Interaction of Anions with the Active Site of Carboxypeptidase A†

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ABSTRACT: Studies of azide inhibition of peptide hydrolysis catalyzed by cobalt(II) carboxypeptidase A identify two anion binding sites. Azide binding to the first site (K_I = 35 mM) inhibits peptide hydrolysis in a partial competitive mode while binding at the second site (K_I = 1.5 M) results in competitive inhibition. The cobalt electronic absorption spectrum is insensitive to azide binding at the first site but shows marked changes upon azide binding to the second site. Thus, azide elicits a spectral change with new λ_{max} (ϵ_M) values of 590 (330) and 540 nm (190) and a K_D of 1.4 M, equal to the second kinetic K_I value for the cobalt enzyme, indicating that anion binding at the weaker site involves an interaction with the active-site metal. Remarkably, in the presence of the C-terminal products of peptide or ester hydrolysis or carboxylate inhibitor analogues, anion (e.g., azide, cyanate, and thiocyanate) binding is strongly synergistic; thus, K_D for azide decreases to 4 mM in the presence of L-phenylalanine. These ternary complexes have characteristic absorption, CD, MCD, and EPR spectra. The absorption spectra of azide/carboxylate inhibitor ternary complexes with Co(II)CPD display a near-UV band between 305 and 310 nm with ϵ_M values around 900–1250 M^{-1} cm^{-1}. The λ_{max} values are close to the those of the charge-transfer band of an aquo Co(II)–azide complex (310 nm), consistent with the presence of a metal azide bond in the enzyme complex. The absorption is well resolved in the corresponding MCD spectra; for example, in the Co(II)CPD·L-Phe·N_3^- complex $\lambda_{extremum}$ = 323 nm and $\Delta\epsilon_M$ = +0.207 M^{-1} cm^{-1} T^{-1}. The absorption band is also present in thiocyanate-containing complexes; for the Co(II)CPD·D-Phe·SCN^- complex λ_{max} = 316 nm and ϵ_M = 760 M^{-1} cm^{-1}. MCD and EPR spectra of the ternary complexes indicate a slightly distorted tetrahedral coordination sphere around the active-site Co(II). The near-UV band in the visible and MCD spectra of a Co(II)CPD·β-phenylpropionate (β-PP)·N_3^- complex is lost on addition of excess β-PP, suggesting that a second molecule of β-PP interacts with the active-site metal ion displacing azide. The steady-state ES_2 intermediate of peptide hydrolysis does not permit binding of azide to the metal, consistent with the proposal that peptides coordinate directly with the metal ion in the ES_2 intermediate.

Replacement of the active-site zinc by cobalt(II) in zinc metalloenzymes gives active chromophoric derivatives that provide a powerful probe of metalloenzyme active-site structure (Vallee & Holmquist, 1980; Auld et al., 1984; Bicknell et al., 1986). It has long been known that binding of inhibitors to cobalt(II)-substituted zinc metalloenzymes often perturbs the spectral properties of the active-site Co(II) ion (Latt & Vallee, 1971).

In contrast to carbonic anhydrase (Lindskog, 1963), the absorption spectrum of cobalt(II) CPD[1] is relatively insensitive to inorganic anions at neutral pH (Latt & Vallee, 1971). However, chemical modification of the Glu-270 residue of carboxypeptidase A gives rise to anion sensitivity at pH 7, and it has been proposed that the metal-coordinated water is stabilized by hydrogen bonding to deprotonated Glu-270 at pH values above 6 (Geoghegan et al., 1983b). This stabilization is lost when Glu-270 is either protonated or chemically modified, and the absorption spectrum of cobalt(II) CPD becomes anion sensitive.

The present study shows that at pH 7 the Co(II)CPD binding of the C-terminal products of peptide and ester hydrolysis, i.e., both amino acids and their alcohol analogues, and some carboxylate inhibitors allows access of anions to the metal coordination sphere. The presence of characteristic near-UV absorption bands suggests that small inorganic anions have entered the coordination sphere of the active-site cobalt(II) in the ternary complexes. It is known from NMR studies that the C-terminal product, L-Phe, does not bind to the metal in the ternary complex (Luchinat et al., 1988). Kinetic studies further show that the spectrally sensitive metal–anion interaction results in competitive inhibition toward peptide substrate hydrolysis which is distinctly different from the partially competitive anion binding site previously identified (Williams & Auld, 1986).

Materials and Methods

CPD A, prepared according to the method of Cox et al. (1964) and supplied by Sigma Chemical Co., was purified by affinity chromatography on CABS-Sepharose (Bicknell et al., 1985) and converted to cobalt(II) CPD by published procedures (Auld & Holmquist, 1974). *N*-(2-Mercaptoacetyl)-D-Phe was prepared as previously described (Holmquist &

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[1] Abbreviations: CPD A, carboxypeptidase A; Co(II)CPD, cobalt carboxypeptidase; CD, circular dichroism; MCD, magnetic circular dichroism; EPR, electron paramagnetic resonance; LMCT, ligand to metal charge transfer; β-PP, β-phenylpropionate; L-OPhe, L-β-phenyllactate; L-Phe, L-phenylalanine; D-Phe, D-phenylalanine; CMC, 1-cyclohexyl-3-[2-(*N*-methylmorpholino)ethyl]carbodiimide; Sarc, sarcosyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Z, carbobenzoxy; CABS-Sepharose, [*N*-(ε-aminocaproyl)-*p*-aminobenzyl]succinyl-Sepharose 4B.

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Vallee, 1979). Model cobalt(II) anion complexes were prepared by addition of anion (1 M) to cobalt(II) chloride (1 mM) in 0.05 M Hepes and 1 M NaCl at pH 7. Plastic labware was used whenever possible, and standard precautions were taken to remove adventitious metal ions from all solutions (Thiers, 1957).

