Interaction of Zinc Ions with Arsanilazotyrosine-248 Carboxypeptidase A

Junzo Hirose,* Masahide Noji, and Yoshinori Kidani

Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori 3-1 Mizuho-ku, Nagoya 467, Japan

Ralph G. Wilkins

Department of Chemistry, New Mexico State University, Las Cruces, New Mexico 88003

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ABSTRACT: The interaction between arsanilazotyrosine-248 carboxypeptidase A ([(Azo-CPD)Zn]) and excess zinc ions has been studied by stopped-flow and spectrophotometric methods at pH 8.2 and 7.7, $I = 0.5$ M (NaCl), and 25 °C. 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 of the enzyme, changes to yellow with the inhibition of peptidase activity of the enzyme. Excess zinc ions have two binding sites for arsanilazotyrosine-248 carboxypeptidase A, and the binding constants of the first site (3.9×10^5 M^{-1} at pH 8.2; 7.1×10^4 M^{-1} at pH 7.7) are much larger than those of the second site (1.8×10^3 M^{-1} at pH 8.2; 7×10^2 M^{-1} at pH 7.7). The binding of excess zinc ions to the first site is completely correlated with the inhibition of the enzyme peptidase activity and the color change of the enzyme. The results can be understood in terms of zinc ions reacting with only one of three conformational states of arsanilazotyrosine-248 carboxypeptidase A [Harrison, L. W., Auld, D. S., & Vallee, B. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4356]. The second-order rate constants for the binding of excess zinc ions to arsanilazotyrosine-248 carboxypeptidase A ([(Azo-CPD)Zn] + Zn^{2+} → [(Azo-CPD)-Zn]·Zn^{2+}) are 4.3×10^6 and 8.4×10^5 M^{-1} s^{-1}, at pH 8.2 and 7.7, respectively, and the first-order rate constants for the dissociation of zinc ions from the adduct between zinc ions and arsanilazotyrosine-248 carboxypeeptidase A ([(Azo-CPD)Zn]·Zn^{2+} → [(Azo-CPD)Zn] + Zn^{2+}) are 11 and 12 s^{-1}, respectively. It was proved that excess zinc ions promoted the inhibition of the peptidase activity and the color change from red to yellow through binding specifically to one conformational state of arsanilazotyrosine-248 carboxypeptidase A.

X-ray diffraction study of carboxypeptidase A which is bound to the pseudosubstrate Gly-L-Tyr shows that the Tyr-248 residue interacts with Gly-L-Tyr through a hydrogen bond (Lipscomb et al., 1968). However, in the absence of Gly-L-Tyr, X-ray diffraction has localized the Tyr-248 residue at the surface of the enzyme some 12 Å removed from the proposed catalytic site (Reeke et al., 1967; Lipscomb et al., 1968). The remarkable movement of Tyr-248 to the active site of binding of Gly-L-Tyr has been cited as an example of the induced fit theory.

Arsanilazotyrosine-248 carboxypeptidase A ([(Azo-CPD)Zn])[1] (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 particularly useful for studying the conformations of the enzyme. In pH 7.5–8.8, the arsanilazotyrosine-248 residue forms an intramolecular complex with the active site zinc (Scheule et al., 1977; Bachovchin et al., 1982) and gives rise to a 510-nm peak. The extent of the complexation is estimated to be about 78% (Harrison et al., 1975) by an optical method and 55% (Bachovchin et al., 1982) from ^{15}N NMR studies at pH 8.8. From these characteristics of the arsanilazotyrosine-248 residue, Vallee et al. proposed that the tyrosine-248 residue was present near the zinc ion of the active site (Bachovchin et al., 1982; Scheule et al., 1980; Johansen & Vallee, 1971). An intramolecular complex (red color) of the arsanilazotyrosine-248 residue with the zinc ion in the active site of the enzyme is very sensitive to ligand interaction and the conformational changes of the enzyme (Harrison et al., 1975; Quiocho et al., 1972).

