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A Probe of the Active Site Acidity of Carboxypeptidase A

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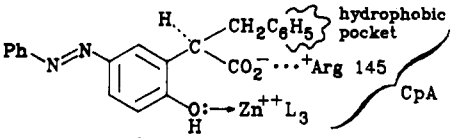
ABSTRACT: The substrate analogue 2-(1-carboxy-2-phenylethyl)-4-phenylazophenol is a potent competitive inhibitor of carboxypeptidase A. Upon ligation to the active site, the azophenol moiety undergoes a shift of pK_a from a value of 8.76 to a value of 4.9; this provides an index of the Lewis acidity of the active site zinc ion. Examination of the pH dependence of K_i for the inhibitor shows maximum effectiveness in neutral solution (limiting $K_i = 7.6 \times 10^{-7}$ M), with an increase in K_i in acid (pK_1 = 6.16) and in alkaline solution (pK_2 = 9.71, pK_3 = 8.76). It is concluded that a proton-accepting enzymic functional group with the lower pK_a (6.2) controls inhibitor binding, that ionization of this group is also manifested in the hydrolysis of peptide substrates (k_{cat}/K_m), and that the identity of this group is the water molecule that binds to the active site metal ion in the uncomplexed enzyme ($H_2OZn^{2+}L_3$). Reverse protonation state inhibition is demonstrated, and conventional concepts regarding the mechanism of peptide hydrolysis by the enzyme are brought into question.

Although details of the mechanism of peptide hydrolysis by the prototypical metalloenzyme carboxypeptidase A are controversial, it is widely accepted that the active site zinc ion functions by coordination to the oxygen atom of the substrate scissile carboxamide, thereby serving to render the peptide linkage more susceptible to nucleophilic addition. This interpretation is indicated strongly by crystallographic evidence for enzyme–pseudosubstrate complexes (Quiocho & Lipscomb, 1971) and by the inactivity of the Co^{3+}- (ligand-exchange inert) substituted enzyme in comparison with other metal ion modified derivatives of carboxypeptidase A (Van Wart & Vallee, 1978). Recently, a synergism test has been applied, in which the pattern of reactivity observed for normal and for thiocarboxamide substrates, acted upon by native and Cd^{2+}-substituted enzymes, substantiated a productive interaction between active site metal ion and substrate carbonyl group (Mock et al., 1981). Upon an assumption that the carboxamide cleavage mechanism for carboxypeptidase A does involve a Lewis acid role for the active site metal ion, it is reasonable next to inquire as to whether a *quantitative assessment* may be obtained for the magnitude of this interaction; i.e., how acidic is the active site zinc ion? This article provides an answer to that question by direct measurement with a suitably designed probe. As a bonus, additional evidence is provided that suggests a reverse protonation mechanism for activation of peptides by this enzyme.

The idea underlying the research to be described was construction of an active site directed agent (inhibitor) for carboxypeptidase A, which would serve as an *indicator* for the electron deficiency of the metal ion known to be present. L-Benzylsuccinic acid, $C_6H_5CH_2CH(CO_2H)CH_2CO_2H$, has been shown to be an avidly bound inhibitor for this enzyme, due to its successful mimicry of the specificity factors associated with good substrates for carboxypeptidase A (Byers & Wolfenden, 1973). Consequently, an analogue, 2-(1-carboxy-2-phenylethyl)-4-phenylazophenol, was synthesized and resolved to provide the appropriate L enantiomer (**1**).



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When fitted as a surrogate substrate as depicted, **1** presents a *phenolic hydroxyl* as a potential ligand to the active site metal ion. This oxygen atom corresponds spatially to the carboxamide oxygen of an *N*-acylphenylalanine substrate.

As we subsequently describe, the special virtues of this molecule are that the correct (L) enantiomer of **1** is indeed a good competitive inhibitor and the azophenol visible absorbance undergoes a characteristic perturbation upon interaction with the metal ion. The relevant evidence to be developed concerns the pH dependence of the electronic spectrum of a complex between **1** and carboxypeptidase A, as well as a variation with pH in the value of the kinetically determined inhibition constant K_i for **1**.

Materials and Methods

Carboxypeptidase A (CpA),[1] was supplied by Sigma Chemical Co. (no. C 0386). The Allan form was chosen for its reported greater solubility. It was recrystallized by dialysis according to established procedures before use (Mock & Chen, 1980). Buffers employed in this work for kinetic analysis were (0.05 M each) as follows: Ammediol, pH 8.75–10; Tris, pH 7.25–8.75; Mes, pH 5.3–7.25; 2,6-pyridinedimethanol, pH

[1] Abbreviations: CpA, bovine pancreatic carboxypeptidase A; FAPP, *N*-(2-furanacryloyl)-L-phenylalanyl-L-phenylalanine; Ammediol, 2-amino-2-methyl-1,3-propanediol; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Me_2SO, dimethyl sulfoxide.

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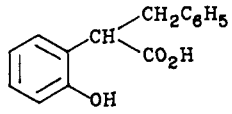
4.2–5.3. The last substance is a new buffer (obtained from Aldrich Chemical Co.), useful at low pH with carboxypeptidase A. It was shown to be noninhibitory at the concentrations used, as is the case for the other buffers. All enzyme work was done with solutions 1.0 F in sodium chloride. Solutions at pH of 6.4 or lower were made (0.5–1.0) × 10^{-4} M in additional zinc ion. The assay substrate for kinetic work was *N*-(2-furanacryloyl)-L-phenylalanyl-L-phenylalanine (FAPP; Riordan & Holmquist, 1984) from Sigma Chemical Co., for which a K_m value of 0.92 (±0.06) × 10^{-4} M was obtained (25 °C, pH 7.47) by spectrophotometric (328 nm) kinetic analysis, with initial rates at various substrate concentrations fitted directly to the Michaelis equation by an iterative nonlinear least-squares procedure.