Absorption spectra of the cobalt enzyme were recorded with a Varian Cary 219 spectrophotometer. The spectrophotometer was interfaced to an Apple IIe computer to enable data storage on floppy disks and routine spectral manipulation. K_D and ϵ_M values were estimated from the intercepts of double-reciprocal plots of the dependence of the absorbance of the enzyme–inhibitor complex on the concentration of inhibitor. Between 5 and 15 inhibitor concentrations were used for each determination.

CD and MCD spectra were recorded at 25 °C with a Cary 61 spectropolarimeter equipped with a Varian 4145 superconducting magnet and a Varian 4106 superconducting power supply. MCD spectra were corrected for CD absorption. A magnetic field of 4 T was used in MCD measurements. X-band EPR spectra were obtained with a Varian E-109 EPR spectrometer. An Air Products Heli-Tran liquid helium transfer line was employed to obtain sample temperatures of 4 K. Atomic absorption analysis was performed with a Perkin-Elmer 5000 graphite furnace spectrophotometer.

Zinc CPD concentrations were determined from the value of A_{278} by using a molar absorptivity of 6.42×10^4 M^{-1} cm^{-1} (Simpson et al., 1963). Co(II)CPD concentrations were determined from the value of A_{550} by using a molar absorptivity of 150 M^{-1} cm^{-1} (Latt & Vallee, 1971). Stock solutions of anions were prepared in buffer and extracted with 0.001% dithizone in carbon tetrachloride immediately before use. The pH of all solutions was checked following titration. If the pH deviated from 7 by more than ±0.1 of a pH unit, the result was rejected.

Kinetic parameters were determined from complete progress curves by the half-time method of Wharton and Szawelski (1982). A good fit to the experimental points was taken as evidence for Michaelis–Menten kinetics. Atomic absorption analysis showed that adventitious zinc contamination gave rise to less than 10% zinc(II) CPD in the Co(II)CPD assays. Inhibition constants were determined from Dixon plots of initial rates (356 nm) with furanacryloyl-L-Phe-L-Phe (Riordan & Holmquist, 1984) as substrate.

The peptide steady-state ES_2 intermediate during hydrolysis of Z-Sarc-L-Phe was prepared by addition of Z-Sarc-L-Phe (20 mM) to Co(II)CPD (0.66 mM) in 1 M NaCl and 0.1 M Hepes, pH 7 and 5 °C (Geoghegan et al., 1986).

RESULTS

(A) Kinetics of Azide Inhibition of Carboxypeptidase A. Figure 1 shows Dixon plots of azide inhibition of the cobalt carboxypeptidase catalyzed hydrolysis of furanacryloyl-L-Phe-L-Phe in 1 M NaCl at pH 7. There was very little change in the rate of hydrolysis with azide concentrations between 0.3 and 0.5 M, showing that, as previously reported for the zinc enzyme (Williams & Auld, 1986), inhibition was partially competitive toward peptide hydrolysis over the azide concentration range 0.025–0.5 M. The K_I value (35 mM) is slightly less for the cobalt enzyme than that determined with this substrate for the zinc enzyme (65 mM) (data not shown).

At azide concentrations greater than 0.5 M, a second mode of inhibition is detected. Inhibition is competitive (Figure 1b), and the K_I for the cobalt enzyme, 1.5 M, is essentially the same as the spectral K_D, 1.4 M, identifying an interaction of the inhibitor with the active-site metal (see below).

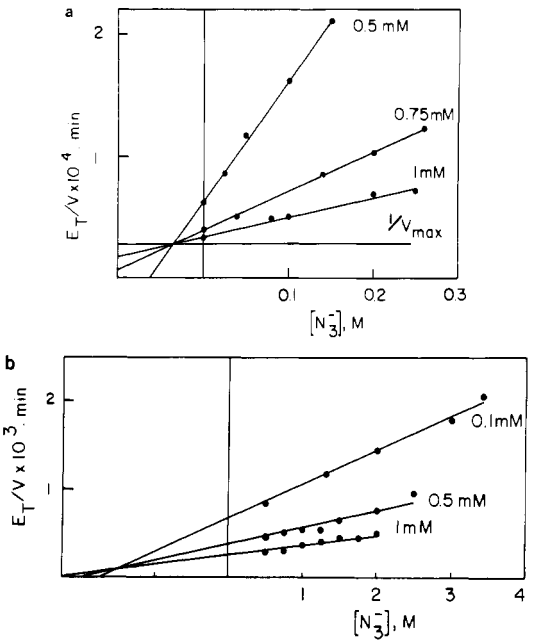

FIGURE 1: Dixon plots of the inhibition by azide of the cobalt carboxypeptidase catalyzed hydrolysis of furanacryloyl-L-Phe-L-Phe at low (≤0.25 M) (a) and high (b) concentrations of azide. Conditions: 50 mM Hepes, 1 M NaCl, pH 7, and 25 °C. The substrate concentration for each assay is given in the figure.

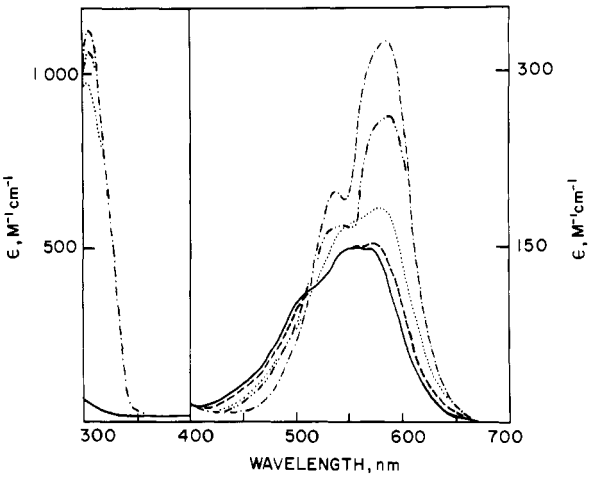

FIGURE 2: Electronic absorption spectrum of cobalt(II) carboxypeptidase A (—) and of the enzyme in the presence of azide (0.1 M) (---). Spectra are also shown of the ternary complexes formed by carboxypeptidase A and azide with L-phenylalanine (20 mM) (-·-), L-β-phenyllactate (10 mM) (-·····), and β-phenylpropionate (0.5 mM, equivalent to enzyme concentration) (···). The azide concentration was 0.1 M for the L-phenylalanine complex and 0.5 M for the L-β-phenyllactate and β-phenylpropionate complexes. Other conditions as in Figure 1. Spectra below 330 nm are corrected for trailing protein absorption by subtraction of the spectrum of the metal-free enzyme.

