Perry, L. J., & Wetzel, R. (1987) *Protein Eng. 1*, 101–105.
Remington, S. J., Anderson, W. F., Owen, J., Ten Eyck, L. F., Grainger, C. T., & Matthews, B. W. (1978) *J. Mol. Biol. 118*, 81–89.
Streisinger, G., Mukai, F., Dreyer, W. J., Miller, B., & Horiuchi, S. (1961) *Cold Spring Harbor Symp. Quant. Biol. 26*, 25–30.
Streisinger, G., Okada, Y., Emrich, J., Newton, J., Tsugita, A., Terzahi, E., & Inouye, M. (1966) *Cold Spring Harbor Symp. Quant. Biol. 31*, 77–84.
van Egmond, J., Kohler, B. E., & Chan, I. Y. (1975) *Chem. Phys. Lett. 34*, 423–426.
Vedenkina, N. S., Troitskill, A. V., & Burshtein, E. A. (1984) *Mol. Biol. (Engl. Transl.) 18*, 362–369.
Weaver, L. H., & Matthews, B. W. (1987) *J. Mol. Biol. 193*, 189–199.
Zang, L.-H., Ghosh, S., & Maki, A. H. (1988) *Biochemistry 27*, 7820–7825.

Characterization of the Inhibitor Complexes of Cobalt Carboxypeptidase A by Electron Paramagnetic Resonance Spectroscopy†

Richard A. Martinelli,‡ Graeme R. Hanson,§ Jeffery S. Thompson,∥ Barton Holmquist, John R. Pilbrow,⊥ David S. Auld, and Bert L. Vallee*

Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, and Brigham and Women's Hospital, Boston, Massachusetts 02115

Received August 10, 1988; Revised Manuscript Received October 26, 1988

ABSTRACT: The metal coordination sphere of cobalt-substituted carboxypeptidase A and its complexes with inhibitors has been characterized by X-band electron paramagnetic resonance (EPR) spectroscopy. The temperature dependence of the EPR spectrum of cobalt carboxypeptidase and the g anisotropy are consistent with a distorted tetrahedral geometry for the cobalt ion. Complexes with L-phenylalanine, a competitive inhibitor of peptide hydrolysis, as well as other hydrophobic L-amino acids all exhibit very similar EPR spectra described by three g values that differ only slightly from that of the cobalt enzyme alone. In contrast, the EPR spectra observed for the cobalt enzyme complexes with 2-(mercaptoacetyl)-D-Phe, L-benzylsuccinate, and L-β-phenyllactate all indicate an approximately axial symmetry of the cobalt atom in a moderately distorted tetrahedral metal environment. Phenylacetate, β-phenylpropionate, and indole-3-acetate, which exhibit mixed modes of inhibition, yield EPR spectra indicative of multiple binding modes. The EPR spectrum of the putative 2:1 inhibitor to enzyme complex is more perturbed than that of the 1:1 complex. For β-phenylpropionate, partially resolved hyperfine coupling (122×10^{-4} cm^{-1}) is observed on the $g = 5.99$ resonance, possibly indicating a stronger metal interaction for this binding mode. The structural basis for the observed EPR spectral perturbations is discussed with reference to the existing crystallographic kinetic and electronic absorption, nuclear magnetic resonance, and magnetic circular dichroic data.

A detailed description of the catalytic role of the essential active-site zinc ion in carboxypeptidase A (CPD-A)[1] (EC 3.4.17.1) requires an understanding of the alterations in the metal coordination sphere accompanying the binding of ligands. Substitution of the active-site zinc atom in CPD-A with cobalt produces a metallo derivative of the enzyme that is active toward both peptide and depsipeptide substrates (Auld & Holmquist, 1974), and X-ray crystallography has shown crystalline Co(II)CPD to have a metal binding site structure similar to that of the native enzyme (Hardman & Lipscomb, 1984). This metal substitution allows the binding of inhibitors and substrates to be monitored by electronic absorption, MCD, EPR, and NMR spectroscopy (Latt & Vallee, 1971; Holmquist et al., 1975; Vallee & Holmquist, 1980; Geoghegan et al., 1983, 1986; Auld et al., 1984, 1986; Kuo & Makinen, 1985; Makinen et al., 1985; Bertini & Luchinat, 1986; Bertini et al., 1988; Bicknell et al., 1988; Luchinat et al., 1988).

The structural characterization of the complexes of Co(II)CPD with various ligands by spectral means has relied primarily on electronic spectroscopy including absorbance, CD, and MCD. The correlation of the spectral features of the cobalt d–d transitions with overall metal geometry of cobalt complex ions has enabled assignments to be made for cobalt-substituted enzymes (Latt & Vallee, 1971; Holmquist & Vallee, 1978). On this basis, the metal environment in Co(II)CPD and some of its inhibitor complexes have been classified as tetrahedral-like. MCD, a technique that allows

† Supported in part by grants from the Australian Research Grants Scheme. J.S.T. was supported by a postdoctoral fellowship from the American Cancer Society. G.R.H. was a recipient of a Research Fellowship from the Scheme.

‡ Permanent address: Ciba-Corning Diagnostics Corp., 63 North St., Medfield, MA 02052.
§ Permanent address: Department of Chemistry, University of Queensland, St. Lucia, Queensland, Australia 4067.
∥ Permanent address: Central Research and Development Department, E. I. du Pont de Nemours & Co., Experimental Station, Wilmington, DE 19898.
⊥ Permanent address: Physics Department, Monash University, Clayton, Victoria, Australia 3168.

[1] Abbreviations: CPD-A, native zinc carboxypeptidase A; Co(II)-CPD, cobalt substituted carboxypeptidase; apo, metal-free; MCD, magnetic circular dichroism; EPR, electron paramagnetic resonance; CD, circular dichroism; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; LSE, measure of the least-squares error between the simulated and experimental EPR spectra.

0006-2960/89/0428-2251$01.50/0

the empirical assignment of an overall coordination number, has confirmed these assignments (Kaden et al., 1974; Holmquist et al., 1975). However, more detailed information on the metal environments of Co(II)CPD and its complexes could not be derived from these electronic spectra since it has not been possible to assign the specific d–d transitions that give rise to the absorption and MCD spectra with any certainty. This results in part from the low-symmetry metal environments encountered in metalloenzymes (Vallee & Galdes, 1984), limiting the degree to which metal complex ions can be compared to metalloenzymes. The spectral properties of low-symmetry Co(II) complexes have been considered in some depth within the framework of the angular overlap model of the ligand field (Banci et al., 1982).