2-(1-Carboxy-2-phenylethyl)-4-phenylazophenol (**1**). Benzylisocumaranone (the lactone of **2**) was prepared by



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catalytic hydrogenation of benzalisocumaranone (Czaplicki et al., 1909). For azo coupling, 1.12 g (5.0 mmol) of this material was dissolved in 4.5 mL of 10% sodium hydroxide by warming. The solution was vigorously stirred and cooled at 0–5 °C while a cold solution of benzenediazonium chloride (from 5.0 mmol of aniline and 5.4 mmol of sodium nitrite in excess hydrochloric acid) was added dropwise, keeping the temperature below 10 °C by addition of crushed ice. During the course of addition, a yellow-orange precipitate separated. Upon completion of the addition, the reaction mixture was allowed to stand in an ice bath for 30 min with occasional stirring. Product was collected by suction filtration and dried, yielding a gummy solid. Repeated recrystallization from chloroform–hexane yielded an orange powder: mp 142–144 °C; UV (H_2O, pH 7) λ_{max} 355 nm (ϵ 2.1 × 10^4 M^{-1} cm^{-1}); IR (KBr) 1590, 1600, 1700, 3300 cm^{-1}; ^1H NMR (Me_2SO-d_6/$CDCl_3$) δ 2.93–4.43 (m, 3 H), 7.20 (s, 5 H), 6.87–7.70 (m, 8 H), and 8.8 ppm (m, 2 H).

Resolution of **1** into D and L enantiomers was accomplished by repeated recrystallization of the salt formed with enantiomerically pure methylbenzylamine (Aldrich Chemical Co.). To a stirred solution of 1.77 g (5 mmol) of crude racemic acid **1** in 50 mL of ether was added 1.22 g (10 mmol) of (*S*)-(−)-α-methylbenzylamine, yielding a yellow precipitate. After refrigeration of the mixture overnight, yellow crystals of the amine salt of **1** were collected by suction filtration and washed with ether, mp 170–183 °C. The filtrate was concentrated by evaporation, and an oil was obtained, which crystallized from chloroform–hexane, giving a yellow solid, mp 161–167 °C.

The initial precipitate was extracted with chloroform, and the insoluble residue (660 mg) was fractionally recrystallized from acetone to yield 315 mg of the pure (*S*)-(−)-α-methylbenzylamine salt of L-**1**: mp 188.5–189.5 °C; $[\alpha]_D$ −22.0° (*c* 2.0, MeOH). The chloroform extract was concentrated under reduced pressure to give a precipitate with mp 158–167 °C, which was combined with the more soluble residues from the previous step. These were partitioned between aqueous hydrochloric acid and chloroform. The organic phase was evaporated, and the residue was taken up in ether and filtered. Addition of (*R*)-(+)-α-methylbenzylamine as before, with fractional recrystallization from acetone, yielded 294 mg of the pure amine salt of D-**1**: mp 189.5–190.5 °C; $[\alpha]_D$ +23.3° (*c* 2.1, MeOH).

Methylbenzylamine was found to influence the kinetics of carboxypeptidase A. The free acids of D- and L-**1** were recovered as before ($CHCl_3/H_2O$–HCl extraction) but were found to crystallize poorly. Treatment with an equivalent of methanolic acetamidine (obtained from the hydrochloride with potassium hydroxide) gave upon evaporation a residue that was repeatedly recrystallized from acetone to give the acetamidine salt of L-**1** [mp 192–193.5 °C; $[\alpha]_D$ −21.0° (*c* 5.05, MeOH)] and the acetamidine salt of D-**1** [mp 192–193.5 °C; $[\alpha]_D$ +21.8° (*c* 5.5, MeOH)].

The material subsequently referred to as **1** in this article is the levorotatory form of the acetamidine salt of **1**. Acetamidine hydrochloride was shown not to be inhibitory. The dextrorotatory form, D-**1**, was found to have an inhibition constant 70 times greater than that for L-**1** at pH 7.5. From this, we conclude (a) that L-**1** has the same configuration as L-phenylalanine (hence its designation as L) and (b) that the optical rotation of the D form corresponds to ≥98.6% enantiomeric purity (i.e., the observed inhibition by D-**1** either may be intrinsic or may be due to residual L-**1**). The precursor **2** [2-(1-carboxy-2-phenylethyl)phenol] was not resolved but was used for inhibition as the racemate. It is assumed that only the L form inhibits, and K_i values for **2** have been adjusted accordingly. Anal. Found for **1** ($C_{21}H_{18}N_2O_3 \cdot C_2H_6N_2$): C, 67.77; H, 6.09; N, 13.76.

Spectrophotometric Titration. For measurement of the pK_a of (L-) **1** (≥0.51 × 10^{-4} M) in the presence of carboxypeptidase A (≥1.01 × 10^{-4} M, calculated from an extinction coefficient of 6.4 × 10^4 M^{-1} cm^{-1} at 280 nm), a series of spectra (pH 4.2–6.5) in 0.02 M 2,6-pyridinedimethanol, 0.02 M Mes, 1.0 F NaCl, and 10^{-4} M $ZnSO_4$ were obtained. (Equivalent results were obtained with buffering provided only by the enzyme itself.) As necessary, solutions were clarified by filtration through glass wool in order to remove traces of precipitated protein after acidification. The enzyme is denatured very rapidly in the more acidic solutions. A similar procedure gave pK_a values for **1** and **2** in 1.0 F sodium chloride solution in the absence of enzyme [absorbance change at λ_{max} 437 (355) and 294 nm, respectively].

Inhibition Kinetics. Competitive inhibition by **1** was demonstrated by determination of Michaelis parameters for FAPP hydrolysis by CpA at a pH of 7.52 in the presence of concentrations of **1** of 0.0, 0.781 × 10^{-6}, and 1.25 × 10^{-6} M. The pH dependence of K_i was determined in buffers previously listed with FAPP as substrate at an initial concentration of 0.1K_m (necessarily at a somewhat higher concentration in acidic solution). Enzyme concentration was maintained well below inhibitor concentration in kinetic studies (100-fold). The initial rate method was employed with spectrophotometric analysis (328 nm, 2–10-cm path length). Temperature was maintained at 25.0 (±0.1) °C. Values for K_i were obtained at each pH by a nonlinear least-squares fit of data to a hyperbolic inhibition curve. All pH values in this article are calibrated pH meter readings uncorrected for ionic strength effects. Tolerances indicated in this article are for the most part standard errors from least-squares analysis.

