Characterization of an Inhibitory Metal Binding Site in Carboxypeptidase A

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ABSTRACT: The specificity of metal ion inhibition of bovine carboxypeptidase A ([(CPD)Zn]) catalysis is examined under stopped-flow conditions with use of the fluorescent peptide substrate Dns-Gly-Ala-Phe. The enzyme is inhibited competitively by Zn(II), Pb(II), and Cd(II) with apparent K_I values of 2.4×10^{-5}, 4.8×10^{-5}, and 1.1×10^{-2} M in 0.5 M NaCl at pH 7.5 and 25 °C. The k_{cat}/K_m value, 7.3×10^6 M^{-1} s^{-1}, is affected less than 10% at 1×10^{-4} M Mn(II) or Cu(II) and at 1×10^{-2} M Co(II), Ni(II), Hg(II), or Pt(IV). Zn(II) and Pb(II) are mutually exclusive inhibitors. Previous studies of the pH dependence of Zn(II) inhibition [Larsen, K. S., & Auld, D. S. (1989) *Biochemistry 28*, 9620] indicated that [(CPD)Zn] is selectively inhibited by a zinc monohydroxide complex, $ZnOH^+$, and that ionization of a ligand, LH, in the enzyme's inhibitory site (pK_{LH} 5.8) is obligatory for its binding. The present study allows further definition of this inhibitory zinc site. The ionizable ligand (LH) is assigned to Glu-270, since specific chemical modification of this residue decreases the binding affinity of [(CPD)Zn] for Zn(II) and Pb(II) by more than 60- and 200-fold, respectively. A bridging interaction between the Glu-270-coordinated metal hydroxide and the catalytic metal ion is implicated from the ability of Zn(II) and Pb(II) to induce a perturbation in the electronic absorption spectrum of cobalt carboxypeptidase A ([(CPD)Co]). The spectra of the transient [(CPD)Co]·Zn and stable [(CPD)Co]·Pb dimetallic enzyme species are both characterized by a maximum at 560 nm ($\Delta\epsilon_{560} = +60$ M^{-1} cm^{-1}) and shoulders at 520 and 585 nm. Spectral titrations of the [(CPD)Co]·Pb complex with azide show that Pb(II) prevents azide binding to the catalytic Co(II) ion, providing further support for the idea that the inhibitory metal monohydroxide binds to the catalytic metal ion. The marked metal ion specificity of the inhibitory site can in part be accounted for by the stabilities of monohydroxide complexes of the metal ions examined. On the basis of charge considerations in the active site, the metal monohydroxide complex likely also carries one anion (Cl^-) from the supporting electrolyte; the K_I values for the neutral complexes Zn(OH)Cl and Pb(OH)Cl are $\sim 5 \times 10^{-7}$ M. If the inhibitory metal monohydroxide complex is charge neutralized by a multidentate ligand, an even lower inhibition constant can be anticipated. Such inhibitory complexes could be very important to regulatory and/or toxicological processes of zinc enzymes.

The protein ligands of several catalytic zinc binding sites are known, and putative zinc binding sites can now be tentatively assigned on the basis of consensus sequences and the knowledge gained from the structural standards of reference (Vallee & Auld, 1990). Several zinc proteinases are known to be inhibited by excess zinc [e.g., carboxypeptidase A (Larsen & Auld, 1989a); thermolysin (Holmquist & Vallee, 1974) and other neutral endopeptidases (Kerr & Kenny, 1974; Pangburn & Walsh, 1975); angiotensin-converting enzyme (Bünning et al., 1983); collagenase (Mallya & Van Wart, 1989)], but in contrast to catalytic zinc binding sites, the ligands constituting the inhibitory zinc binding sites have not been identified.

The purpose of this study is the characterization of the zinc inhibitor binding site in carboxypeptiase A ([(CPD)Zn])[1] and the examination of the specificity of metal ion inhibition. The metal ion specificity of the inhibitory site is determined by stopped-flow methodology and a high turnover fluorescent substrate, Dns-Gly-Ala-Phe. This approach allows acquisition of kinetic data within a time period that is short compared to the rate of exchange of the catalytic metal ion with the inhibitory metal ion.

In a previous kinetic study it was suggested that a zinc monohydroxide complex, $ZnOH^+$, selectively inhibits [(CPD)-Zn] and that the inhibitory complex bridges the Glu-270 carboxylate and the catalytic zinc ion (Larsen & Auld, 1989a). In the present study, the mode of interaction for inhibitory metal ions with Glu-270 is investigated by the specific chemical modification of Gly-270 and subsequent studies of direct metal binding to the modified enzyme. The proposed interaction between the hydroxide moiety of the inhibitory complex and the catalytic metal ion is probed through the effect of inhibitory metal ion binding on the electronic absorption spectrum of [(CPD)Co]. A preliminary account of this work has been reported (Larsen & Auld, 1989b).