Of the spectral techniques available for the study of high-spin Co(II) complexes, EPR spectroscopy, despite its early use (Kennedy et al., 1972), has only recently been emphasized in the study of Co(II)CPD (Kuo & Makinen, 1982, 1985; Geoghegan et al., 1983; Auld et al., 1984; Makinen et al., 1985). Owing to the sensitivity of the g and hyperfine principal values to variations in the metal environment, EPR spectroscopy has the demonstrated capability of providing useful structural information about the metal coordination sphere in Co(II) complexes (Banci et al., 1982). In this paper we have examined a variety of inhibitor complexes of Co(II)CPD using EPR spectroscopy assisted, where possible, by quantitative computer simulation. These results have been integrated with the available electronic absorption, CD, MCD, and kinetic data.

Materials and Methods

Carboxypeptidase A, prepared by the method of Cox et al. (1964), was received as a crystalline suspension from Sigma Chemical Co. and was recrystallized twice prior to use.

Apocarboxypeptidase was prepared from the native enzyme by a slight modification of the published procedure (Auld & Holmquist, 1974; Auld, 1988). The crystals were washed with a solution of 10 mM 1,10-phenanthroline (Aldrich Chemical Co.) in 10 mM Mes, pH 7.0, for 1 h at 4 °C and collected by centrifugation, and the procedure was repeated twice more. The 1,10-phenanthroline was removed by repeated washing with 10 mM Mes, pH 7.0, until the absorbance at 320 nm was negligible ($\ll$0.01). Co(II)CPD was prepared from the apoenzyme crystals by incubating the apoenzyme with 0.2 mM cobalt chloride (Specpure grade, Johnson Matthey Chemicals, Ltd.) in 10 mM Mes, pH 7.0, for 1 h, centrifuging the crystals and repeating this a further two times. Excess cobalt was removed by washing the crystals repeatedly with 10 mM Mes, pH 7.0, buffer. Enzyme concentration was determined by using $\epsilon_{278} = 6.4 \times 10^4$ M^{-1} cm^{-1}.

The L-amino acids and L-β-phenyllactate were obtained from Sigma, and phenylacetic acid was from Chemical Dynamics Corp. Indole-3-acetic acid and β-phenylpropionate were obtained from Eastman Organic Chemical Co. and recrystallized prior to use. L-Benzylsuccinate was prepared from the D,L mixture (Burdrick & Jackson) by the method of Byers and Wolfenden (1973). Mercaptoacetyl-D-Phe was synthesized according to a literature procedure (Holmquist & Vallee, 1979). Buffer solutions were extracted with dithizone (Fisher Scientific), 0.01% in CCl_4, to remove trace metal contaminants (Holmquist, 1988).

X-band EPR spectra were obtained with a Varian E-9 spectrometer equipped with a dual cavity operating in the TE_{104} mode at a microwave frequency of $\simeq$9.35 GHz, allowing concurrent magnetic field calibration with a Varian strong pitch sample (g = 2.0027). An Air Products LTD-3-110 Heli-Tran liquid helium transfer line was employed to obtain optimal sample temperatures of 4 K. Some of the spectra were also obtained at a microwave frequency of $\simeq$9.28 GHz by using similar equipment at the National Biomedical ESR Center, Medical College of Wisconsin, Milwaukee. EPR spectra of the inhibitor complexes of Co(II)CPD and the native enzyme were recorded at a microwave power of 200 mW, modulation frequency 100 kHz, and a modulation amplitude of 10 G.

Calibration of the magnetic field and microwave frequency was independently checked by remeasuring the spectra on a Varian E-12 EPR spectrometer at Monash University equipped with an Oxford Instruments helium flow cryostat, where magnetic fields were directly calibrated against proton magnetic resonance. Both proton resonance frequencies and the microwave frequency were measured with an EIP 548A (DC-26.5 GHz) frequency counter.

EPR samples were prepared by using zwitterionic buffers (Hepes, Mes) to avoid large changes in pH with temperature (Williams-Smith et al., 1977) and were frozen by immersion in liquid nitrogen prior to insertion into the EPR cavity.

The signal to noise ratios for the EPR spectra of Co(II)CPD and the inhibitor complexes are quite low as a result of their inherently large line widths (see Table III). Computer averaging to improve the signal to noise ratio was carried out on an Apple IIe computer or on a PDP 11/34 computer. Spectra obtained on the E-12 instrument at Monash University were collected on an LSI 11/23 computer linked to the VAX network at the Monash Computer Center. The spin Hamiltonian parameters were determined from computer simulation studies.

Computer-simulated EPR spectra of Co(II)CPD and its complexes with inhibitors were obtained by using (Dougherty et al., 1985)

$$S(\nu_c, B) = \sum_{\theta=0°}^{90°} \sum_{\Phi=0°}^{90°} \sum_{M_1=-7/2}^{+7/2} \bar{g}_1^2 f(\nu_c - \nu_0(B), \sigma_\nu) \Delta \cos\theta \Delta\Phi \quad (1)$$

where $\bar{g}_1^2$ is the power-averaged expression for the transition probability (Pilbrow, 1969) and f, the lineshape function, is assumed to be Gaussian. In f, $\nu_0(B)$ is the actual energy difference between the energy levels and is evaluated by using second-order perturbation theory (Lin, 1973; Freeman, 1973). To determine the approximate magnetic field at which each transition occurs, the conventional second-order perturbation field expressions are retained in the simulation program, but these are not used to calculate the line shape. They are used in setting up the "windows" for the calculation of each individual line. The $1/g$ factor introduced by Aasa and Vanngard (1975) is not required in eq 1 since the calculation of the line-shape function is carried out directly in terms of frequency rather than field. In a field-swept experiment it is ν_c, the microwave frequency, that is constant and $\nu_0(B)$ that varies with the magnetic field.

The line widths, σ_ν, used in the simulations are based on a model developed for copper(II) complexes by Froncisz and Hyde (1980). In this model it is assumed that the dependence of line width on both microwave frequency and M_1 in frozen solution EPR of metal complexes is due to random strain. Such strains, which occur upon freezing, are believed to be responsible for strongly correlated distributions of g and A values. Thus, for the principal directions in the molecule, where $i = x$, y, or z, the line width, in frequency units, is of the form (Dougherty et al., 1985)

$$\sigma_i = [\sigma_{Ri}^2 + (C_{1i}\nu_0(B) + C_{2i}M_I)^2]^{1/2} \quad (2)$$

Table I: EPR Parameters of the Inhibitor Complexes of Cobalt Carboxypeptidase A[a]

inhibitor	concn (mM)	g_x	g_y	g_z	A_x[b]	A_y[b]	A_z[b]
Co(II)CPD (alone)		5.40	2.84	1.90	0[c]	0	0
mercaptoacetyl-D-Phe	1	5.06	3.67	2.05	54	103	58
L-benzylsuccinate	1.1	4.13	3.29	1.71	0	0	50
L-β-phenyllactate[d]	10	4.40	3.49	1.99	0	0	50
L-phenylalanine[e]	10	6.03	2.17	1.68			
L-leucine[e]	100	5.46	3.88	2.12			
L-valine[e]	218	5.82	2.55	1.85			
L-tryptophan[e]	45	5.94	3.71	1.96			
phenylacetate[d,g]							
(1)	2	5.30	2.88	2.00	0	0	0
(2)	20	5.77[f]		1.31[f]			
β-phenylpropionate[d,g]							
(1)	20	5.29	2.41	1.65	0	199	200
(2)	20	5.99	2.48	1.68	122	0	0
indole-3-acetate[e,g]							
(1)	0.9	6.32	3.49	1.91			
(2)	18	6.03	3.59	1.96			

