Carboxypeptidase A: Mechanism of Zinc Inhibition†

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ABSTRACT: Zinc ions competitively inhibit carboxypeptidase A from bovine pancreas. The state(s) of hydroxylation of zinc and their possible site(s) of interaction with the enzyme have been investigated by determining the strength of zinc inhibition over the pH range 4.6–10.5. The inhibition kinetics were recorded under stopped-flow conditions using the α-Val isozyme and the peptide substrate Dns-Gly-Ala-Phe in 0.5 M NaCl at 25 °C. The pH dependence of pK_I follows a pattern which indicates that the enzyme is selectively inhibited by zinc monohydroxide, $ZnOH^+$ ($K_I = 7.1 \times 10^{-7}$ M). The formation of the inhibitory $ZnOH^+$ complex from fully hydrated Zn^{2+} is characterized by an ionization constant of 9.05, and the consecutive conversion of $ZnOH^+$ to $Zn(OH)_2$, $Zn(OH)_3^-$, and $Zn(OH)_4^{2-}$ complexes takes place with ionization constants of 9.75, 10.1, and 10.5, respectively. Ionization of a ligand, LH, in the enzyme's inhibitory site (pK_{LH} 5.8) is obligatory for binding of the $ZnOH^+$ complex. The enzymatic activity (k_{cat}/K_m) is influenced by three ionizable groups: pK_{EH_2} 5.78, pK_{EH} 8.60, and pK_E 10.2. Since the values of pK_{LH} and pK_{EH_2} are virtually identical, it is possible that the inhibitory $ZnOH^+$ complex interacts with the group responsible for pK_{EH_2}. Previous studies have suggested that pK_{EH_2} reflects the ionization of Glu-270 and its interaction with a water molecule coordinated to the catalytic zinc ion. It is proposed that the inhibitory zinc ion binds to the carboxylate of Glu-270 and that the inhibition process is specific for zinc monohydroxide because it allows the formation of a stabilizing hydroxide bridge between the inhibitory and catalytic zinc ions.

Several studies have indicated that the zinc metalloenzyme carboxypeptidase A ([(CPD)Zn])[1] from bovine pancreas is inhibited by zinc ions, but the mechanism of inhibition has not been determined. Thus, within a few years of finding that carboxypeptidase A is a zinc protein (Vallee & Neurath, 1954), it was reported that a 1 mM concentration of zinc inhibits its enzymatic activity (Vallee et al., 1960). Equilibrium dialysis experiments indicate that [(CPD)Zn] loosely binds a second equivalent of zinc in the pH range from 7 to 10 (Coleman & Vallee, 1960), and kinetic studies show that the zinc inhibition is strongest in the alkaline pH region (Auld & Vallee, 1970). The degree of zinc inhibition has also been correlated to one of three conformational states of an arsanilazotyrosine derivative of carboxypeptidase A, [(Azo-CPD)Zn], and it has been proposed that the inhibition of [(Azo-CPD)Zn] by zinc ions is due to stabilization of a conformational state of the enzyme to which substrate cannot bind (Hirose et al., 1985, 1987).

The determination of the mechanism by which an inhibitor acts requires knowledge of both its ionization state and its possible sites of interaction within the active center of the enzyme. In this regard, water ligands associated with fully hydrated Zn^{2+} ionize to form zinc hydroxides [e.g., $ZnOH^+$, $Zn(OH)_2$, $Zn(OH)_3^-$, and $Zn(OH)_4^{2-}$]. The examination of the effect of pH on the inhibitory behavior of zinc would, therefore, be necessary in deciphering its mechanism of inhibition. The pH dependence of the zinc inhibition was determined in this study in order to gain information about the ionization state of the inhibitory zinc complex and the ionizable protein ligands in the inhibitory binding site. In a preliminary account, we have reported that zinc ions inhibit the activity of [(CPD)Zn] toward peptide and ester substrates in a competitive mode (Larsen et al., 1986), and identical observations have been described recently for both [(CPD)Zn] and [(Azo-CPD)Zn] (Hirose et al., 1987).

Materials and Methods

Purification of α-Val Isozyme. [(CPD)Zn] prepared from bovine pancreas according to the method of Cox et al. (1964) was obtained from Sigma Chemical Co., St. Louis, MO (catalog no. C-0261, lot 24F-8081). β-Phenylpropionic acid (βPP) was purchased from Lancaster Synthesis Ltd. (Windham, NH) and crystallized twice from petroleum ether. The α-Val isozyme used in this study was isolated by a modification of the procedure developed by Pêtra and Neurath (1969). Crystalline enzyme (700 mg) was dissolved in 5 mL of 2 M Tris (HCl), pH 7.5, and diluted to 200 mL with 50 mM βPP/1 mM Tris, pH 7.5. The solution was applied to a column (2.6 × 30 cm) of DEAE-Sephacel (Pharmacia LKB Biotechnology, Piscataway, NJ) equilibrated with 50 mM βPP/50 mM Tris, pH 7.5, and the isozymes were fractionated by a linear gradient (0–0.175 M) of LiCl in 2 × 1 L of the equilibration solvent. The solvents used for the ion-exchange chromatography were pH adjusted with LiOH. The ion-exchange column irreversibly binds yellow-brown contaminants present in the starting material and yields close to quantitative recovery of the enzymatic activity. The inhibitory βPP used in the chromatography solvents was partly removed from the re-

