Articles

^{1}H NMR Spectroscopic Characterization of Binary and Ternary Complexes of Cobalt(II) Carboxypeptidase A with Inhibitors

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ABSTRACT: The binding of L- and D-phenylalanine and carboxylate inhibitors to cobalt(II)-substituted carboxypeptidase A, Co(II)CPD (E), in the presence and absence of pseudohalogens (X = N_3^-, NCO^-, and NCS^-) has been studied by ^{1}H NMR spectroscopy. This technique monitors the proton signals of histidine residues bound to cobalt(II) and is therefore sensitive to the interactions of inhibitors that perturb the coordination sphere of the metal. Enzyme–inhibitor complexes, E·I, E·I_2, and E·I·X, each with characteristic NMR features, have been identified. Thus, for example, L-Phe binds close to the metal ion to form a 1:1 complex, whereas D-Phe binds stepwise, first to a nonmetal site and then to the metal ion to form a 2:1 complex. Both acetate and phenylacetate also form 2:1 adducts stepwise with the enzyme, but β-phenylpropionate gives a 2:1 complex without any detectable 1:1 intermediate. N_3^-, NCO^-, and NCS^- generate E·I·X ternary complexes directly with Co(II)CPD·L-Phe and indirectly with the D-Phe and carboxylate inhibitor 2:1 complexes by displacing the second moiety from its metal binding site. The NMR data suggest that when the carboxylate group of a substrate or inhibitor binds at the active site, a conformational change occurs that allows a second ligand molecule to bind to the metal ion, altering its coordination sphere and thereby attenuating the bidentate behavior of Glu-72. The ^{1}H NMR signals also reflect alterations in the histidine interactions with the metal upon inhibitor binding. Isotropic shifts in the signals for the C-4 (c) and N protons (a) of one of the histidine ligands are readily observed in all of these complexes. These signals are relatively constant for all E·I and E·I·X complexes, indicating that this ligand is in a relatively fixed or "buried" conformation. However in the 2:1 carboxylate inhibitor (E·I_2) complexes, both signals are shifted upfield ca. 10 ppm, suggesting a disturbance in the interaction of this histidine with the metal. The other histidine ligand may be more exposed to solvent since its NH is not observed in E, E·I, or most E·I·X and E·I_2 complexes and, thus, is presumably in rapid exchange. In addition, the C-4 proton signal, d, for this histidine residue varies from 42 to 65 ppm for the binary and ternary complexes, likely reflecting a more labile metal–histidine interaction. In conjunction with crystallographic data, signals a and c are assigned to His-69 and signal d is assigned to His-196.

Proton NMR[1] spectroscopy of isotropically shifted signals in cobalt(II)-substituted metalloenzymes provides an excellent tool for detecting even minor structural changes within the active site (Bertini & Luchinat, 1983a, 1986). Moreover, such cobalt-substituted enzymes are generally catalytically active. In fact, the cobalt(II) derivative of bovine carboxypeptidase A (EC 3.4.17.1, CPD-A) is as active toward peptide substrates as the native zinc enzyme (Vallee et al., 1983). As a consequence, the cobalt atom has served as a very useful spectroscopic probe for investigating structural changes that occur around the metal atom during the catalytic process (Vallee & Holmquist, 1980; Auld et al., 1984; Makinen et al., 1984).

Much attention has been devoted to the study of the adducts between CPD-A and inhibitors such as D- and L-amino acids and their derivatives (Latt & Vallee, 1971; Holmquist & Vallee, 1979; Vallee et al., 1983), as well as carboxylate anions (Coleman & Vallee, 1964; Auld et al., 1972, 1986a,b; Palmer et al., 1982; Bunting & Myers, 1973), in order to elucidate their modes of binding to the protein. In particular, it has been observed that both carboxylate anions and amino acids display a synergistic interaction with respect to pseudohalogen binding, giving rise to mixed ternary complexes (Bicknell et al., 1985, 1988; Bertini et al., 1985; Luchinat et al., 1988).

In the present work, we have investigated the interaction between Co(II)CPD and β-PhPr, D- and L-Phe, PhAc, and acetate using ^{1}H NMR spectroscopy; formation of ternary complexes with pseudohalogens has also been investigated. The structural features of the above complexes have been analyzed, the common characteristics of binary and ternary complexes have been defined, and a general model of anion interaction has been proposed.

Experimental Procedures

Bovine CPD-A prepared by the method of Cox et al. (1964) was purchased from Sigma Chemical Co. (St. Louis, MO) and further purified according to standard procedures (Bazzone et al., 1979). All reagents used were of analytical grade.

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[1] Abbreviations: CPD or CPD-A, bovine carboxypeptidase A; D-Phe, D-phenylalanine; L-Phe, L-phenylalanine; β-PhPr, β-phenylpropionate; PhAc, phenylacetate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance; MCD, magnetic circular dichroism; EPR, electron paramagnetic resonance.

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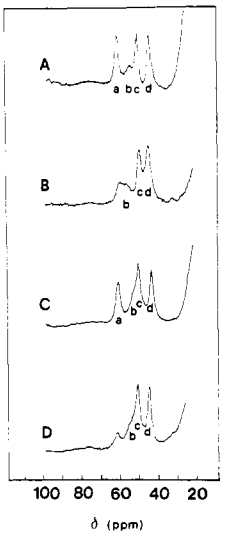

FIGURE 1: ^{1}H NMR spectra of Co(II)CPD at pH 6 in H_2O (A) and D_2O (B) and at pH 7.5 in H_2O (C) and D_2O (D). No other signal is observed beyond the spectral range shown. Conditions: 90 MHz; 300 K; [Co(II)CPD], 1 mM; [NaCl], 1 M; [Mes], 10 mM; pH 6. See text for identification of a, b, c, and d.