We have previously studied the kinetics of the interaction of a variety of cobalt(II) and zinc(II) complexes with apoarsanilazotyrosine-248 carboxypeptidase A ([(Azo-CPD)]) (Hirose & Wilkins, 1984). In this reaction, the uptake of Zn^{2+} by apoazoprotein is triphasic. The very fast reaction is the binding of zinc ions to the active site of apoazoprotein ([(Azo-CPD)] + Zn^{2+} → [(Azo-CPD)Zn]), and the last two stages represent the interaction between excess zinc ions and azoenzyme:

$$[(\text{Azo-CPD})\text{Zn}] \xrightarrow{Zn^{2+}} [(\text{Azo-CPD})\text{Zn}]\cdot Zn^{2+} \xrightarrow{Zn^{2+}} [(\text{Azo-CPD})\text{Zn}]\cdot Zn_2^{2+}$$

Spectral examination showed that the red color which is characteristic of the intramolecular complex of the arsanilazotyrosine residue changed to yellow in the presence of excess zinc ions. This behavior indicates that a conformational change of arsanilazotyrosine-248 carboxypeptidase A occurs in the azoenzyme. An inhibition of native enzyme activity by excess Zn^{2+} has been reported (Vallee et al., 1960; Auld & Vallee, 1970) and is presumably due to excess zinc binding to the protein with concomitant changes at the active site. Therefore, in this paper, we studied the interaction between excess zinc ions and azoprotein ([(Azo-CPD)Zn]) by equilibrium and kinetic methods.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; [(Azo-CPD)Zn], arsanilazotyrosine-248 carboxypeptidase A; [(Azo-CPD)], apoarsanilazotyrosine-248 carboxypeptidase A; MES, 2-(*N*-morpholino)ethanesulfonic acid; CBZ-Gly-Phe, *N*-carbobenzoxyglycyl-L-phenylalanine; EDTA, ethylenediaminetetraacetic acid.

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Materials and Methods

Carboxypeptidase A_α (Cox) was obtained from Sigma as a crystalline suspension and used without further purification. Arsanilazotyrosine-248 carboxypeptidase A ([(Azo-CPD)Zn]) was prepared by treatment of carboxypeptidase crystals with diazotized *p*-arsanilic acid (Johansen & Vallee, 1971). Apoarsanilazotyrosine-248 carboxypeptidase ([(Azo-CPD)]) was prepared by suspending crystals of the arsanilazotyrosine-248 carboxypeptidase A (5 mg/mL) in 10^{-2} M, 1,10-phenanthroline and 0.01 M MES buffer at pH 7.0 and 25 °C for 6 h followed by washing for 0.5 h with 0.01 M MES buffer at pH 7.0. The process was repeated 4 times (Auld & Holmquist, 1974; Bachovchin et al., 1982). In our hands, the demetallation took longer than described in the literature. The product showed 5–10% activity toward CBZ-Gly-Phe substrate (Coleman & Vallee, 1960). The enzyme activity was measured by a spectrophotometric method (at 223 nm) and using CBZ-Gly-Phe (10^{-3} M, 0.01 M Tris-HCl buffer, pH 7.5, and 0.5 M NaCl) as substrate (Billo et al., 1978, Urdea & Legg, 1979). Protein concentration was determined spectrally [$\lambda_{278} = 7.32 \times 10^4$ M^{-1} cm^{-1} (Johansen & Vallee, 1971)]. All other reagents were reagent grade and used without further purification. Buffer solutions were treated with 0.005% dithizone in CCl_4 to remove traces of metal ions. Stock solutions of Zn^{2+} ions were standardized by EDTA. The interaction of zinc ions with arsanilazotyrosine-248 carboxypeptidase A was measured on a Union Giken stopped-flow apparatus interfaced with a data-collecting system for data acquisition and manipulation. The formation and dissociation of zinc ions from the adduct between [(Azo-CPD)Zn] and excess Zn^{2+} were monitored at 510 nm. All studies were carried out at pH 8.2, I = 0.5 M, and 25 °C.