RESULTS

pK_a of Enzyme–Inhibitor. The free, uncomplexed azocarboxylate **1** undergoes typical phenolic ionization in alkaline solution. The absorbance of the uncharged azophenol moiety (λ_{max} 355 nm) shifts strongly to the visible (λ_{max} 437 nm) upon deprotonation. By spectrophotometric titration, a pK_a value of 8.76 (±0.02) was obtained in buffered 1.0 F sodium chloride solution (the medium employed for enzymic investigations). Such a value corresponds to that recorded for azophenol itself

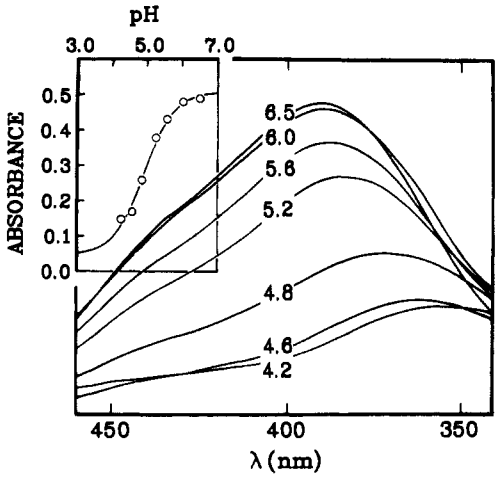
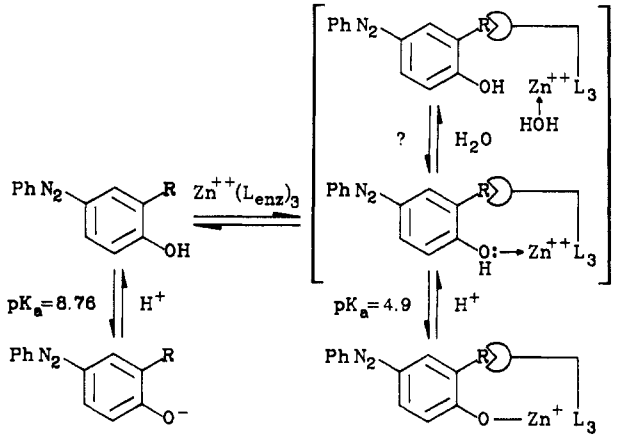


FIGURE 1: Absorbance spectrum of **1** in the presence of excess carboxypeptidase A as a function of pH. (Inset) Least-squares fit of A_{390} to a sigmoidal expression; $pK_a = 4.9$ (±0.1). See Materials and Methods for conditions.

Scheme I



(Farmer, 1901). A pH-dependent inflection in absorbance intensity attributable to the carboxyl group of **1**, $pK_a = 4.35$ (±0.07), was also observed at 355 nm.

However, in the presence of an excess (greater than 1 molar equiv) of carboxypeptidase A, the phenol ionization is perturbed. The complex of **1** with the enzyme yields an absorbance at 390 nm, which is unchanged in passing from neutral to alkaline solution, strongly suggesting that this is a metal–phenoxide absorbance. Only upon lowering the pH below 6 does evidence appear for protonation of the coordinated phenolate (λ_{max} decrease in intensity, shift to 355 nm, reversible on pH increase). By careful acidification of buffered solutions of the complex, a spectrophotometric pK_a of 4.9 (±0.1) was obtained (Figure 1). With reference to Scheme I, the effect of coordination of the zinc ion to the phenol group of **1** is to *increase* the apparent acidity of the phenolic hydroxyl by nearly 4 pK units, or by a factor of 10^4. This constitutes a direct measure of the Lewis acidity of the active site zinc ion, correlated with the Brønsted acidity scale (that which is most applicable in aqueous solution). The special relevance of this number to the mechanism of carboxypeptidase A will be developed under Discussion. However, we would note that this technique for estimation of metal ion acidities is quite general and is potentially widely applicable in enzymology.

As noted in Scheme I, the consequence of protonation of **1** in the complex may well be release of the phenol from the metal inner coordination sphere (i.e., replacement of ArOH by a more basic H_2O ligand). This would yield a falsely high

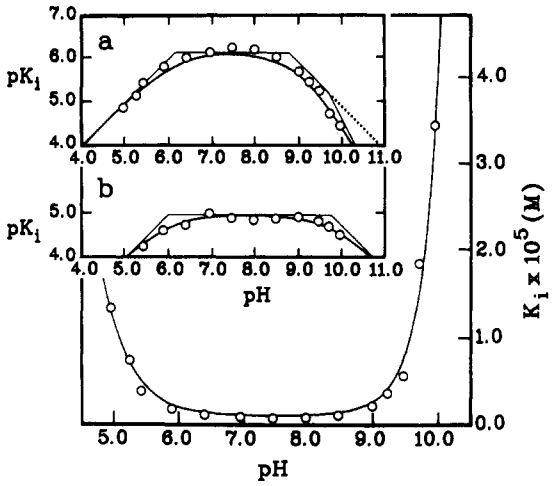


FIGURE 2: Plot of competitive inhibition constant K_i vs. pH for **1**. Line is a least-squares fit to an expression given in the text. (Inset) (a) Same data for **1** with logarithmic vertical axis (pK_i vs. pH). Breaks in asymptotic straight lines denoted pK_a values from the previous curve fit. (b) Data from the fit of K_i vs. pH for **2**. Presentation is as in (a).

apparent pK_a for the metal-bound phenol. The fact that the spectrum of free **1** in weakly acidic medium is similar to that produced from the complex below a pH of 4.9 permits such an interpretation, although K_i measurements suggest that **1** remains mostly bound to the active site at the apparent pK_a, presumably by interactions involving its benzyl group and the carboxylate (R in Scheme I). Since in this pH region carboxylate groups (as present in both inhibitor and enzyme) commence to undergo protonation, and also metal ion release from the enzyme becomes rapid, there is some uncertainty as to the nature of the proton attachment yielding the spectral perturbation. These ambiguities do not adversely affect the conclusion regarding Lewis acidity of $Zn^{2+}L_3$; the factor of 10^4 is a *minimum* estimate of its ability to acidify the phenolic hydroxyl. Stated differently (but equivalently), the active site zinc ion of carboxypeptidase A *stabilizes* a coordinated phenolate anion by a factor of $\geq 10^4$, thereby suppressing its proton affinity by ≥4 pK units.

pH Dependence of K_i. As has been emphasized (Cleland, 1977), K_i values for inhibitors are especially valuable for investigation of enzyme mechanisms. Inhibition constants represent pure equilibrium binding of ligands to an enzyme, unlike kinetic K_m values, which frequently are a complex quantity incorporating rate constants for multiple steps within the reacting enzyme–substrate assemblage.