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[1] Abbreviations: [(CPD)Zn], carboxypeptidase A; [(Azo-CPD)Zn], arsanilazotyrosine-248 carboxypeptidase A; βPP, β-phenylpropionic acid; Homopipes, 1,4-diazacycloheptane-*N*,*N*′-bis(2-ethanesulfonic acid); Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; Ampso, 3-[[dimethyl(hydroxymethyl)methyl]amino]-2-hydroxypropanesulfonic acid; Caps, 3-(cyclohexylamino)propanesulfonic acid; Dns- or dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl.

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chromatographed protein by dialysis against 2 M NaCl at pH 7.5. The remaining βPP and trace amounts of proteins other than the α-Val isozyme were finally eliminated by affinity chromatography (Bazzone et al., 1979). The enzyme preparation was done at 4 °C.

Substrate Preparation. The substrate Dns-Gly-Ala-Phe was synthesized by dansylation of glycine followed by stepwise coupling of L-Ala and L-Phe (Sigma) with dicyclohexylcarbodiimide (Fluka)/*N*-hydroxysuccinimide (Sigma). The final reaction mixture was fractionated by reversed-phase chromatography on a column (19 × 150 mm) of Microbondapak C18 (Waters Associates, Milford, MA) eluted with 22% CH_3CN (J. T. Baker) and 0.1% trifluoroacetic acid at 4.8 mL min^{-1}. Each run yielded approximately 10 mg of pure Dns-Gly-Ala-Phe.

Enzyme Kinetics. The kinetics of [(CPD)Zn]-catalyzed hydrolysis of Dns-Gly-Ala-Phe were recorded on a Durrum stopped-flow spectrophotometer, Model D110, equipped as described previously (Hanahan & Auld, 1980).

The substrate dansyl chromophore was excited either at 337 nm or with the intense mercury lines at 365 and 405 nm. The fluorescence envelope was monitored through a 430 nm cut-on optical filter. In this recording configuration, the intensity of the dansyl fluorescence (F) of the substrate (Dns-Gly-Ala-Phe) is 10–25% higher relative to the hydrolysis product (Dns-Gly-Ala + Phe) depending on the excitation wavelength, the bandwidth, and the pH of the reaction mixture. This signal change is sufficiently large to follow the substrate hydrolysis continuously. Since [ES] is <0.1% of [S], under the conditions employed here, there is essentially no interference from differences in the fluorescent properties of free and enzyme-bound substrate (Lobb & Auld, 1984). All reactions were recorded to completion in order to establish the relationship between the change in fluorescence intensity and the initial substrate concentration.

The kinetics were performed in 0.5 M NaCl in order to maintain constant ionic strength and to solubilize the enzyme. Further, chloride inhibits the activity of [(CPD)Zn] but is relatively insensitive to small concentration changes around 0.5 M (Williams & Auld, 1986). On the basis of the chloride stability constants for zinc (Ahrland, 1979) and mass action considerations, it is expected that inclusion of up to 0.2 M $ZnCl_2$ in the reaction medium (0.5 M NaCl) will have only a minor effect on the concentration of free chloride and on the ionic strength.

The reaction mixtures were buffered by zwitterionic compounds. The buffers used are all alkyl sulfonic acids, and the buffering group is either a secondary or a tertiary amine. The degree to which these compounds form complexes with zinc is unknown but is expected to be insignificant at the concentrations used (Good et al., 1966). Nevertheless, we have examined the strength of zinc inhibition at various buffer concentrations (10 or 50 mM) and used neighboring buffers in overlapping pH ranges: Homopipes (pH 4.6–5.5), Mes (pH 5–7), Mops (pH 6.5–7.5), Hepes (pH 7–8), Taps (pH 7.5–9), Ampso (pH 8.5–10), or Caps (pH 9–10.5). The buffers were purchased from Research Organics Inc. (Cleveland, OH). Homopipes, Mes, and Mops were crystallized from water/ethanol, and Caps was crystallized from water.