[a] Samples were prepared in 2 M NaCl and 50 mM Hepes, pH 7.5. Effective g and A values were determined from computer-simulated spectra unless otherwise stated. For fitting errors see text. [b] To convert A values in units of 10^{-4} cm^{-1} to MHz multiply by 2.9979. [c] Hyperfine values are assigned zero since if given a finite value the corresponding σ_R's would then reduce. [d] Sample prepared in 2 M NaCl and 50 mM Mes, pH 6.0. [e] g values are apparent, calculated from the experimental spectrum. [f] Value measured for sharp (low field) and broad (high field) features. [g] The numbers in parentheses refer to the putative 1:1 and 2:1 complexes, respectively, or in the case of β-phenylpropionate the two simulated species at 20 mM.

Table II: Line-Width Parameters for Cobalt Carboxypeptidase and Inhibitor Complexes

inhibitor	σ_{Rx} (MHz)	σ_{Ry} (MHz)	σ_{Rz} (MHz)	C_{1x} (MHz)	C_{1y} (MHz)	C_{1z} (MHz)	C_{2x} (MHz)	C_{2y} (MHz)	C_{2z} (MHz)
Co(II)CPD (alone)	79	164	312	0.058	0.148	0.045			
mercaptoacetyl-D-Phe	142	206	201	0.022	0.026	0.0	35	61	64
L-benzylsuccinate	83	86	190	0.10	0.10	0.03	22	22	9
L-phenyllactate	89	91	223	0.10	0.10	0.03	22	22	12
phenylacetate									
(1)	107	196	280	0.10	0.25	0.08	22	22	12
β-phenylpropionate									
(1)	141	300	900	0.0011	0.16	0.0	0	10	80
(2)	45	484	529	0.014	0.05	0.05	−9	0	0

where $C_{1i} = \sigma_{gi}/g$ and $C_{2i} = \sigma_{Ai}$, where σ_{gi} and σ_{Ai} are the half-widths of the distributions of the ith components of g and A, respectively. The residual line widths, the σ_{Ri}'s, result from all or some of the following effects: homogeneous broadening from spin–lattice relaxation and spin–spin interactions, unresolved metal ion hyperfine structure, and unresolved ligand hyperfine structure. A unique set of line-width parameters cannot be determined for resonances in which hyperfine coupling is unresolved. The angular variation, for purposes of frozen solution spectral simulations, is assumed to behave in a similar manner to hyperfine structure, viz.

$$\sigma_\nu^2 = \sum(\sigma_i g_i l_i)^2 \qquad i=x,y,z \tag{3}$$

The l_i's are the direction cosines of the magnetic field direction with the principal magnetic axes of the cobalt center in the protein.

The use of eq 1–3, couched in terms of frequency variables, conforms to the general principles discussed previously (Pilbrow, 1984) and provides a model in which a minimum number of assumptions are made about the nature of the intrinsic line shapes. In this case the line shape in the frequency domain is chosen to be either Gaussian or Lorentzian; at any magnetic field and any orientation, and for any particular value of the nuclear quantum number, M_I, only one parameter is needed to specify the width, i.e., the value of σ_ν given by eq 3. The simulation program then calculates the contribution to $S(\nu_c, B)$ (eq 1) for each individual line at each output field within a window centered about its resonance field. The Gaussian or Lorentzian line is truncated in the wings so as to minimize computer-generated noise in the simulated spectrum.

The output from the EPR spectrometer is the first derivative of $S(\nu_c, B)$ with respect to magnetic field and is obtained by using finite levels of magnetic field modulation at 100 kHz; it turns out to be accurate to use finite difference differentiation of eq 1. The differentiation step chosen is approximately the same magnitude as the field modulation amplitude.

Computer simulations were carried out on the VAX network at the Monash Computer Center by using FORTRAN program EPR50F, an extension of an earlier program MONOQF (Dougherty et al., 1985), which is capable of simulating spectra arising from metal ions with triclinic symmetry. Use of the least-squares computer simulation program EPR50FIT has automated the fitting procedure, which enables simulations of a higher quality to be obtained in a shorter time.

An estimation of the quality of fit of a simulation (S) to an experimental spectrum (E) was provided by the least-squares error parameter (LSE), defined in

$$\mathrm{LSE} = [(E/I_E) - (S/I_S)]^2/N \tag{4}$$

where I_E and I_S are the normalization factors (doubly integrated intensities) and N is the number of points in both the simulated and experimental spectra. This normalization ensures comparison of commensurate values inside the brackets of eq 4. A menu-based file handling and plotting package developed at Monash University was used to carry out plotting of spectra, spectral subtractions, and calculation of the least-squares error parameters, where appropriate.

Results

The EPR spectrum of Co(II)CPD in 2 M NaCl and 50 mM Hepes, pH 7.5, is characterized by three resonances with g values $g_x = 5.40$, $g_y = 2.84$, and $g_z = 1.90$ determined from computer simulation (Figure 1 and Tables I–III).[2] The in-

Table III: EPR Line Widths for Cobalt Carboxypeptidase A and Inhibitor Complexes[a,b]

inhibitor	σ_x (MHz)	σ_y (MHz)	σ_z (MHz)	W_x (G)	W_y (G)	W_z (G)
Co(II)CPD (alone)	545	1386	522	73	349	196
mercaptoacetyl-D-Phe	164	208	204	23	40	71
	357	500	300[c]	50	97	105
L-benzylsuccinate	857	857	312	148	185	130
	1010	1010	364	174	219	221
L-phenyllactate	857	857	325	139	175	116
	1010	1010	390	164	206	140
phenylacetate	859	2256	755	115	559	270
(1)	1013	2409	834	136	597	298
β-phenylpropionate	140	1483	900	19	439	642
(1)	1552	942			459	573
(2)	168	671	704	20	193	300
	108			38		

[a] Upper and lower values indicated whenever the C_2's of Table II are non-zero. [b] The relation between W's in gauss and σ's in megahertz is $\sigma = 1.3996gW$ [c] Since $C_{1z} = 0$ in this case, the actual values are 301, 257, 223, 204, 204, 223, 257, and 301 MHz.