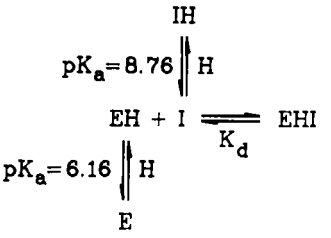
As might be expected from its substrate mimicry, **1** is a good competitive inhibitor of carboxypeptidase A. Values for K_i were measured by perturbation of the second-order rate constant, k_{cat}/K_m, for cleavage of the Phe–Phe bond of the peptide substrate *N*-(2-furanacryloyl)-L-phenylalanyl-L-phenylalanine (FAPP). Figure 2 shows an experimentally obtained, inverted bell-shaped dependence of K_i upon pH. Binding of **1** is tightest at neutrality, with diminished affinity for the enzyme in either acidic or alkaline solution. The deviation in basic solution presumably reflects deprotonation of **1** (phenolic pK_a of 8.76). By nonlinear least-squares curve fitting, it was determined that the abrupt increase in K_i at high pH was greater than first order in hydroxide (i.e., it could not be accommodated by only considering the phenolic ionization of **1**). We infer that there is a second deprotonation, necessarily occurring on the enzyme, which also affects binding of **1**. Likewise, the increase in K_i at low pH apparently must be attributed to an enzymic protonation. The curve shown in Figure 2 corresponds to a best

fit to the equation $K_i(app) = K_i(1 + [H]/K_1)(1 + K_2/[H])(1 + K_3/[H])$, in which K_3 is specified as the value of the spectrophotometric ionization constant for **1**; $K_3 = 10^{-8.76}$ N. Unless K_3 is defined, K_2 cannot be ascertained, since their values nearly coincide. The resulting parameter values for the lower curve in Figure 2 are $K_i = 7.6\ (\pm 1.7) \times 10^{-7}$ M, $pK_1 = 6.16\ (\pm 0.11)$, and $pK_2 = 9.71\ (\pm 0.17)$, in which the latter two constants presumably refer to deprotonations of the enzyme, influencing binding of **1**. Also shown in Figure 2 (inset) is a log plot of the same data; the intersections of the conventional asymptotic lines denote the pK_a values previously listed. The inset also contains a similar log plot of K_i dependence for **2**, the synthetic precursor of **1**, which lacks an arylazo substituent on the phenol ring. The phenolic ionization constant for this substance (spectrophotometric determination in the absence of enzyme) is $pK_a = 10.68\ (\pm 0.02)$, a value outside of the range of collectable data in Figure 2. For this inhibitor, the parameters (obtained as for **1**) are $K_i = 1.14\ (\pm 0.08) \times 10^{-5}$ M, $pK_1 = 6.02\ (\pm 0.05)$, and $pK_2 = 9.75\ (\pm 0.08)$. It appears that although **2** is less tightly bound than **1**, enzymic pK_a values bracketing the limiting K_i values for **1** and **2** correspond. In particular, the somewhat uncertain second deprotonation on the base limb for **1** is supported by its unambiguous occurrence on the base limb for **2**. It should be evident that all ionization constants alluded to in this section pertain to the *free* enzyme (K_1 and K_2) and inhibitor (K_3) and not to their complex, since data were obtained from perturbations of k_{cat}/K_m, with K_i increasing from a limiting value on the acid and base limbs (Cleland, 1977). In principle, the K_i curve for **1** might be expected to level off in acid solution (pH <4.9), although experimentally such an effect is neither demonstrated nor disproven in this case (i.e., the data fit equally well to such an interpretation).

Reverse Protonation. A peculiarity within the pH dependence of K_i for **1** is immediately apparent. The active form of the inhibitor (insofar as tight binding to the enzyme) certainly contains the phenolate anion (ArO^-). This would lead to an expectation that K_i should achieve its extreme, or minimum, corresponding to firmest ligation, at pH values *above* 8.76. Under high dilution (the conditions for measurement of a competitive K_i), the inhibitor is largely uncomplexed. Its affinity for the enzyme should substantially decrease at pH values below the pK_a value for the free inhibitor, due to reduced concentrations of phenolate anion. The protonated (phenolic) form would have much reduced capacity (10^4-fold) to coordinate with the active site zinc ion. However, the experimental data is just the opposite of this naive expectation. While it seems certain that the first base-limb break in the pH profile for **1** (Figure 2, inset, $pK_3 = 8.76$) corresponds to ionization of the inhibitor, it appears that the phenol is active and the phenolate is *not*.

The only plausible rationalization for this behavior is that a proton-accepting functional group *on the enzyme* also controls binding of inhibitor in an absolute fashion. The evidence indicates that, for effective attachment of inhibitor to enzyme, an active site residue with a pK_a of 6.16 must be *protonated* and that its deprotonation adversely affects binding of **1**. The situation may be illustrated as in Scheme II, wherein EH is the obligatory protonated form of the enzyme and I corresponds to the phenolate form of the inhibitor. At pH values between 6.16 and 8.76, both EH and I are *minor* species; the preponderant form of inhibitor below a pH of 8.76 will be IH, and the major form of enzyme above a pH of 6.16 will be E. In the vicinity of neutrality, the concentration of EH will decrease linearly with diminishing hydrogen ion concentration,

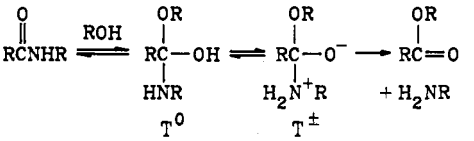
Scheme II



but the concentration of I will increase proportionately. The net result will be *pH independence* for K_i in this region ($K_i = [E][HI]/[EHI]$). The true measure of affinity of **1** for carboxypeptidase A ($K_d = [EH][I]/[EHI]$, Scheme II) is not the apparent limiting value of K_i but, when correction is made for diminished concentrations of reactants, is $K_d = (K_i \times 10^{-8.76})/10^{-6.16} = 1.9 \times 10^{-9}$ M. The phenomenon described may be categorized as *reverse* (or *minor*) *protonation state inhibition* (Cleland, 1977). The nature of the enzyme functional group with pK_a of 6.16 is not identified by the evidence cited, but in view of the strong, specific interaction of **1** with the enzyme, it must be equated with the corresponding pK_a noted kinetically in the pH profile for k_{cat}/K_m with peptide substrates (Auld & Vallee, 1970). With the substrate here employed (FAPP), we find pK_a values of 6.24 (±0.07) and 9.22 (±0.06) bracketing the active range (k_{cat}/K_m) for carboxypeptidase A (Allan). It appears that a 0.5 pK unit separates the enzymic base-limb ionization constants for inhibition and catalysis, a difference which may or may not be real. However, the lower (acid-limb) pK_a values match satisfactorily at 6.2. It is suggested that the latter congruity has great significance for interpretation of the enzyme mechanism.