Metal-free stock solutions of Hepes, Taps, Ampso, Dns-Gly-Ala-Phe, and NaCl were prepared by extraction with dithizone in either carbon tetrachloride or chloroform at neutral pH (Holmquist, 1988). The pH of the solvents used in the kinetic studies was adjusted with concentrated NaOH. The pH measurements were done with a glass–calomel combination electrode (Radiometer, GK2321 type C) and a Markson pH meter, Model 90, calibrated with buffer standards from Fisher Scientific (Medford, MA). The alkaline pH readings were corrected for the electrode's sodium ion error according to the manufacturer's bulletin. Kinetics and pH measurements were performed at 25.0 ± 0.1 °C. Zinc chloride was purchased from Johnson Matthey Inc. (Seabrook, NH), and the concentrations of stock solutions were determined by flame atomic absorption spectrometry on a Perkin-Elmer atomic absorption spectrometer, Model 2280. Purified water was used throughout this study (Milli-Q/Milli-RO water purification system, Millipore, Bedford, MA).

Data Analysis. Initial rates either were calculated from the initial linear slope of the change in fluorescence intensity versus time or were obtained by fitting the entire progress curve to an integrated form of the Michaelis–Menten rate equation (Pollack & Auld, 1982). Steady-state parameters were determined from Lineweaver–Burk plots by least-squares analysis. The plots consisted of at least four different substrate concentrations for which the initial rate was the average of three to five determinations. The mode of inhibition was determined from the effect of zinc on Lineweaver–Burk plots. Linear regression analysis of the relationship between the apparent K_m/k_{cat} values and the corresponding zinc concentrations (3–10) served to obtain both the K_I values and the K_m/k_{cat} values in the absence of zinc. The apparent K_m/k_{cat} values were determined from Lineweaver–Burk plots from pH 6.0 to 9.3 and from first-order rate constants ($k_{cat}/K_m[E]_T$) outside this pH range. First-order rate constants were obtained by linear regression of log ($F_t - F_\infty$) versus time under conditions where $[S] \ll K_m$.

The pH dependence[2] of log (k_{cat}/K_m) and pK_I could be fitted to eq 1 and 2, respectively (see Results). The choice of the logarithmic function form (eq 1 and 2) is dictated by the fact that both the catalytic activity and the strength of inhibition are governed by thermodynamic functions in which the observed activities and inhibition constants appear in logarithmic format. It was assumed that all the experimental error can be attributed to the ordinate and that there are no systematic errors (pH meter calibration, concentration determinations, and temperature control). Theoretical pH profiles were calculated by allowing trial parameters to vary over predetermined ranges. The "best fit" is defined here as the set of parameters which give the smallest overall absolute deviation between calculated and observed values (Auld & Vallee, 1970). The error ranges on each parameter were estimated as follows: Theoretical pH profiles were calculated by allowing the parameter being examined to vary while fixing the other previously determined "best fit" parameters. The parameter was restricted to assume values yielding theoretical curves which fall within the boundary of three standard deviations of the mean values of the data points. This analysis yields asymmetrical error ranges, and the estimates of error reported here (Table I) represent the largest single-sided deviation.

Results

The chromatography system used in this study yields the same fractionation pattern of the [(CPD)Zn] isozymes as that previously described by Pĕtra and Neurath (1969): peak I (α-Val), peak II (α-Leu and β-Val), peak III (β-Leu), peak IV (γ-Val), and peak V (γ-Leu) (Figure 1). Peak I is not

[2] The pH values used in the calculation of pK values were the sodium ion corrected pH meter readings in 0.5 M NaCl after calibration of the electrode with buffer standards traceable to the pH scale defined by the National Bureau of Standards.

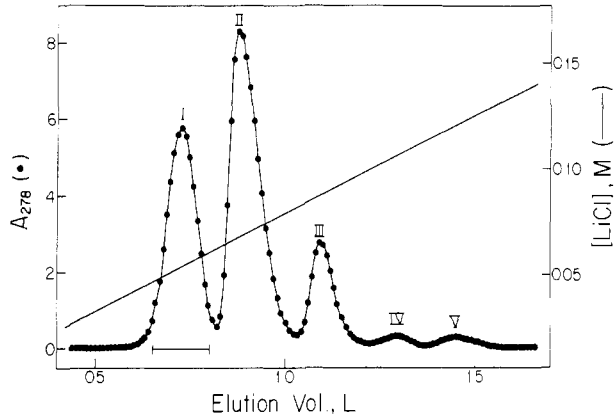

FIGURE 1: Fractionation of [(CPD)Zn] isozymes (700 mg) on a column (2.6 × 30 cm) of DEAE-Sephacel. The column was eluted with a linear gradient (0–0.175 M) of LiCl in the presence of 50 mM βPP and 50 mM Tris at pH 7.5. Peak I was pooled as indicated and rechromatographed to homogeneity in the same system.