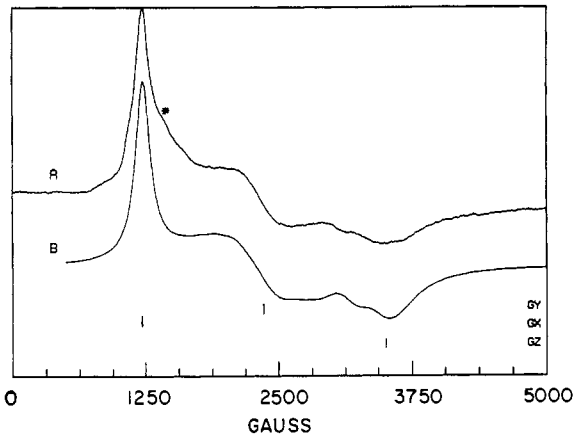

FIGURE 1: (A) EPR spectrum of Co(II)CPD, 2 mM. Sample was prepared in 2 M NaCl and 50 mM Hepes, pH 7.5. Microwave frequency was 9.356 GHz. (B) Computer simulation of (A), LSE = 0.00344.

ability of the simulation to account for features around 1360 G (labeled with an asterisk in Figure 1A) suggests the presence of a broad, underlying spectrum of unknown origin. In the present study the g factors are given in decreasing order as g_x, g_y, and g_z. This is not of fundamental significance; in some cases it will mean that, e.g., $g_x \sim 6$ corresponds to the unique axis in the axial limit, but for the other doublet $g_z \sim 2$ corresponds to the same direction in the molecule. At pH 7.5, neither a change in NaCl concentration from 1 to 4.5 M nor a change in salt from NaCl to NaF, NaBr, or NaI has an effect upon the spectrum (data not shown). The line widths of the resonances in the EPR spectrum of Co(II)CPD are extremely sensitive to temperature, and the spectra are virtually unobtainable above 15 K, implying rapid relaxation.

The binding of mercaptoacetyl-D-Phe, L-benzylsuccinate, or L-β-phenyllactate to Co(II)CPD perturbs the EPR spectrum of Co(II)CPD in a similar manner. The spectra of these complexes (Figure 2A,C,E) have a strong feature at low magnetic field (the sum of an absorption and a derivative peak) and a weak absorption peak at high field, indicative of an approximate axial symmetry for the cobalt atom at the active site. Computer simulation of these spectra (Figure 2B,D,F) provides the g values (Table I) and indicates some departure from true axial symmetry.

The EPR spectrum of the cobalt enzyme is perturbed in a different manner upon binding of L-Phe (Figure 3) and other L-amino acids. The EPR spectra of these complexes exhibit three resonances with larger g_x values (Table I) and broader resonances for g_y and g_z than those observed in the spectrum of Co(II)CPD (Figure 3).

A third group of inhibitors includes phenylacetate, β-phenylpropionate, and indole-3-acetate. The EPR spectra of their complexes with the cobalt enzyme exhibit spectral features arising from two distinct species whose relative amounts depend upon the inhibitor concentration. At 2 mM phenylacetate, approximately a 2-fold molar excess over the cobalt enzyme, the EPR spectrum consists of contributions from two distinct metal species attributable to the putative 1:1 and 2:1 phenylacetate–Co(II)CPD complexes (Figure 4A). ^{13}C NMR and electronic absorption studies have indicated the 1:1 inhibitor to enzyme complex is converted to a 2:1 species over the concentration range 1–20 mM at pH 6.0 and 1 M NaCl (Bertini et al., 1988). Addition of phenylacetate to Co(II)CPD to a concentration of 20 mM (Figure 4B) yields a spectrum predominantly that of the 2:1 complex with a minor contribution from the 1:1 complex. The EPR spectrum of the 1:1 complex is obtained by spectral subtraction (Figure 5A). The orthorhombic EPR spectrum of the 1:1 complex is remarkably similar to that of the native enzyme, having $g_x = 5.30$, $g_y = 2.88$, and $g_z = 2.00$ (Table I). The computer simulation of this spectrum is shown in Figure 5B.

The features of the line shape of the EPR spectrum associated with the putative 2:1 complex (Figure 4B) includes a sharp low-field resonance ($g_x = 5.77$) and a broad resonance at high field ($g_z = 1.31$). This broad resonance may be a combination of a first derivative and a negative absorption peak. Alternatively, the high-field resonance may be just a first derivative resonance, g_y, in which case the third resonance with a negative absorption shape, g_z, would be at higher field and so broad as to preclude resolution under the present conditions.

The EPR spectrum of Co(II)CPD, 1.1 mM, in the presence of β-phenylpropionate, 20 mM, is shown in Figure 6A. The presence of two distinct complexes is particularly apparent from inspection of the complex EPR features in the g_x region of the spectrum. The simulation of one of the species was made on the assumption that the observed hyperfine splitting in g_x is due to this species as well as the other strong resonances

[2] The main source of error in the principal g and A (hyperfine) values and line-width parameters listed in Tables I and II, respectively, arise from the very large line widths (Table III). g values are reported to two decimal places, although for some of the simulations the error is ±0.005. For spectra in which hyperfine coupling is unresolved, errors of A values are not given because of the interaction between them and the line-width parameters for the unresolved features. Table II contains values of the σ_R's, C_1's, and C_2's, while Table III contains values (in megahertz and gauss) of maximum and minimum line widths across the eight-line pattern due to x, y, and z orientations. All line-width parameters are calculated by using eq 2 and converted to gauss as shown in the footnote to Table III. These values are reported to define the simulations.