Discussion

Lewis Acidity of $Zn^{2+}L_3$. Measurement of the electron deficiency of the active site zinc ion in carboxypeptidase A has certain immediate implications for the mechanism of hydrolysis of peptide bonds by the enzyme. To summarize, the direct spectrophotometric evidence is that coordination of that metal ion to an azophenolate stabilizes the oxyanion by at least 10^4-fold (a 4-unit shift in pK_a). The relevance of this number may be seen by examination of intermediates in the generic addition–elimination mechanism for cleavage of the amide linkage. Regardless of the nature of the nucleophile



ROH or of the details of timing for proton transfers, a zwitterion such as the tetrahedral adduct $T^{\pm}$ is the most probable penultimate intermediate in neutral solution, since it is most explicitly poised for collapse to the final fragments produced in the reaction. The proton affinity of the *oxyanion* within $T^{\pm}$ may be estimated with reasonable confidence, on the basis of mutually reinforcing extrapolations from model substances. Other investigators have independently concluded that the pK_a for *hydroxyl* ionization of the protonated form of $T^{\pm}$ should be about 8.4 (±0.5) (Barnett & Jencks, 1969; Fox & Jencks, 1974; Satterthwait & Jencks, 1974). The cumulative effect of substituent-produced inductive and electrostatic electron withdrawal for such a hydroxyl renders it comparably acidic as the phenolic hydroxyl of inhibitor **1**. It follows that the metal ion of carboxypeptidase A should be able to stabilize intermediate $T^{\pm}$ by an equivalent amount,

namely, *≥10⁴-fold.* It might be argued that a phenol ring and a tetrahedral adduct are structurally unrelated, so that the comparison is suspect. However, the zinc ion at the active site merely engages an oxyanion of a certain basicity. Insofar as the *localized interaction* is concerned, the comparison is completely valid. The function of an enzyme such as carboxypeptidase A is to stabilize active intermediates and the transition states associated with them. To the extent that transition states resemble $T^{\pm}$, the role of the zinc ion in the mechanism may be quantitatively assessed; conservatively, it provides a kinetic acceleration in the neighborhood of 10 000-fold. Depending upon the timing of proton transfers within the appropriate transition states, the actual contribution to kinetics might differ by 1 or 2 orders of magnitude. However, for the balance of the enzymic kinetic acceleration for peptide hydrolysis, one should look to other active site functionality.

The aforementioned estimate of tetrahedral adduct stabilization may be derived in another way. Good peptide substrates for carboxypeptidase A (e.g., FAPP) typically have K_s values of 10^{-4} M (at pH values <6 for CpA, where K_m necessarily equals K_s since K_m is pH-invariant but k_{cat} is not; Cornish-Bowden, 1976). The enzyme complex of **1** was shown to have a true dissociation constant of 2×10^{-9} M. The ratio K_s/K_d, namely, (5×10^4)-fold, may be attributed largely to a stronger linkage by the phenoxide to the metal ion as compared to the corresponding coordination by a less basic substrate carboxamide. In other words, the shift in pK_a correlates with the incremental binding energy of substrate and intermediate/inhibitor, as it should according to our interpretation.

Brønsted Acidity of $H_2OZn^{2+}L_3$. Regarding the pH dependence of K_i, there is a clear indication that an enzymic functional group with a pK_a of 6.2 controls inhibitor binding, as well as catalytic activity. We and others have suggested that the water molecule which is known to be bound to the active site zinc ion in the absence of substrate has a pK_a of 6.2. Although not universally accepted (Geoghegan et al., 1983; King & Fife, 1983; Spratt et al., 1983; Vallee et al., 1983; Shoham et al., 1984), evidence for this is quite convincing. Substitution of other metal ions (Co^{2+}, Ni^{2+}, Mn^{2+}) for Zn^{2+} yields active enzymes in which this pK_a (noted in k_{cat}/K_m) is systematically perturbed (but not the kinetically observed higher pK_a of ~9; Auld & Vallee, 1970; King & Fife, 1983). Cryoenzymological investigations have also led to the conclusion that the metal-bound water is relatively acidic (Makinen et al., 1979). Exchange of ligand chloride on this metal occurs rapidly only below a pH of ~6 (Stephens et al., 1974; Geoghegan et al., 1983). To these may now be added the results of the present investigation. Whereas a *water* molecule *can* be dislodged from $H_2OZn^{2+}L_3$, *hydroxide cannot* be replaced in $HOZn^{+}L_3$ by the phenolate of the inhibitor **1**. As the pH is increased above a value of 6.2, $H_2OZn^{2+}L_3$ becomes a (diminishing) minor species. Similarly, as the pH is decreased below 8.76, the concentration of the phenolate of **1** (I in Scheme II) dwindles. Since these are the active forms for effective binding ($K_d = 2 \times 10^{-9}$ M), the net result is an invariant value for K_i in the region of neutrality (Figure 2). Above a pH of 8.76, the inhibitor approaches complete ionization; affinity for the enzyme then decreases because of further diminution of the already miniscule amount of $H_2OZn^{2+}L_3$ in solution. Likewise, below a pH of 6.2, all of the free enzyme becomes susceptible to inhibitor, yet K_i increases because even less azophenoxide becomes available in more acidic solution. Clearly, what is occurring is *reverse protonation inhibition.*

This interpretation is entirely substantiated by the inhibition characteristics of **2** (Figure 2). This inhibitor, which contains a less acidic phenol, is apparently bound 15-fold less avidly than **1** (comparison of K_i values). However, this is entirely attributable to the higher pK_a of the phenol of **2** ($pK_a = 10.7$). Even less of the required minor protonation state species are available at an intermediate pH value in this case. When an appropriate correction is made for reverse protonation, the K_d value for **2** actually becomes smaller than for **1**. Incidentally, this interpretation also provides explanation for the strong pH dependence in the binding of benzylsuccinic acid (Byers & Wolfenden, 1973; Palmer et al., 1982) as we have previously noted (Mock & Chen, 1980).