Materials and Methods

Enzyme Preparation. [(CPD)Zn] from bovine pancreas, prepared according to the method of Cox et al. (1964), was obtained from Sigma Chemical Co., St. Louis, MO (C-0261, Lot 57F-8110), and the α-Val isozyme used in this study was isolated by a combination of ion-exchange and affinity chromatography (Larsen & Auld, 1989a). [(CPD)Co] was prepared in the crystalline state and contained ≤1% zinc (Auld, 1988b). Enzyme chemically modified at Glu-270 was prepared by treatment of [(CPD)Zn], 1.5×10^{-4} M, with 2×10^{-2} M CMC metho-*p*-toluenesulfonate (Aldrich, Milwaukee, WI)

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[1] Abbreviations: [(CPD)Zn], carboxypeptidase A; [(CPD)Co], cobalt(II)-substituted carboxypeptidase A; CMC-[(CPD)Zn], CMC-modified carboxypeptidase A; CMC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Dns (dansyl), 5-(dimethylamino)naphthalene-1-sulfonyl; RET, radiationless energy transfer; K_D, dissociation constant.

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in 1 M NaCl/0.05 M Mes, pH 6.0, at 4 °C for 1 h (Riordan & Hayashida, 1970; Nau & Riordan, 1975). Excess reagent was removed by gel filtration on Bio-Gel P-6 (Bio-Rad, Richmond, CA), and the derivative modified at Glu-270 was subsequently obtained by affinity chromatography (Cueni et al., 1980). Enzyme concentrations were determined by amino acid analysis or from A_{278} by using an extinction coefficient of 6.42×10^4 M^{-1} cm^{-1} (Simpson et al., 1963).

Equilibrium Dialysis. Metal-free dialysis tubing (Spectrapor membrane tubing No. 1; Spectrum Medical Industries Inc., Los Angeles, CA) was prepared as described (Auld, 1988a). Native and CMC-modified enzymes (5×10^{-5} M) were dialyzed against solutions containing either Zn(II) [(0–3.3) $\times 10^{-4}$ M] or Pb(II) [(0–2) $\times 10^{-4}$ M] in 0.5 M NaCl/0.05 M Hepes, pH 7.5. After 12 h, the dialysates were centrifuged (2 min at 2000*g*), and the supernatants were analyzed for protein and metal content. The zinc and lead concentrations in the centrifuged retentate and the dialysate were determined by graphite furnace atomic absorption spectrometry (Perkin-Elmer, Model 5000).

Spectroscopic Measurements. The effects of Zn(II) and Pb(II) on the electronic absorption spectrum of [(CPD)Co] were measured in 1 M NaCl/0.05 M Hepes, pH 7.5. The interaction of Zn(II) with [(CPD)Co] was monitored with a rapid-scanning silicon diode array spectrophotometer (EG&G, optical multichannel analyzer, Model 1214) coupled to a rapid-mixing stopped-flow instrument (Hanahan & Auld, 1980; Geoghegan et al., 1983). The light source was a 200-W quartz–iodine lamp (General Electric, Q6.6A/T4/CL), and the scan time was 8.3 ms for the 435–700-nm wavelength range. Spectral titrations of [(CPD)Co] with Pb(II) and/or NaN_3 were performed with use of a diode array spectrophotometer (Hewlett-Packard, Model 8451A).

Enzyme Kinetics. The kinetics of [(CPD)Zn]-catalyzed hydrolysis of the peptide substrate Dns-Gly-Ala-Phe was recorded on a Durrum stopped-flow spectrophotometer (Model 13000) equipped as described previously (Hanahan & Auld, 1980). A 250–400-nm ultraviolet band-pass filter (Schott Optical Glass Inc., Duryea, PA; Model UG-11) was placed in the exit mirror box in order to eliminate low-intensity visible stray light from the optical system. The reactions were monitored through radiationless energy transfer (RET), which arises from quenching of the enzyme tryptophan fluorescence by the dansyl chromophore of bound substrate (excitation at 285 nm and fluorescence emission from 320 to 390 nm) (Lobb & Auld, 1984). A standard RET-based assay ([E] = 1×10^{-6} M, [S] = 1×10^{-4} M) was used to survey the effect of metal ions on the enzymatic activity in 0.5 M NaCl/0.05 M Hepes, pH 7.5. The metal ion concentratons were 5×10^{-5} and 1×10^{-4} M for Mn(II), Cu(II), and Pb(II) and 1.5×10^{-3}, 5×10^{-3}, and 1×10^{-2} M for Hg(II), Ni(II), Pt(IV), and Co(II). In these experiments, the metal ion being studied was contained only in the substrate drive syringe.