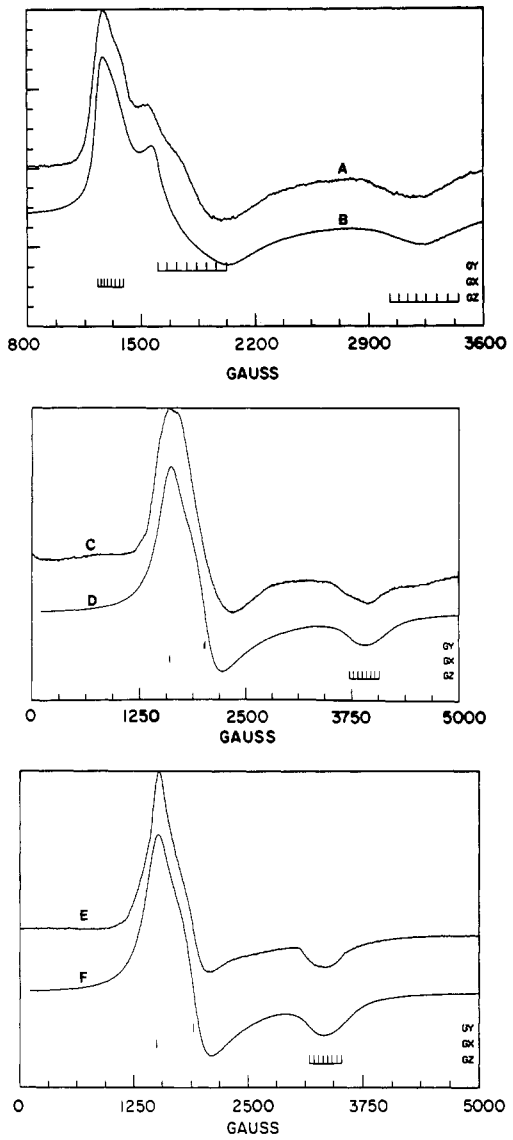

FIGURE 2: (A) EPR spectrum of Co(II)CPD, 0.8 mM, and mercaptoacetyl-D-Phe, 1 mM. Microwave frequency was 9.331 GHz. (B) Computer simulation of (A), LSE = 0.0043. (C) EPR spectrum of Co(II)CPD, 0.7 mM, and L-benzylsuccinate, 1.1 mM. Microwave frequency was 9.353 GHz. (D) Computer simulation of (C), LSE = 0.0088. (E) EPR spectrum of Co(II)CPD, 1.1 mM, and L-phenyllactate, 10 mM. Identical spectra were obtained at pH 7.5 and pH 6.0. Microwave frequency was 9.357 GHz. Sample was prepared in 2 M NaCl and 50 mM Mes, pH 6.0. (F) Computer simulation of (E) LSE = 0.0041. All samples were prepared as described in Figure 1 except where indicated.

for g_y and g_z (Figure 6B). This complex of β-phenylpropionate with Co(II)CPD contains resolved hyperfine coupling (122 $\pm$ 6 $\times$ 10^{-4} cm^{-1}) on the low-field resonance (g = 5.99) corresponding to a hyperfine splitting of 44 G (Figure 6B, Table I). This spectrum was subtracted from that of Figure 6A to yield a sharp low-field peak and a very broad feature toward the high-field portion of the spectrum. Figure 6C is a computer simulation of the latter β-phenylpropionate–Co(II)CPD species using the EPR parameters given in Tables I and II. The two large hyperfine principal values for the g_y and g_z resonances of this species are estimates since they cannot be resolved; the total line width is the convolution of the large line widths (Table III) for this example and the effective line width from the unresolved hyperfine structure. The simulations of Figure 6B,C are added together as described in the figure caption to produce Figure 6D to be compared with the original experimental spectrum (Figure 6A).

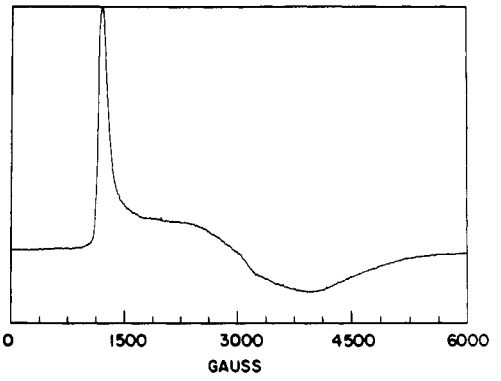

FIGURE 3: EPR spectrum of Co(II)CPD, 1.1 mM, in the presence of L-Phe, 10 mM. Sample was prepared as in Figure 1. Microwave frequency was 9.357 GHz. Computer simulation not shown, LSE = 0.0238.

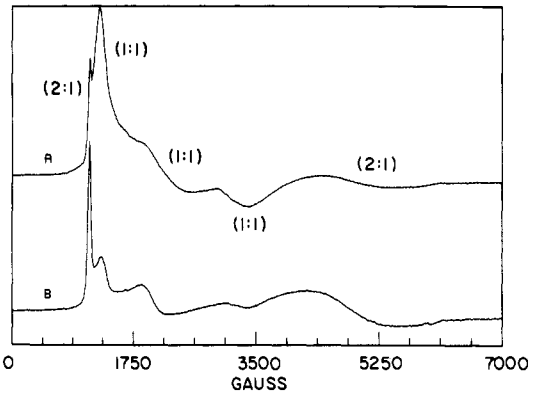

FIGURE 4: (A) EPR spectrum of Co(II)CPD, 1.1 mM, and phenylacetate, 2 mM. Microwave frequency was 9.287 GHz. (B) EPR spectrum of Co(II)CPD, 1.1 mM, and phenylacetate, 20 mM. Microwave frequency was 9.285 GHz. Samples A and B were prepared in 2 M NaCl and 50 mM Mes, pH 6.0. The resonances attributed to the putative 1:1 and 2:1 species are designated.

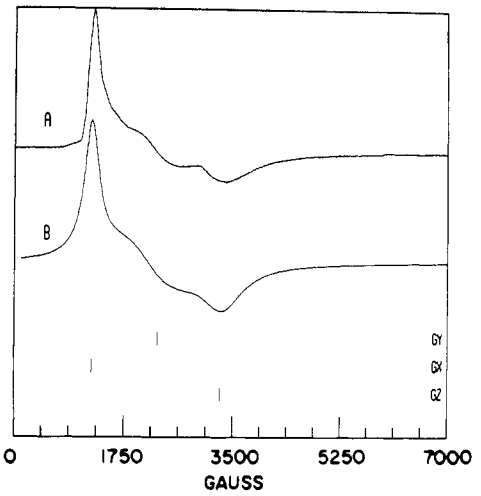

FIGURE 5: (A) EPR spectrum of the putative 1:1 phenylacetate–Co(II)CPD complex obtained by subtraction of 0.34 of (4B) from (4A). (B) Computer simulation of (A), LSE = 0.0066.