The realization that an H_2O molecule bound to the active site metal ion is especially acidic explains one puzzling feature of the original X-ray crystallographic structure determination for carboxypeptidase A (Quiocho & Lipscomb, 1971). The metal ion was initially reported to be tetracoordinate [but see Rees et al. (1981, 1983)], although in aqueous solution Zn^{2+} ordinarily bears five or six ligands of the kind found in carboxypeptidase A [also see Horrocks et al. (1982)]. Recognition that a strongly electron-donating hydroxide would be the coordinating species rather than H_2O under conditions employed for the structure determination helps to explain why the Lewis acidity of the tetrahedral metal ion may be satisfied without an enhancement of ligation number. It is expected that there should be a direct correlation between the electron deficiency of a metal ion and the pK_a of a ligand water molecule. Hence, the evident low pK_a of $H_2OZn^{2+}L_3$ constitutes a further index of the Lewis acidity of the metallic species in the active site of carboxypeptidase A.

Cooperative Ionization. The statement that $H_2OZn^{2+}L_3$ in this metalloenzyme has a pK_a of 6.2 requires one qualification. According to crystallographic evidence, the side-chain carboxylate of Glu-270 is sufficiently close to the metal-bound H_2O (or OH^-) as to be able to hydrogen bond to it (Lipscomb, 1974). In consequence, the ionization of these groups should be cooperative in nature; i.e., there will be reciprocal perturbations of their microscopic ionization constants (Dixon, 1976). However, this does not materially affect the foregoing conclusions. It is the *second* deprotonation of the cooperative system that is under consideration; the first ionization of Glu^{270}–CO_2H···$H_2OZn^{2+}L_3$ presumably occurs at a pH normal for a carboxylic acid (or even with a lower pK_a). Nevertheless, any interaction between these groups means that a nonspectroscopic probe cannot distinguish between them. For example, the rate of chemical modification of Glu^{270}–CO_2H by O-alkylation with a (bromomethyl)carbonyl-containing substrate analogue appears to be controlled by an enzyme group with pK_a of 6–6.5 (Hass & Neurath, 1971). Such evidence does not exclude the second ionization of a cooperative system as being responsible. Indeed, a strong Glu^{270}–CO_2^-···$H_2OZn^{2+}L_3$ hydrogen bond should depress the nucleophilicity of the carboxylate, such that reaction would occur faster at pH >6, as is observed. For sake of clarity, the enzyme pK_a of 6.2 is elsewhere attributed solely to $H_2OZn^{2+}L_3$ in this article.

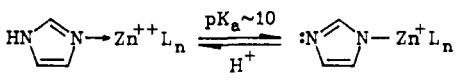
Mechanistic Implications. The pH dependence of k_{cat}/K_m for peptide substrates hydrolyzed by carboxypeptidase A is also bell shaped, with pK_a values of 6.2 and 9.2 (this study) bracketing the region of maximum enzyme activity. Several investigators have concluded that the pK_a of 6.2 is attributable to active site residue glutamic acid-270 and that the value of 9.2 should be assigned to $H_2OZn^{2+}L_3$. According to such an interpretation, the pK_a of 9.2 controls binding of substrates (a K_m or K_s effect), and the pK_a of 6.2 indicates that the

carboxylate of Glu-270 is essential for catalysis (a k_{cat} effect, presumably general base or nucleophilic participation). Our results are incompatible with this interpretation. Clearly, it is the group with pK_a of 6.2 that has the major effect upon binding and not a group with pK_a of 9.2. An apparent perturbation of K_i for **1** and **2** arising from an enzymic group with pK_a of 9.75 was in fact noted. However, we have doubts that the same enzyme functionality as in k_{cat}/K_m is responsible, and the effect with respect to K_i is not large at accessible pH values, when compared with that of the group with pK_a of 6.2. The alkaline behavior of the enzyme will be considered subsequently.

Regarding the conventional interpretation of the k_{cat}/K_m vs. pH profile, it is implausible that the ionization state of Glu^{270}–CO_2H should have such a gross effect upon binding of **1**. Although ionization of the side-chain carboxylic acid might produce an inflection in the K_i vs. pH profile (if its pK_a were abnormally high in the free enzyme—an unnecessary assumption), it is unlikely that the carboxylate could exclude inhibitor absolutely, at pH values even up to 10, as the experimental evidence indicates. Furthermore, if the ionization state of the Glu-270 side chain were the controlling factor in binding of inhibitor (and substrates), the evidence for reverse protonation would require that the active form (EH in Scheme II) be the *carboxylic acid* (Glu^{270}–CO_2H). This fact is not easily reconciled with proposals that Glu^{270}–CO_2^- is the obligatory species that functions as a base or nucleophile in the subsequent hydrolysis of peptides. Proponents of the latter mechanisms are now faced with a harsh paradox; if it is Glu^{270}–CO_2^- that causes rejection of substrate at the active site, then the carboxylate anion can scarcely be essential in that form for subsequent action in the catalytic cycle! (If the anionic natures of I and Glu^{270}–CO_2^- causes a unique electrostatic repulsion compared with peptide substrate, how is it that I binds $>10^4$ times more firmly than does substrate?)

Regarding the enzymic pK_a values of 9.2 (catalysis) and 9.75 (inhibition), several interpretations are possible. The responsible functional groups may be one and the same, since the difference represents only a factor of 4 in the underlying ionization constants (the outer limit of experimental error) and shifted or "kinetically miraged" pK_a values may in fact occur in k_{cat}/K_m (Cleland, 1977). Upon the tentative assumption of a real numerical difference, it should be noted that the pK_a of 9.2 has previously been associated with Tyr^{248}–C_6H_4OH (Mock & Chen, 1980). Were this functional group (whatever its identity) to be active in the *first committed step* within the enzyme–substrate complex (the first event in the catalytic cycle for which the flux in the reverse direction is negligible in the steady state), it would show up in k_{cat}/K_m but not necessarily in K_i nor in k_{cat}. Support for its assignment came from a systematic perturbation of the base-limb pK_a upon selective nitration of the tyrosine side chain in the enzyme. More recent results involving mutant forms of carboxypeptidase A cast this interpretation seriously into doubt (Gardell et al., 1985).