(B) Synergistic Interaction of Anions and C-Terminal Products of Peptide and Ester Hydrolysis and Their Carboxylate Analogues with Co(II)CPD. Spectral studies of Co(II)CPD at pH 7 in 1 M NaCl show that azide binds weakly to the enzyme (K_D = 1.4 M). The spectrum of Co(II)CPD in the presence of azide, 0.1 M, at pH 7 in 1 M NaCl does not differ greatly from that of the Co(II)CPD alone, indicating the first kinetically detected azide binding site is spectrally silent (Figure 2). However, subsequent addition of L-Phe (20 mM) results in a new spectrum with λ_{max} (ϵ_M) values of 590 (330) and 540 (190), which differ markedly from that of Co(II)CPD or Co(II)CPD plus L-Phe in the absence

Table I: Stabilities of Cobalt(II) CPD Inhibitor Complexes at Neutral pH[a]

(A) Anion Apparent Stability Constants					
complex	azide K_D (mM)	complex	cyanate K_D (mM)	complex	thiocyanate K_D (mM)
N_3^- alone	1400	OCN^- alone	143	SCN^- alone	(>3000)[b]
N_3^-/L-Phe (5)	4	OCN^-/L-Phe (5)	5	SCN^-/L-Phe (5)	500
N_3^-/β-PP (0.5)[c]	77	OCN^-/β-PP (0.5)[c]	140	SCN^-/D-Phe (1)[d]	100
N_3^-/L-OPhe (5)	59	OCN^-/L-OPhe (5)	146		
N_3^-/D-Phe (1)[d]	25	OCN^-/D-Phe (1)[d]	3.7		

(B) Organic Inhibitor Apparent Stability Constants					
complex	L-Phe K_D (mM)	complex	L-OPhe K_D (mM)	complex	β-PP K_D (mM)
L-Phe alone	1.67	L-OPhe alone	0.5	β-PP alone	<0.5
L-Phe/N_3^- (100)	0.07	L-OPhe/N_3^- (600)	0.08	β-PP/N_3^- (600)	0.018
L-Phe/OCN^- (100)	0.13				

[a] Conditions: 50 mM Hepes/1 M NaCl at pH 7. For ternary complexes parentheses indicate the concentration (mM) of the second inhibitor. [b] No detectable spectral perturbation up to a concentration of 3 M SCN^-, at which point the enzyme started to precipitate. [c] For β-PP-containing complexes 1 equiv of β-PP was added to the enzyme to obtain the 1:1 complex, which was then titrated with the anion. [d] For D-Phe-containing complexes, the D-Phe concentration (1 mM) was chosen to give the 1:1 enzyme–D-Phe complex (G. R. Hanson, personal communication). The 1:1 complex was then titrated with the anion.

Table II: Wavelength Maxima and Molar Absorptivities for the Visible Absorption Spectra of Various Ternary Complexes Formed between Cobalt(II) CPD, Monovalent Anions, and Inhibiting Amino Acids or Carboxylates[a]

anion	inhibitor	λ_{max} (nm)	ϵ_M (M^{-1} cm^{-1})	λ_{max} (nm)	ϵ_M (M^{-1} cm^{-1})	λ_{max} (nm)	ϵ_M (M^{-1} cm^{-1})
N_3/pH 7	none	592	430			535	398
N_3	L-OPhe	590	261			540	165
N_3	β-PP	586	188	556 (s)[b]	176	522	165
N_3	L-Phe	590	330			540	190
N_3	D-Phe	592	294			540	192
OCN	none	575	254			540	200
OCN	L-Phe	592	248	578 (s)[b]	240	539	171
OCN	D-Phe	589	307			540	207
SCN	L-Phe	590	200			535	140
SCN	D-Phe	591	284			534	211

[a] Buffer: 50 mM Hepes, 1 M NaCl, pH 7. [b] (s) indicates a shoulder.

of azide, 510 (130), 555 (200), 574 (205), and 610 (140) (Latt & Vallee, 1971). This spectrum therefore corresponds to that of a ternary complex formed from Co(II)CPD, azide, and L-Phe. Both azide and L-Phe bind much tighter to the enzyme in the ternary complex (Figure 3). Thus, $K_D(N_3^-)$ is 4 mM at 5 mM L-Phe, and K_D(L-Phe) is 70 μM at 100 mM azide compared to a $K_D(N_3^-)$ of 1.4 M and a K_D(L-Phe) of 1.7 mM for Co(II)CPD alone (Table I).

Only those amino acids that are either products or inhibitors of peptide hydrolysis give rise to similar ternary complexes (e.g., D-Phe, L-Tyr, L-Leu, L-Val, and, weakly, L-Ala). L-Arginine does not form a ternary complex in the presence of azide. The visible absorption spectra of the ternary complexes with different amino acids are remarkably similar both to each other (data not shown) and to that of the binary complex of the enzyme with azide alone (Table II). In each case a near-UV absorption band is present; e.g., the Co(II)CPD·L-Phe·azide complex has a λ_{max} at 308 nm and ϵ_M = 1250 M^{-1} cm^{-1} (Figure 2).

