Excess Zinc Ions Are a Competitive Inhibitor for Carboxypeptidase A

Junzo Hirose,* Sonomi Ando, and Yoshinori Kidani
Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori 3-1, Mizuho-ku, Nagoya 467, Japan
Received March 17, 1987; Revised Manuscript Received June 1, 1987

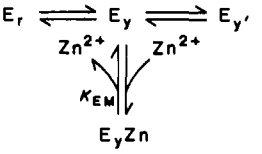
ABSTRACT: The mechanism for inhibition of enzyme activity by excess zinc ions has been studied by kinetic and equilibrium dialysis methods at pH 8.2, $I = 0.5$ M. With carboxypeptidase A (bovine pancreas), peptide (carbobenzoxyglycyl-L-phenylalanine and hippuryl-L-phenylalanine) and ester (hippuryl-L-phenyl lactate) substrates were inhibited competitively by excess zinc ions. The K_i values for excess zinc ions with carboxypeptidase A at pH 8.2 are all similar [$K_i = (5.2–2.6) \times 10^{-5}$ M]. The apparent constant for dissociation of excess zinc ions from carboxypeptidase A was also obtained by equilibrium dialysis at pH 8.2 and was 2.4×10^{-5} M, very close to the K_i values above. With arsanilazotyrosine-248 carboxypeptidase A ([(Azo-CPD)Zn]), hippuryl-L-phenylalanine, carbobenzoxyglycyl-L-phenylalanine, and hippuryl-L-phenyl lactate were also inhibited with a competitive pattern by excess zinc ions, and the K_i values were $(3.0–3.5) \times 10^{-5}$ M. The apparent constant for dissociation of excess zinc ions from arsanilazotyrosine-248 carboxypeptidase A, which was obtained from absorption changes at 510 nm, was 3.2×10^{-5} M and is similar to the K_i values for [(Azo-CPD)Zn]. The apparent dissociation and inhibition constants, which were obtained by inhibition of enzyme activity and spectrophotometric and equilibrium dialysis methods with native carboxypeptidase A and arsanilazotyrosine-248 carboxypeptidase A, were almost the same. This agreement between the apparent dissociation and inhibition constants indicates that the zinc binding to the enzymes directly relates to the inhibition of enzyme activity by excess zinc ions. Excess zinc ions were competitive inhibitors for both peptide and ester substrates. This behavior is believed to arise by the excess zinc ions fixing the enzyme in a conformation to which the substrates cannot bind.

Carboxypeptidase A ([(CPD)Zn],[1] EC 3.4.17.1) catalyzes the hydrolysis of the carbonyl-terminal residue from peptide or ester substrates by cleavage of the peptide or ester bond.

Crystallographic studies indicate that in the enzyme the side-chain oxygen of Tyr-248 is about 17 Å away from the Zn atom and that the phenolic oxygen moves about 12 Å toward the active site as substrates bind (Lipscomb et al., 1968; Reeke et al., 1967). Recent high-resolution crystal structures of native carboxypeptidase A indicate that in the pH range of 7.5–9.5 the enzyme structure is practically unchanged and that the flexible side chain of Tyr-248 remains in the "up" position, away from the zinc ion in the active site, throughout the pH range (Shoham et al., 1984). Kinetics, chemical modification, and X-ray crystallographic data have suggested that tyrosine-248 is one of the catalytic groups, but recent studies of mutagenically modified carboxypeptidase A demonstrate that Tyr-248 is not required for catalysis of substrate cleavage but is important for substrate binding (Hilvert et al., 1986).

Arsanilazotyrosine-248 carboxypeptidase A ([(Azo-CPD)Zn]) (Johansen & Vallee, 1973, 1975; Alter & Vallee, 1978; Harrison & Vallee, 1978) is a derivative with the selectivity modified chromophoric arsanilazotyrosine-248 residue introduced without significantly affecting the enzyme's catalytic properties. It has proven to be particularly useful for studying the conformations of the enzyme (Harrison et al., 1975; Hirose & Wilkins, 1984; Hirose et al., 1985a,b). In the pH range from 7.5 to 8.8, the arsanilazotyrosine residue forms an intramolecular complex with the active site zinc and has a 510-nm peak (Scheule et al., 1977; Bachovchin et al., 1982). The extent of the complex formation is estimated to be about 78% by an optical method (Harrison et al., 1975) and 55% from ^{15}N NMR studies at pH 8.8 (Bachovchin et al., 1982). From these characteristics of the arsanilazotyrosine-248 residue, Vallee et al. proposed that the tyrosine-248 residue was near the zinc ion of the active site (Bachovchin et al., 1982; Scheule et al., 1980; Johansen & Vallee, 1971).