Metal removal and cobalt(II) replacement were performed as previously described (Auld & Holmquist, 1974). Protein crystals were dissolved in 0.01 M Mes buffer, pH 6, in the presence of 1 M NaCl. Formation of the cobalt(II)-substituted enzyme was monitored through electronic absorption spectroscopy by comparing absorbance values at 280 and 575 nm, with molar absorptivities of 6.42×10^4 (protein) and 150 (cobalt) M^{-1} cm^{-1}, respectively.

NMR enzyme samples were usually 0.5–1 mM; deuteriation was performed by Amicon exchange with deuteriated buffers; pH was changed by addition of small amounts of sodium hydroxide. ^{1}H NMR measurements were performed at 90 MHz on a Bruker CXP instrument at 300 K using the modified driven equilibrium Fourier transform pulse sequence (Hochmann & Kellerhals, 1980; Bertini et al., 1984). Spectra typically consisted of 100 000–200 000 scans taken over a period of a few hours. Chemical shifts are referenced to $(CH_3)_4Si$, with downfield shifts taken as positive. T_1 measurements were also performed with the modified driven equilibrium Fourier transform sequence (Hochmann & Kellerhals, 1980).

RESULTS

Co(II)CPD. As previously reported (Bertini et al., 1982), the ^{1}H NMR spectrum of Co(II)CPD is well-defined. Three sharp signals are clearly observed in H_2O (pH 6, 10 mM Mes, 1 M NaCl) at 62 (a), 52 (C), and 45 (d) ppm downfield from $(CH_3)_4Si$ with a somewhat broader one at 56 ppm (b) (Figure 1A). In D_2O (Figure 1B), signal a markedly decreases in agreement with the previous assignment of this signal to a coordinated histidine NH. The other two sharp signals (c and d) are assigned to the C-4 protons of the two coordinated histidines. There is no evidence for the second His NH, perhaps due to fast exchange between NH and solvent protons, which would either bring the shift within the diamagnetic region or abolish the intensity. The spectra also show evidence of further broad and barely detectable signals at about 75 and 40 ppm downfield. It is reasonable to assign the latter signals and that found at 56 ppm (b) to two C-2 histidine protons and a γ-CH_2 proton of Glu-72. The spectra at pH 7.5 in H_2O and D_2O (Figure 1C,D) reveal only minor variations with respect to that at pH 6.

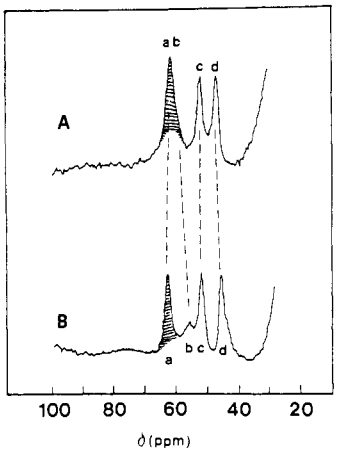

FIGURE 2: ^{1}H NMR spectrum of Co(II)CPD·L-Phe (A) and of Co(II)CPD alone (B), both at pH 6. The dashed lines relate the signals of the Co(II)CPD·L-Phe adduct to those of the cobalt enzyme alone and are deduced from ^{1}H NMR titration of (B) with increasing amounts of L-Phe. Shaded signals disappear in D_2O. Final concentration of L-Phe in (A) is 0.1 M; for other conditions, see Figure 1.

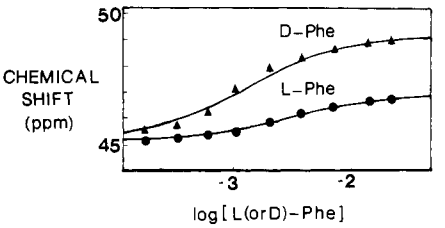

FIGURE 3: Titration of the chemical shift of signal d (Figure 2) as a function of increasing concentrations of L-Phe (●) and D-Phe (▲). The conditions are as in Figure 2. The lines are theoretical with values of 45.2 ppm for Co(II)CPD and 47.4 ppm for Co(II)CPD·L-Phe and K_B = 300 M^{-1} for L-Phe and of 45.2 ppm for Co(II)CPD and 49.2 ppm for Co(II)CPD·D-Phe and K_B = 700 M^{-1} for D-Phe.[2]

Binary Co(II)CPD·L-Phe Complex. The ^{1}H NMR spectrum of Co(II)CPD at pH 6 changes only slightly upon saturation with L-Phe (Figure 2). Only the broad signal at 56 ppm (b) and signal d undergo appreciable shifts. The affinity constant K of L-Phe for Co(II)CPD is estimated to be 300 M^{-1} from ^{1}H NMR titration of the chemical shift of signal d (Figure 3), comparable to a value of 290 M^{-1} from ^{13}C NMR (Luchinat et al., 1988) and 300–600 M^{-1} from electronic spectroscopy (Latt & Vallee, 1971; Bicknell et al., 1988).

Ternary Co(II)CPD·L-Phe·Anion Complexes. Both N_3^- and NCO^- form ternary complexes with Co(II)CPD·L-Phe (Bertini & Luchinat, 1984; Bicknell et al., 1985, 1988). The ^{1}H NMR spectra of the ternary adducts with azide and cyanate (Figure 4A,B) are similar to one another in shape, providing evidence that the two histidines are still coordinated and that their overall structural relationship to one another is not significantly changed. A ^{1}H NMR titration of the Co(II)CPD·L-Phe adduct with azide indicates that the binary and the ternary complexes are in fast exchange on the NMR time scale and that the signals at 73, 64, 55, and 43 ppm in the azide-containing ternary complex correspond to signals b, a, c, and d in the cobalt(II) enzyme, respectively (Figure 4A). The chemical shift values for the cyanate derivative are 69, 60, 52, and 42 ppm (Figure 4B).