Discussion

The EPR spectrum of cobalt carboxypeptidase (Figure 1A) is characterized by an anisotropic effective g matrix (Table I) and the absence of resolved hyperfine coupling on any of the resonances. The necessity for recording the EPR spectrum of Co(II)CPD at liquid helium temperatures implies a rapid relaxation (i.e., short T_1) as is observed for many high-spin cobalt complexes with low-lying excited states. Zero-field splitting of the spin degenerate orbital singlet ground state [$^4A_2$

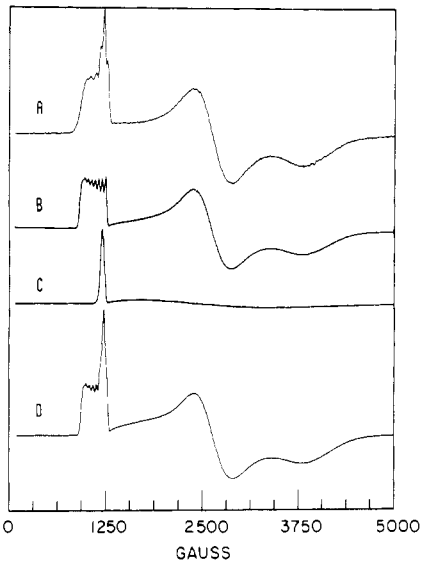

FIGURE 6: (A) EPR spectrum of Co(II)CPD, 1.1 mM, and β-phenylpropionate, 20 mM. Sample was prepared in 50 mM Mes and 2 M NaCl, pH 6.0. Microwave frequency was 9.285 GHz. (B and C) Computer simulations of the two β-phenylpropionate complexes. (D) Computer simulation of (A) obtained by adding 0.54 of (B) to (C), LSE = 0.0048.

for high-spin tetrahedral Co(II) complexes] produced by the combined effect of spin orbit coupling and low-symmetry ligand fields gives rise to two Kramers doublets. If the magnitude of the zero-field splitting is somewhat greater than the applied microwave frequency (0.32 cm^{-1}), then only transitions within one doublet are expected. Computer simulation of the EPR spectrum of Co(II)CPD, assuming an effective spin of $^1/_2$, confirms that EPR transitions are indeed only observed within one doublet and further that the zero-field splitting is indeed greater than 0.32 cm^{-1}. The observed g matrix for Co(II)CPD, with three principal g values, indicates an orthorhombic or lower symmetry environment for the cobalt ion.

A distorted tetrahedral environment had previously been assigned to the cobalt atom in Co(II)CPD on the basis of electronic absorption and MCD spectra (Latt & Vallee, 1971; Holmquist et al., 1975). The EPR spectrum of Co(II)CPD strongly resembles that of cobalt carbonic anhydrase–sulfonamide complexes, which have also been assigned a tetrahedral geometry (Cockle et al., 1974). Finally, the X-ray crystal structures of the native zinc- and cobalt-substituted carboxypeptidases have identified His-69, His-196, Glu-72 (possibly two oxygens), and water as metal ligands thought to result in a coordination number of four or five (Rees et al., 1983; Hardman & Lipscomb, 1984).

The binding of inhibitors markedly affects the EPR spectra because of alterations in the metal coordination sphere. The EPR spectra of the complexes of the cobalt enzyme with L-benzylsuccinate, mercaptoacetyl-D-Phe, and L-β-phenyllactate are similar and approximately axially symmetric (Figure 2). The similar EPR spectral features of these complexes imply analogies in their modes of binding to Co(II)CPD.

Binding of mercaptoacetyl-D-Phe to Co(II)CPD produces distinctive perturbations of its electronic absorption spectrum: the appearance of a sulfur to cobalt charge-transfer band and a dramatic increase in the intensity of the d–d transitions in the visible region (Holmquist & Vallee, 1979). These features are consistent with ligation of the thiolate group to the metal and a shift toward a more regular tetrahedral arrangement of ligands. The perturbation of the orthorhombic EPR spectrum of Co(II)CPD (Figure 1) toward axial symmetry (Figure 2A) upon binding of mercaptoacetyl-D-Phe is consistent with direct metal coordination of one of the functional groups of this compound and a more symmetric arrangement of ligands. With the thiolate group coordinating to the metal, the carboxylate and phenyl side chains of mercaptoacetyl-D-Phe are available for interaction with other active-site loci, e.g., Arg-145 and the hydrophobic pocket, provided there are no stereochemical impediments.

A binding mode analogous to that for mercaptoacetyl-D-Phe is suggested for L-benzylsuccinate based on the axial appearance of the EPR spectrum of its Co(II)CPD complex (Figure 2C). One mole of L-benzylsuccinate binds per mole of enzyme. It was postulated that one of the carboxylates coordinates to the metal in this single binding mode. Since L-benzylsuccinate also binds to apocarboxypeptidase, the other carboxylate and/or the aromatic side chain must be able to interact with other regions of the active site (Byers & Wolfenden, 1973). L-Benzylsuccinate and the phenylalanyl-like portions of peptide and ester substrates are structurally similar, and this agent competitively inhibits hydrolysis of these substrates (Byers & Wolfenden, 1973). On the basis of these facts, the other sites of L-benzylsuccinate interaction may be tentatively identified as Arg-145 and the hydrophobic specificity pocket of CPD.

The metal environment in L-benzylsuccinate–Co(II)CPD was judged to be pentacoordinate or even octahedral (Kuo & Makinen, 1985) on the basis of the magnitude of the zero-field splitting determined for this complex (51 cm^{-1}) from power saturation measurements of the relaxation time determined for the Orbach process and the apparent empirical relationship between this parameter and coordination number. The values of this parameter for four, five, and six coordination were $\leq$13, 20–50, and $>$50 cm^{-1}, respectively (Makinen et al., 1985). However, theoretical calculations have demonstrated that large distortions from tetrahedral symmetry may produce anomalously large zero-field splittings (Banci et al., 1982). Such anomalous values have been observed experimentally, even in the relatively small selection of cobalt model compounds that have been studied (Makinen et al., 1985). The metal environments encountered in proteins are generally severely distorted from idealized tetrahedral or octahedral symmetry (Vallee & Galdes, 1984), often precluding an unambiguous assignment. For this reason, it is necessary to integrate the available structural data rather than to rely on any single technique to provide a definitive conclusion. The assignment here of an approximate tetrahedral geometry for the metal environment in the L-benzylsuccinate–Co(II)CPD complex is consistent with visible absorption and MCD data (unpublished data).

The approximate axially symmetric EPR spectrum of the complex of Co(II)CPD with L-β-phenyllactate (Figure 2E) suggests interactions at the active site analogous to those described for the mercaptoacetyl-D-Phe and L-benzylsuccinate complexes. Although L-benzylsuccinate and mercaptoacetyl-D-Phe have been shown to provide a ligand to the metal, there is no experimental evidence that the hydroxyl or carboxyl group of L-β-phenyllactate coordinates to the metal, though the similarity of their EPR spectra suggests that an oxygen atom may be close, even if not directly bound, to the metal. In addition, kinetic (Byers & Wolfenden, 1973) as well as spectral data with the azo-Tyr-248 derivative of CPD-A (Scheule et al., 1981) indicate that the binding of L-benzylsuccinate and L-β-phenyllactate to CPD-A is mutually exclusive; hence, it is likely that these two inhibitors share at least some common binding loci.

These three inhibitors may also interact with other active-site loci, notably those moieties that confer the enzyme's specificity: Arg-145 and the hydrophobic pocket at the back of the active site. Such nonmetal active-site interactions may be crucial in the orientation of the metal coordinating group necessary to produce the observed approximate axially symmetric EPR spectra for these complexes.