We have previously studied the interaction between arsanilazotyrosine-248 carboxypeptidase A ([(Azo-CPD)Zn]) and excess zinc ions by stopped-flow and spectrophotometric methods (Hirose et al., 1985b). When excess zinc ions bind to arsanilazotyrosine-248 carboxypeptidase A, the characteristic red color, which arises from the intramolecular complex of the arsanilazotyrosine-248 residue with the active site zinc atom of the enzyme, changes to yellow as the arsanilazotyrosine residue is released from the coordination sphere of the zinc ion. The mechanism for binding of excess zinc ions is shown in eq 1 on the basis of the kinetic and equilibrium



(1)

data for [(Azo-CPD)Zn] (Hirose et al., 1985b), where E_r is the red coordination complex, E_y is a yellow azophenol, and $E_{y'}$ is a second yellow conformational state indistinguishable spectrally from E_y. Excess zinc ions react with only the E_y form of the enzyme: The $E_{y'}$ and E_r forms, which are different conformational states, do not bind to zinc ions. Thus zinc binding to [(Azo-CPD)Zn] is a very selective reaction (Hirose et al., 1985b).

The absorbance changes associated with binding of zinc ions to [(Azo-CPD)Zn] were completely consistent with the amount of inhibition of CBZ-Gly-Phe peptidase activity with the following conditions: 1.0 mM CBZ-Gly-Phe at pH 8.2

[1] Abbreviations: [(CPD)Zn], carboxypeptidase A; [(Azo-CPD)Zn], arsanilazotyrosine-248 carboxypeptidase A; CBZ-Gly-Phe, carbobenzoxyglycyl-L-phenylalanine; HPA, hippuryl-L-phenylalanine; HPLA, hippuryl-L-β-phenyl lactate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/87/0426-6561$01.50/0

and 7.7 (0.05 M Tris-HCl, 0.5 M NaCl). The inhibition of native carboxypeptidase A by zinc ions was similar to that of [(Azo-CPD)Zn] (Hirose et al., 1985b). It is known that excess zinc ions inhibit the activity of native enzyme (Vallee et al., 1960; Auld et al., 1970), but the mechanism of inhibition of enzyme activity by excess zinc ions has not been studied.

Therefore, we were interested in the mechanism for inhibition by excess zinc ions of arsanilazotyrosine-248 carboxypeptidase A and native carboxypeptidase A and in the correlation between the color change and the inhibition of the enzyme by excess zinc ions. In this paper, a more detailed study of these relationships is reported.

Materials and Methods

Carboxypeptidase A (bovine pancreas) (COX) ([(CPD)Zn]) was obtained from Sigma as a crystalline suspension. Arsanilazotyrosine-248 carboxypeptidase A ([Azo-CPD)Zn]) was prepared by treatment of carboxypeptidase A crystals with diazotized *p*-arsanilic acid (Johansen & Vallee, 1971) and purified by affinity column chromatography (Bazzone et al., 1979). The enzyme concentration was determined spectrophotometrically by utilizing the following molar absorptivities: [(CPD)Zn], 6.41×10^4 M^{-1} cm^{-1} at 278 nm; [(Azo-CPD)Zn], 7.32×10^4 M^{-1} cm^{-1} at 278 nm (Johansen & Vallee, 1971). Enzyme preparations of lot 24F-8080 were used in all experiments. Carbobenzoxyglycyl-L-phenylalanine (Peptide Institute Inc. Japan) (CBZ-Gly-Phe), hippuryl-L-phenylalanine (Merck) (HPA), and hippuryl-L-β-phenyl lactate (Sekagaku Kogyo Co. Ltd.) (HPLA) were used without further purification. The kinetics of hydrolysis of the substrates by native carboxypeptidase A and arsanilazotyrosine-248 carboxypeptidase A were determined on a Hitachi 557 recording spectrophotometer equipped with a thermostated cell compartment. All kinetic experiments were performed at 25 °C. The wavelength used in following the various reactions were 220–236 nm for CBZ-Gly-Phe, 250–258 nm for HPLA, and 281–289 nm for HPA (Whitaker et al., 1966; Urdea & Legg, 1979; Davies et al., 1978). Infinity values were determined for each reaction solution in order to check the stoichiometry. The enzyme activity kinetics were measured according to a slight variation of the method of Whitaker et al. (1966).

For HPLA kinetics, a 5-cm cuvette was used for low substrate concentrations [$(3.5–9.5) \times 10^{-5}$ M]. All other kinetic experiments were performed in a 1-cm cuvette. Initial rates were calcualted from the linear initial slopes of the change in absorbance vs. reaction time curves where the amount of substrate consumed was always less than 10%. K_m and k_{cat} were determined by computer analysis according to the nonlinear least-squares method (Hashino & Tashiro, 1985).