D-Phe Complexes with Co(II)CPD. In contrast to L-Phe, D-Phe forms both 1:1 and 2:1 complexes with Co(II)CPD. A ^{1}H NMR titration of the chemical shift signal d (Figures 3 and 5) indicates that D-Phe binds to the protein with an initial affinity constant of ~700 M^{-1}, comparable to the value of the 670 M^{-1} obtained by ^{13}C NMR (Luchinat et al., 1988). Thus,

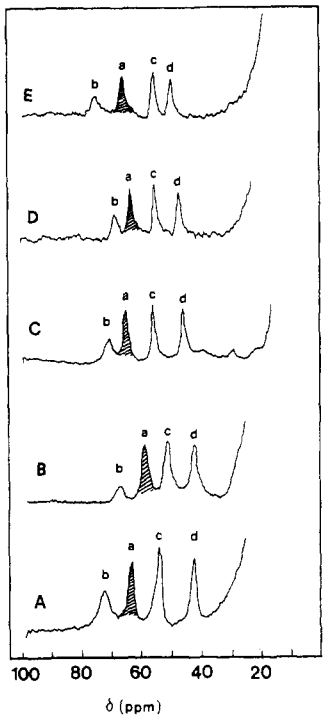

FIGURE 4: ^1H NMR spectra of ternary adducts: Co(II)CPD·L-Phe·N_3^- (A); Co(II)CPD·L-Phe·NCO^- (B); Co(II)CPD·D-Phe·N_3^- (C); Co(II)CPD·D-Phe·NCO^- (D); Co(II)CPD·D-Phe·NCS^- (E). Shaded signals disappear in D_2O. [Co(II)CPD], 1 mM; [L-Phe], 10 mM; [D-Phe], 5 mM; [N_3^-], 0.1 M; [NCO^-], 0.2 M; [NCS^-], 0.2 M. For other conditions, see Figure 1.

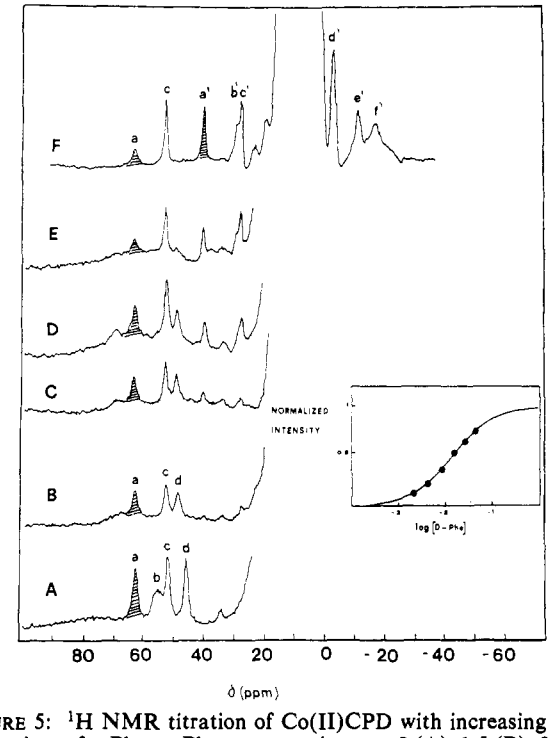

FIGURE 5: ^1H NMR titration of Co(II)CPD with increasing concentrations of D-Phe. D-Phe concentrations are 0 (A), 1.5 (B), 5 (C), 10 (D), 40 (E), and 80 mM (F). Intermediate points of the titrations are not shown. In (F) the upfield part of the spectrum is also shown where the new signals d′, e′, and f′ appear. Shaded signals disappear in D_2O. Other conditions as in Figure 1. (Inset) Titration of the normalized intensity of signal a′ [(height of signal a′)/(height of signal c)] as a function of increasing D-Phe concentration. The line is theoretical with normalized intensity values of 0 for Co(II)CPD and 1.0 for Co(II)CPD·(D-Phe)$_2$ and K_B = 73 M^{-1}.

at the start of the titration the ^1H NMR spectrum of Co(II)CPD undergoes small variations similar to those observed

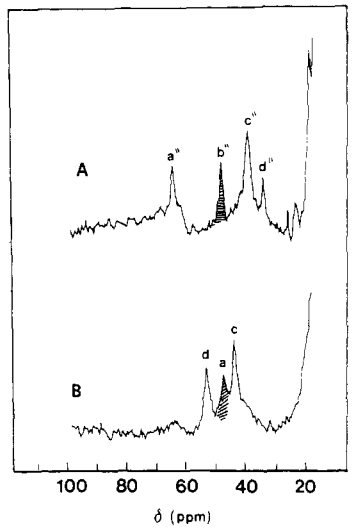

FIGURE 6: ^1H NMR spectra of Co(II)CPD·(β-PhPr)$_2$ (A) and Co(II)CPD·(PhAc)$_2$ (B). Inhibitor concentrations are 20 mM β-PhPr and 50 mM PhAc. Shaded signals disappear in D_2O. The other conditions are as in Figure 1.

on binding L-Phe; i.e., the signal at 56 ppm (b) broadens and disappears while signal d moves 4 ppm downfield (Figure 5B).[2] When the D-Phe concentration exceeds 1.5 mM (Figure 5C–F), drastic changes occur; new signals appear in the spectra that increase in intensity with increasing D-Phe concentration (a′ to f′) whereas signal d progressively decreases and eventually vanishes and signal a broadens. This behavior is typical of slow exchange (on the NMR time scale) between free and bound D-Phe. A reasonable explanation for these results is that the second D-Phe molecule binds to the protein in a different position, possibly the metal site, thereby distorting the chromophore. From a best fit of the variation of the intensities of signal a′/c with D-Phe concentration, an affinity constant of 73 M^{-1} is estimated for the binding of the second D-Phe (Figure 5, inset).