The metal salts $CoCl_2$, $NiCl_2$, $CuCl_2$, $ZnCl_2$, $CdCl_2$, $HgCl_2$, and $PbCl_2$ were purchased from Johnson Matthey Inc., Seabrook, NH ("Puratronic grade"), and $MnCl_2$ and H_2PtCl_6 were obtained from Fisher Scientific. The concentrations of $HgCl_2$ and H_2PtCl_6 were based on weight, and the concentrations of the other metal ions were determined by flame atomic absorption spectrometry (Perkin-Elmer, Model 2280). Metal-free solutions were prepared as described (Holmquist, 1988). Kinetics and pH measurements were performed at 25.0 ± 0.1 °C, and purified water was used throughout this study (Milli-Q/Milli-RO water purification system; Millipore, Bedford, MA).

Table I: Inhibitory Effect[a] of Metal Ions on the Activity of [(CPD)Zn] and Concentration of Their Monohydroxide Complexes,[b] Ionic Radii,[c] and Hydration Energies[c]

metal cation	[(CPD)Zn] K_I (mM)	[$MeOH^+$] (μM)	ionic radius (Å)	ΔG_{hydr} (kJ/mol)
Mn(II)	≫0.1	0.8	0.80	−1770
Co(II)	≫10	0.07	0.72	−1922
Ni(II)	≫10	0.04	0.70	−1998
Cu(II)	>0.1	6.3	0.70	−2016
Zn(II)	0.024	3.3	0.74	−1963
Cd(II)	11	2.6	0.97	−1736
Pt(IV)	≫10			
Hg(II)	≫10	0.002	1.10	−1766
Pb(II)	0.048	37.6	1.32	−1434

[a] The K_I values are determined from the relationship between K_m/k_{cat} and the metal ion concentration in 0.5 M NaCl/0.05 M Hepes, pH 7.5 and 25.0 °C. The standard error around the mean is less than 10% for the K_I values for Zn(II), Cd(II), and Pb(II). Estimates of $K_I \gg 0.1$ mM or $K_I \gg 10$ mM are based on assays in which the level of activity (k_{cat}/K_m) determined in the absence of excess metal ion is affected less than 10% by metal ion concentrations of 0.1 or 10 mM, respectively. [b] The concentrations of metal(II) monohydroxide complexes, [$MeOH^+$], are calculated for a total metal(II) ion concentration of 0.1 mM at pH 7.5, 25.0 °C with use of the acid dissociation constants compiled by Baes and Mesmer (1976) for both mono- and polynuclear species. The calculation did not incorporate complexation with NaCl, since the chloride stability constants for mixed-metal hydroxide chloride complexes are not available for all the metal ions examined. [c] Ionic radius of the metal cation and absolute hydration energy (Marcus, 1985).

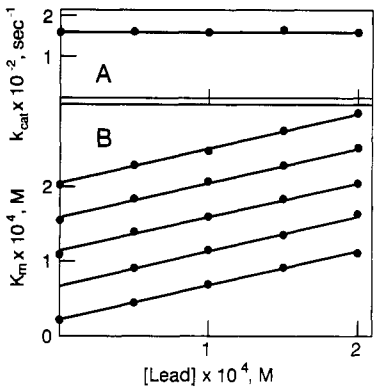

FIGURE 1: Effect of Pb(II) and Zn(II) on the steady-state parameters for [(CPD)Zn] acting on the peptide substrate Dns-Gly-Ala-Phe in 0.5 M NaCl/0.05 M Hepes, pH 7.5 at 25 °C: (A) k_{cat} versus Pb(II) in the absence of added Zn(II); (B) K_m versus Pb(II) in the presence of 0, 0.5, 1.0, 1.5, and 2.0×10^{-4} M zinc (bottom to top).

RESULTS

Effect of Metal Ions. The effect of various metal ions on the activity of [(CPD)Zn] toward Dns-Gly-Ala-Phe was examined in 0.5 M NaCl/0.05 M Hepes, pH 7.5 at 25 °C, where Zn(II) is known to exhibit a K_I value of 2.4×10^{-5} M (Larsen & Auld, 1989a). The K_I value for Pb(II) is of similar magnitude, 4.8×10^{-5} M, whereas Cd(II) is a weak inhibitor with a K_I value of 1.1×10^{-2} M (Table I). The k_{cat}/K_m value, 7.3×10^6 M^{-1} s^{-1}, is affected less than 10% at 1×10^{-4} M Mn(II) or Cu(II) and at 1×10^{-2} M Hg(II), Ni(II), Pt(IV), or Co(II). Estimates of the K_I values for these metals are given in Table I.

Mode of Metal Ion Inhibition. Pb(II) is a competitive inhibitor of [(CPD)Zn] catalysis, since the k_{cat} values remain constant (Figure 1A) and the K_m values increase in proportion to metal ion concentration (Figure 1B). Identical results were obtained with or without preincubation of [(CPD)Zn] with Pb(II) for 10–30 min.