The equilibrium dialysis cells (capacity 1 mL; Hirose et al., 1981, 1982, 1985a) were assembled by use of a sheet of dialysis membrane. The typical procedure for these experiments was as follows. Approximately 0.8 mL of 1.1×10^{-4} M [(CPD)Zn] in a buffer solution at pH 8.2 was placed in compartment A, and the same volume of various concentrations of zinc ions dissolved in the same buffer was placed in compartment B. Dialysis was allowed to proceed for about 2–3 days on the rotator in a cold room (10 °C). At various intervals, a certain volume of the solution was removed from both compartments, and the zinc content in both compartments was measured by an atomic absorption spectrophotometer, Shimazu AA-630-12.

Results

Inhibition Kinetics of Carboxypeptidase A and Arsanilazotyrosine-248 Carboxypeptidase A by Excess Zinc Ions.

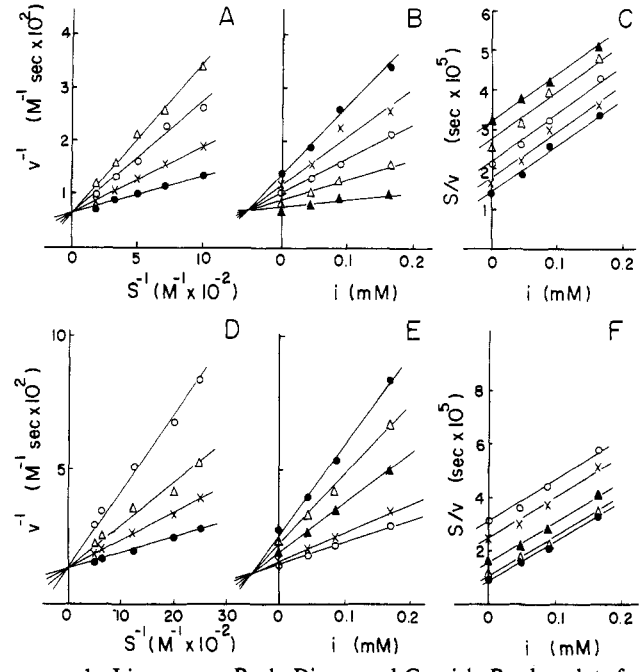


FIGURE 1: Lineweaver–Burk, Dixon, and Cornish–Borden plots for the inhibition of [(Azo-CPD)Zn]- and [(CPD)Zn]-catalyzed hydrolysis of hippuryl-L-phenylalanine by excess zinc ions (pH 8.2, 0.05 M Tris-HCl–0.5 M NaCl buffer, 25 °C). (A) [(CPD)Zn]. Zinc concentrations of (●) 0, (×) 41.7, (○) 83.3, and (△) 167 μM. (B and C) [(CPDZn]. Substrate concentrations of (●) 1, (×) 1.4, (○) 2, (△) 3, and (▲) 4.5 mM. (D) [(Azo-CPD)Zn]. Zinc concentrations of (●) 0, (×) 41.6, (△) 83.3, and (○) 167 μM. (E and F) [(Azo-CPD)Zn]. Substrate concentrations of (●) 0.4, (△) 0.5, (▲) 0.8, (×) 1.5, and (○) 2.0 mM.

Table I: Kinetic Constants for Peptide and Ester Hydrolysis by Arsanilazotyrosine-248 Carboxypeptidase A and Carboxypeptidase A[a]

	[(CPD)Zn]		[(Azo-CPD)Zn]	
	K_m (×10^3 M)	k_{cat} (×10^{-1} s^{-1})	K_m (×10^3 M)	k_{cat} (×10^{-1} s^{-1})
HPA	1.6 ± 0.4	18.2 ± 1.0	0.51 ± 0.04	7.3 ± 1.0
CBZ-Gly-Phe	7.2 ± 0.5	14.2 ± 0.6	1.65 ± 0.3	4.3 ± 0.3
HPLA	0.069 ± 0.02	61.9 ± 7.1	0.053 ± 0.006	7.2 ± 0.3

[a] Assay performed in 0.05 M Tris-HCl containing 0.5 M NaCl (pH 8.2) at 25 °C.

The enzyme activity of [(CPD)Zn] and [(Azo-CPD)Zn] in the presence of excess zinc ions was determined for peptide (HPA and CBZ-Gly-Phe) and ester (HPLA) substrates. Figure 1 shows one set of Lineweaver–Burk, Dixon, and Cornish-Bowden plots for the hydrolysis of HPA from initial rate data, in the absence and presence of excess zinc ions for [(CPD)Zn] and [(Azo-CPD)Zn].