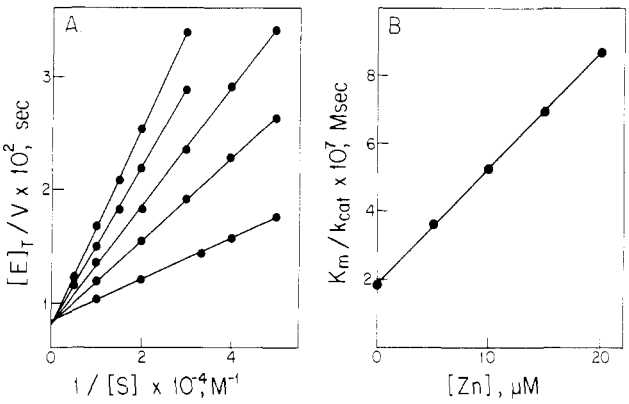

FIGURE 2: (A) Effect of zinc on the [(CPD)Zn]-catalyzed hydrolysis of Dns-Gly-Ala-Phe in 0.5 M NaCl/10 mM Taps, pH 8.2 at 25 °C. The concentrations of zinc added to the assays were 0, 5, 10, 15, and 20 μM (bottom to top). The data points are average values of three to five determinations. Values of k_{cat}^{-1} (ordinate intercept) and K_m/k_{cat} (slope) were calculated for each zinc concentration by linear least-squares analysis. (B) Plot of the calculated K_m/k_{cat} values versus the individual zinc concentrations. A K_I value of 5.3×10^{-6} M was determined from least-squares analysis of the proportional relationship between K_m/k_{cat} and the zinc concentration.

fractionated to base-line level and was therefore rechromatographed to homogeneity in the same system (see Materials and Methods). The amino acid composition and amino- and carboxy-terminal sequences of the enzyme purified from peak I are in agreement with the characteristics of the α-Val isozyme (Pětra & Neurath, 1969). On the basis of the enzymatic activity, the α-Val isozyme constituted approximately 30% of the starting material. We have found that some lots of commercial Cox preparation contain significantly less of the α-isozymes and more of the β-isozymes.

Mode of Zinc Inhibition. The effect of zinc ions on the hydrolysis of Dns-Gly-Ala-Phe catalyzed by the α-Val isozyme of [(CPD)Zn] was examined in 0.5 M NaCl/10 mM Taps, pH 8.2, 25 °C. The kinetic parameters are obtained from initial rate determinations rather than from the analysis of the progress curve because the product, L-phenylalanine, inhibits (Auld et al., 1972) and is expected to form complexes with zinc ions (Perkins, 1953).

Zinc acts as a competitive inhibitor since the k_{cat} values remain unaffected (reciprocal ordinate intercepts, Figure 2A) and the observed K_m/k_{cat} values increase in proportion to the zinc concentration (Figure 2B). The derived inhibition constant ($K_I = 5.3 \times 10^{-6}$ M) is approximately 10-fold lower than

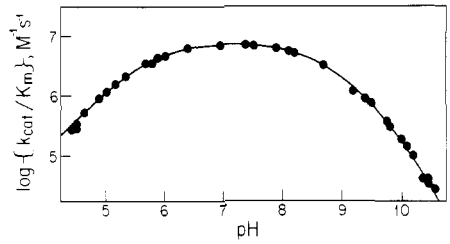

FIGURE 3: pH dependence of the activity of [(CPD)Zn] toward Dns-Gly-Ala-Phe in the presence of 0.5 M NaCl at 25 °C. The limiting activity and the ionization constants were determined from the best fit to eq 1. The theoretical curve was calculated by using eq 1 and the parameters in Table I. The average percent deviation between calculated and observed k_{cat}/K_m values is 9.06%.

Table I: Limiting Parameters and Ionization Constants Influencing the Activity and the Zinc Inhibition of [(CPD)Zn]-Catalyzed Hydrolysis of Dns-Gly-Ala-Phe in 0.5 M NaCl at 25 °C

catalytic activity[a]		zinc inhibition[b]	
parameter	value	parameter	value
$(k_{cat}/K_m)_{lim}$ ($\times 10^6$ M^{-1} s^{-1})	7.59 ± 0.51	$K_{I,lim}$ ($\times 10^{-7}$ M)	7.08 ± 0.57
pK_{EH_2}	5.78 ± 0.04	pK_{LH}	5.80 ± 0.10
pK_{EH}	8.60 ± 0.05	pK_1	9.05 ± 0.04
pK_E	10.20 ± 0.15	pK_2	9.75 ± 0.05
		pK_3	10.10 ± 0.25
		pK_4	10.50 ± 0.40

[a] From the best fit of the data shown in Figure 3 to eq 1. The nomenclature was adapted from Auld and Vallee (1970). [b] From the best fit of the data in Figure 4 to eq 2. The individual parameters are defined as dissociation constants (Figure 5).