Results

Effect of Excess Zinc Ions on the Absorption of Arsanilazotyrosine-248 Carboxypeptidase A. The addition of excess zinc ions to arsanilazotyrosine-248 carboxypeptidase A ([(Azo-CPD)Zn]) affected its absorption spectrum. The spectra that resulted when various concentrations of excess zinc ions were added to [(Azo-CPD)Zn] at pH 7.7, 8.2, and 9.0 are shown in Figure 1. The absorbance around 510 nm decreased with increasing concentration of free zinc ions. At pH 9.0, λ_{max} was shifted to 485 nm. In each spectrum, there is a single isosbestic point at 445 (pH 7.7), 455 (pH 8.2) and 475 nm (pH 9.0). These results indicate that two interconvertible species are present with these conditions. To determine the reaction ratio between [(Azo-CPD)Zn] and zinc ions, the molar ratio method was used and is shown in Figure 1C. In Figure 1C, the absorbance changes at 510 nm in pH 9.0 were plotted vs. the ratio of (added zinc concentration)/(concentration of [(Azo-CPD)Zn]). Data at pH 9.0 were used, because the binding constant of zinc ions to [(Azo-CPD)Zn] increased with increasing pH. The molar ratio method showed that the molar ratio between excess zinc ions and [(Azo-CPD)Zn] was 1/1 in the main reaction. Therefore, the following equation holds for the main reaction.

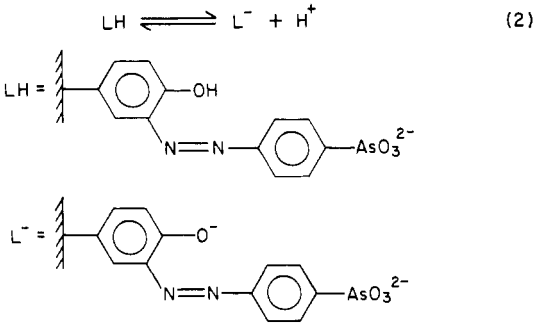
$$[(\text{Azo-CPD})\text{Zn}] + Zn^{2+} \rightleftarrows [(\text{Azo-CPD})\text{Zn}]\cdot Zn^{2+} \quad (1)$$

In Figure 2, the absorbance change ratios ($\Delta A/\Delta A_{total}$ at 510 nm) at pH 7.7 and 8.2 were plotted as a function of concentration of free zinc ions. At pH 9.0, the binding constant of excess zinc ions to [(Azo-CPD)Zn] was large, so that it is impossible to calculate accurate concentrations of free zinc ions. Therefore, the relationship between $\Delta A/\Delta A_{total}$ and free zinc concentration at pH 9.0 is not shown in Figure 2.

Inhibition of Peptidase Activity of Arsanilazotyrosine-248 Carboxypeptidase A and Carboxypeptidase A by Zinc Ions. The peptidase activity of the enzyme was measured by using CBZ-Gly-Phe as a substrate (1.0 mM CBZ-Gly-Phe and 0.05 M Tris-HCl buffer (0.5 M NaCl), 25 °C) at pH 7.7, 8.2, and 9.0. The (activity with zinc ions)/(activity without zinc ions) ratios are plotted as a function of free zinc concentrations in Figure 2. At pH 7.7 and 8.2, the inhibitions of carboxypeptidase A and [(Azo-CPD)Zn] activities by zinc ions were directly proportional with absorbance changes at 510 nm of [(Azo-CPD)Zn]. This result clearly indicates that the binding of zinc ions to the enzyme correlates with the inhibition of the enzyme activity.

The inhibition behavior of native carboxypeptidase A was completely consistent with that of [(Azo-CPD)Zn] (Figure 2). This result implies that the arsanilazotyrosine residue in [(Azo-CPD)Zn] does not affect the binding of excess zinc ions to the enzyme.