K_m and k_{cat} values for various substrates for [(Azo-CPD)Zn] and [(CPD)Zn], which were calculated by microcomputer, are shown in Table I. With peptide substrates (HPA, CBZ-Gly-Phe), the K_m values for [(Azo-CPD)Zn] are slightly smaller than those of [(CPD)Zn], and the k_{cat} values for [(Azo-CPD)Zn] are one-third of those of [(CPD)Zn]. With ester substrates (HPLA), the K_m of [(Azo-CPD)Zn] was almost the same as that of [(CPD)Zn], but k_{cat} of [(Azo-CPD)Zn] was almost the same as that of [(CPD)Zn], but k_{cat} of [(Azo-CPD)Zn] was one-tenth of that of [(CPD)Zn]. This behavior indicates that the binding strengths of the substrates in [(Azo-CPD)Zn] are almost the same as those in [(CPD)Zn] but that the rates of turnover of substrates by [(Azo-CPD)Zn] (k_{cat}) are slower than those of native carboxypeptidase A. K_m and k_{cat} values of HPLA in [(Azo-CPD)Zn] and [(CPD)Zn]

Table II: Inhibition of Carboxypeptidase A and Arsanilazotyrosine-248 Carboxypeptidase A Catalyzed Hydrolysis of Peptide and Ester Substrates by Excess Zinc Ions and Dissociation Constants of Zinc Ions for Carboxypeptidase A and Arsanilazotyrosine-248 Carboxypeptidase A Obtained by Equilibrium Dialysis and Spectrophotometric Methods at pH 8.2[a]

	[(CPD)Zn]		[(Azo-CPD)Zn]	
	$K_i \pm$ SD (μM)	mode	$K_i \pm$ SD (μM)	mode
(I) enzyme activity				
HPA	52 ± 18	competitive	35 ± 10	competitive
CBZ-Gly-Phe	52 ± 16	competitive	30 ± 6	competitive
HPLA	26 ± 3	competitive	31 ± 5	competitive

	$K_{d(CPD)} \pm$ SD (μM)	$K_{d(Azo\text{-}CPD)} \pm$ SD (μM)
(II) spectrophotometric	20 ± 5	32 ± 5 27 ± 6 (10 °C)
(III) equilibrium dialysis	24 ± 5 (10 °C) 6.3 ± 2 (10 °C, pH 9.0)	

[a] All experiments were performed in 0.05 M Tris-HCl containing 0.5 M NaCl (pH 8.2) at 25 °C.

are similar to those obtained by Urdea and Legg ([(CPD)Zn], $K_m = 4.7 \times 10^{-5}$ M and $k_{cat} = 5.83 \times 10^2$ s^{-1}; [(Azo-CPD)Zn], $K_m = 4.2 \times 10^{-5}$ M and $k_{cat} = 1.4 \times 10^2$ s^{-1}).

Table II contains the values of K_i and the mode of inhibition by zinc ions of [(CPD)Zn] and [(Azo-CPD)Zn]. Intersection points for different sets of straight lines (HPA (Figure 1), HPLA, and CBZ-Gly-Phe) in Lineweaver–Burk plots of [(CPD)Zn] and [(Azo-CPD)Zn] were on the $1/v$ axis, and the Cornish–Borden plots of the same data had apparently parallel lines. This behavior indicates that zinc ion is a competitive inhibitor for both peptide (HPA, CBZ-Gly-Phe) and ester (HPLA) substrates with the native enzyme ([(CPD)Zn]) and the arsanilazo enzyme ([(Azo-CPD)Zn]). The K_i values for zinc ions from Dixon plots are shown in Table II. These values are similar to those obtained from Lineweaver–Burk plots. The K_i values for zinc ions for HPLA, HPA, and CBZ-Gly-Phe with [(CPD)Zn] vary little [$K_i = (5.2\text{–}2.6) \times 10^{-5}$ M]. The K_i values for zinc ions with HPLA, HPA, and CBZ-Gly-Phe in [(Azo-CPD)Zn] are also nearly the same [$K_i = (3.0\text{–}3.5) \times 10^{-5}$ M]. Finally, K_i values for [(CPD)Zn] are similar to those of [(Azo-CPD)Zn].

Constants for Dissociation of Excess Zinc Ions from [(CPD)Zn] and [(Azo-CPD)Zn]. Vallee proposed that [(Azo-CPD)Zn] consists of three different conformations at pH 7.5–8.5 (E_r, E_y, E_y') (Harrison et al., 1975). In the previous paper, the mechanism in eq 1 for zinc binding to [(Azo-CPD)Zn] was proposed on the basis of kinetic and equilibrium data.