In view of the above assignments it is most interesting that the binding of the competitive product inhibitors of peptide hydrolysis, L-Phe and other L-amino acids, perturbs the EPR spectrum of Co(II)CPD in a less drastic manner (Figure 3), reflecting a weak interaction with the metal. These inhibitor complexes exhibit anisotropic g matrices that vary slightly from that of Co(II)CPD (Table I). At pH 7.5, the amino group of L-Phe is fully protonated and would not be expected to interact strongly with the metal, if at all. This aspect of the binding of L-Phe distinguishes it from the binding of the first group of inhibitors analyzed, although interactions of the carboxylate groups with Arg-145 and of the phenyl moiety with the hydrophobic pocket, as discussed for the first group of inhibitors, have been proposed (Lipscomb et al., 1968).

The sensitivity of the EPR spectral parameters to even slight changes in the metal environment allows subtle differences in the binding modes of inhibitors to be discerned. For example, the X-ray crystal structures of the complexes of L-β-phenyllactate and L-phenylalanine at 6-Å resolution indicate very similar binding modes for these two compounds (Lipscomb et al., 1968). Yet the EPR spectra for these two complexes differ distinctly (Figures 2C and 3), implying different metal interactions for these two inhibitors. Relaxation kinetics of the native enzyme have also indicated that the structurally similar compounds, L-Phe, L-β-phenyllactate, and β-phenylpropionate, all interact differently with the enzyme (French et al., 1974).

The inhibitors that have been discussed thus far appear to bind to carboxypeptidase in a single mode. The other inhibitors investigated, phenylacetate, β-phenylpropionate, and indole-3-acetate, induce complex perturbations of the EPR spectrum of Co(II)CPD that clearly indicate multiple binding modes (Figures 4–6). The EPR spectral manifestations of multiple binding modes for these inhibitors are entirely consistent with the existing spectral and kinetic data on their multiple modes of interaction with CPD-A. Phenylacetate and β-phenylpropionate exhibit multiple modes of inhibition toward carboxypeptidase catalysis of peptide hydrolysis (Auld et al., 1986b). Separate binding modes have been postulated to account for the competitive and noncompetitive components of their inhibition. Thus, for phenylacetate it was found that the competitive mode of inhibition hardly varies with pH; in contrast, the noncompetitive component is pH dependent, and binding becomes tighter with decreasing pH (Auld et al., 1972). These results have been thought to reflect that the inhibitor binds most tightly to the EH_2 form of the enzyme (Auld et al., 1972, 1986b). The EPR data for the binding of phenylacetate to Co(II)CPD (Figure 4) are in agreement with these kinetic data.

Multiple binding modes for phenylacetate, β-phenylpropionate, and indole-3-acetate have been well established by electronic absorption, CD, and NMR studies (Latt & Vallee, 1971; Bertini et al., 1988). At low concentrations these inhibitors act noncompetitively toward peptide hydrolysis and alter the Co(II)CPD absorption spectrum only slightly, while at high concentrations they act competitively and induce a more marked effect on the visible absorption spectrum. Perturbation of the CD spectra of Co(II)CPD and that of the azo-Tyr-248 derivative of CPD-A is also complex and concentration dependent (Latt & Vallee, 1971; Johansen et al., 1976).

^{13}C NMR studies of cobalt carboxypeptidase also provide evidence that acetate and phenylacetate bind at two distinct sites (Bertini et al., 1988). The cobalt $^{13}COO^-$ distances, calculated by means of the Solomon equation, indicate that the second acetate molecule binds directly to the metal ion while the first binds to a protein group at a distance 5–8 Å from the metal ion, consistent with its interacting with one or more of the arginyl residues, Arg-145, Arg-127, or Arg-71.

These EPR spectra show that the binding of the first equivalent of inhibitor perturbs the spectrum of Co(II)CPD only slightly. Thus, the line shape of the EPR spectrum and the effective g matrix for the 1:1 phenylacetate–Co(II)CPD complex differ only slightly from that of Co(II)CPD (Figure 5 and Table I), whereas the binding of the second inhibitor molecule produces a complex with markedly different spectral properties (Figure 4, Table I). Similarly, for β-phenylpropionate, the unusual feature [for complexes of Co(II)CPD] of resolved hyperfine coupling observed on the low-field (g = 5.99) resonance may indicate a stronger metal interaction for this binding mode (Figure 6).

The EPR data reported here both complement and extend the existing data on the binding of inhibitors to carboxypeptidase A. Even though some of the EPR parameters cannot be determined uniquely, as explained earlier, the g values are unambiguous as is the hyperfine splitting on the g = 5.99 peak of the β-phenylpropionate–Co(II)CPD complex. Furthermore, the hyperfine constants for the high-field features of the L-benzylsuccinate and L-β-phenyllactate complexes with Co(II)CPD are also well established. The catalog of spectral perturbations and the inhibitor–enzyme interactions inducing them provide a frame of reference for the investigation of the spectral properties of other complexes of this enzyme. This framework will be utilized in the structural characterization of reaction intermediates of Co(II)CPD with peptide and ester substrates.

ACKNOWLEDGMENTS

We thank Professor James S. Hyde for making available the facilities at the National Biomedical ESR Center, Medical College of Wisconsin.

REFERENCES

Aasa, R., & Vanngard, T. (1975) *J. Magn. Reson. 19*, 308.
Auld, D. S. (1988) *Methods Enzymol. 158*, 71.
Auld, D. S., & Holmquist, B. (1974) *Biochemistry 13*, 4355.
Auld, D. S., Latt, S. A., & Vallee, B. L. (1972) *Biochemistry 11*, 4994.
Auld, D. S., Galdes, A., Geoghegan, K. F., Holmquist, B., Martinelli, R. A., & Vallee, B. L. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5041.
Auld, D. S., Geoghegan, K., Galdes, A., & Vallee, B. L. (1986a) *Biochemistry 25*, 5156.
Auld, D. S., Larsen, K. S., & Vallee, B. L. (1986b) in *Zinc Enzymes* (Bertini, I., Luchinat, C., Maret, W., & Zeppezauer, M., Eds.) p 133, Birkhauser Verlag, Basel.
Banci, L., Bencini, A., Benelli, C., Gatteshci, D., & Zanchini, C. (1982) *Struct. Bonding 52*, 37.
Bertini, I., & Luchinat, C. (1986) *NMR of Paramagnetic Molecules in Biological Systems*, Benjamin Cummings, Boston.
Bertini, I., Monnanni, R., Pellacani, G. C., Sola, M., Vallee, B. L., & Auld, D. S. (1988) *J. Inorg. Biochem. 32*, 13.