Spectra of the Arsanilazotyrosine-248 Carboxypeptidase A–Zn^{2+} Adduct at Various pHs. To clarify the spectral changes of [(Azo-CPD)Zn] with increasing excess zinc ion concentrations, the spectra of the adduct between [(Azo-CPD)Zn] and Zn^{2+} were measured at various pH's (7.6–12.0) in the presence of excess zinc ions (1.2×10^{-4} M). Figure 3A shows the spectral changes with pH. There was a single isosbestic point at 420 nm, and λ_{max} was at 485 nm. This result indicates that only two interconvertible species are present with these conditions. The shape of the spectrum, λ_{max} (485 nm) and isosbestic point (420 nm), was exactly that which was reported for apoarsanilazotyrosine-248 carboxypeptidase A [[(Azo-CPD)]] (Johansen & Vallee, 1973). The relationship between the absorbance change of the [(Azo-CPD)Zn]·Zn^{2+} adduct and pH is shown in Figure 3B. The pK_a value of the [(Azo-CPD)Zn]·Zn^{2+} adduct is 8.9 (0.05 M Tris-HCl buffer and 0.5 M NaCl). The pK_a of [(Azo-CPD)] was also measured under the same conditions, and it is 9.3. This pK_a value is almost the same as that of the [(Azo-CPD)Zn]·Zn^{2+} adduct. Therefore, in the presence of excess of zinc ions, the arsanilazotyrosine residue is released from the coordination sphere of the active site zinc of [(Azo-CPD)Zn], so that the changes in the spectra of the [(Azo-CPD)Zn]·Zn^{2+} adduct with pH resemble those of [(Azo-CPD)], and the pK_a value, the isosbestic point, and the shape of the spectra in th [(Azo-CPD)Zn]·Zn^{2+} adduct are similar to those for the apoenzyme ([(Azo-CPD)]). This behavior is expressed by the equation:



Kinetics of Zinc Binding to Arsanilazotyrosine-248 Carboxypeptidase A. The kinetics of excess zinc binding to [(Azo-CPD)Zn] was monitored by the stopped-flow method. When zinc ions were added to [(Azo-CPD)Zn], two reactions were observed at pH 7.7 and 8.2 (Figure 4). In the fast stage, only a decreasing absorbance was observed, and the reaction was pseudofirst order as shown in Figure 4A. The pseudo-first-order rate constants of the fast stage increased with in-