On the basis of eq 1, the apparent constant for dissociation of zinc ions from [(Azo-CPD)Zn]Zn^{2+} is given by eq 2.

$$K_{d(Azo\text{-}CPD)} = \frac{([E_r] + [E_y] + [E_y'])[Zn^{2+}]}{[E_yZn]} = \frac{([E_T] - [E_yZn])[Zn^{2+}]}{[E_yZn]} \quad (2)$$

$K_{d(Azo\text{-}CPD)}$ was easily obtained by the spectral titration methods (Table II). The value, 3.2×10^{-5} M, is similar to K_i [$(3.5\text{–}2.4) \times 10^{-5}$ M] obtained by the enzyme inhibition method for [(Azo-CPD)Zn] and is almost independent of temperature (Table II).

In order to probe the binding of zinc ions to [(CPD)Zn], the apparent dissociation constant ($K_{d(CPD)}$) of excess zinc ions

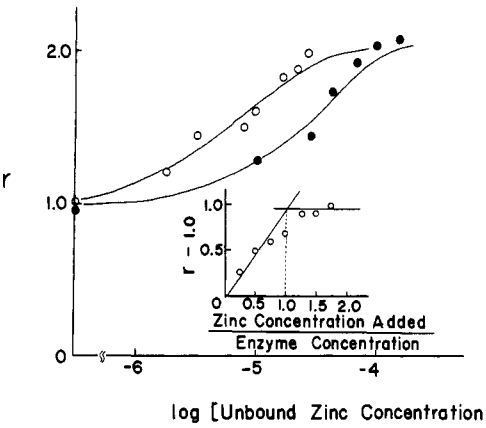


FIGURE 2: Relationship between r and the concentration of unbound zinc ion. Conditions: 0.05 M Tris-HCl, 0.5 M NaCl, and 10 °C. (○) pH 9.0; (●) pH 8.0.

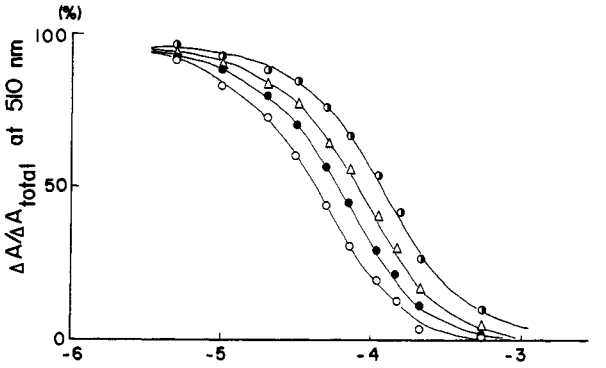


FIGURE 3: Competitive reaction between [(Azo-CPD)Zn] and [(CPD)Zn] for zinc binding at pH 8.2. [(Azo-CPD)Zn], 2.42×10^{-5} M; 0.05 M Tris-HCl buffer containing 0.5 M NaCl, 25 °C. [(CPD)Zn] concentration of (○) 0, (●) 2.07×10^{-5}, (△) 4.14×10^{-5}, and (◑) 8.28×10^{-5} M.

from the native enzyme ([(CPD)Zn]$Zn^{2+} \rightleftharpoons$ [(CPD)Zn] + Zn^{2+}) was determined by equilibrium dialysis. These experiments were performed at pH 8.2 and 9.0, and the data are shown in Figure 2. At pH 9.0, the apparent dissociation constant was very small, so that the molar ratio method showed a good inflection point and the molar ratio between excess zinc ions and [(CPD)Zn] is 1:1 (Figure 2). The relationship between the logarithm of the concentration of unbound zinc obtained from the zinc concentration in the ligand chamber and r (the number of zinc ions bound to 1 mol of enzyme), which was calculated from the difference between the zinc concentrations in the enzyme and ligand chambers, is sigmoidal as shown in Figure 2. The theoretical curve calculated on the basis of one zinc ion binding to [(CPD)Zn] was in very good agreement with the data points obtained by the equilibrium dialysis method. The apparent dissociation constant was adjusted so as to obtain the best fit between the theoretical curve and experimental data and is shown in Table II. The apparent dissociation constant of zinc ions from [(CPD)Zn]Zn^{2+} at pH 9.0 was larger than that at pH 8.2. The pH dependence of the apparent dissociation constant of the zinc ions is almost the same as that for [(Azo-CPD)Zn] and indicates that few protons participate in the binding of zinc ions to the enzyme.