The apparent K_m values for Pb(II) inhibition in the presence of different concentrations of Zn(II) fall on parallel lines

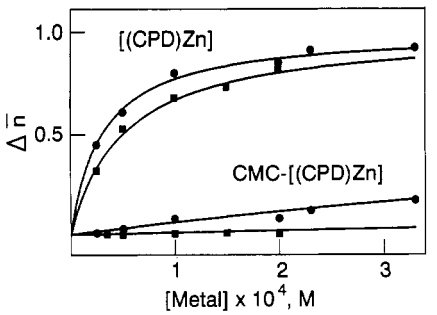

FIGURE 2: Change in metal coordination number ($\Delta\bar{n}$) for native and CMC-modified [(CPD)Zn] as a function of the dialysate concentration of Zn(II) (●) and Pb(II) (■). The data were obtained by equilibrium dialysis in 0.5 M NaCl/0.05 M Hepes, pH 7.5 at 25 °C.

(Figure 1B). This type of kinetic behavior is indicative of mutually exclusive inhibitors (Wong, 1975).

The results of assays performed with the depsipeptide substrate Dns-Gly-Ala-OPhe indicate that the mode and potency of Zn(II) and Pb(II) inhibition are the same as those determined with the peptide substrate Dns-Gly-Ala-Phe.

Equilibrium Dialysis. The apparent dissociation constant (Figure 2) for binding a second zinc ion to native [(CPD)Zn] is 3×10^{-5} M, and that for binding a lead ion, 5×10^{-5} M, in reasonable agreement with the kinetic results (Table I). Dialysis of [(CPD)Zn] against Pb(II) does not affect the Zn(II) content of the protein. This result can be attributed to strong Zn(II) binding to the catalytic site (3×10^{-11} M) (Coleman & Vallee, 1961); the zinc content of the enzyme is expected to be unaffected by dialysis against zinc-free solvent under the present conditions.

The binding of inhibitory zinc and lead to the CMC-modified zinc enzyme (Figure 2) is greatly diminished. The dissociation constant for Zn(II) is estimated to be 1.5×10^{-3} M and that for Pb(II) is $>1 \times 10^{-2}$ M. This residual weak affinity of modified enzyme for Zn(II) and Pb(II) may result from the contribution of additional constituent ligands within the inhibitory metal binding site (see below) or from nonspecific binding.

In the dialysis experiments, exposure to excess zinc or lead produces a small but perceptible irreversible precipitation of protein, even though the experiments are performed at protein concentrations >5-fold below the solubility limit of [(CPD)Zn]. The extent of precipitation increases with time and with increasing amounts of excess metal ion. This phenomenon does not critically affect the results, since the precipitate amounts to <2% of the remaining soluble protein based on A_{278} measurements. In the case of lead, absorption of CO_2 from the air no doubt produces some water-insoluble $PbCO_3$ (Kragten, 1978).

Interaction of Zn(II) and Pb(II) with [(CPD)Co]. Electronic absorption spectra (435–700 nm) of 8.1×10^{-5} M [(CPD)Co] were recorded in the absence and presence of 1.0×10^{-4} M Zn(II) by rapid-scanning stopped-flow spectrophotometry. A marked and rapid absorbance increase takes place within the 0.02-s response time of the instrument (Figure 3). The [(CPD)Co]·Zn species is characterized by a maximum at 560 nm ($\Delta\epsilon_{560} = +60$ M^{-1} cm^{-1}) and shoulders at 525 and 585 nm. The [(CPD)Co]·Zn species is stable for at least 0.85 s (Figure 3, inset). A displacement reaction occurs after the initial spectral perturbation (Figure 4, top to bottom) since the zinc stability constant for the catalytic site is more than 300 times higher than that of cobalt (Coleman & Vallee, 1961). The displacement takes >900 s (Figure 4, inset). The observed exchange rate likely reflects the off-rate of Co(II)

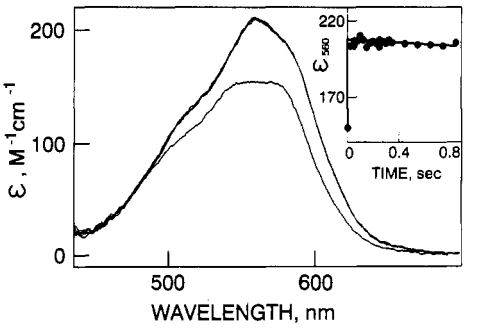

FIGURE 3: Electronic absorption spectra of [(CPD)Co], 8×10^{-5} M, recorded in the absence (bottom) and presence of 1×10^{-4} M Zn(II) at 0.02, 0.05, 0.25, 0.45, and 0.85 s after mixing (top). The dimetallic complex, [(CPD)Co]·Zn, is characterized by a λ_{max} at 560 nm. The inset (ϵ_{560} obtained from 20 spectra versus time) shows that the complex persists for at least 0.85 s. The spectra were recorded in 1 M NaCl/0.05 M Hepes, pH 7.5, and the sample solvent was used as the absorbance reference.