The spectra in H_2O and D_2O of the final Co(II)CPD·(D-Phe)$_2$ adduct are shown in Figure 5F. Two exchangeable NH signals are now observed (signal a and a′ at 63 and 40 ppm) which are assigned to the His-196 and His-69 NH protons. Three more sharp signals are present at 52, 28, and 27 ppm (signals c, b′, and c′). They are assigned to two His C-4 (at least signal c as in the cobalt enzyme alone) and to either the α-CH of D-Phe or the γ-CH_2 of Glu-72. Three additional signals appear in the upfield region at −7, −15, and −21 ppm (d′, e′, and f′). These may be due to protein protons near the metal ion. Significantly, the positions of signals a and c remain unchanged.

The binding of N_3^- is competitive with the second D-Phe molecule which it displaces to give a ternary product (Figure 4C) whose ^1H NMR spectrum is very close to that of the Co(II)CPD·L-Phe·N_3^- complex. The same adduct can also be obtained directly by addition of azide to the 1:1 Co(II)-CPD·D-Phe complex. NCS^- and NCO^- also form analogous ternary complexes with Co(II)CPD·D-Phe (Figure 4D,E). The spectral variations among the three anion–D-Phe ternary complexes are minimal.

[2] A slightly better fit can be obtained from the sequential binding model

$$\text{Co(II)CPD} \overset{K_1}{\rightleftharpoons} \text{Co(II)CPD·D-Phe} \overset{K_2}{\rightleftharpoons} \text{Co(II)CPD·(D-Phe)}_2$$

in which the theoretical line is derived with values of 45.2 ppm for Co(II)CPD, 48.8 ppm for Co(II)CPD·D-Phe (K_1 = 1250 M^{-1}), and 49.2 ppm for Co(II)CPD·(D-Phe)$_2$ (K_2 = 73.0 M^{-1}).

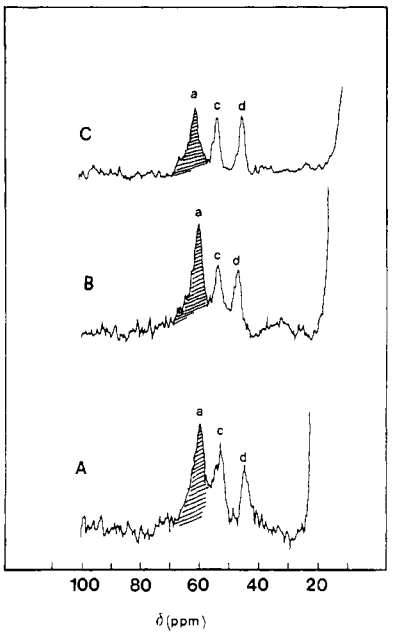

FIGURE 7: ^{1}H NMR spectra of the ternary adducts: Co(II)CPD·β-PhPr·N_3^- (A), Co(II)CPD·PhAc·N_3^- (B), and Co(II)CPD·acetate·N_3^- (C). Shaded signals disappear in D_2O. [β-PhPr], 2 mM; [PhAc], 5 mM; [acetate], 10 mM; [N_3^-], 0.2 M. For other conditions, see Figure 1.

β-Phenylpropionate (β-PhPr) Complexes with Co(II)CPD. Addition of β-PhPr at pH 6 causes a marked change in the ^{1}H NMR spectrum of Co(II)CPD. The variation is essentially complete at 20 mM β-PhPr. In particular, signals a, b, c, and d of Co(II)CPD disappear progressively, and new signals appear at 65, 48, 39, and 34 ppm (signals a″, b″, c″, and d″; Figure 6A). Equilibration is slow on the NMR time scale. In D_2O, signal b″ vanishes, allowing it to be assigned to the NH proton of a coordinated histidine. The other signals are assigned to two C-4 protons of coordinated histidines and possibly to the α-CH_2 protons of bound β-PhPr. The data suggest formation of a 2:1 adduct between β-PhPr and Co(II)CPD (results not shown). In contrast to D-Phe, there is no detectable stepwise binding of the inhibitor (i.e., first a 1:1 then a 2:1 complex).

Addition of sodium azide, 50 mM, to a solution 2 mM in β-PhPr and 1 mM in cobalt enzyme results in the formation of a Co(II)CPD·β-PhPr·N_3^- adduct (Bicknell et al., 1988). Its ^{1}H NMR spectrum is quite distinct from that of the Co(II)CPD·(β-PhPr)$_2$ adduct (Figures 6A and 7A). Titration of Co(II)CPD·(β-PhPr)$_2$ with azide to form Co(II)CPD·β-PhPr·N_3^- indicates that the equilibrium is slow on the NMR time scale. The signal at 59 ppm in the latter spectrum disappears in D_2O (signal a) and is therefore assigned to a His NH proton. The other two sharp signals at 52 and 44 ppm (signals c and d) are assigned to two C-4 His protons. By analogy with the carboxylate ternary complexes where signal assignment can be performed through ^{1}H NMR titration (see Figure 7), the three signals at 59, 52, and 44 ppm, respectively, are related to signals a, c, and d of the cobalt enzyme alone (Figure 1). Moreover, in the ^{1}H NMR spectrum of Co(II)CPD·β-PhPr·N_3^- as well as in those of Co(II)CPD·PhAc·N_3^- and Co(II)CPD·acetate·N_3^-, signal a is markedly more intense than signals c and d; it disappears completely in D_2O, thus ruling out the presence of underlying broad signals which could alter the intensity ratios (Figure 7).