The apparent dissociation constant for the excess zinc ions from [(CPD)Zn]Zn^{2+} was also spectrophotometrically measured by use of competition between [(CPD)Zn] and [(Azo-CPD)Zn] for excess zinc ions. Figure 3 shows that a higher concentration of excess zinc ions was needed to obtain the same absorbance change on addition to [(Azo-CPD)Zn] in the

presence of a higher concentration of [(CPD)Zn]. In eq 3,

$$[\text{(Azo-CPD)Zn}]\text{Zn}^{2+} \underset{-[\text{(Azo-CPD)Zn}]}{\overset{+[\text{(Azo-CPD)Zn}]}{\rightleftharpoons}} \underset{K_{d(\text{Azo-CPD})}}{\text{Zn}^{2+}} \underset{-[\text{(CPD)Zn}]}{\overset{+[\text{(CPD)Zn}]}{\rightleftharpoons}} \underset{K_{d(\text{CPD})}}{[\text{(CPD)Zn}]\text{Zn}^{2+}} \quad (3)$$

the apparent dissociation constant of excess zinc ions ($K_{d(\text{Azo-CPD})}$) from [(Azo-CPD)Zn]Zn^{2+} was known, so that the $K_{d(\text{CPD})\text{Zn}}$ is easily calculated from the data in Figure 3 and is shown in Table II. This value is very similar to that obtained by equilibrium dialysis methods.

Discussion

The apparent constants for dissociation and inhibition by zinc ions of [(Azo-CPD)Zn] and [(CPD)Zn] are shown in Table II. The $K_{d(\text{Azo-CPD})}$ obtained by the spectrophotometric method is 3.2×10^{-5} M and is similar to the K_i [$(3.0–3.5) \times 10^{-5}$ M] obtained by the kinetic method of enzyme activity in [(Azo-CPD)Zn]. The apparent constant for dissociation of excess zinc ions from [(CPD)Zn]Zn^{2+} obtained by the equilibrium dialysis and competitive reaction method is also similar to the K_i values [$(5.2–2.6) \times 10^{-5}$ M]. This agreement between K_i and K_d in [(Azo-CPD)Zn] and [(CPD)Zn] indicates that the zinc binding to the enzymes directly correlates with inhibition of the enzyme activity and suggests that the zinc binding site for inhibition is on the enzyme and not on the substrates.

In the previous paper (Hirose et al., 1985b), it was indicated that the absorbance changes associated with zinc ions binding to [(Azo-CPD)Zn] could be related directly to the extent of inhibition of enzyme activity toward CBZ-Gly-Phe (1.0 mM of CBZ-Gly-Phe was used in this experiment) by the zinc ions. This was a fortuitous result, because K_m (Table I) for CBZ-Gly-Phe is coincidently larger than the substrate concentration used in the enzyme activity measurement, so the apparent correlation was observed.

From the enzyme kinetics, the inhibition pattern by excess zinc ions was competitive for both ester and peptide substrates. This requires that the enzyme, to which zinc ions have bound, cannot bind the peptide and ester subtrates. Many competitive inhibitors that are substrate analogues (Ludwig & Lipscomb, 1973; Galardy & Kortylewicz, 1984, 1985) are known for carboxypeptidase A. A zinc ion is a very different type of competitive inhibitor, because it is a metal ion and competitive for both ester and peptide substrates. Why is excess zinc ion a competitive inhibitor for the substrates? In the previous paper, it was found that zinc ions can only bind to the E_y form of the enzyme and thus fix the enzyme in the one conformational state (E_y form in [(Azo-CPD)Zn]). If the substrates cannot bind to E_yZn, then zinc ions would be a competitive inhibitor for the substrates. Why cannot E_yZn bind to the substrates? There are two possibilities: one is that the excess zinc ions bind to some residues in the active site that are very important for substrate binding and therefore block their binding; another is that the excess zinc ions bind to residues that are far from the active site and change the conformation of the enzyme to one to which the substrates cannot bind.

In carboxypeptidase G_2, the zinc complexes of some triazine dyes form a ternary complex with some residues in the active site of the enzyme (Hughes et al., 1984). The ternary complex is enzymatically inactive, the Zn–dye acting as a competitive inhibitor. In acid proteases, the zinc complex of pyridine-2-azo-*p*-dimethylaniline forms a quaternary complex and is bound to two carboxylate groups in the active site, which are part of the substrate-binding site of the enzyme (Nakatani et al., 1975). From the evidence presented in this paper, it is impossible to decide the binding site of the excess zinc ions.

In [(Azo-CPD)Zn], the E_y form only can bind to zinc ions as shown in eq 1, and the proportion of the three conformational states will directly influence the binding constant for the excess zinc ions to the enzyme. The apparent dissociation constant for the zinc ions in [(CPD)Zn]Zn^{2+} is almost the same as that in [(Azo-CPD)Zn]Zn^{2+}. This behavior indicates that the conformational states (E_y, E_r, and $E_{y'}$) in [(Azo-CPD)Zn] are also present in [(CPD)Zn]. Therefore, it is suggested that the arsanilazo residue does not influence the conformational states of the enzyme. Recent ^{113}Cd NMR data also suggest that there are several conformational states present in Cd carboxypeptidase A (Gettins, 1986). It is possible that the tyrosine-248 residue is near the zinc ion in the active site to the same extent.