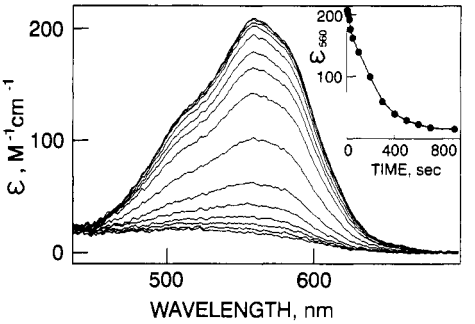

FIGURE 4: Electronic absorption spectra of the conversion of the [(CPD)Co]·Zn complex into [(CPD)Zn] and free cobalt ions recorded over the time period 1–900 s after mixing (top to bottom). The inset shows the time course of this interconversion monitored at ϵ_{560}. The experimental conditions are as given in the legend to Figure 3.

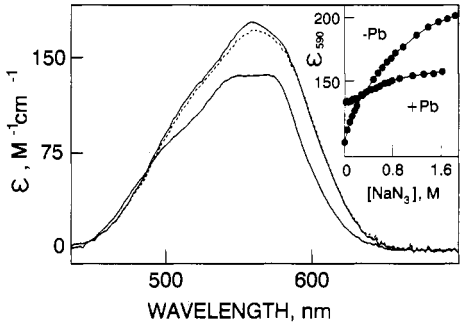

FIGURE 5: Electronic absorption spectra of 5×10^{-4} M [(CPD)Co] in the absence (bottom) and presence of 7×10^{-4} M Pb(II) (top). Addition of 0.1 M NaN_3 has little effect (dashed line) on the spectrum of the dimetallic [(CPD)Co]·Pb complex. The inset shows the effect of NaN_3 on ϵ_{590} for [(CPD)Co] in the absence and presence of Pb(II). The spectra were recorded in 1 M NaCl/0.05 M Hepes, pH 7.5, and equimolar concentrations of [(CPD)Zn] in sample solvent were used as the absorbance reference.

dissociating from [(CPD)Co]. Since the concentration of [(CPD)Co] is linked to the concentrations of [(CPD)Co]·Zn, [(CPD)Zn], and [(CPD)Zn]·Zn, it is not surprising that the time dependence of ϵ_{560} does not conform to a single-exponential decay process.

Addition of Pb(II) to [(CPD)Co] perturbs the cobalt spectrum (Figure 5) to essentially the same degree as does Zn(II) (Figure 3). The Pb(II) concentration used is several times beyond the solubility limit of Pb(II) at pH 7.5 (the concentrated $PbCl_2$ stock solution was pH ~ 5). As a result the reaction mixture is initially turbid due to local precipitation of Pb(II) but becomes clear after sufficient mixing, since

[(CPD)Co] acts as a solubilizing chelator for Pb(II). In contrast to Zn(II), Pb(II) does not appear to displace the catalytic Co(II) ion, and the mixed dimetallic [(CPD)Co]·Pb species is stable for several days.

The nearly identical spectral properties of [(CPD)Co]·Zn and [(CPD)Co]·Pb suggest that Zn(II) and Pb(II) interact with the catalytic Co(II) in a similar fashion. The spectrum of [(CPD)Co]·Pb is essentially insensitive to 0.1 M NaN_3 (Figure 5) in marked contrast to the ternary complexes observed with L-phenylalanine and L-β-phenyllactate, which are characterized by an intense absorption band centered at 590 nm (Bicknell et al., 1988). Spectral titrations of [(CPD)Co] and [(CPD)Co]·Pb with azide (Figure 5, inset) show that the Pb(II) ion competes with azide for binding to the catalytic Co(II) ion. The dissociation constant, K_D, for NaN_3 is 0.83 M toward [(CPD)Co] and >10 M toward the [(CPD)Co]·Pb species at the Pb(II) concentrations used. It was not possible to derive a precise binding constant for NaN_3 toward the [(CPD)Co]·Pb species because the interaction is extremely weak. The quantitative analysis of this spectral titration is further hampered by the concomitant formation of lead azide complexes.

Both the [(CPD)Co] and the [(CPD)Co]·Pb species are unstable in NaN_3. A few minutes after addition of ≥0.5 M NaN_3, the spectra (300–900 nm) show a slow formation of additional Co(II) azide complexes and later Co(III) azide complexes. This observation suggests that NaN_3 is also capable of extracting Co(II) from the catalytic site. The absorbance measurements (Figure 5) are not influenced by these subsequent reactions.

