used in

Table I. Structural and Electronic Spectral Data for Copper(II) Complexes

	Donor set	Structure[a] (θ)	ν_{max} (cm^{-1})	ϵ_m
$Na_4Cu(NH_3)_4\{Cu(S_2O_3)_2\}H_2O$[b]	N_4	Pl	19,200	
$Na_4Cu(NH_3)_4\{Cu(S_2O_3)_2\}NH_3$[b]	N_5	SPy	17,700	
$[Cu(en)_2](BF_4)_2$[c]	N_4	Pl	19,400	
$[Cu(en)_2NH_3](BF_4)_2$[d]	N_5	SPy	17,400	
$Cu(H(pa))_2$[e]	N_4	Pl	19,600	~75
$Cu(n\text{-}Bu(pa))_2$[f]	N_4		18,940	~110
$Cu(i\text{-}Pr(pa))_2$[f]	N_4		18,600	~110
$Cu(t\text{-}Bu(pa))_2$[f,g]	N_4	P-T (60°)	15,000	~160
$Cu(HPhHMe)_2$[h]	N_2O_2	Pl	17,500	~75
$Cu(CH_3PhHMe)_2$[h]	N_2O_2		16,000	~95
$Cu(i\text{-}PrPhHMe)_2$[h]	N_2O_2		13,800	~110
$Cu(N\text{-}n\text{-}PrSal)_2$[i]	N_2O_2	Pl[j]	16,600	~30
$Cu(N\text{-}n\text{-}BuSal)_2$[i]	N_2O_2	Pl[j]	15,750	~25
$Cu(N\text{-}i\text{-}PrSal)_2$[i]	N_2O_2	P-T (60°)[k]	14,700	~40
$Cu(N\text{-}PhSal)_2$[i]	N_2O_2	Pl[l]	14,100	~45
$Cu(N\text{-}t\text{-}BuSal)_2$	N_2O_2	P-T (54°)[m]	12,900[i]	~90[i]
			12,740[n]	~115[n]
$Cu(o\text{-}phen)Cl_2$[o]	N_2Cl_2	P-T (79°)	13,800	
$[(n\text{-}C_3H_7)_4N]Cu(cat)_2$[p]	O_4	Pl	~15,000	
$Cu(facfac)_2$[q,r]	O_4	Pl	13,500	~40
$Cu^{II}CPA$[s]	N_2O_2?	P-T?	12,580	124
$Cu^{II}CPA\cdot\beta PP$[s]	N_2O_2?	P-T?	11,400	180
$Cu^{II}CA$[t]	N_3O?	P-T?	13,900	110

[a] Abbreviations: Pl = planar; P-T = pseudo tetrahedral; SPy = square pyramidal. [b] B. J. Hathaway and F. Stephens, *J. Chem. Soc. A*, 884 (1970). [c] I. M. Procter, B. J. Hathaway, and P. Nicholls, *ibid.*, 1678 (1968). [d] A. G. Tomlinson and B. J. Hathaway, *ibid.*, 1685 (1968). [e] A. Chakravorty and T. S. Kannan, *J. Inorg. Nucl. Chem.*, 29, 1691 (1967); pa = pyrrole-2-aldimino. [f] R. H. Holm, A. Chakravorty, and L. J. Theriot, *Inorg. Chem.*, 5, 625 (1966). [g] R. H. Holm and M. J. O'Connor, *Prog. Inorg. Chem.*, 14, 325 (1971). [h] D. H. Gerlach and R. H. Holm, *Inorg. Chem.*, 9, 589 (1970); R-PhHMe = N-R-phenylmethyl-β-ketimine. [i] This work; spectrum in toluene at 25°. [j] G. Bombieri, C. Panattoni, E. Fursellini, and R. Graziani, *Acta Crystallogr., Sect. B*, 25, 1208 (1969). [k] P. L. Orioli and L. Sacconi, *J. Am. Chem. Soc.*, 88, 277 (1966). [l] L. Wei, R. M. Stogsdill, and E. C. Lingafelter, *Acta Crystallogr.*, 17, 1058 (1964). [m] T. P. Cheesman, D. Hall, and T. N. Waters, *J. Chem. Soc. A*, 685 (1966). [n] This work, spectrum in 1,1,2,2-tetrachloroethane solution at 25°. [o] G. F. Kokoszka, G. W. Reimann, and H. C. Allen, Jr., *J. Chem. Phys.*, 71, 121 (1967). [p] F. Röhrscheid, A. L. Balch, and R. H. Holm, *Inorg. Chem.*, 5, 1545 (1966). [q] J. P. Fackler, Jr., F. A. Cotton, and D. W. Barnum, *Inorg. Chem.*, 2, 97 (1963). [r] J. A. Bertrand and R. I. Kaplan, *Inorg. Chem.*, 5, 489 (1966). [s] This work; spectrum in D_2O buffer (7–12°). [t] S. Lindskog and P. O. Nyman, *Biochim. Biophys. Acta*, 85, 462 (1964).