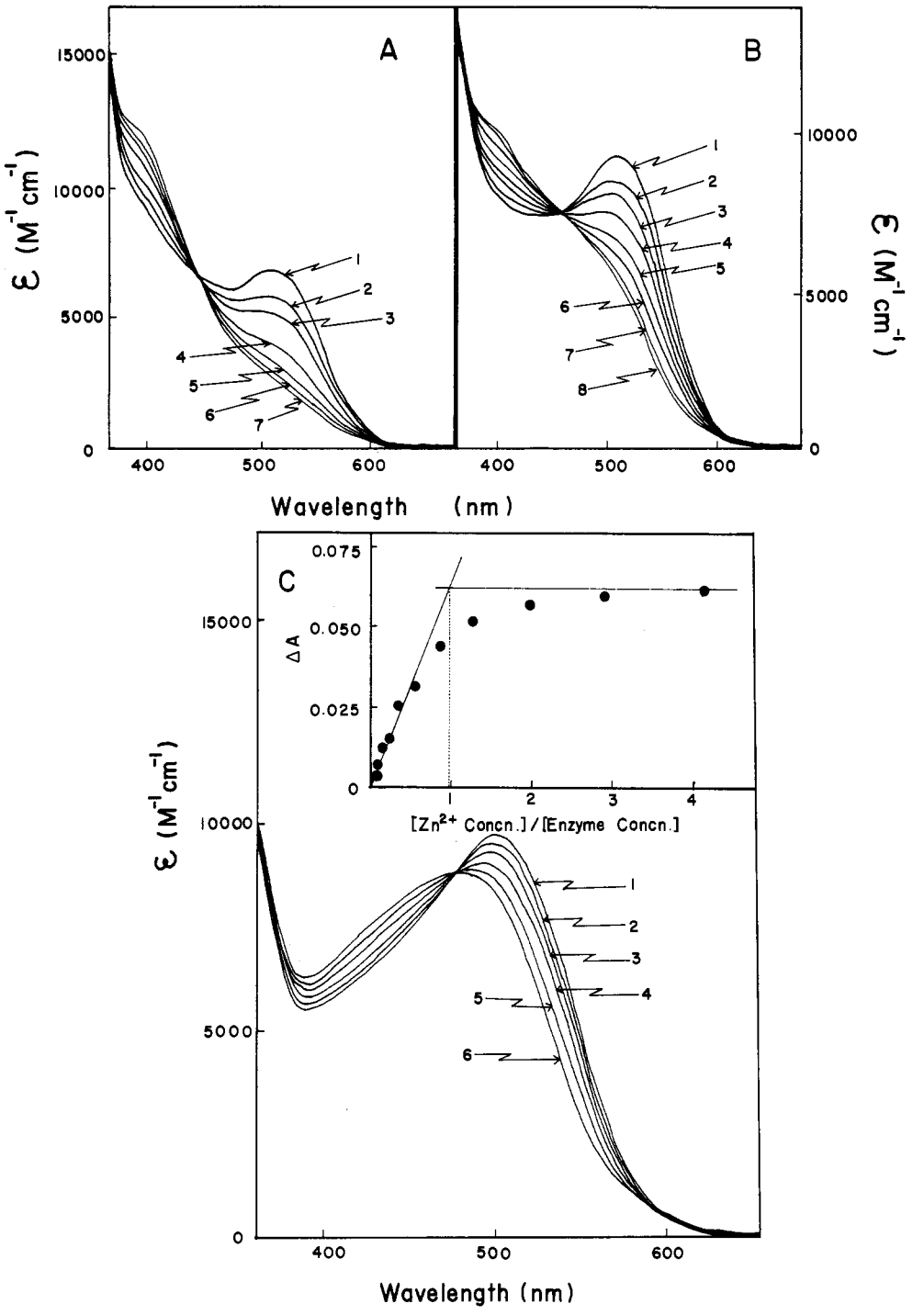


FIGURE 1: Effect of zinc ions on the absorption spectrum of arsanilazotyrosine-248 carboxypeptidase A. (A) pH 7.7, 0.05 M Tris-HCl buffer (0.5 M NaCl). (1) Without zinc ions; (2) $[Zn^{2+}] \doteq 2.0 \times 10^{-5}$ M; (3) $[Zn^{2+}] = 4.0 \times 10^{-5}$ M; (4) $[Zn^{2+}] = 6.0 \times 10^{-5}$ M; (5) $[Zn^{2+}] = 1.4 \times 10^{-4}$ M; (6) $[Zn^{2+}] = 1.8 \times 10^{-4}$ M; (7) $[Zn^{2+}] = 3.8 \times 10^{-4}$ M; [[(Azo-CPD)Zn] = 2.45×10^{-5} M. Temperature 25 °C. (B) pH 8.2, 0.05 M Tris-HCl buffer (0.5 M NaCl). (1) Without zinc ions; (2) $[Zn^{2+}] = 10^{-5}$ M; (3) $[Zn^{2+}] = 2 \times 10^{-5}$ M; (4) $[Zn^{2+}] = 3.0 \times 10^{-5}$ M; (5) $[Zn^{2+}] = 5.0 \times 10^{-5}$ M; (6) $[Zn^{2+}] = 7.0 \times 10^{-5}$ M; (7) $[Zn^{2+}] = 1.5 \times 10^{-4}$ M; (8) $[Zn^{2+}] = 3.5 \times 10^{-4}$ M; [[(Azo-CPD)Zn]] = 3.4×10^{-5} M. Temperature 25 °C. (C) pH 9.0, 0.05 M Tris-HCl buffer (0.5 M NaCl). (1) Without zinc ions; (2) $[Zn^{2+}] = 2.0 \times 10^{-6}$ M; (3) $[Zn^{2+}] = 6.0 \times 10^{-6}$ M; (4) $[Zn^{2+}] = 1.1 \times 10^{-5}$ M; (5) $[Zn^{2+}] = 2.6 \times 10^{-5}$ M; (6) $[Zn^{2+}] = 7.6 \times 10^{-5}$ M; [[(Azo-CPD)Zn]] = 3.0×10^{-5} M. Temperature 25 °C.

creasing zinc concentration (Figure 5A). On the other hand, the slow stage was complex (Figure 4B). At low zinc concentration, an increase in absorbance at 510 nm was observed (Figure 4B), but the ΔA decreased with increasing zinc concentration. Finally, at high concentration of excess zinc ions, a decrease in absorbance was observed. The slow reaction was pseudo first order ($k_{obsd(slow)}$) and was almost independent of the zinc concentration (Figure 5B). A portion of raw data for these reactions is shown in Table I.