that reported by Hirose et al. (1987) at the same NaCl concentration, temperature, and pH. In part, the difference is likely due to the different buffers used. The present reactions are buffered with 10 mM Taps, a secondary amine, while the previous ones (Hirose et al., 1987) used 50 mM Tris, a primary amine. The latter, like many other primary amines, forms zinc complexes and will therefore make the strength of inhibition appear weaker compared to data obtained in the presence of a secondary amine with lower zinc affinity. Quite similar K_I values are obtained when the data of Hirose et al. (1987) are recalculated for complexation of Tris base with zinc ions (Bologni et al., 1983).

pH Dependence of Substrate Hydrolysis. The pH dependence of the activity of [(CPD)Zn] toward Dns-Gly-Ala-Phe is shown in Figure 3. The present data conform to the three protonation state model ($EH_2 \rightleftharpoons EH \rightleftharpoons E$) over the pH interval from 4.5 to 9.5 (Auld & Vallee, 1970). Above pH 9.5, an improved fit was achieved by extending the three protonation state model by one additional ionization ($E \rightleftharpoons E^-$). The ionization constants in the four protonation state model are obtained by fitting the data shown in Figure 3 to eq 1. Table I summarizes the limiting activity and the ionization constants obtained. A previous study performed with a mixture of isozymes cited ionization constants of 6.0 ± 0.1 and 9.0 ± 0.1 for the free enzyme (Auld & Vallee, 1970), in reasonable agreement with 5.78 ± 0.04 and 8.60 ± 0.05 obtained here for the purified α-Val isozyme.

$$\log (k_{cat}/K_m) = \log (k_{cat}/K_m)_{lim} - \log [10^{pK_{EH_2}-pH} + 1 + 10^{pH-pH_{EH}} + 10^{2pH-pK_{EH}-pK_E}] \quad (1)$$

pH Dependence of Zinc Inhibition. Zinc inhibition of [(CPD)Zn]-catalyzed hydrolysis of Dns-Gly-Ala-Phe is strongly pH dependent (Figure 4). The inhibition is influenced minimally by five protons since the slope of pK_I versus pH approaches +2 and −3 at extremes of low and high pH values (Figure 4).

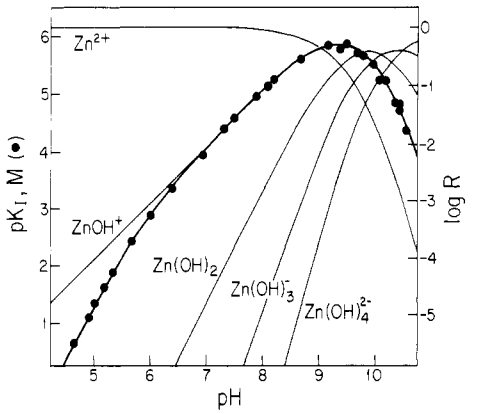

FIGURE 4: pH dependence of the pK_I values for zinc inhibition (●) and the relative distribution (R) of mononuclear zinc complexes (thin lines) in 0.5 M NaCl at 25 °C. The limiting inhibition constant and the ionization constants (Table I) determined from the best fit to eq 2 were used to calculate the theoretical curve (thick line). The average percent deviation between calculated and observed K_I values is 9.49%. The pH dependence of the relative distribution of zinc complexes was calculated on the basis of the stability constants derived from the inhibition profile.

The inhibition pattern of a pK_I–pH profile depends on changes in the state of ionization of both the inhibitor and the protein under investigation (Dixon & Webb, 1964). Over the pH range examined, the inhibitor is distributed among a minimum of five protonation states [Zn^{2+}, $ZnOH^+$, $Zn(OH)_2$, $Zn(OH)_3^-$, and $Zn(OH)_4^{2-}$], and activity–pH profiles (Figure 3) detect four protonation states of the enzyme (EH_2, EH, E, and E^-). Thus, theoretical ionization schemes can be postulated to account for the pH dependence of pK_I by assigning selective interactions between various combinations of the protonation states of the inhibitor and the enzyme.

Over the pH range 6.5–8, the pH does not greatly affect the activity of the enzyme (Figure 3) since the catalytically active form, EH, predominates. It is also known that the logarithmic concentration of the $ZnOH^+$ complex depends linearly on pH in this region, since the hydrated Zn^{2+} complex is converted to the $ZnOH^+$ complex with a pK_a of approximately 9 (Sillén & Martell, 1964). It is, therefore, reasonable to assign the slope of +1 for this pH interval of the pK_I–pH profile to the preferential interaction of $ZnOH^+$ with the EH form of the enzyme (Figure 4). The rapid decrease of pK_I above the pH maximum of 9.3 is consistent with the conversion of the inhibitory $ZnOH^+$ ion to the noninhibitory $Zn(OH)_2$, $Zn(OH)_3^-$, and $Zn(OH)_4^{2-}$ complexes. There is the possibility that part of the rapid decrease in pK_I above pH 9.3 is due to a lower than anticipated concentration of soluble zinc due to the limited solubility product of the neutral $Zn(OH)_2$. However, the solubility limit should be evident in the plots of K_m/k_{cat} versus the zinc concentration. Attempts to go beyond the solubility limit would result in an abrupt change from a proportional increase in K_m/k_{cat} to a situation where K_m/k_{cat} becomes independent of the zinc concentration (due to a phase transition from soluble to insoluble zinc). This behavior was not observed over the zinc concentration ranges used to determine the pK_I values in this study.