Phenylacetate (PhAc) Complexes with Co(II)CPD. Both ^{13}C NMR and electronic spectroscopy have shown that Co(II)CPD binds 2 equiv of PhAc (Latt & Vallee, 1971; Bertini et al., 1988). Formation of the 2:1 complex is complete at a PhAc concentration of 10 mM at pH 6.0. The ^{1}H NMR spectrum of this complex is shown in Figure 6B. Three sharp signals at 56, 50, and 46 ppm are superimposed on a broad resonance at 43 ppm and a further broad resonance at 66 ppm; the signal at 50 ppm is abolished in D_2O and is therefore assigned to a His NH proton (signal a). Titration with PhAc indicates that the equilibrium is fast on the NMR time scale and allows signal assignment (see Figure 6B). Progressive formation of the 1:1 and then the 2:1 Co(II)CPD·PhAc complex is readily observed by ^{1}H NMR titration. The overall pattern of signals is similar to that observed with acetate (see below) but differs significantly from those of Co(II)CPD·(β-PhPr)$_2$. Addition of excess azide to the final adduct changes the ^{1}H NMR spectrum substantially, indicating formation of a Co(II)CPD·PhAc·N_3^- ternary complex (Figure 7B). Interestingly, the resulting spectrum is quite similar to those of the ternary complexes Co(II)CPD·acetate·N_3^- and Co(II)CPD·β-PhPr·NH_3^- (Figure 7A,C). The signal at 59 ppm disappears in D_2O and is therefore assigned to signal a whereas the other two sharp signals at 52 and 45 ppm are assigned to signals c and d, respectively, by analogy with the Co(II)CPD·acetate·N_3^- complex (see below).

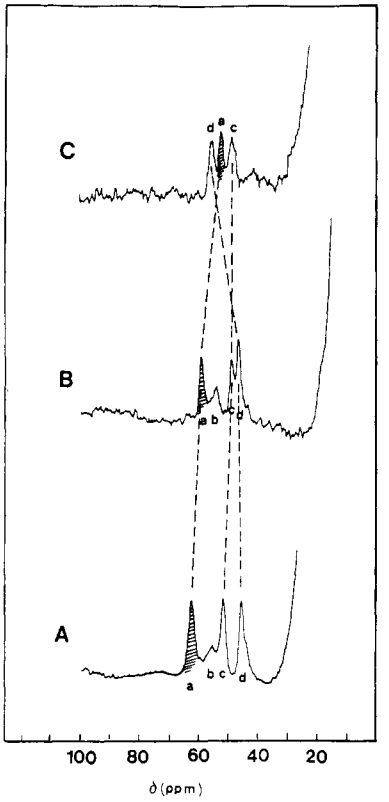

FIGURE 8: ^{1}H NMR spectra of the 1:1 (B) and 2:1 (C) Co(II)CPD·acetate adducts compared to that of the cobalt enzyme alone (A). Shaded signals disappear in D_2O; dashed lines relate the signals of the acetate adducts to those of the pure cobalt enzyme as deduced from ^{1}H NMR titrations. [Acetate], 10 mM (B) and 2 M (C). For other conditions, see Figure 1.

Acetate Complexes with Co(II)CPD. Titration of Co(II)CPD at pH 6 with up to relatively high concentrations (10 mM) of acetate barely alters the ^{1}H NMR spectrum. Only signal c moves 2.5 ppm upfield (Figure 8B). ^{13}C NMR studies show that binding of the first acetate molecule is complete (Bertini et al., 1988) at this acetate concentration. Hence, the first acetate moiety, analogous to other carboxylate inhibitors, does not affect the cobalt(II) chromophore substantially, and it must bind at a site remote from the para-

Table I: T_1 Values for Some Binary and Ternary Complexes of Co(II)CPD[a]

inhibitor complex	signals (ms)[b]			
	a (NH)	b (CH-2)	c (CH-4)	d (CH-4)
none	4.9	2.4	6.3	6.0
L-Phe	5.4	c	6.4	6.3
L-Phe·N_3^-	5.7	1.5	5.2	3.4
D-Phe	5.8	c	7.7	6.8
D-Phe·N_3^-	5.7	1.3	7.6	3.3
(D-Phe)$_2$	c		9.7	
β-PhPr·N_3^-	5.9		5.2	4.2
(D-Phe)$_2$	9.8 (a′)	8.5 (b′)	8.0 (c′)	
(β-PhPr)$_2$	4.6 (a″)	7.0 (b″)	6.4 (c″)	5.7 (d″)

[a] The estimated error in T_1 values is ~10%. [b] For the assignment, see the text. [c] Not estimated.

magnetic center. Further increases in acetate concentration, however, induce marked changes in the ^{1}H NMR spectrum that can be explained by assuming an equilibrium in fast exchange between two species [Co(II)CPD·acetate ⇌ Co(II)CPD·(acetate)$_2$] with a formation constant of about 10 M^{-1}. This is consistent with the binding of a second acetate moiety with a low affinity constant (4 M^{-1}) proposed on the basis of electronic spectroscopy (Bertini et al., 1988). Assignment of the signals is easily made by examination of the entire titration; the two coordinated histidines are not displaced by the binding of the second acetate moiety, but they undergo slight distortions. The NH signal at 62 ppm moves 10 ppm upfield (signal a), whereas the two C-4 signals at 52 and 47 ppm (signals c and d) are shifted to 48 and 57 ppm, respectively (Figure 8C).

Addition of excess azide to Co(II)CPD·acetate induces ^{1}H NMR spectral changes consistent with the formation of a ternary Co(II)CPD·acetate·N_3^- complex (Figure 7C). The equilibrium is fast on the NMR time scale. Titration with azide permits assignment of the signals at 60, 53, and 44 ppm in Co(II)CPD·acetate·N_3^-, respectively, to signals a, c, and d in the enzyme alone (see Figure 7C). As expected from its assignment to an exchangeable histidine NH, signal a is abolished in D_2O.