Acknowledgments

We thank Professor H. Ikezawa at Nagoya City University and Professor R. G. Wilkins at New Mexico State University for many helpful comments. We also thank Professor I. Bertini and Dr. R. Monnani at Firenze University and Dr. S. Nagai and Professor J. Sakakibara at Nagoya City University for many suggestions for preparing the affinity chromatography column for carboxypeptidase A.

Registry No. CPD, 11075-17-5; HPA, 744-59-2; HPLA, 3675-74-9; Cbz-Gly-Phe, 1170-76-9; Zn, 7440-66-6.

References

Alter, G. M., & Vallee, B. L. (1978) *Biochemistry 17*, 2212–2218.

Auld, D. S., & Vallee, B. L. (1970) *Biochemistry 9*, 4352–4359.

Bachovchin, W. W., Kanamori, K., Vallee, B. L., & Roberts, J. D. (1982) *Biochemistry 21*, 2885–2892.

Bazzone, T. J., Sakolovsky, M., Vallee, B. L., & Roberts, J. D. (1979) *Biochemistry 18*, 4362–4366.

Davies, R. C., Riordan, J. F., Auld, D. S., & Vallee, B. L. (1968) *Biochemistry 7*, 1090–1099.

Galady, R. E., & Kortylewicz, Z. P. (1984) *Biochemistry 23*, 2083–2087.

Galady, R. E., & Kortylewicz, Z. P. (1985) *Biochemistry 24*, 7607–7617.

Gettins, P. (1986) *J. Biol. Chem. 261*, 15513–15518.

Harrison, L. W., & Vallee, B. L. (1978) *Biochemistry 17*, 4359–4363.

Harrison, L. W., Auld, D. S., & Vallee, B. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4356–4360.

Hashino, H., & Tashiro, M. (1985) *Kagaku (Kyoto) 40*, 1–8.

Hilvert, D., Gradell, S. J., Rutter, W. J., & Kaiser, E. T. (1986) *J. Am. Chem. Soc. 108*, 5298–5304.

Hirose, J., & Wilkins, R. G. (1984) *Biochemistry 23*, 3149–3153.

Hirose, J., Iwatsuka, K., & Kidani, Y. (1981) *Biochem. Biophys. Res. Commun. 15*, 58–65.

Hirose, J., Ohhira, T., Hirata, H., & Kidani, Y. (1982) *Arch. Biochem. Biophys. 718*, 179–186.

Hirose, J., Hayakawa, C., Noji, M., & Kidani, Y. (1985a) *Inorg. Chim. Acta 107*, L7–L10.

Hirose, J., Noji, M., Kidani, Y., & Wilkins, R. G. (1985b) *Biochemistry 24*, 3495–3502.

Hughes, P., Sherwood, R. F., & Lowe, C. R. (1984) *Eur. J. Biochem. 144*, 135–142.

Johansen, J. T., & Vallee, B. L. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2532–2535.

Johansen, J. T., & Vallee, B. L. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2006–2010.

Johansen, J. T., & Vallee, B. L. (1975) *Biochemistry 14*, 649–659.
Lipscomb, W. N., Hartsuck, J. A., Reeke, G. N., Quincho, F. A., Betnge, P. H., Ludwig, M. L., Steiz, T. A., Muirhead, H., & Coppola, J. C. (1968) *Brookhaven Symp. Biol. 21*, 24–90.
Ludwig, M., & Lipscomb, W. N. (1973) *Inorganic Biochemistry*, Vol. 1, pp 438–487, Elsevier, Amsterdam.
Nakatani, H., Hiromi, K., Satoi, S., Oda, K., Murano, S., & Ichishima, E. (1975) *Biochim. Biophys. Acta 391*, 415–421.
Reeke, G. N., Hartsuck, J. A., Ludwing, M. L., Quiocho, F. A., Steitz, T. A., & Lipscomb, W. N. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 2220–2226.
Scheule, R. K., VanWart, H. E., Vallee, B. L., & Scheraga, H. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3273–3277.
Scheule, R. K., VanWart, H. E., Vallee, B. L., & Scheraga, H. A. (1980) *Biochemistry 19*, 759–766.
Shoham, G., Rees, D. C., & Lipscomb, W. N. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7767–7771.
Urdea, S. M., & Legg, J. I. (1979) *Biochemistry 18*, 4985–4991.
Vallee, B. L., Rupley, J. A., Coombs, T. L., & Neurath, H. (1960) *J. Biol. Chem. 235*, 64–67.
Whitaker, J. R., Menger, F., & Bender, M. (1966) *Biochemistry 5*, 386–392.