the parallel and perpendicular regions. Although the values $|A_\perp| = 15$ and $\Delta_\perp = 25$ G provide an excellent fit in the perpendicular region, equally good agreement with experiment can be obtained by assuming a slightly larger line width and a smaller hyperfine splitting or vice versa ($\Delta_\perp = 25 \pm 3$; $|A_\perp| = 15 \pm 3$ G). The perpendicular region is further complicated by the presence of an angular anomaly[17] at $\theta = 79°$. As there is large absorption due to the angular anomaly so close to $\theta = 90°$, we cannot rule out the possibility of a small rhombic distortion. That such a distortion may be present was noted in a preliminary report of a single-crystal EPR study of $Cu^{II}CPA$, where three principal g values were observed.[9]

The values ($g_\parallel = 2.327$; $|A_\parallel| = 124$ G (13.5 mK)) we find for the parallel components of the g and A tensors for $Cu^{II}CPA$ differ significantly from those ($g_\parallel = 2.24$; $|A_\parallel| = 182$ G (19.0 mK)) reported earlier by Malmström and Vänngård.[10] The probable reason for the discrepancy lies in the fact that the latter parameters were measured on samples that were freeze-dried or were in solution at pH 5.5. Freeze-drying reduces the specific activity of native CPA by more than 50%,[19] whereas dialysis of the protein against pH 5.0 buffer has been employed as a means of preparation of apoCPA.[3] Thus it is reasonable to conclude that Malmström and Vänngård observed the EPR signal of cupric ion that either was bound nonspecifically to the protein or was complexed by solvent molecules. In this regard, we note that we have found $g_\parallel = 2.24$ and $|A_\parallel| = 185$ G (19.3 mK) for Cu(II) in 1:1 Tris buffer–ethylene glycol at 77°K, in very close agreement with the parameter values reported previously for $Cu^{II}CPA$.[10]

Electronic absorption spectral data for an extensive selection of copper(II) model complexes containing N_xO_y donor atom sets are summarized in Table I. The series of Cu(N-R-Sal)$_2$ complexes is of particular interest, as direct structural information on each member is available. X-Ray structural studies have shown that the distortion from planarity in these $Cu^{II}N_2O_2$ systems, as measured by the angle (θ) between the two CuNO planes, is determined by the bulkiness of the imine side chain.[20-24] The complexes which are significantly distorted ($\theta \geq 50°$) will be referred to as having a "pseudo tetrahedral" geometry. Electronic spectra of the pseudo-tetrahedral complex Cu(N-*t*-BuSal)$_2$ in toluene and 1,1,2,2-tetrachloroethane solutions are compared in Figure 4.

EPR spectra of the Cu(N-R-Sal)$_2$ complexes in frozen toluene–methylcyclohexane solutions (77°K) are axial in nature and exhibit well-resolved parallel regions. It is apparent from an inspection of Table II that the value of $|A_\parallel|$ decreases with increasing distortion toward tetrahedral geometry. Both the pseudo-tetrahedral complex Cu(N-*t*-BuSal)$_2$ and the Cu^{2+}-doped sample of Zn(*o*-phen)Cl_2[25] exhibit relatively small $|A_\parallel|$ values, for example. The significant decrease in $|A_\parallel|$ on changing from toluene to 1,1,2,2-tetrachloroethane solution indicates that Cu(N-*t*-BuSal)$_2$ is more severely distorted toward tetrahedral geometry in the latter medium.

Discussion

It has been shown that the relative magnitudes of the g values for copper(II) complexes can be used to distinguish certain types of coordination environments.[26] For example,