Discussion

Carboxypeptidase A, like several other zinc proteases, binds zinc tightly for catalytic activity but is inhibited in the concentration range 10^{-6}–10^{-4} M. The properties of several catalytic zinc binding sites have been elucidated in detail (Argos et al., 1978; Christianson & Alexander, 1989; Vallee & Auld, 1989, 1990), but little is known about the properties of inhibitory zinc binding sites despite their common occurrence. The pH dependence of zinc inhibition of [(CPD)Zn] has been interpreted as ionization of a single protein ligand (LH) being required for binding a zinc monohydroxide complex, $ZnOH^+$, to the EH ionization state of the enzyme (Larsen & Auld, 1989a). The fact that identical ionization constants (pK_a's of 5.8) are found for pK_{LH} and pK_{EH_2} provides an important clue in the search for the inhibitory metal binding site, since it has already been suggested that the entity responsible for pK_{EH_2} is either Glu-270 or the juxtaposed H_2O which resides on the catalytic metal ion (Auld et al., 1986). The anionic Glu-270 carboxylate is the most logical binding site for a cationic inhibitory metal ion. The survey of several metal ions (Table I) reveals that only Pb(II) inhibits [(CPD)-Zn] with a potency comparable to that of Zn(II). Zn(II) and Pb(II) compete with each other and the substrate for a site on the enzyme (Figure 1).

Chemical modification of Glu-270 with CMC abolishes the enzymatic activity (Nau & Riordan, 1975). The strength of Zn(II) and Pb(II) binding to [(CPD)Zn] and CMC-modified [(CPD)Zn] was determined by equilibrium dialysis rather than by inhibition of enzymatic activity. The equilibrium dialysis data for [(CPD)Zn] confirm the kinetic observations in demonstrating the ability of the enzyme to bind two metal ions, either two zinc ions (one inhibitory, one catalytic) or one lead ion and one zinc ion (Figure 3). The greatly diminished binding of Zn(II) and Pb(II) to the CMC-modified enzyme is consistent with the proposed binding of inhibitory metal ions to Glu-270. Since anion binding to this derivative is enhanced (Geoghegan et al., 1983), the greatly weakened metal ion binding is not solely due to steric constraints imposed on the enzyme by the bulky nature of the chemical agent.

A direct interaction between the inhibitory and catalytic metal ions does not seem likely because of a strong charge repulsion. However, the metal ions could interact through a hydroxide moiety to form a dimetallic monohydroxide, in analogy with the propensity of many metal ions to polymerize through such hydroxide bridges at high concentrations (Baes & Mesmer, 1976).

The marked effect of both zinc and lead on the electronic absorption spectrum of [(CPD)Co] is apparent as an increase in the overall absorbance intensity and formation of a new maximum at 560 nm (Figures 3–5). The spectra of the [(CPD)-Co]·Zn and [(CPD)Co]·Pb species are remarkably similar and are consistent with a hydroxide-mediated interaction. A slightly higher intensity and a band shift to 592 nm has been observed for the ternary complex [(CPD)Co]·D-Phe·N_3^- in which the α-amino group of D-Phe is thought to form an ion pair with the Glu-270 carboxylate and the azide anion coordinates directly with the catalytic cobalt ion, as evidenced by a charge-transfer band (Bicknell et al., 1988). Recent X-ray crystallographic studies of the D-Phe complex of the zinc enzyme are said to be consistent with such a mechanism for azide binding to the metal in the presence of amino acids (Christianson et al., 1989). By analogy, it could be postulated that binding of a metal ion (Zn^{2+} or Pb^{2+}) to the Glu-270 carboxylate would promote the binding of chloride or other anions to the catalytic metal ion. However, spectral titrations of [(CPD)Co)]·Pb with NaN_3 yielded no evidence for a ternary complex. Quite to the contrary, it appears that lead competes with azide for binding to the catalytic metal ion.

If the inhibitory and catalytic metal ions are cross-linked by a hydroxide anion, then charge neutrality is likely maintained by either a hydrated chloride anion, Cl^-(aq), or a chloride anion that has penetrated the coordination sphere of the inhibitory metal ion. The latter species, Pb(OH)Cl, has a net charge of 0, the same as the neutral water molecule (Wat-571) that it displaces in the free enzyme (Figure 6A,B). This cross-linking would effectively prevent the azide anion from binding to the catalytic metal.