For the pK_a of 9.75 in the K_i vs. pH profile, another possibility merits consideration. It has been established in other ligand–metal systems that *imidazole* rings coordinated to a transition metal ion have pK_a of 9–12, values that are intermediate between that of imidazole (pK_a ~ 14) and imidazolium ion (pK_a ~ 7) (Sundberg & Martin, 1974). Two of



the enzyme-attached ligands for the active site zinc ion in carboxypeptidase A are imidazoles (His-69, His-196). We would merely note that proton dissociation from one of these groups should have a predictable consequence on K_i for **1** or **2**. Specifically, the Lewis acidity of the metal center $Zn^{2+}L_3$ would *decrease* upon deprotonation of one of the ligands L. The strength of the attachment of a coordinated phenoxide (i.e., **1**) would therefore weaken, and K_i would increase, as observed. Since the effect is anticipated and since K_i represents an unambiguous equilibrium binding phenomenon, this interpretation of the base–limb region of the pH dependence of K_i commands consideration. Association of the high pK_a of carboxypeptidase A with the metal ion in this manner is independently supported by pH profiles for Zn^{2+} binding to the apoenzyme (Coleman & Vallee, 1961; Billo et al., 1978) and by pH-dependent spectral perturbations of the Co^{2+}-substituted enzyme (Latt & Vallee, 1971).

If the conventional interpretation of the carboxypeptidase A mechanism is in doubt, what alternatives are allowed by the realization of a pK_a value of 6.2 for $H_2OZn^{2+}L_3$? One possibility is the so-called carbonic anhydrase type mechanism, in which it is a metal-bound hydroxide that adds across the substrate carbonyl, yielding T^0 (R = Zn^{2+}) and subsequently a $T^{\pm}$ adduct by *N*-protonation. Our previous conclusions regarding stabilization of $T^{\pm}$ are equally valid in this scheme, since *thermodynamically it would matter not how that metal ion adduct was formed.* Such a mechanism is not supported by crystallographic evidence and may be discounted for peptide substrates by the synergism test (Mock et al., 1981). In the latter investigation, a substrate analogue was prepared in which the scissile carboxamide linkage had been replaced by a thioamide. The thiocarbonyl-containing substrate was not cleaved by Zn-CpA, but was hydrolyzed normally by Cd-CpA. Ordinary peptides are *not* cleaved by Cd-CpA (but are by Zn-CpA). The conclusion that follows is that substrate activation involves coordination of (thio)carbonyl to metal ion; the cited behavior arises from hard–soft acid–base interactions (Pearson, 1973). As far as we know, the carbonic anhydrase type mechanism *is* viable for hydrolysis of *ester* substrates by carboxypeptidase A. Specific acid activation of an ester by Lewis-type coordination should be much more difficult to achieve, since the Brønsted basicities of R(C=O)NHR and R(C=O)OR differ by a conservatively estimated 5 pK units (for carbonyl protonation). Therefore, an ester would be much less able to compete with hydroxide for coordination to $Zn^{2+}L_3$ but should be rather more susceptible to addition by the oxygen of $HOZn^+L_3$. A scheme with a common type intermediate [$T^{\pm}\cdot Zn^{2+}L_3$ or $RC(OH)(OR)O^-\cdot Zn^{2+}L_3$], obtained by two different paths, provides a convenient rationale for the oft-cited differences in ester and amide hydrolysis by carboxypeptidase A. It explains why specificity should be the same for both types of substrate in spite of different pathways to an activated complex.

However, we have in earlier articles shown that it is also possible to reconcile a low value for the pK_a of $H_2OZn^{2+}L_3$ with the conventional Lewis acid role for the active site zinc ion in the cleavage of peptides. The crucial concept is *reverse protonation* as demonstrated for the binding of **1**. As already noted, it formerly appeared kinetically feasible that the phenolate of Tyr-248 might obligatorily function catalytically in the first committed step as a general base for peptide hydrolysis (Mock, 1976; Mock & Chen, 1980). But that is not the only possible version of a reverse protonation mechanism. For example, protonation of the imidazole (histidine) ligands of the active site zinc ion may be critical. This could be the case if the first committed step (which is not necessarily the k_{cat} step) within the enzyme–substrate complex were replacement

of zinc-bound water by substrate carbonyl: $HO-Zn^{+}(L_2)\leftarrow NC_3H_2RNH$ (inactive); $H_2O\rightarrow Zn^{+}(L_2)-NC_3H_2RN$ (active). That is, rapid release of H_2O in a (probably) associative process requires a less electron-deficient zinc ion with a deprotonated imidazole ligand of $pK_a = 9-10$. In order to accommodate the experimental kinetics, all that is required according to this hypothesis is that a water molecule then adds to the carbonyl of actively bound substrate (leading to $T^{\pm}$ and hydrolysis) *faster than it readds to* Zn^{2+}. Subsequent steps may be relatively slow, allowing for an accumulation of intermediates (Galdes et al., 1983). The addition of water to substrate would likely be aided by a general base functionality and might involve the same H_2O released from Zn^{2+} (but this need not be so). In summary, we argue for a reverse protonation *activation* of peptide substrates.