The carboxylate products of depsipeptide (ester) hydrolysis and their carboxylate analogues also give ternary complexes with cobalt carboxypeptidase and azide. The visible λ_{max} values (590 and 586 nm) and the near-UV bands (310 and 305 nm) of the ternary complexes with L-phenyllactate or 1 equiv of β-phenylpropionate are closely similar to those containing the C-terminal products of peptide hydrolysis, but the d–d transitions at 590 and 540 nm are spectrally less intense (Figure 2 and Table II). The spectral intensity decreases in the order L-Phe > L-OPhe > β-PP. Although the K_D values for azide are significantly greater for the L-OPhe (59 mM) and β-PP (77 mM) complexes than for L-Phe (4 mM), they are a factor of 20–30-fold less than with enzyme alone (1.4 M).

β-Phenylpropionate is an example of several carboxylate inhibitors that exhibit two modes of binding to CPD A (Steitz et al., 1967; Auld & Vallee, 1970; Latt & Vallee, 1971; Auld et al., 1972, 1986). Further addition of β-phenylpropionate (up to 25 mM) converts the visible absorption and MCD spectra of the azide ternary complex into that of the cobalt enzyme with two molecules of β-phenylpropionate bound. Concurrently, the near-UV absorbance is lost. Spectral titration gives a K_D of 2 ± 0.5 mM for the weaker binding mode of β-phenylpropionate. K_I for the weaker (competitive with peptide hydrolysis) mode is approximately 1 mM for the zinc enzyme at pH 6 (Auld et al., 1986).

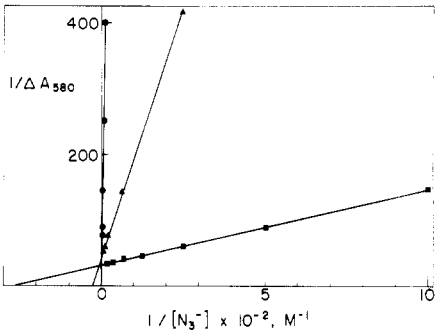

FIGURE 3: Synergistic binding of L-Phe and azide to Co(II)CPD at pH 7.0. The respective L-Phe concentrations are 0 (●), 0.1 (▲), and 5 (■) mM. The corresponding azide apparent K_D values are 1.4 M, 31 mM, and 4 mM, respectively. Conditions as in Figure 1.

CD, MCD, and EPR spectra of the ternary complex formed between L-Phe, azide, and Co(II)CPD are shown in Figure 4. The CD spectrum (Figure 4a) shows three bands at 528, 576, and 602 nm with $\Delta\epsilon_M$ values of +0.164, −0.087, and +0.072 M^{-1} cm^{-1}. However, the near-UV absorption band is not resolved clearly in the CD spectrum (data not shown). In contrast, the MCD spectrum (Figure 4b) shows a well-resolved extremum at 323 nm ($\Delta\epsilon_M$ = +0.207 M^{-1} cm^{-1} T^{-1})

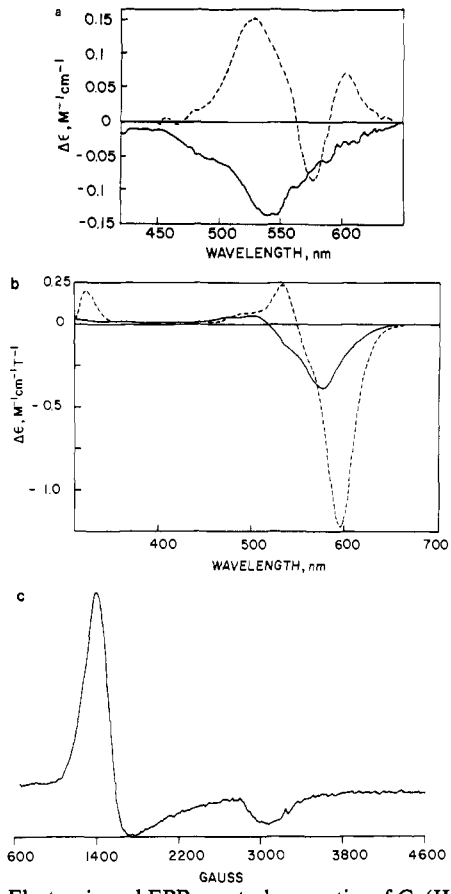

FIGURE 4: Electronic and EPR spectral properties of Co(II)CPD and of the ternary complex of cobalt(II) carboxypeptidase A, L-phenylalanine, and azide (a) CD and (b) MCD spectra of Co(II)CPD (—) and of the ternary complex (---). (c) EPR spectrum of the ternary complex. Conditions as in Figure 1. The EPR spectrum was recorded on a frozen sample at 4 K. EPR spectrometer settings were microwave frequency 9.228 GHz, microwave power 200 mW, modulation amplitude 10 G, and modulation frequency 100 kHz. Three scans were averaged to obtain the final spectrum.

and in addition a weak shoulder at 486 nm ($\Delta\epsilon_M$ = +0.056 M^{-1} cm^{-1} T^{-1}) and three bands corresponding to those present in the CD spectrum at 532, 570 (s), and 597 nm with $\Delta\epsilon_M$'s of +0.24, −0.25, and −1.23 M^{-1} cm^{-1} T^{-1}, respectively. The X-band EPR spectrum (Figure 4c) has two resonances with g values at 4.68 ($g_\perp$) and 2.14 ($g_\parallel$).