The slow interconversion of [(CPD)Co]·Zn into [(CPD)Zn] plus free cobalt provides further support for this mechanism (Figure 4). Although zinc binding to the catalytic site is much stronger than cobalt binding, the exchange of zinc for cobalt at this site is very slow at high concentrations of the cobalt enzyme. If the hydroxide moiety of $ZnOH^+$ binds to the catalytic cobalt ion, then the exchange reaction would be expected to be slow because the bound $ZnOH^+$ prevents the catalytic cobalt ion from being released. It is likely that the metal-displacement reaction proceeds through an apoenzyme intermediate. If the apoenzyme is an obligatory intermediate, the rate of the metal-exchange reaction should decrease with increasing zinc concentration. Thus, increasing the concentration of zinc would result in a decreasing amount of free [(CPD)Co] and thereby a decreasing amount of apoenzyme to which zinc can bind.

The initial spectrum of [(CPD)Co] in the presence of Pb(II) does not change with time (several days) and suggests that Pb(II) binding toward the catalytic site is much weaker compared with Co(II) (Figure 5). The dissociation constant, K_D, for [(CPD)Co] is approximately 10^{-6} M for Co(II) with respect to enzyme, and the apparent lack of Pb(II) exchange

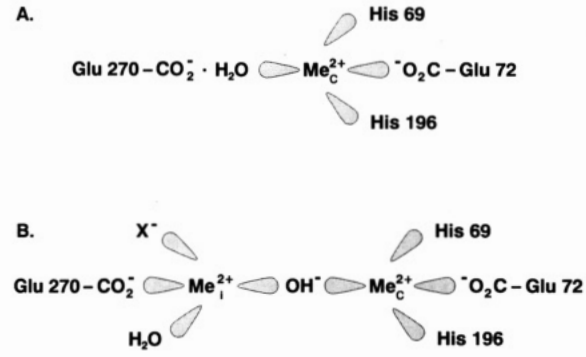

FIGURE 6: (A) Schematic diagram of the immediate surrounding of the catalytic metal ion (Me_c^{2+}) in carboxypeptidase A. The carboxylates of Glu-270 and Glu-72 provide local charge neutralization of the catalytic metal cation in the EH ionization state. The EH form of the enzyme productively binds substrates and is susceptible to metal ion inhibition. (B) Schematic diagram of metal ion inhibited enzyme. The inhibitory metal cation (Me_i^{2+}) is coordinated to Glu-270 and shares a hydroxide anion with the Me_c^{2+}. The excess positive charge centered on the Me_i^{2+} is neutralized by an anion (X^-) from the supporting electrolyte.

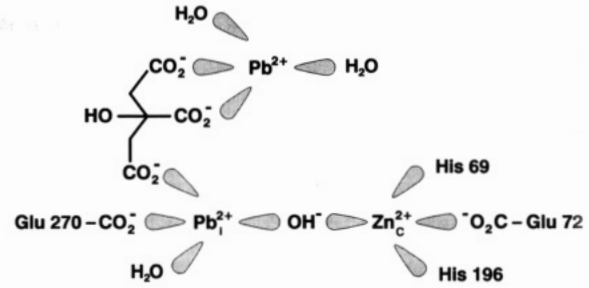

FIGURE 7: Schematic diagram of [(CPD)Zn]·Pb_2·citrate complex. The inhibitory Pb^{2+}·OH^- species cross-links Glu-270 and the catalytic zinc. Local charge neutrality is maintained by a terminal citrate carboxylate coordinated to the inhibitory Pb^{2+} cation. The citrate coordination in turn anchors a second lead ion to the enzyme through a nonprotein lead–citrate interaction.

predicts of K_D value of $>10^{-4}$ M for [(CPD)Pb]. Thus, while Zn(II) and Pb(II) have similar potency toward the inhibitory site in [(CPD)Zn], their stability constants for the catalytic site differ by a factor of more than 10^7.

The binding site for inhibitory Zn(II) has not yet been examined by X-ray crystallography, but two lead atoms have been observed in the low-resolution electron density map of crystalline [(CPD)Zn] (Lipscomb et al., 1966). The two lead atoms form a triangle with the catalytic zinc in which the two lead atoms are located 4 and 6 Å from the catalytic zinc. This lead binding pattern was only observed when the crystals were soaked in 0.01 M citrate. In the absence of citrate, exposure to solutions containing 1 or 3×10^{-3} M Pb(II) failed to produce intensity changes in the electron density map. Later data recorded to a resolution of 2.0 Å revealed that one lead atom binds to or near the active-site residue Glu-270 and that the second site was lead citrate (Lipscomb et al., 1968). These early observations can now be rationalized based upon the present data and principles of charge neutrality.