Effects of pH on Allosteric and Catalytic Properties of the Guanosine Cyclic 3′,5′-Phosphate Stimulated Cyclic Nucleotide Phosphodiesterase from Calf Liver

Hideo Wada,[‡] James C. Osborne, Jr.,[§] and Vincent C. Manganiello*,[‡]

Laboratory of Cellular Metabolism and Molecular Disease Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892, and Spinco Division, Beckman Instruments, Inc., Palo Alto, California 94304

Received December 26, 1986; Revised Manuscript Received May 8, 1987

ABSTRACT: We have investigated effects of pH on the catalytic and allosteric properties of the cGMP-stimulated cyclic nucleotide phosphodiesterase purified from calf liver. In the "activated" state, i.e., with 0.5 μM [^{3}H]cAMP plus 1 μM cGMP or at saturating substrate concentrations (250 μM [^{3}H]cAMP or [^{3}H]cGMP), hydrolysis was maximal at pH 7.5–8.0 in assays of different pH. Hydrolysis of concentrations of substrate not sufficient to saturate regulatory sites and below the apparent Michaelis constant (K_m^{app}), i.e., 0.5 μM [^{3}H]cAMP or 0.01 μM [^{3}H]cGMP, was maximal at pH 9.5. Although hydrolysis of 0.5 μM [^{3}H]cAMP increased with pH from 7.5 to 9.5, cGMP stimulation of cAMP hydrolysis decreased. As pH increased or decreased from 7.5, Hill coefficients (n_{app}) and V_{max} for cAMP decreased. Thus, assay pH affects both catalytic (V_{max}) and allosteric (n_{app}) properties. Enzyme was therefore incubated for 5 min at 30 °C in the presence of $MgCl_2$ at various pHs before assay at pH 7.5. Prior exposure to different pHs from pH 6.5 to 10.0 did not alter the V_{max} or cGMP-stimulated activity (assayed at pH 7.5). Incubation at high (9.0–10.0) pH did, in assays at pH 7.5, markedly increase hydrolysis of 0.5 μM [^{3}H]cAMP and reduce K_m^{app} and n_{app}. After incubation at pH 10, hydrolysis of 0.5 μM [^{3}H]cAMP was maximally increased and was similar in the presence or absence of cGMP. Thus, after incubation at high pH, the phosphodiesterase acquires characteristics of the cGMP-stimulated form. Activation at high pH occurs at 30 °C but not 5 °C, requires $MgCl_2$, and is prevented but not reversed by ethylenediaminetetraacetic acid. These results indicate that incubation at high pH in the absence of substrates and/or effectors promotes allosteric transitions (n_{app}) and a decrease in K_m^{app} in the absence of changes in V_{max} and suggest independent regulation of topographical features and domains responsible for these properties.

A cGMP-stimulated cyclic nucleotide phosphodiesterase (PDE)[1] has been purified from bovine heart, adrenal, and liver tissue (Martins et al., 1982; Yamamoto et al., 1983a). The enzyme hydrolyzes both cAMP and cGMP with positively cooperative kinetics. At substrate concentrations well below the apparent K_m (K_m^{app}), hydrolysis of one cyclic nucleotide is stimulated by the other; cGMP is the preferred substrate or effector (Martins et al., 1982; Yamamoto et al., 1983a; Moss et al., 1977). Earlier studies have suggested that substrates and effectors induce allosteric transitions that lead to enhanced catalytic activity as well as increased susceptibility to proteolytic attack (Moss et al., 1977). As might be expected from the cooperative behavior of the PDE, certain substrate analogues and competitive inhibitors can, like substrates, induce allosteric transitions that result in "activation" of the PDE and stimulation of hydrolysis of low substrate concentrations. Substrate analogues and inhibitors can also compete with substrate at catalytic sites and competitively inhibit hydrolysis of substrate by the activated form of the enzyme (Moss et al.,

* Correspondence should be addressed to this author.
‡ Laboratory of Cellular Metabolism, National Institutes of Health.
§ Molecular Disease Branch, National Institutes of Health, and Spinco Division, Beckman Instruments, Inc.

[1] Abbreviations: PDE, phosphodiesterase; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; IBMX, isobutylmethylxanthine; K_m^{app}, apparent Michaelis constant; n_{app}, apparent number of cooperatively interacting sites (Hill coefficient); K_I, inhibition constant; EDTA, ethylenediaminetetraacetic acid; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BICINE, *N*,*N*-bis(2-hydroxyethyl)glycine; MES, 4-morpholineethanesulfonic acid.