In the pH region below 6.5, the strength of the zinc inhibition is suppressed[3] below the level expected solely for the formation of $ZnOH^+$. However, this is the pH region in which the enzyme form EH is converted to EH_2 (Figure 3, Table I). If $ZnOH^+$ binds to the EH form but not the EH_2 form of the enzyme, then the strength of inhibition would be expected to decrease as pH is decreased. For the purpose of fitting the pK_I–pH profile,[2] the value of the ionization constant pK_{LH} for the ionizable ligand LH in the inhibitory site (Figure 5) is not assumed to be identical with pK_{EH_2} (Table I). The following expression for zinc inhibition of carboxypeptidase A catalysis can therefore be formulated based on the scheme in Figure 5:

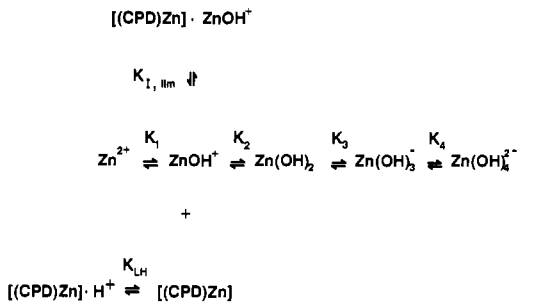

FIGURE 5: Minimal scheme that accounts for the observed pH dependence of zinc inhibition of [(CPD)Zn]. The scheme shows that ionization of a ligand (LH) in the enzyme inhibitory site is obligatory for binding of the zinc monohydroxide complex. The individual zinc complexes are defined only with respect to their number of hydroxide ligands. For instance, zinc monohydroxide, $ZnOH^+$, represents a class of zinc complexes which all contain one hydroxide ligand, but no distinction is made with regard to the composition of additional water and chloride ligands.

$$pK_I = -\log K_{I,lim} - \log (10^{pK_1-pH} + 1 + 10^{pH-pK_2} + 10^{2pH-pK_2-pK_3} + 10^{3pH-pK_2-pK_3-pK_4}) - \log (1 + 10^{pK_{LH}-pH}) \quad (2)$$

The parameters in eq 2 are determined by successive approximation. First values of pK_1, pK_2 and $pK_{I,lim}$ are obtained from the data in the pH range 7–10. The value of pK_{LH} is determined by including the data from pH 4.6 and using the previous values of pK_1, pK_2, and $pK_{I,lim}$. Finally, pK_3 and pK_4 are estimated from the data above pH 10 and the parameters determined previously. Several combinations of pK_3 and pK_4 can satisfy the data. The best fit of the data in Figure 4 to eq 2 yields the parameters listed in Table I. Using these ionization constants, it is possible to derive a zinc distribution diagram at 0.5 M NaCl and 25 °C for this system. The results, indicated by the thin lines, are superimposed on the data in Figure 4. The distribution diagram was offset such that the $ZnOH^+$ curve coincides with the inhibition profile.

Discussion

Hydrated Zn^{2+} ionizes to form $ZnOH^+$, $Zn(OH)_2$, $Zn(OH)_3^-$, and $Zn(OH)_4^{2-}$ over the pH range 4.6–10.5. The very strong and distinctive pH dependence of the zinc inhibition (Figure 4) suggests that the enzyme's inhibitory site discriminates between the various zinc complexes. The stability constants for all the pertinent zinc complexes are not determined as definitely as are the ionization constants for amino acid and carboxylate inhibitors of carboxypeptidase A studied previously (Auld et al., 1972, 1986). The interpretation of the data, therefore, is developed most easily from the shape of the inhibition profile followed by correlating the results of the fit to what is known about the ionization constants for the inhibitor and the enzyme's active-site residues.

The fact that the pK_I increases with a slope of +1 from pH 6.5 to the maximum at pH 9.3 and decreases rapidly above the maximum is consistent with $ZnOH^+$ being the only in-

[3] Some of decreased inhibition might be due to suppression of the relative $ZnOH^+$ concentration, because of the formation of polynuclear hydroxides [e.g., Zn_2OH^{3+}, $Zn_2(OH)_6^{2-}$]. However, on the basis of available stability constants (Schorsch, 1964, 1965) and zinc concentrations of ≤0.1 M, the formation of polynuclear hydroxides will not significantly affect the concentration of mononuclear zinc complexes below pH 7.