Bicknell, R., Schäffer, A., Bertini, I., Luchinat, C., Vallee, B. L., & Auld, D. S. (1988) *Biochemistry 27*, 1050.
Byers, L. D., & Wolfenden, R. (1973) *Biochemistry 12*, 2070.
Cockle, S. A., Lindskog, S., & Grell, E. (1974) *Biochem. J. 143*, 703.
Cox, O. J., Bovard, F. C., Bargetzi, J. P., Walsh, K. A., & Neurath, H. (1964) *Biochemistry 3*, 44.
Dougherty, G., Pilbrow, J. R., Skorobogaty, A., & Smith, T. D. (1985) *J. Chem. Soc., Faraday Trans. 2 81*, 1739.
Freeman, T. E. (1973) Ph.D. Thesis, Monash University.
French, T. C., Yu, N.-T., & Auld, D. S. (1974) *Biochemistry 13*, 2877.
Froncisz, W., & Hyde, J. S. (1980) *J. Chem. Phys. 73*, 3123.
Geoghegan, K. F., Galdes, G., Martinelli, R. A., Holmquist, B., Auld, D. S., & Vallee, B. L. (1983) *Biochemistry 22*, 2255.
Geoghegan, K. F., Galdes, A., Hanson, G., Holmquist, B., Auld, D. S., & Vallee, B. L. (1986) *Biochemistry 25*, 4669.
Hardman, K. D., & Lipscomb, W. N. (1984) *J. Am. Chem. Soc. 106*, 463.
Holmquist, B. (1988) *Methods Enzymol. 158*, 6.
Holmquist, B., & Vallee, B. L. (1978) *Methods Enzymol. 49*, 149.
Holmquist, B., & Vallee, B. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6216.
Holmquist, B., Kaden, T. A., & Vallee, B. L. (1975) *Biochemistry 14*, 1454.
Johansen, J. T., Klyosov, A. A., & Vallee, B. L. (1976) *Biochemistry 15*, 296.
Kaden, T. A., Holmquist, B., & Vallee, B. L. (1974) *Inorg. Chem. 13*, 2585.
Kennedy, F. S., Hill, H. A. O., Kaden, T. A., & Vallee, B. L. (1972) *Biochem. Biophys. Res. Commun. 48*, 1533.
Kuo, L. C., & Makinen, M. W. (1982) *J. Biol. Chem. 257*, 24.
Kuo, L. C., & Makinen, M. W. (1985) *J. Am. Chem. Soc. 107*, 5255.
Latt, S. A., & Vallee, B. L. (1971) *Biochemistry 10*, 4263.
Lin, W. C. (1973) *Mol. Phys. 25*, 247.
Lipscomb, W. N., Hartsuck, J. A., Reeke, G. N., Jr., Quiocho, F. A., Bethge, P. H., Ludwig, M. L., Steitz, T. A., Muirhead, H., & Cappola, J. C. (1968) *Brookhaven Symp. Biol. 21*, 24.
Luchinat, C., Monnanni, R., Roelens, S., Vallee, B. L., & Auld, D. S. (1988) *J. Inorg. Biochem. 32*, 1.
Makinen, M. W., Kuo, L. C., Yim, M. B., Wells, G. B., Fukuyama, J. M., & Kim, J. E. (1985) *J. Am. Chem. Soc. 107*, 5245.
Pilbrow, J. R. (1969) *J. Mol. Phys. 16*, 307.
Pilbrow, J. R. (1984) *J. Magn. Reson. 58*, 186.
Rees, D. A., Lewis, M., & Lipscomb, W. N. (1983) *J. Mol. Biol. 169*, 367.
Scheule, R. K., Han, S. L., Van Wart, H. E., Vallee, B. L., & Scheraga, H. A. (1981) *Biochemistry 20*, 1778.
Vallee, B. L., & Holmquist, B. (1980) in *Methods for Determining Metal Ion Environments in Proteins, Structure and Function of Metalloproteins* (Darnell, D. W., & Wilkins, R. G., Eds.) Vol. 2, pp 27–73, Elsevier, New York.
Vallee, B. L., & Galdes, A. (1984) *Adv. Enzymol. Relat. Areas Mol. Biol. 56*, 283.
Williams-Smith, D. L., Bray, R. C., Barber, M. J., Tsopanakis, A. D., & Vincent, S. P. (1977) *Biochem. J. 167*, 593.

Time-Resolved Absorption, Circular Dichroism, and Emission of tRNA. Evidence That the Photo-Cross-Linking of 4-Thiouridine in tRNA Occurs from the Triplet State†

Steven J. Milder, Peter S. Weiss, and David S. Kliger*
Department of Chemistry, University of California, Santa Cruz, California 95064
Received August 2, 1988; Revised Manuscript Received November 1, 1988

ABSTRACT: The time-resolved optical density (TROD) and time-resolved circular dichroism (TRCD) spectra of the lowest triplet state of 4-thiouridine (4t-Urd) in aqueous solutions of tRNA are reported. The TROD spectrum is consistent with the triplet state being primarily in the thione tautomer. The intersystem crossing yield to the triplet is 0.35 and 0.27 (±10%), respectively, with and without 10^{-2} Mg^{2+} added to the solution. Upon addition of increasing amounts of I^- to solutions of tRNA, the initial triplet yield decreases, the rate of the observed triplet decay increases, and the quantum yield of internal photo-cross-linking decreases for the 4t-Urd chromophore. The results show quantitatively that the near-UV-induced photo-cross-linking reaction in tRNA occurs from the triplet state of 4t-Urd. From the TRCD spectrum the dissymmetry factor ($\Delta\epsilon/\epsilon$) of some of the triplet–triplet absorption bands is shown to be significantly larger than for any of the ground-state absorption bands. Two CD transitions are seen in the triplet–triplet spectrum which are obscured in the TROD spectrum by the strong ground-state bleaching signal near 335 nm. This shows that TRCD may be useful, in some cases, in locating electronic transitions that are not observed in TROD spectra.

Transfer ribonucleic acid (tRNA)[1] has been shown to be the photoactive species in the near-UV inhibition of the growth of *Escherichia coli*. The chromophore responsible for absorption of this light is the somewhat rare base 4-thiouridine (4t-Urd) (Favre & Thomas, 1981; Jagger, 1975; Peak et al., 1983; Caldeira de Araujo & Favre, 1985). For tRNA in solution, 4t-Urd has been shown to photo-cross-link with a

† We thank the National Institutes of Health for financial support under Grant GM 35158.

[1] Abbreviations: CD, circular dichroism; CPL, circularly polarized luminescence; Cyt, cytidine; DMTU, 1,3-dimethyl-4-thiouracil; OMA, optical multichannel analyzer; PMMA, poly(methyl methacrylate); tRNA, transfer ribonucleic acid; TRCD, time-resolved circular dichroism; TROD, time-resolved optical density; 4t-Urd, 4-thiouridine.

0006-2960/89/0428-2258$01.50/0