Table II. EPR Data for Copper(II) Complexes

Complex	θ, deg	$g_\parallel$	$\lvert A_\parallel \rvert$, mK
Cu^{2+} in $Ni(Sal)_2$[a]	0	2.20	18.5
Cu(N-*n*-PrSal)$_2$	0[b]	2.23	18.6[c]
Cu(N-*n*-BuSal)$_2$	0[b]	2.23	18.3[c]
Cu(N-PhSal)$_2$	0[d]	2.23	17.2[c]
Cu(N-biPh/2-Sal)$_2$	37[e]	2.24	17.6[c]
Cu(N-*i*-PrSal)$_2$	60[f]	2.25	16.4[c]
Cu(N-*t*-BuSal)$_2$	54[g]	2.28	14.7[c]
		2.27	11.9[h]
Cu^{2+} in Zn(N-*i*-PrSal)$_2$	>70[i]	2.29	12.5[j]
Cu^{2+} in Zn(N-*t*-BuSal)$_2$	>70[i]	2.29	12.5[k]
Cu^{2+} in Zn(*o*-phen)Cl_2[l]	79	2.30	12.3
Cu^{II}CPA[m]		2.327	13.5
Cu^{II}CA[n]		2.314	13.3

[a] A. H. Maki and B. R. McGarvey, *J. Chem. Phys.*, 29, 35 (1958). [b] G. Bombieri, C. Panattoni, E. Fursellini, and R. Graziani, *Acta Crystallogr., Sect. B*, 25, 1208 (1969). [c] This work; spectrum measured in 1:1 methylcyclohexane–toluene frozen glass (77°K). [d] L. Wei, R. M. Strogsdill, and E. C. Lingafelter, *Acta Crystallogr.*, 17, 1058 (1964). [e] T. P. Cheesman, D. Hall, and T. N. Waters, *Proc. Chem. Soc., London*, 379 (1963). [f] P. L. Orioli and L. Sacconi, *J. Am. Chem. Soc.*, 88, 277 (1966). [g] T. P. Cheesman, D. Hall, and T. N. Waters, *J. Chem. Soc. A*, 685 (1966). [h] Y. Nonaka, T. Tokii, and S. Kida, *Bull. Chem. Soc. Jpn.*, 47, 312 (1974); spectrum measured in 1,1,2,2-tetrachloroethane (137°K). [i] E. Frasson and C. Panattoni, *Z. Kristallogr. Kristallgeom., Kristallphys., Kristallchem.*, 116, 154 (1961). [j] H. P. Fritz, B. M. Golla, and H. Keller, *Z. Naturforsch., Teil B*, 21, 1015 (1960). [k] *ibid.*, 23, 876 (1968). [l] G. F. Kokoszka, C. W. Reimann, and H. C. Allen, Jr., *J. Phys. Chem.*, 71, 121 (1967). [m] This work; spectrum measured in 1:1 buffer–ethylene glycol frozen glass (85°K). [n] S. Lindskog and P. O. Nyman, *Biochim. Biophys. Acta*, 85, 462 (1964).

tetragonal distortion from octahedral symmetry may occur either by axial elongation or compression. In the former case, $g_\parallel > g_\perp > 2.00$, whereas $g_\perp > g_\parallel > 2.00$ for the latter. According to theory, the g value pattern for axial compression should be exactly the same as that for trigonal bipyramidal coordination. Thus on the basis of the g values alone, both trigonal bipyramidal and axially compressed tetragonal coordination can be eliminated as possibilities for Cu^{II}CPA.

An undistorted square planar coordination geometry is also highly unlikely for Cu^{II}CPA. The near-infrared and visible absorption spectra of square planar Cu(II) complexes containing N_xO_y donor-atom sets all have principal d-d bands in the region 14,000–20,000 cm^{-1} (Table I). The main d-d band in Cu^{II}CPA peaks well below the lower limit of this range. It may be noted at this point that the position (13,900 cm^{-1}) of the principal d-d band in the Cu(II) derivative of carbonic anhydrase (CA) is also too low to be consistent with square planar coordination involving a probable N_3O[27] donor set.

Examination of the absorption spectral data for the various Cu(II) model complexes set out in Table I reveals two alternative explanations for the relatively low energy, moderately intense d-d maximum in Cu^{II}CPA. Addition of axial ligands to square planar Cu(II), giving first a square pyramidal and then an elongated tetragonal structure, shifts the main absorption band to lower energy. The shift to lower energy is accompanied by an intensity increase, and is sometimes called the "pentaammine effect". This effect was first observed[28] on further addition of ammonia to a solution containing $Cu(NH_3)_4(H_2O)_2^{2+}$ and can be considered to occur generally when a stronger field ligand replaces a weaker field one in an axial position. However, EPR results rule against a pentaammine-like structural situation in Cu^{II}CPA, as its $\lvert A_\parallel \rvert$ of 13.5 mK falls below the range of 15–20 mK observed for tetragonal Cu(II) in protein type II sites and in a variety of low molecular weight