Table II: Recorded Mean Values of the Cumulative Acid Dissociation Constant for the Zn(II) Cation in Water at 25 °C[a]

pK_1	pK_1K_2	$pK_1K_2K_3$	$pK_1K_2K_3K_4$	medium	ref
9.05	18.80	28.90	39.40	0.5 M NaCl	*b*
	20.10	28.83	38.72	3 M $NaClO_4$	*c*
		27.77	40.51	0 correction	*d*
7.69	16.81	27.69	38.30	1 M $NaClO_4$	*e*
9.12				2 M NaCl	*f*
9.01				2 M KCl	*g*
9.25				3 M NaCl	*g*
9.26				3 M KCl	*g*
8.96				0 correction	*h*

[a] Zinc hydroxide stability constants were converted to acid dissociation constants using a value of $pK_w = 14$ unless the water ionization product was deteremined in the individual reaction media. [b] This study. [c] Sekine (1965). [d] Fulton & Swinehart (1954). [e] Gubeli & Ste-Marie (1967). [f] Schorsch (1964). [g] Schorsch (1965). [h] Perrin (1962).

hibitory species. In addition, the pK_1 value of 9.05 determined in this study in 0.5 M NaCl is in good agreement with the values determined electrochemically: 8.96 (extrapolation to zero ionic strength), 9.12 in 2 M NaCl, and 9.25 in 3 M NaCl (Table II). The rapid decrease in pK_1 above the pH maximum at 9.3 indicates that the higher hydroxides [$Zn(OH)_2$, $Zn(OH)_3^-$, and $Zn(OH)_4^{2-}$] are formed in rapid succession and do not inhibit (Figure 5). This observation is expressed as very small increments between the values of pK_2, pK_3, and pK_4 obtained from the data fit to eq 2 (Table I). Literature values for these ionization constants vary considerably (Sillén & Martell, 1964). However, several studies are in qualitative agreement with the cumulative values of the acid dissociation constants (Table II). The cooperative nature of hydroxylation of zinc along with a solubility limit below 10 μM for $Zn(OH)_2$ is in part the reason why the stability constants have been difficult to determine by established techniques.

The present study did not aim at the determination of the stability constants of zinc, but it has not escaped our attention that the enzymatic system could serve that purpose. A distribution diagram of the various zinc species is superimposed on the data in Figure 4. The relative concentrations of the individual zinc complexes are calculated on the basis of the ionization constants (Table I) derived from the data fit to eq 2. Above pH 6.5, only the distribution of $ZnOH^+$ follows a pattern similar to that of the inhibition curve with a slope of +1 below the maximum and a slope of −3 above the maximum.

The values of pK_{EH_2} (5.78) and pK_{LH} (5.8) are virtually identical within the accuracy of determination, suggesting that they reflect the same group, and thus constitute a site of interaction common for both the substrate and the inhibitory zinc complex. Thus, ionization of EH_2 ($EH_2 \rightleftharpoons EH$) is essential both for the catalytic activity of the enzyme (Figure 3) and for binding the inhibitory $ZnOH^+$ complex (Figure 4).

The selective interaction of $ZnOH^+$ with the enzyme form EH ($pK_{I,lim} = 7.1 \times 10^{-7}$ M) is sufficient to give an excellent fit to the data (Figure 4). The kinetically equivalent interaction of $Zn(OH)_2$ with EH_2 is also possible, although it involves physically distinct protons. However, in the case where $EH_2 \cdot Zn(OH)_2$ is the only inhibited adduct, one obtains a limiting inhibition constant of 7.1×10^{-11} M, a value 4 orders of magnitude lower than that calculated for the $EH \cdot ZnOH^+$ adduct (Table I). The inhibition constant for the $EH_2 \cdot Zn(OH)_2$ adduct makes it an unrealistic case since most of the data (Figure 4) were obtained with an enzyme concentration of 1×10^{-7} M.

The present fitting procedure does not give E or E^- any role in the inhibitory process. It is clear that a selective interaction of particular zinc complexes with either of these forms of the enzyme will not allow a theoretical fit to the experimental data. Since pK_E is 10.2 (Table I), a role for E^- in the decreased zinc inhibition at pH values above 10 is not excluded, but it is clear that the ionization of either or both EH and E alone cannot account for the rapid decrease of pK_I above pH 9.3 (Figure 4).