Vallee proposed that [(Azo-CPD)Zn] consists of three different conformations in pH 7.5–8.5 (E_r; E_y; E_y'). E_r is a red coordination complex, and E_y is a yellow azophenol. E_y' is the second yellow conformational state indistinguishable spectrally from E_y. For these species, eq 3 was proposed. In

$$E_r \underset{\text{very fast}}{\overset{K_A}{\rightleftharpoons}} E_y \underset{\text{slow}}{\overset{K_y'}{\rightleftharpoons}} E_y' \qquad (3)$$

eq 3, the equilibrium between E_r and E_y is very fast and that

Table I: Reaction between [(Azo-CPD)Zn] and Zinc Ions at 0.05 M Tris-HCl Buffer (pH 7.7, 0.5 M NaCl)[a]

final Zn^{2+} concn (10^{-5} M)	fast phase		slow phase		no.	free zinc concn[b] (10^{-5} M)
	k_{obsd} (s^{-1})	ΔA	k_{obsd} (s^{-1})	ΔA		
3.0	28.3	−0.013	0.64	+0.0048	1	2.35
6.0	33.5	−0.022	0.57	+0.0048	2	5.06
9.0	39.4	−0.029	0.54	+0.0040	3	7.92
12.0	54.0	−0.035	0.69	+0.0028	4	10.8
24.0	96.9	−0.082	1.00	+0.0016	5	22.5
36.0	127.6			0.0	6	34.5
64.0	292			−0.0024	7	62.6
100.0			0.52	−0.003	8	98.5

[a] [(Azo-CPD)Zn] (3.0×10^{-5} M) solution was mixed with Zn^{2+} solutions of various concentrations at 25 °C. Absorbance was monitored at 510 nm, T = 25 °C. [b] Free zinc concentration was calculated as the following: final zinc concentration minus [(Azo-CPD)Zn]·Zn^{2+} concentration.

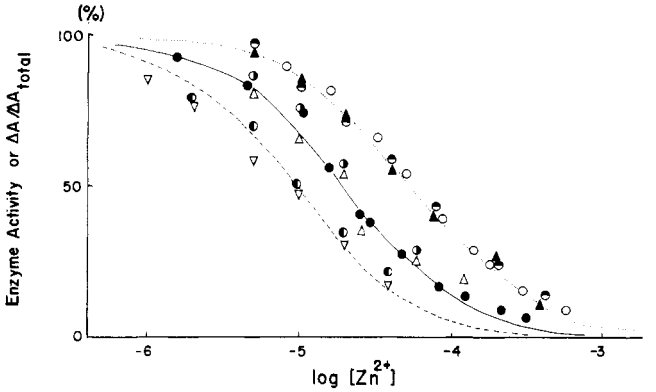
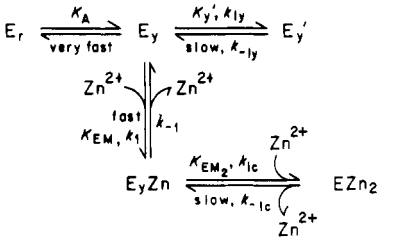


FIGURE 2: Relationship between free zinc concentration and absorbance change (at 510 nm) or enzyme peptidase activity. For [(Azo-CPD)Zn]: absorbance change (510 nm) at (○) pH 7.7 and (●) 8.2; peptidase activity at (▲) pH 7.7, (△) 8.2, and (▽) 9.0. For carboxypeptidase A: peptidase activity at (◓) pH 7.7, (◑) 8.2, and (◐) 9.0.

between E_y and E_y' is slow. The equilibrium constant between E_r and E_y is pH dependent, whereas that between E_y and E_y' is pH independent (Harrison et al., 1975). K_A and K_y' are equilibrium constants measured by Harrison et al. as shown in Table II. When excess zinc ions are added to [(Azo-CPD)Zn], one plausible explanation of the binding is shown by the equations:



(4)

$$K_A = [E_y]/[E_r] \quad (5)$$

$$K_y' = k_{1y}/k_{-1y} \quad (6)$$

$$K_{EM} = k_1/k_{-1} = [E_yZn]/([E_y][Zn^{2+}]) \quad (7)$$

$$K_{EM_2} = [E_yZn_2]/([E_yZn][Zn^{2+}]) = k_{1c}/k_{-1c} \quad (8)$$

where E_yZn and E_yZn_2 are 1:1 and 1:2 [(Azo-CPD)Zn]–zinc adducts. There are two binding sites for excess zinc ions in [(Azo-CPD)Zn]. The first binding site has a much larger binding constant than the second one. In eq 4, the fast reaction with a decrease of absorbance at 510 nm takes place among only three species (E_r, E_y, and E_yZn).

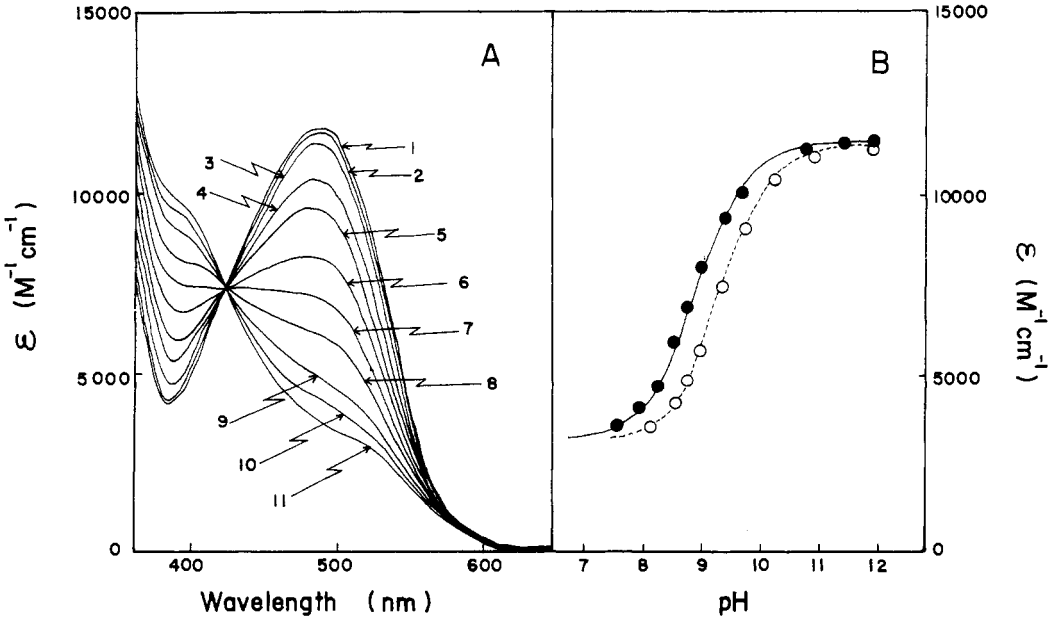


FIGURE 3: Absorption spectrum of [(Azo-CPD)Zn]·Zn^{2+} adduct at various pH values. (A) Absorption spectra: 0.05 M Tris-HCl (0.5 M NaCl); [[(Azo-CPD)Zn]] = 3.0×10^{-5} M and [Zn^{2+}] = 1.2×10^{-4} M. (1) pH 12.0; (2) pH 11.5; (3) pH 10.8; (4) pH 9.75; (5) pH 9.46; (6) pH 9.02; (7) pH 8.76; (8) pH 8.54; (9) pH 8.25; (10) pH 7.96; (11) pH 7.6. (B) The relationship between pH and absorbance change at 485 nm. (●) [(Azo-CPD)Zn] in the presence of excess zinc ions; same conditions as in (A). (○) [(Azo-CPD)]; 0.05 M Tris-HCl, 0.5 M NaCl, and [[(Azo-CPD)]] = 2.51×10^{-5} M.

$$E_r \underset{\text{very fast}}{\overset{K_A}{\rightleftharpoons}} E_y \overset{Zn^{2+}}{\underset{Zn^{2+}}{\overset{k_1}{\underset{k_{-1},\ \text{fast}}{\rightleftharpoons}}}} E_yZn \qquad (9)$$