In this conjunction, it is worth considering the consequences of the observation of a very high value for k_{cat}/K_m (6.7×10^6 M^{-1} s^{-1} for FAPP at neutral pH; Riordan & Holmquist, 1984) upon the feasibility of a reverse protonation mechanism. One might be tempted to argue that a minor protonation-state scheme may be excluded on grounds of kinetic incompetency (Cleland, 1977). For example, at a pH of 9.2 the encounter frequency of enzyme with substrate would have to be at least 10^3 times greater than the apparent value of k_{cat}/K_m (3.3×10^6 M^{-1} s^{-1}) in order to allow for the fact that only 0.1% of the enzyme is in the form of $H_2OZn^{2+}L_3$ at this pH (i.e., the actual velocity of productive complexation must be increased over the apparent value by the ratio of the acid dissociation constants of the two enzymic functional groups). The resulting number exceeds the generally accepted diffusion limit; therefore, reverse protonation appears to be kinetically incompetent. However, this argument ignores the consequences of productive binding upon the enzymic functional group with pK_a of 6.2. Coordinative addition of a weakly basic carboxamide oxygen to $HOZn^{+}L_3$ ought to increase the proton affinity of the bound hydroxide by a substantial amount. For example, binding of the inhibitor (*R*)-2-benzyl-3-(*p*-methoxybenzoyl)propionic acid to carboxypeptidase A apparently causes the pK_a of $H_2OZn^{2+}L_3$ to increase from a value of 6.2 to a value of 7.56 [Spratt et al. (1983), see Figure 3 therein. Caution: the authors erroneously attributed the higher pK_a in their study to the free enzyme and thereby misidentified the functionality responsible; for the correct interpretation of K_i vs. pH profiles, see Cleland (1977)]. Actually, this substrate analogue apparently inhibits and produces its pK_a shift without coordination to the metal, for the most part (Christianson et al., 1985). A similar perturbation of pK_a was noted (and properly interpreted) for binding of *N*-(bromoacetyl)phenylalanine (Hass & Neurath, 1971). A corresponding shift of pK_a attendant upon binding of regular peptide substrates to the hydroxy-ligated form of carboxypeptidase A would mean that $H_2OZn^{2+}(S)L_3$ could be formed in sufficient abundance by subsequent protonation so as to be a kinetically competent intermediate. The corrected rate constant in such a case (10^7-10^8 M^{-1} s^{-1}) also corresponds to the exchange rate of water ligands upon Zn^{2+} (Eigen, 1963) so that this is a reasonable first committed step, as previously proposed. (The inherent cooperativity of the HO–Zn–imidazole system should be appreciated—protonation of ligand hydroxide would cause the pK_a of ligand imidazole to decrease, by arguments previously adduced, so that there need be no diffusion limit constraint at low pH as well. Implicit in the preceding analysis is rapid-equilibrium proton transfer within the active site, although a slow proton shift could have equivalent net consequences.)

Our interpretation makes sense from the standpoint of biological evolution. We have argued that the enzyme's ability to stabilize $T^{\pm}$ and thus speed the hydrolysis reaction should be proportionate to the Lewis acidity of $Zn^{2+}L_3$, at least unto the degree whereby retardation of decay of the (stabilized) intermediate becomes a constraint upon k_{cat}. The situation with carboxypeptidase A is that pressure of natural selection to enhance the Lewis acidity of the active site zinc ion, by successive mutations favoring greater efficiency, has concurrently generated Brønsted acidity in $H_2OZn^{2+}L_3$, until as an unavoidable consequence inhibition by *hydroxide* has become a limiting factor in the peptide hydrolysis rate at low substrate concentration, even at neutral pH. This appears to be true regardless of the actual details of the process of amide bond scission. We conclude that the complete mechanism of carboxypeptidase A is still very much of an unresolved matter and that reverse protonation possibilities should not continue to be ignored.

Registry No. FAPP, 83661-95-4; DL-**1**, 101493-07-6; D-**1**, 101627-05-8; D-**1** [(*R*)-(+)-α-methylbenzylamine salt], 101692-56-2; L-**1**, 101627-06-9; L-**1** [(*S*)-(−)-α-methylbenzylamine salt], 101692-55-1; **2** (lactone), 73406-74-3; carboxypeptidase A, 11075-17-5.

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Biochemical Characterization of the Phospholipase A_2 Purified from the Venom of the Mexican Beaded Lizard (*Heloderma horridum horridum* Wiegmann)†

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ABSTRACT: A phospholipase A_2 was isolated from the venom of the mexican beaded lizard (*Heloderma horridum horridum*) by phenyl-Sepharose chromatography followed by Sephadex G-75 gel filtration and two additional steps on ion exchange resins (DE-32 cellulose). The affinity chromatographic method (PC–Sepharose 4B) reported for the isolation of other phospholipases [Rock, Ch. O., & Snyder, F. (1975) *J. Biol. Chem. 250*, 2564–2566; King, T. P., Alagon, A. C., Kwan, J., Sobotka, A. K., & Lichteinstein, L. M. (1983) *Mol. Immunol. 20*, 297–308; King, T. P., Kochoumian, L., & Joslyn, A. (1984) *Arch. Biochem. Biophys. 230*, 1–12] was uneffective for the separation of this enzyme. The monomeric form of the *Heloderma* phospholipase has an apparent M_r of 18 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and 19 060 as calculated from amino acid analysis. It also contains on the order of 7% carbohydrates per mole of enzyme. The N-terminal amino acid sequence was shown to be very different from that of phospholipases isolated from mammalian pancreas and crotalids and elapids snake venoms. The first 39 amino acid residues at the N-terminal region have 56% homology with bee venom phospholipase but differ from the bee phospholipase in that its isoelectric point is acidic (pI = 4.5), instead of basic, and it has approximately 50 amino acid residues more in the molecule. The specificity of the enzyme is mainly A_2 type with possible residual B-type activity. The enzymatic activity is Ca^{2+}-dependent. Half-cystine alignment of the *Heloderma* phospholipase sequence with those of other known phospholipases shows the lack of an octadecapeptide at the N-terminal region, the existence of an extra hexapeptide at positions 42–47, and an exact correspondence of *Heloderma* Gly-12, Gly-14, His-36, and Asp-37 with Gly-30, Gly-32, His-48, and Asp-49 from other phospholipases shown to be important for Ca^{2+} binding [Dijkstra, B. W., Drenth, J., Kalk, K. H., & Vandermaalen, P. J. (1978) *J. Mol. Biol. 124*, 53–60]. The *Heloderma* phospholipase like the bee phospholipase has a Trp at position 10 corresponding to Tyr-28 of other phospholipases, also claimed to be important for calcium binding. Although the present enzyme is structurally very different, it could be sharing similar peptide sequences around the Ca^{2+} binding site of other phospholipases A_2 reported, thus far.

The enzymes with phospholipase A_2 (EC 3.1.1.4) activity are calcium-dependent esterases. They hydrolyze the 2-acyl bond of 3-*n*-phosphoglycerides. Most of the phospholipases A_2 isolated so far, from pancreas, snake venoms, and bee venoms, are relatively small and rigid molecules, having 6–7 disulfide bridges in a protein approximately 125 residues in length (Verheij et al., 1981; Heinrikson, 1982).

Comparison of the primary structure of more than 30 phospholipases has revealed a high degree of homology in the amino acid sequence of these enzymes. Two classes of phospholipases have been proposed: group I comprises phospholipases from pancreatic juice, elapids (cobras), and hydrophids (sea snakes) and group II is composed by phospholipases from many crotalids (rattlesnakes) (Heinrikson et al., 1977). The two different structural groups were separated on the basis of specific disulfide bridges of the molecules.

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