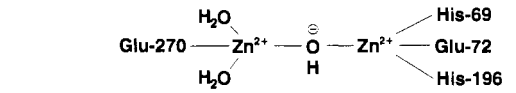

FIGURE 6: Schematic diagram of proposed interactions in the inhibited enzyme. The inhibitory zinc ion coordinates to Glu-270, and the hydroxide moiety is shared between the inhibitory and catalytic zinc ions. The catalytic zinc ion remains bound to His-69, Glu-72, and His-196. It is possible that water molecules associated with the inhibitory $ZnOH^+$ complex contribute to its binding through hydrogen bonding to adjacent amino acid residues.

The group responsible for the pK_{EH_2} observed for peptide hydrolysis has not been identified conclusively. On the basis of kinetic (Auld & Vallee, 1970, 1971) and spectroscopic studies (Stephens et al., 1974; Geoghegan et al., 1983) and crystallographic data (Lipscomb, 1974), it has been proposed that the ionization is due to the ionization of the Glu-270 carboxylate and its subsequent interaction with the water molecule (Wat-571) coordinated to the catalytic zinc ion (Auld et al., 1986). Binding of $ZnOH^+$ to the carboxyl group of Glu-270 alone cannot explain the limiting inhibition constant of 7.1×10^{-7} M observed in this study, since the stability constants for complexation of zinc ions with carboxyl groups are substantially weaker (Archer & Monk, 1964). Thus, additional ligands are needed to account for the observed strength of the zinc inhibition. The close proximity between Glu-270 and the catalytic zinc ion (Lipscomb, 1974) may allow the hydroxide moiety of the inhibitory $ZnOH^+$ complex to coordinate to the catalytic zinc ion in direct analogy to zinc's ability to form polynuclear hydroxides (Schorsch, 1964, 1965) (Figure 6). This interaction could also account for the disruptive nature of excess zinc ions on the intramolecular coordination of the 3-arsanilazotyrosine-248 residue and the catalytic zinc ion (Hirose et al., 1985, 1987).

The enzyme's high specificity for $ZnOH^+$ suggests that only this zinc complex can provide a structure which is complementary to constituents of the enzyme's active site. The potency of inhibition ($K_{I,lim} = 7.1 \times 10^{-7}$ M) may in part be due to similarities between the structure of the inhibitory adduct (Figure 6) and tetrahedral complexes in the enzyme's catalytic pathway. The inability of the higher zinc hydroxide complexes to inhibit the enzyme is possibly due to unfavorable electrostatic repulsions, steric constraints, and a reduction in the strength of the ionic interaction between the carboxylate of Glu-270 and the inhibitory zinc ion. Alternatively, the selectivity for $ZnOH^+$ may be due to stabilizing hydrogen bonding between water ligands associated with the inhibitory zinc ion and the adjacent protein constituents [e.g., the carbonyl oxygen of Ser-197 (Rees et al., 1983) and Arg-71/Arg-127 (Shoham et al., 1988)].

Zinc is known to inhibit other zinc hydrolyses (Auld, 1987), and it is possible that the mode of interaction also involves coordination of the hydroxide moiety of $ZnOH^+$ to their catalytic zinc ions. Minimally, the data presented here demonstrate that minute changes in pH drastically affect the potency of zinc inhibition, and it is thus possible that zinc could have a regulatory role in the activity of zinc hydrolyses.

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Evidence for the Formation of Strand-Break Precursors in Hydroxy-Attacked Thymidine 5′-Monophosphate by the Spin Trapping Method

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ABSTRACT: A method combining spin trapping, ESR, and HPLC was employed to obtain evidence for the formation of sugar radicals in OH-attacked TMP with special emphasis on the detection of strand-break precursors of DNA. OH radicals were produced by irradiating an N_2O-saturated aqueous solution with X-rays. When an N_2O-saturated aqueous solution containing TMP and a spin trapping reagent, MNP, was irradiated with X-rays, it was estimated on the basis of theoretical calculations using rate constants that 94% of the TMP radicals were induced by OH radicals. Since several spin adducts between TMP radicals and MNP, as well as the byproducts of the spin trapping reagent itself, were produced, reverse-phase HPLC was used to separate them. The presence of six spin adducts was confirmed by ESR examination. Further examination of these spin adducts by UV absorbance spectrophotometry showed the presence of a chromophore at 260 nm in three adducts. Since a gradual increase in the release of unaltered base from these adducts was observed when they were allowed to stand for 0–22 h at room temperature, they could be regarded as the spin adducts of sugar radicals and MNP. ESR spectra from the spin adducts were consistent with hydrogen abstraction radicals at the C1′, C4′, and C5′ positions of the sugar moiety. These radicals appeared to be precursors of AP sites and strand breaks. In addition to these spin adducts, ESR spectra that were consistent with the spin adducts of base radicals (the C5 and C6 radicals) and MNP were observed.

Hydroxy radicals, which are the most reactive species among the active oxygens, react with DNA to induce strand breaks, AP[1] sites, and base modification (Têoule, 1987; von Sonntag, 1987; Teebor et al., 1988). TMP is a DNA con-

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