The results of electronic absorption studies indicate that both cyanate (OCN^-) and thiocyanate (SCN^-) form L-Phe containing ternary complexes (Figure 5 and Table I). Cobalt(II) azide and thiocyanate model complexes display near-UV, i.e., charge-transfer, bands at 310 and 316 nm, respectively. However, a cobalt(II) cyanate model complex does not show a charge-transfer absorption beyond 300 nm. Similarly, there is no detectable charge-transfer absorption in the enzyme complex with cyanate. In contrast, the thiocyanate, D-Phe containing complex has a well-resolved near-UV absorption band (λ_{max} = 316 nm, ϵ_M = 760 M^{-1} cm^{-1}; Figure 5).

In contrast to the aforementioned anions, cyanide and cysteine, inhibitors of carboxypeptidase (Smith & Hanson, 1949) remove the metal from Co(II)CPD. Titration of the cobalt enzyme, 0.2 mM, with a Chelex-100-extracted cyanide stock solution at pH 7 gives rise to progressive loss of the absorption associated with Co(II)CPD at 550 nm and appearance of an absorption corresponding to the aquo cobalt(II) cyanide complex at lower wavelength (λ_{max} = 370 nm). The isosbestic point at 478 nm is constant within 0.002 absorbance units, and the process is complete at a cyanide concentration

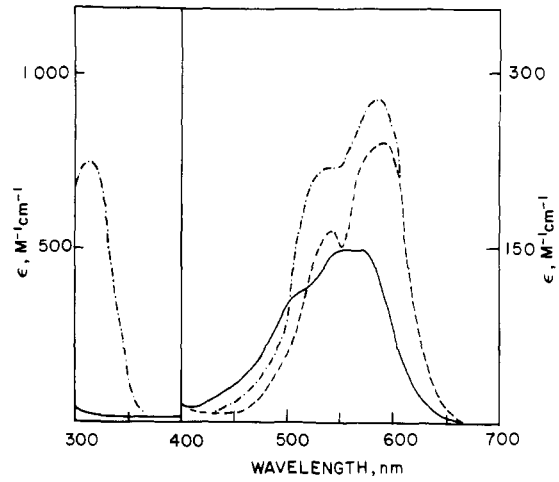

FIGURE 5: Electronic absorption spectra of Co(II)CPD (—) and of the ternary complexes with L-phenylalanine (5 mM) and cyanate (0.25 M) (---) and D-phenylalanine (1 mM, to give a 1:1 complex) and thiocyanate (0.75 M) (-·-). Conditions as in Figures 1 and 2.

of 20 mM. No other species is detected, even when the titration is carried out in the presence of L-Phe (20 mM). Cysteine at a concentration of 5 mM removes the metal from the enzyme, 0.2 mM, and again there is no spectral evidence for complexation with the enzyme.

Neither the product of ester hydrolysis, L-β-phenyllactate, nor the analogue, β-phenylpropionate, promotes tighter binding of cyanate to Co(II)CPD. Thus, the presence of L-β-phenyllactate (10-fold above K_D) has no effect on K_D for cyanate (Table I), and the final spectrum is identical with that seen with cyanate in the absence of the carboxylate inhibitor.

(C) Interaction of D-Phenylalanine and Anions with Cobalt(II) Carboxypeptidase A. D-Phe (1 mM) promotes 5-fold tighter binding of thiocyanate to cobalt carboxypeptidase than does L-Phe (Table I). In contrast to L-Phe, there are two binding sites for D-Phe, and the visible absorption, CD, MCD, and EPR spectra of the binary and ternary D-Phe complexes are distinct from each other and from that of the cobalt enzyme (G. R. Hanson, unpublished data). As observed with β-phenylpropionate (section B), addition of excess D-Phe causes the absorption spectrum of the Co(II)CPD·SCN^-·D-Phe complex to collapse to that of the Co(II)CPD·D-Phe·D-Phe complex, suggesting that the weaker binding D-Phe and SCN^- compete for the same site. The Co(II)CPD·SCN^-·D-Phe complex shows a prominent near-UV absorption at 316 nm (ϵ_M = 760 M^{-1} cm^{-1}), confirming that as in the azide-containing ternary complexes the anion enters the metal coordination sphere (Figure 5).

(D) Inhibitors That Do Not Allow Concurrent Azide Binding to Cobalt(II) Carboxypeptidase A. The inhibitors pyrophosphate (10 mM), L-benzyl succinate (1 mM), and the pseudosubstrate Gly-L-Tyr (10 mM) result in characteristic absorption spectra with Co(II)CPD (0.34 mM) at pH 7 in 1 M NaCl (data not shown). These enzyme–inhibitor spectra are insensitive to the addition of either azide or cyanate at concentrations up to 1 M.

Similarly, the enzyme–inhibitor complex of Co(II)CPD and *N*-(2-mercaptoacetyl)-D-Phe (6 mM) in which the thiol group of the inhibitor displaces the metal-bound water (Holmquist & Vallee, 1979) does not permit concurrent binding of azide at concentrations of azide up to 4 M.

(E) Effect of the Addition of Phenylacetate on the Spectrum of the Co(II)CPD·L-Phe Complex. Phenylacetate concentrations up to 5 mM do not affect the binary complex Co(II)CPD·L-Phe spectrum. Between 5 and 20 mM phenyl-

acetate the spectrum changes into that characteristic of Co(II)CPD with two molecules of phenylacetate bound. The K_I (zinc enzyme) values for phenylacetate at pH 7 are 1 (noncompetitive) and 10 mM (competitive) (Auld et al., 1972). Jointly, this suggests that L-Phe and phenylacetate cannot bind concurrently to Co(II)CPD in the manner that two molecules of phenylacetate may bind.