The T_1 Values. T_1 values have been measured for several derivatives (Table I). For NH and C-4 His protons these values are between 3 and 7 ms (except for D-Phe) whereas for the often-observed C-2 His proton they are between 1 and 2 ms. In cobalt(II) carbonic anhydrase the proton T_1 values have been related to the coordination number in that values of 7–12 ms for NH and C-4 His protons have been associated with pentacoordination of the metal whereas values of 3–5 ms have been associated with tetracoordination (Bertini & Luchinat, 1983b; Bertini et al., 1983a, 1981). In Co(II)CPD the values are less definitive. This is not surprising considering that pentacoordination[3] here is attributed to bidentate ligation by Glu-72 on the basis of X-ray data for both the zinc and cobalt(II)-substituted enzymes (Rees et al., 1983, 1986; Hardman & Lipscomb, 1984). The bidentate coordination behavior of Glu-72 apparently changes to monodentate when the various inhibitors bind to Co(II)CPD (Christianson & Lipscomb, 1985, 1986a,b). It is not possible to observe this coordination change in solution by T_1 measurements although there is a tendency for high-intensity spectra (namely, those formed with N_3^- and amino acids) to have lower average T_1 values for the C-4 proton. Indeed, nuclear T_1 values depend on electronic relaxation times which in turn depend on the energy levels of the cobalt(II) ion. The mono- and bidentate behavior of a carboxylate is therefore more likely to be reflected in spectral intensity than in the pattern of energy levels (Cotton et al., 1963).

[3] The use of the terms tetra- and pentacoordination in this paper is operational, being a summation of the number of oxygen and nitrogen atoms within liganding distance of the zinc atom. It is not meant to imply a geometry. Pentacoordination, e.g., where the ligands to the metal are the two oxygens of Glu-72, the nitrogens of His-69 and His-196, and the oxygen of a water molecule, could likely result in a metal coordination sphere which is best represented as a distorted tetrahedron.

Discussion

Some general criteria for interpreting the patterns of the interaction between Co(II)CPD and inhibitors emerge from this ^{1}H NMR investigation. Co(II)CPD may form three different kinds of adducts with inhibitors and pseudohalogens, namely, Co(II)CPD·I, Co(II)CPD·I_2, and Co(II)CPD·I·X complexes. These findings are in agreement with previous descriptions of multiple binding sites for substrates and modifiers in the active site region of CPD-A (Vallee et al., 1968; Alter & Vallee, 1978).

Co(II)CPD·I Complexes. Binary 1:1 complexes are clearly formed with relatively low concentrations of L- and D-Phe, acetate, and PhAc. Only minor variations relative to the enzyme alone are apparent in the respective ^{1}H NMR or electronic absorption spectra (Latt & Vallee, 1971) indicating that the first inhibitor moiety neither binds to the metal nor alters its general chromophoric features. The cobalt–$^{13}COO^-$ distances calculated by ^{13}C NMR for the Co(II)CPD 1:1 complexes of L-Phe, D-Phe, and acetate are consistent with binding of their carboxylates to one of the three arginine residues in the active site (Bertini et al., 1988; Luchinat et al., 1988). For L-Phe, D-Phe, and PhAc, it is tempting to suggest that their carboxylate group binds to Arg-145 and the phenyl ring occupies the S_1' hydrophobic pocket. High-resolution X-ray data have shown this to be the case for several CPD-A adducts with inhibitors bearing a benzylpropionate group (Christianson & Lipscomb, 1986a). A low-resolution X-ray investigation of the CPD·L-Phe adduct also suggests that L-Phe binds in the S_1' site. The α-amino group of bound L-Phe or D-Phe could interact with either Glu-270 (Christianson & Lipscomb, 1986b) or Tyr-248 (Lipscomb, 1982).

Co(II)CPD·I_2 Complexes. The present ^{1}H NMR data together with previous spectral results indicate that while L-Phe only forms a 1:1 complex with Co(II)CPD, D-Phe, β-PhPr, PhAc, and acetate can also form 2:1 complexes. ^{1}H NMR titrations of Co(II)CPD with acetate reveal that binding of the first inhibitor moiety hardly perturbs the ^{1}H NMR and electronic absorption spectra (Bertini et al., 1988). Binding of the second equivalent, however, induces marked changes in both spectra (Latt & Vallee, 1971; Bertini et al., 1988). Since azide competes with the binding of the second inhibitor molecule (see below), it is proposed that the carboxylate group of the latter binds to the metal site. Analysis of the dependence of ^{13}C NMR line width on ^{13}C-enriched acetate concentration showed that Co(II)CPD binds two acetate molecules with markedly different affinity constants; in both cases the equilibrium is fast on the NMR time scale (Bertini et al., 1988). It was proposed that the first binding site for acetate involves an Arg residue situated in the active site cavity whereas the second site is the metal. The ^{1}H NMR and electronic spectra support this view and indicate that acetate binding does not change the overall cobalt(II) coordination.

Although both D-Phe and acetate form 2:1 complexes with Co(II)CPD, their titrations and final ^{1}H NMR spectra are dissimilar, suggesting differences in the structure of the final adducts. The electronic absorption and MCD spectra of the Co(II)CPD·(D-Phe)$_2$ complex indicate hexacoordinate cobalt (G. Hanson and B. Holmquist, unpublished observations)

suggesting that the metal-bound water may be retained in this complex in contrast to the Co(II)CPD·(acetate)$_2$ complex where it is likely displaced. For the (acetate)$_2$ complex the ligands are in fast exchange on the NMR time scale, while for the (D-Phe)$_2$ complex they are in slow exchange. It would appear that binding of the second D-Phe moiety causes some conformational change which retards the overall exchange process.

The large spread of the signals in Co(II)CPD·(D-Phe)$_2$ is a unique feature indicating large magnetic anisotropy and therefore large dipolar shifts. The observation of several upfield signals is consistent with this hypothesis. These are possibly due to protons of nonbound residues close to the paramagnetic center. The emergence of a second His NH signal (Figure 5, signal a′) indicates that the exchange rate of the second coordinated histidine is somewhat slower in the 2:1 complex.