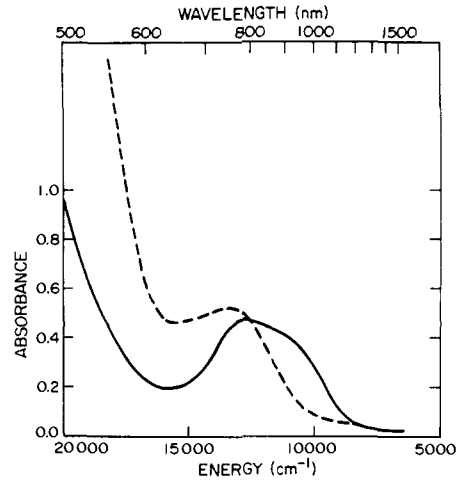

Figure 4. Absorption spectra of Cu(N-*t*-BuSal)$_2$ at 25°: (—) in 1,1,2,2-tetrachloroethane; (- - - -) in toluene.

model species.[29]

Red shifts and intensity enhancements of d-d electronic absorption bands also accompany changes from square planar toward tetrahedral geometry. Thus a very attractive structural model for Cu^{II}CPA is the pseudo-tetrahedral complex Cu(N-*t*-BuSal)$_2$, whose electronic absorption spectrum in 1,1,2,2-tetrachloroethane exhibits a principal maximum at 785 nm (12,740 cm^{-1}) with an ϵ_m of 115 (Figure 4). The spectrum also reveals a prominent low-energy shoulder (950 nm; 10,500 cm^{-1}). The positions and intensities of both the maximum and the shoulder strikingly resemble the electronic spectral features observed for Cu^{II}CPA and Cu^{II}CPA·βPP (Figure 1). Furthermore, both the $g_\parallel$ and $\lvert A_\parallel \rvert$ values of Cu^{II}CPA are in reasonable agreement with those found for certain of the pseudo-tetrahedral Cu(II) complexes (Table II). Again, the data for Cu(N-*t*-BuSal)$_2$ are in particularly close accord with the Cu^{II}CPA results. The weight of the available evidence, then, points clearly to a pseudo-tetrahedral geometry for Cu(II) coordination in CPA. A similar conclusion concerning structural assignment may be reached for Cu^{II}CA.

It must be emphasized that the $\lvert A_\parallel \rvert$ for Cu^{II}CPA is still greater than typical values (3–10 mK range) for type I Cu(II) proteins.[29] A distorted tetrahedral coordination geometry is also a prime candidate for type I Cu(II),[30] but the situation is probably not strictly comparable because of the likelihood of Cys-S binding to Cu(II) in the "blue" proteins.[30]

The substantial increase in the intensity of the main d-d absorption band in the Cu^{II}CPA·βPP complex over Cu^{II}CPA cannot be explained by ligand exchange (e.g., substitution of carboxylate for water) alone. Such exchange would be expected to produce a red shift, as carboxylate is below water in the spectrochemical series, but little or no intensity enhancement. It is probable, therefore, that ligand substitution is accompanied by a small conformational change that produces a more tetrahedral-like environment. Such a conformational change could be caused by interaction of the aromatic part of the inhibitor with groups in the enzyme. Structural changes associated with βPP^- binding to Ni^{II}CPA have also been postulated, based on spectroscopic results.[7]

Formation constants for reaction of β-phenylpropionate with several M^{II}CPA derivatives are summarized in Table III. It is striking that K_I for the one inactive derivative, Cu^{II}CPA, fails well over an order of magnitude below the rather narrow range measured for the active enzymes. Clearly, the presence of cupric ion significantly alters the

Table III. Binding Constants of β-Phenylpropionate to M^{II}CPA Derivatives

M^{II}CPA	K_I ($10^4 M^{-1}$)
Mn(II)[a]	0.28
Co(II)[b]	>0.5
Ni(II)[c]	0.37
Cu(II)[d]	0.014
Zn(II)[e]	0.53

[a] G. Navon, R. G. Shulman, B. J. Wyluda, and T. Yamane, *J. Mol. Biol.*, 51, 15 (1970). [b] J. E. Coleman and B. L. Vallee, *Biochemistry*, 3, 1874 (1964). [c] R. C. Rosenberg, C. A. Root, and H. B. Gray, *J. Am. Chem. Soc.*, 97, 21 (1975). [d] This work. [e] D. S. Auld and B. L. Vallee, *Biochemistry*, 9, 602 (1970).

inhibitor binding site. Such alteration could mean that certain of the mechanistically important protein side chains have been shifted from their optimal positions. This suggestion derives some support from the low resolution X-ray work on the Gly-L-Tyr complex of Cu^{II}CPA, as in this case the conformational change of Glu 270 associated with substrate binding in the native enzyme is not observed. [4,31]

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