(F) Effect of Azide on the Absorption Spectrum of the Steady-State CPD Intermediate in Peptide Hydrolysis. Further studies were carried out with the slowly hydrolyzed (low k_{cat}) dipeptide, Z-Sarc-L-Phe, 20 mM, at pH 7 and 5 °C that gives rise to a long-lived peptide ES_2 intermediate (Geoghegan et al., 1986). Azide, up to 2 M, has no effect on the cobalt absorption spectrum of the intermediate. However, on incubation with 2 M azide at 5 °C, pH 7.0, the spectrum gradually changes to that of the Co(II)CPD·L-Phe·azide ternary complex. When azide is omitted, no change in the peptide intermediate spectrum occurs over this time period. The slow spectral change in the presence of azide is therefore due to the formation of L-Phe upon hydrolysis and the subsequent displacement of the peptide from the enzyme by the combined binding of L-Phe and azide. This shows that the substrate Z-Sarc-L-Phe and azide do not form a ternary complex analogous to those observed with the C-terminal product and azide.

Addition of Z-Sarc-L-Phe, 20 mM, to Co(II)CPD in the presence of 0.1 M azide (6-fold above the tighter, partially competitive, K_I) gives a spectrum characteristic of a normal peptide intermediate. The formation of a spectrum characteristic of a normal peptide intermediate on addition of Z-Sarc-Phe to Co(II)CPD in which the partially competitive inhibitory mode is saturated with azide shows that anion binding at the partially competitive site (Williams & Auld, 1986) is spectrally insensitive not only in the free cobalt enzyme but also in the peptide, ES_2 intermediate.

Discussion

The spectra of some cobalt(II)-substituted zinc metalloenzymes, e.g., carbonic anhydrase (Lindskog, 1963) and horse liver alcohol dehydrogenase (Maret & Zeppezauer, 1986), are markedly sensitive to anions. In the case of carbonic anhydrase, sensitivity extends over a wide pH range (Lindskog, 1963). In contrast, spectra of, e.g., cobalt(II)-substituted CPD A (Latt & Vallee, 1971), thermolysin (B. Holmquist, personal communication), and β-lactamase II (Baldwin et al., 1980; Bicknell et al., 1986) are not. Some rationalization of this difference was provided by the studies of Geoghegan et al. (1983b) on cobalt(II) CPD, which demonstrated that the enzyme is anion sensitive both at low pH and at neutral pH after chemical modification of the active-site Glu-270. This is consistent with the proposal of Stephens et al. (1974) that chloride displaces water from the active-site zinc atom at low pH due to protonation of Glu-270. These results point to a mechanistically important interaction of Glu-270 with the metal-bound water molecule. The spectroscopically anion-insensitive cobalt(II)-substituted thermolysin and β-lactamase II also have active-site glutamate residues (Kester & Matthews, 1977; Little et al., 1986) that may have a similar stabilizing role to Glu-270 in CPD A. In contrast, the anion-sensitive cobalt carbonic anhydrase does not have a glutamate residue within hydrogen-bonding distance of the metal-bound water.

The relationship between the kinetic and spectral studies of anion interactions has not previously been explored for CPD A. The kinetic data presented here show that azide has two inhibitory binding sites to cobalt(II) CPD A, the tighter of which shows partial competitive inhibition (Figure 1) and is spectrally insensitive in the cobalt enzyme (Figure 2). The weaker binding mode is competitive and the K_I is equivalent to the observed spectral K_D, identifying the coordination sphere of the active-site metal ion as the potential site of interaction. Analogous studies on the zinc enzyme show both a strong (Williams & Auld, 1986, Figure 1b) and a weak (R. Bicknell, unpublished observation) mode of azide inhibition.

While several studies have investigated the interaction of anions such as azide and cyanate with Co(II)CPD (Geoghegan et al., 1983a; Bertini et al., 1985b), it has been recognized only recently (Bicknell et al., 1985) that the presence of an organic inhibitor enhances anion binding to the enzyme at neutral pH. The synergistic binding of either amino acid or carboxylate inhibitors in the presence of anions such as azide and cyanate suggests an opening up of the metal coordination site upon binding of the organic inhibitor. The azide ternary complex spectra (Figures 2 and 5) are very similar to those of the binary complex between azide and the CMC-modified enzyme at neutral pH (Geoghegan et al., 1983b). This remarkable similarity between the azide-containing complexes suggests that the amino acids and carboxylate inhibitors disrupt the metal–H_2O–Glu-270 interaction, as does CMC modification of Glu-270 or low pH (Geoghegan et al., 1983b).

The cobalt(II) electronic absorption spectra of the ternary complexes are closely similar to those of undistorted tetrahedral complexes [see, for example, Dennard and Williams (1966)]. A characteristic feature of the azide- and thiocyanate-containing ternary complex is a near-UV absorption band centered at 308 nm (1250 M^{-1} cm^{-1}) for azide and at 316 nm (760 M^{-1} cm^{-1}) for thiocyanate (Figures 2 and 5). The wavelength maxima of the bands in the azide complexes are close to the charge-transfer band of the aqueous cobalt(II) azide complex (310 nm); however, the λ_{max} at 316 nm of the enzyme thiocyanate complex is some 40 nm greater than that of the aqueous cobalt(II) thiocyanate complex (274 nm). The longer wavelength maxima of the band in the enzyme-bound thiocyanate complex compared to that of the free cobalt thiocyanate complex probably indicates a weaker metal–anion interaction in the enzyme–inhibitor complex (Banci et al., 1982). Metal azide charge-transfer absorptions have not previously been reported in cobalt(II) complexes but have been observed with copper(II) in complexes of azide with copper(II) containing native laccase (Allendorf et al., 1985) and in the dicopper–dicobalt derivative of superoxide dismutase (Bertini et al., 1985a). A sulfur → cobalt(II) charge-transfer absorption (λ_{max} = 338 nm and ϵ_M = 960 M^{-1} cm^{-1}) has, however, been observed in the Co(II)CPD complex with the inhibitor *N*-(2-mercaptoacetyl)-D-Phe (Holmquist & Vallee, 1979).