The behavior of β-PhPr is similar to that of D-Phe in that it has the same stoichiometry and a slow exchange rate. However, its final ^{1}H NMR spectrum is also different from that observed for the D-Phe complex. In addition, the first and second equilibria cannot be differentiated, possibly due to a positive binding cooperativity. The electronic spectra also suggest the formation of a 2:1 adduct between Co(II)CPD and β-PhPr with a high affinity constant (Latt & Vallee, 1971; Bicknell et al., 1988). Since the values of the electronic molar absorptivity of the d–d transitions do not change greatly upon formation of the 1:1 or 2:1 β-PhPr complexes, the cobalt(II) coordination likely does not change. It is concluded that two β-PhPr molecules bind to the enzyme in the active site region, possibly at both the S_1' site and the metal. The results of [^{14}C]-β-PhPr binding studies of the native enzyme and apoenzyme have indicated that one mode of β-PhPr binding occurs through the metal atom (Coleman & Vallee, 1964). Stabilization of the Co(II)CPD·(β-PhPr)$_2$ complex could occur through formation of a hydrogen bond between the metal-bound carboxylate group and Tyr-248 as previously proposed for the arsanilazo Tyr-248 CPD-A·(β-PhPr)$_2$ complex (Bachovchin et al., 1982).

Finally, PhAc behaves like acetate since (i) the stoichiometry is 2:1 and binding is sequential, (ii) the final spectra are very similar, (iii) the patterns of the ^{1}H titrations are parallel, and (iv) the exchange rates are fast on the NMR time scale.

Co(II)CPD·I·X Complexes. In the absence of inhibitors such as L- or D-Phe or other carboxylic acids, the electronic spectrum of Co(II)CPD exhibits a marked insensitivity to inorganic anions at neutral pH. It has been proposed (Geoghegan et al., 1983) that the metal-coordinated water cannot be displaced by such anions because it is stabilized by hydrogen bonding to deprotonated Glu-270. Binding of amino acid or carboxylate inhibitors likely disrupts this interaction and thereby opens up the metal coordination site since anions can now bind to the metal. The present ^{1}H NMR results together with electronic spectroscopy data (Bicknell et al., 1985, 1988) provide evidence for the formation of mixed ternary complexes with pseudohalogens. The high intensity of the absorption spectrum of the Co(II)CPD·L-Phe·N_3^- (Bicknell et al., 1988, Bertini et al., 1984) and its negative MCD ellipticity (Bicknell et al., 1988) are entirely consistent with tetracoordination (Rosenberg et al., 1967; Holmquist et al., 1975). The EPR spectrum ($g_\perp$ = 4.68, $g_\parallel$ = 2.14) of the Co(II)CPD·L-Phe·N_3^- complex also indicates a much less distorted tetrahedral complex for Co(II)CPD (Bicknell et al., 1988). Indeed, it is similar to the EPR spectra of inhibitor complexes of carbonic anhydrase in which the metal is thought to occupy a tetrahedral coordination site (Bencini et al., 1981). Direct binding of the anion to the metal is likely, on the basis of the appearance of absorption bands typical of ligand-to-metal charge-transfer transitions (Bicknell et al., 1988). Synergistic interactions between pseudohalogens and inhibitor binding have been demonstrated (Bicknell et al., 1988).

Table II: ^{1}H NMR Signals for Histidyl Ligands of CPD-A in Binary and Ternary Complexes

inhibitor complex	signal a	c	d	a′
none	62	52	45	
L-Phe	61	52	47	
D-Phe	62	52	49	
L-Phe·N_3^-	64	55	43	
L-Phe·NCO^-	60	52	42	
D-Phe·N_3^-	65	56	45	
D-Phe·NCO^-	64	55	47	
D-Phe·NCS^-	66	55	50	
β-PhPr·N^{3-}	59	52	44	
PhAc·N^{3-}	59	52	45	
acetate·N_3^-	60	53	44	
acetate	62	52	47	
(acetate)$_2$	52	48	57	
(PhAc)$_2$	50	46[a]	56[a]	
(β-PhPr)$_2$	48	39[a]	65[a]	
(D-Phe)$_2$	63	52		40

[a] Signal assignment based on acetate titration and final signal pattern observed for PhAc and β-PhPr.

In the presence of D- and L-Phe, the ternary complexes formed with N_3^-, NCO^-, and NCS^- give rise to similar ^{1}H NMR spectra. Signal assignment is easily performed by titrating the binary complex, Co(II)CPD·I, with increasing amounts of the pseudohalogen anion, X. Pseudohalogen binding does not displace histidine from the cobalt(II) chromophore. The electronic absorption spectra show that addition of excess β-PhPr to the Co(II)CPD·β-PhPr·N_3^- adduct causes disruption of the ternary complex and restores the Co(II)-CPD·(β-PhPr)$_2$ adduct (Bicknell et al., 1988). Again, as in the case of D-Phe, there is competition between azide and the inhibitor for the same binding site, i.e., the metal. This behavior is easily monitored through ^{1}H NMR spectroscopy.

Metal–Protein Ligand Interactions. The positions of signals a, 62 ± 2 ppm, and c, 53 ± 1.5 ppm, are essentially unchanged in the binary and ternary complexes or move in the same direction in the dicarboxylate, E·I_2, species (Table II). Since these signals are assigned to His NH and C-4 protons, respectively, they likely belong to the same His residue, and hence, coordination of this residue is probably not greatly affected in the E·I and E·I·X complexes. It may therefore be in a relatively fixed or "buried" conformation. The other His ligand seems to be more exposed to solvent since its NH is not observed in E, E·I, or most of the E·I_2 and E·I·X complexes and thus is presumably in rapid exchange. However, in the E·(D-Phe)$_2$ complex a second exchangeable NH signal, a′, is observed at 40 ppm, suggesting that this second His ligand is now in slower exchange. Moreover, in the enzyme–carboxylate–azide ternary complex, signal a is much larger than usual but still disappears completely in D_2O. The exchange rate in these ternary adducts between the nonobservable His NH proton and solvent is most likely decreased, and its ^{1}H NMR signal is now superimposed on the other His NH signal. Furthermore, the C-4 proton signal for this His residue, d, varies from 42 to 62 ppm for the binary and ternary complexes (Table II), likely indicating a more labile metal–histidine interaction.