In the EH form of the enzyme, local charge neutrality around the catalytic metal cation is maintained by the carboxylates of Glu-270 and Glu-72 (Figure 6A). Upon binding of an inhibitory metal cation to Glu-270, charge neutralization is provided by a hydroxide anion and an anion (e.g., Cl^-) from the supporting electrolyte (FIgure 6B). In the specific case of [(CPD)Zn]·Pb_2·citrate (Figure 7), the Glu-270 carboxylate and the catalytic zinc are cross-linked by Pb^{2+}·OH^- and the excess positive charge on the lead is neutralized by a citrate carboxylate. The remaining citrate carboxylates bind a second lead cation. This network explains why electron densities were observed 4 and 6 Å from the catalytic zinc. This arrangement postulates a sequential binding of the two lead atoms, which further explains why a higher electron density was observed at the Glu-270 site compared with the nonprotein citrate site, as some of the Glu-270-occupied Pb^{2+} will be charge neutralized by Cl^- or citrate instead of lead citrate. It may be possible to prepare a crystalline [(CPD)Zn]·Pb complex at low ionic strength by substituting acetate for citrate. The inhibitory zinc site is likely to be the same as or in close proximity to the lead site or sites, since zinc and lead act as mutually exclusive inhibitors in kinetic assays (Figure 1B).

It has been proposed that Glu-218/His-303 (21 Å from the active site) may be responsible for binding excess, inhibitory zinc (Christianson & Alexander, 1989). Zinc binding distant from the active site would be consistent with a conformation-induced inhibition mechanism (Hirose et al., 1985, 1987). Crystallographic studies showed that both Co(II) and Pt(IV) bind to His-303 (Lipscomb et al., 1968). The fact that we have been unable to detect inhibition by 1×10^{-2} M Co(II) or Pt(IV) (Table I) does not favor Glu-218/His-303 as a potential inhibitory metal binding site.

Other noncatalytic metal binding sites determined by crystallography are His-29 and Lys-84 for Hg(II) and Cys-161, Met-103, and the α-amino of Ala-1 for Pt(IV) (Lipscomb et al., 1968). The lack of inhibition by these metal ions (Table I) excludes these residues as sites for potent metal ion inhibition under rapid equilibrium conditions.

The ionic radius of the metal ions examined does not correlate with the observed metal ion specificity of the inhibitory site (Table I). While the inhibitory potencies of Zn(II) and Pb(II) are of similar magnitude, their ionic radii [0.74 Å for Zn(II) and 1.32 Å for Pb(II)] are quite different. Thus, the size of the metal cation is not an important factor. A much better correlation is obtained by comparing the observed specificity with the relative amount of chloride-free metal monohydroxide complexes under standard assay conditions (Table I). The metal ions with the highest [$MeOH^+$] values, Zn(II), Pb(II), and Cd(II), are inhibitory, whereas those with low values, Mn(II), Co(II), Ni(II), and Hg(II), are not. Since K_I determination for Cu(II) is restricted by a low solubility limit [$\sim 2 \times 10^{-4}$ M Cu(II)], it cannot be excluded that the K_I value for Cu(II) is between those for Pb(II) (4.8×10^{-5} M) and Cd(II) (1.1×10^{-2} M). Alternatively, lack of Cu(II) inhibition could be explained by the very large hydration energy of the Cu(II) cation in comparison with the values for Pb(II) and Cd(II) (Table I).

The quantitative differences likely reflect the energetic contributions of the carboxylate and water ligand components of the inhibitory metallocomplex interacting with the enzyme. If it is assumed that only $ZnOH^+$ and $PbOH^+$ are inhibitory, then limiting inhibition constants of 7.1×10^{-7} and 2.0×10^{-5} M, respectively, are obtained in 0.5 M NaCl (Larsen & Auld, 1989a,b). If the inhibition constants are adjusted for chloride complexation, then very similar K_I values are obtained: 4.4×10^{-7} M for Zn(OH)Cl versus 4.7×10^{-7} M for Pb(OH)Cl.

Lead has no known biological benefit, and its toxic effects are well-known. The use of lead in gasoline, paint, plumbing, and food cans has become a major health issue in the past decade. It is thought that lead brings about physiological imbalance by occupying sites on proteins that are normally reserved for calcium (Taylor, 1990). In the present study, a novel type of lead binding site is proposed that has the potential of inactivating any metalloproteinase that operates with a general base/hydrated catalytic zinc mechanism.

If multidentate ligands should interact with both a metal complex and the enzyme active site, significantly lower inhibition constants can be anticipated. Inhibition by such metal monohydroxide complexes could be very important to regulatory and/or toxicological processes of zinc proteases and possibly zinc proteins in general.

Registry No. [(CPD)Zn], 11075-17-5; Gh, 56-86-0; Mn(II), 7439-96-5; Co(II), 7440-48-4; Ni(II), 7440-02-0; Cu(II), 7440-50-8; Zn(II), 7440-66-6; Cd(II), 7440-43-9; Pt(II), 7440-06-4; Hg(II), 7439-97-6; Pb(II), 7439-92-1.

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