Substitution of a hydrogen in β-phenylpropionate by OH (in L-OPhe) or NH_2 (in L-Phe) promotes a striking increase in the molar absorptivities of the ternary azide complex d–d transitions but little change in the band positions (Figure 2). Proton NMR spectroscopy indicates that none of these inhibitors bind to the metal in the ternary complex (Bertini et al., 1987a). The increase in spectral intensity presumably reflects a minor steric rearrangement of the metal ligands induced by the bulkier hydroxyl and amino groups.

Several inhibitors of CPD A are either known or thought to chelate the active site in the enzyme–inhibitor complex and in so doing displace the zinc-bound water. *N*-(2-Mercaptoacetyl)-D-Phe binds to the metal through its mercaptide group and gives rise to characteristic LMCT bands (Holmquist & Vallee, 1979). L-Benzyl succinate is known from X-ray

crystallographic studies to chelate the active site zinc in thermolysin through a carboxylate group and is thought to bind in a similar manner to CPD A (Bolognesi & Matthews, 1979). A reexamination of the Gly-L-Tyr·crystalline CPD A complex at −9 °C by high-resolution (1.6 Å) X-ray crystallography has now confirmed that this is a nonproductive complex in which the amino-terminal group has displaced the zinc-bound water (Christianson & Lipscomb, 1986a,b). Each of these complexes was insensitive to high concentrations of azide, consistent with loss of the displaceable metal-bound water upon formation of the complex.

Cyanate binds to cobalt(II) in tetrahedral $Co(NCO)_4^{2+}$ exclusively via the nitrogen atom (Cotton & Goodgame, 1961). In view of the close similarity between the spectra of the azide and of the cyanate ternary complexes this may well be the case here. Nevertheless, in the absence of characteristic charge-transfer absorption in cobalt(II) cyanate complexes (Figure 5), chelation to cobalt(II) via the oxygen of the cyanate cannot be excluded. The binding of thiocyanate, weaker relative to that of azide and cyanate, may reflect steric hindrance to accommodation within the active site of the larger sulfur atom.

The removal of cobalt(II) from Co(II)CPD by cyanide is in sharp contrast with cobalt(II) carbonic anhydrase. In the latter case cyanide forms the most stable inhibitor complex currently known for that enzyme (K_I = 2.6 μM; Pocker & Stone, 1967). This different behavior may be a result of weaker cobalt(II) binding at neutral pH by apocarboxypeptidase A compared to that by apocarbonic anhydrase (Lindskog & Nyman, 1964).

The CD spectrum of the Co(II)CPD·L-Phe·N_3^- ternary complex is somewhat unusual for a CPD A derivative, showing three clear bands (Figure 4a) at 528, 576, and 602 nm. The MCD and EPR spectra of the ternary complex (Figure 4b,c) are consistent with a slightly distorted tetrahedral coordination sphere around the active-site metal atom. The EPR spectrum ($g_\perp$ = 4.68, $g_\parallel$ = 2.14) is similar to those of inhibitor complexes of carbonic anhydrase in which the metal is thought to occupy a tetrahedral coordination site (Bencini et al., 1981). There is particularly close similarity to the 1:1 complex of cobalt(II) carbonic anhydrase with cyanate or cyanide (Bencini et al., 1981). In accord with the entatic state hypothesis of Vallee and Williams (1968), the increase in ligand field stabilization energy with loss of distortion in the metal environment upon formation of the ternary complex may well contribute to the synergistically tight binding of anion and inhibitor. A similar loss of distortion has recently been observed on binding of picomolar K_I inhibitors to cobalt(II) angiotensin converting enzyme (Bicknell et al., 1987).

Tetrahedral coordination of the metal in the ternary complex, in which azide is one ligand, makes it unlikely that the third component of the ternary complex, i.e., L-phenylalanine or β-phenylpropionate, coordinates directly to the metal atom. This has been established in the L-phenylalanine ternary complex by ^{13}C NMR spectroscopy, where the calculated Co–$^{13}COO^-$ distance, 4.2 Å, is too great for direct coordination of the carboxylate to the metal (Luchinat et al., 1988). However, binding of a second molecule of β-phenylpropionate is accompanied by the loss of the near-UV absorption, suggesting that it is the second molecule and not the first which interacts with the metal ion. This proposal is consistent with earlier studies on the rate of exchange of zinc in the absence and presence of high concentrations of β-phenylpropionate (Coleman & Vallee, 1964) and the observation that the latter abolish proton relaxation associated with the metal-bound water in manganese(II) CPD (Schulman et al., 1966). The latter observation was assumed to point to displacement of the metal-bound water by the inhibitor. ^{13}C NMR spectroscopy of acetate and phenylacetate binding to the cobalt enzyme also indicates that a carboxylate metal complex is only formed upon binding of the second mole of inhibitor (Bertini et al., 1988b).

Table III: Inhibitors Forming Homo and Hetero Ternary Complexes with Carboxypeptidase A at Neutral pH

inhibitors forming homo complexes	inhibitors forming hetero complexes with anions
D-phenylalanine	D-phenylalanine
β-phenylpropionate	L-phenylalanine
acetate	(L-Tyr, L-Leu, L-Val)
phenylacetate	β-phenylpropionate
azide	acetate
	phenylacetate
	L-β-phenyllactate

Table III summarizes observed inhibitor binding combinations to cobalt(II) CPD at pH 7. It has long been proposed (Latt & Vallee, 1971; Auld et al., 1972) that at least two inhibitor binding sites are present in CPD A, as is confirmed here. The ternary complexes may be described as either "homo" or "hetero", depending on whether two molecules of the same inhibitor or one molecule of different inhibitors is bound in the complex. All inhibitors that form homo complexes also form hetero complexes. It is only L-amino acids and their alcohol analogues that form hetero complexes with anions but do not form homo complexes. The near-UV absorption of anion-containing complexes implicates one site as the active-site metal. The second site will be referred to simply as the "nonmetal" site to indicate inhibitor binding to the enzyme but not markedly perturbing the coordination sphere of the metal ion. This nonmetal site need not be the same locus in the various ternary complexes. The preferential binding of C-terminal products of peptide hydrolysis and the first binding mode of carboxylate inhibitors to a site other than the metal has recently been confirmed by NMR studies (Bertini et al., 1988a,b; Luchinat et al., 1988). Earlier studies have implicated residues other than the metal ion in inhibitor binding; thus, β-phenylpropionate or chloride (a partially competitive inhibitor) afford some protection against arginine modification by butanedione (Riordan, 1973; Williams & Auld, 1986).