X-ray crystallographic studies have demonstrated that the N-3 proton of His-196 is hydrogen bonded to a water molecule

while that of His-69 is hydrogen bonded to a carboxylate oxygen of Asp-142 (Quiocho & Lipscomb, 1971). The His-69 N-3 proton is therefore more likely in a slow proton exchange situation, and hence, signals a and c are assigned to it while signal d is assigned to the N-3 proton of solvent-exposed His-196.

The combined results of electronic absorption, MCD, and EPR measurements also make it be possible to assign a ^1H NMR signal to the γ-CH_2 of Glu-72. Examination of the Co(II)CPD·L-Phe·N_3^- ternary complex by these spectroscopic techniques reveals a much less distorted tetrahedral complex than for Co(II)CPD or Co(II)CPD·L-Phe (Bicknell et al., 1988). If Glu-72 acts as a bidentate ligand in the native enzyme, this would result in a metal coordination sphere best represented as a distorted tetrahedron.[3] The conversion of Glu-72 to a monodentate ligand in the ternary N_3^- complexes involving L-Phe or D-Phe would then be expected to result in a more regular tetrahedral environment for the metal. Such a movement of Glu-72 from a bidentate to monodentate liganding position would likely lead to an alteration in its ^1H NMR signal. The most dramatic shift seen in the ternary complexes occurs with signal b which shifts from 56 ppm in Co(II)CPD to 69–74 ppm in the various ternary complexes (Figures 1 and 4). On this basis, signal b can be tentatively assigned to the γ-CH_2 of Glu-72.

In the β-PhPr and PhAc ternary complexes the metal may still be pentacoordinate since the intensities of their electronic spectra are only slightly elevated (Bicknell et al., 1988). The T_1 values do not provide a conclusive answer regarding cobalt(II) coordination. In the present system the high-intensity electronic spectra and short ^1H NMR T_1 values may reflect a Co(II) complex in which Glu-72 is monodentate resulting in overall tetracoordination whereas low-intensity spectra and long ^1H NMR T_1 values may reflect a cobalt complex in which Glu-72 is bidentate resulting in pentacoordination. Thus, changes in the coordination can occur upon binding of inhibitors and substrates to the cobalt which result in a major or minor movement of the metal atom (Christianson & Lipscomb, 1985, 1986b). Independent of the coordination, the presence of either a zwitterionic or anionic carboxylate ligand facilitates pseudohalogen binding to the metal. It may well be that carboxylate binding disrupts a functionally important electrostatic interaction of Arg-145 which governs substrate recognition and orientation. Binding may also induce a conformational change within the active site which makes the metal site more accessible to the carbonyl group of the substrate. This picture is in reasonable agreement with X-ray structural information on the native crystalline enzyme in which binding of inhibitors induces a large movement of Tyr-248 (Steitz et al., 1967; Rees & Lipscomb, 1982, 1983), and often causes a sizable shift of the zinc(II) ion without appreciably affecting His coordination. In addition, inhibitor binding makes the distances between zinc(II) and the two carboxylate oxygen atoms of Glu-72 nonequivalent (Christianson & Lipscomb, 1986b).

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Proteolytic Modification of Tissue Plasminogen Activator: Importance of the N-Terminal Part of the Catalytically Active B-Chain for Enzymatic Activity†

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ABSTRACT: Native one-chain tissue plasminogen activator (t-PA) was rapidly converted to the two-chain form by trypsin–Sepharose cleavage. This caused an increase in the amidolytic activity on low molecular weight peptide substrates, while plasminogen activation in the presence of fibrin markedly decreased. Cleavage sites were identified by N-terminal sequence analysis of reduced and carboxymethylated peptides. In the B-chain, the expected cleavage at Arg_{278}–Ile_{279} was identified. Furthermore, a specific cleavage site was found at Arg_{302}–Ser_{303}, 24 amino acids from the N-terminus of the B-chain. The peptide released by this cleavage (designated B_{1-24}) remained associated with the activator molecule by strong noncovalent interactions but could be dissociated under denaturing conditions (4 mol/L of guanidine hydrochloride), leading to a 20-fold decrease in amidolytic activity. Addition of purified B_{1-24} peptide to t-PA treated in this manner restored the activity in a concentration-dependent way. In contrast to trypsin, cleavage of the single-chain t-PA molecule with endoproteinase Lys-C generated a two-chain form of the activator, without simultaneous increase in the amidolytic activity. By sequence analysis, a major cleavage was identified at Lys_{280}–Gly_{281}, two residues into the B-chain. Together, the results presented provide additional information on the one-chain to two-chain conversion of t-PA and the role of the free N-terminus of the B-chain.

Plasminogen activators catalyze the activation of plasminogen to the active proteolytic enzyme plasmin. Plasmin in turn degrades fibrin clots to soluble components. Tissue plasminogen activator is sythesized in the endothelial cells as a single-chain molecule but can be converted to a two-chain form by plasmin cleavage (Wallén et al., 1982, 1983). The two chains, held together by a disulfide bond, are designated A-chain, for the polypeptide originating from the N-terminal part of the enzyme, and B-chain, for the polypeptide from the C-terminal part of the enzyme. The primary structure of t-PA[1] has been determined by both protein and cDNA sequence analysis (Pennica et al., 1983; Pohl et al., 1984). For the protease domain, extensive homologies with other proteases have been shown (Strassburger et al., 1983).

Recently, several studies on structure–function relationships in the t-PA molecule using molecular biology techniques such as deletion mutants (van Zonneveld et al., 1986) or site-specific

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[1] Abbreviations: AA, amino acid(s); DFP, diisopropyl fluorophosphate; DTE, dithioerythritol; NaCl/P_i, phosphate-buffered saline (50 mM phosphate, pH 7.3, containing 0.1 g/L Triton X-100, I= 0.15); PAGE, polyacrylamide gel electrophoresis; pNA, *p*-nitroanilide; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; t-PA tissue plasminogen activator.

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