X-ray diffraction analyses have included high-resolution studies of the interaction of the cross-linked enzyme with a number of inhibitors. These have included the potato inhibitor (Ryan et al., 1974) at 2.5 Å (Rees & Lipscomb, 1982), the ketone (*R*)-2-benzyl-3-(*p*-methoxybenzoyl)propionate (Spratt et al., 1983) at 2.8 Å (Rees & Lipscomb, 1981), the aldehyde 2-benzyl-3-formylpropionate (Galardy & Kartylewicz, 1984) at 1.7 Å (Christianson & Lipscomb, 1985), the phosphonamidate inhibitor *N*-[[[(benzyloxycarbonyl)amino]methyl]-hydroxyphosphinyl]-L-phenylalanine, Z-Gly^P-Phe (Jacobsen & Bartlett, 1981), at 1.8 Å (Christianson & Lipscomb, 1986a,b), and indole-3-acetate at 2.3 Å (Rees & Lipscomb, 1981). Several different interactions are observed. Thus, their carboxyl groups interact to various degree with the zinc atom, Arg-145, Arg-127, and Tyr-248. Arg-145, Arg-127, and Arg-71 have all been assigned various roles in catalysis (Lipscomb, 1968; Christianson & Lipscomb, 1986a,b; Nakagawa & Umeyama, 1978). These three residues are the most likely nonmetal binding sites in the enzyme.

Rapid-scanning stopped-flow spectroscopy has characterized the cobalt(II) spectra of IES complexes between Co(II)CPD, a peptide substrate, and a carboxylate inhibitor, e.g., β-phenylpropionate or phenylacetate (Auld et al., 1986). Such inhibitors have at least two binding modes to CPD A[2] (Steitz

et al., 1967; Auld & Vallee, 1970; Latt & Vallee, 1971; Auld et al., 1972, 1986), the tighter of which at pH 7 shows noncompetitive inhibition of peptide hydrolysis. The second binding mode is competitive with peptide hydrolysis. The 1:1 complexes of these inhibitors with the cobalt enzyme readily form azide-containing ternary complexes in which the azide is bound to the metal site. Azide is competitive with the weaker carboxylate binding mode for the metal site; thus, high β-PP concentrations displace the azide from the enzyme to give a homo ternary β-PP complex.

In contrast to the carboxylate inhibitors described above, C-terminal products of peptide hydrolysis (e.g., L-Phe) and C-terminal products of ester hydrolysis, the corresponding alcohol analogues (e.g., L-OPhe) show no evidence for more than a single binding mode to CPD A or for coformation of hetero ternary complexes with inhibitors such as phenylacetate.[2] Nevertheless, they readily form anion-containing hetero complexes in which the anion is again bound in the metal site. Apparently, the amine or hydroxyl group, present in L-Phe and L-OPhe, respectively, but absent from β-phenylpropionate and phenylacetate, prohibits binding of a second inhibitor molecule unless that molecule is small (e.g., azide, cyanate, or thiocyanate).

D-Phe terminal peptides are slowly hydrolyzed by CPD A (Schechter & Berger, 1966). D-Phe is exceptional for a C-terminal product of peptide hydrolysis in that two molecules may bind concurrently to Co(II)CPD, suggesting that in this case the amino group is orientated such that it does not prohibit binding at a second site. It is interesting in this respect that the larger thiocyanate anion binds weakly in the ternary complex with L-Phe but more tightly in that with D-Phe, supporting a steric hindrance argument.

Byproduct inhibitors and substrate esters appear to occupy both binding sites. Thus, inhibitors such as L-benzyl succinate and *N*-(2-mercaptoacetyl)-D-Phe form enzyme complexes with Co(II)CPD, the spectra of which are insensitive to the presence of high azide concentrations. Esters, unlike peptide substrates, do not form IES complexes with inhibitors such as L-OPhe or β-phenylpropionate (Auld et al., 1986), suggesting that like the byproduct inhibitors the esters occupy both binding sites. This result is consistent with the finding that the ester bond is broken in the ES_2 complex, thus forming an $E{\cdot}P_1P_2$ or enzyme byproduct complex (Galdes et al., 1986).

Although the cobalt enzyme is spectrally sensitive to high concentrations of azide (K_D = 1.4 M), the steady-state peptide intermediate, ES_2, is not, up to concentrations of at least 2 M. Higher concentrations of azide (up to 6 M) gave rise to mixed spectra of ES_2 intermediate and Co(II)CPD·azide complex, confirming that although azide does not bind concurrently to the enzyme with Z-Sarc-Phe (excluding binding at the partially competitive site) to give an analogous ternary complex to those seen with inhibitors, it does compete with Z-Sarc-Phe for the metal site. This is consistent with the absence of a metal-coordinated water in the intermediate. The substrate carbonyl oxygen is presumably bound to the metal in ES_2, as has long been proposed to occur during the course of CPD A catalysis (Vallee et al., 1963; Lipscomb et al., 1968).

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[2] A notable exception is L-OPhe, which has only one binding mode to carboxypeptidase A. L-OPhe in contrast to L-Phe does form an IES complex with peptides (Auld et al., 1986), indicating that differences exist between the binding modes of these two inhibitors.

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