The reaction rate of eq 9 is given by the equation (Diven et al., 1965):

$$k_{obsd(fast)} = k_1(\bar{C}_E + \bar{C}_{Zn})/(1 + 1/K_A) + k_{-1} \qquad (10)$$

where $\bar{C}_E$ and $\bar{C}_{Zn}$ are the enzyme and zinc concentration in the equilibrium state, and these values were calculated from the equilibrium data in Figure 2. In eq 10, $k_{obsd(fast)}$ increases with increasing concentration of zinc ions. This behavior is consistent with the results of Figure 5A. In Figure 5A, k_1 and k_{-1} were calculated from the slope and the intercept of the line, respectively. These values are shown in Table II, and the ratio between k_1 and k_{-1} gives the binding constant of zinc ions to the first binding site.

In the slow stage at low zinc concentration, the E_y' species appears in the equilibrium of eq 9.

$$E_r \underset{\text{very fast}}{\overset{K_A}{\rightleftharpoons}} E_y \overset{\text{slow}\ K_y',\ k_{1y}}{\underset{k_{-1y}}{\rightleftharpoons}} E_y' \qquad (11)$$

$$Zn^{2+} + Zn^{2+} \xrightarrow[K_{EM}]{\text{fast}} E_yZn$$

Therefore, the concentration of E_r increases under these conditions, so that the slow stage results in increasing absorbance at 510 nm. On the basis of eq 11 and the fast equilibrium among E_r, E_y, and E_yZn, $k_{obsd(slow)}$ is given by the following equation (Hiromi, 1979):

$$k_{obsd(slow)} = \frac{k_{1y}}{1 + 1/K_A + \bar{C}_{Zn}/(1/K_{EM} + \bar{C}_{E_y})} + k_{-1y} \qquad (12)$$

where $\bar{C}_{Ey}$ is the E_y concentration at equilibrium. In eq 12, K_A is 0.56 at pH 7.7 and 0.18 at pH 8.2 (Table II), k_{1y} is $0.89k_{-1y}$ from eq 6 (Table II), and the term $\bar{C}_{Zn}/(1/K_{EM} + \bar{C}_{E_y})$ is larger than zero, so that $k_{obsd(slow)}$ is almost equal to k_{-1y} at pH 8.2 and 7.7. Because of this, $k_{obsd(slow)}$ is almost independent of the zinc concentration (Table I and Figure 5B).

At high zinc concentration, one more species (E_yZn_2) joins the equilibrium of eq 11, and the final reaction is shown in eq 4. The rate constants equation between E_yZn and E_yZn_2 becomes more complex, so that we cannot interpret the reaction rate that involves E_yZn_2 in the zinc binding studies.

Zinc Removal from the Adduct between Arsanilazotyrosine-248 Carboxypeptidase A and Zinc Ions by Ethylenediaminetetraacetic Acid. In order to check the validity of eq 4, the zinc removal from the [(Azo-CPD)Zn]·Zn^{2+} adduct was studied by using a chelating agent [ethylenediaminetetraacetic acid (EDTA)]. When EDTA was added to the [(Azo-CPD)Zn]·Zn^{2+} adduct, three stages were observed, each approximately pseudo first order (Figure 6A). The theoretical pseudo-first-order reaction curves which are shown in Figure 6A describe the raw data.

In eq 4, it was proposed that E_y, E_y', E_yZn, and E_yZn_2 are spectrally indistinguishable from each other. To confirm this assumption, the zinc removal reaction was followed at various wavelengths. The spectra reconstructed from data obtained immediately after mixing [(Azo-CPD)Zn]·Zn^{2+} adduct with EDTA at pH 7.7 and monitoring at 20-nm intervals between 400 and 610 nm are shown in Figure 6B. The position of λ_{max} of each spectrum remains constant, and a tight isosbestic point is observed at 442 nm, demonstrating that only two chromophoric species are present throughout. These results indicate the validity of the assumption that yellow forms (E_y, E_y', E_yZn, and E_yZn_2) are spectrally indistinguishable from each other.

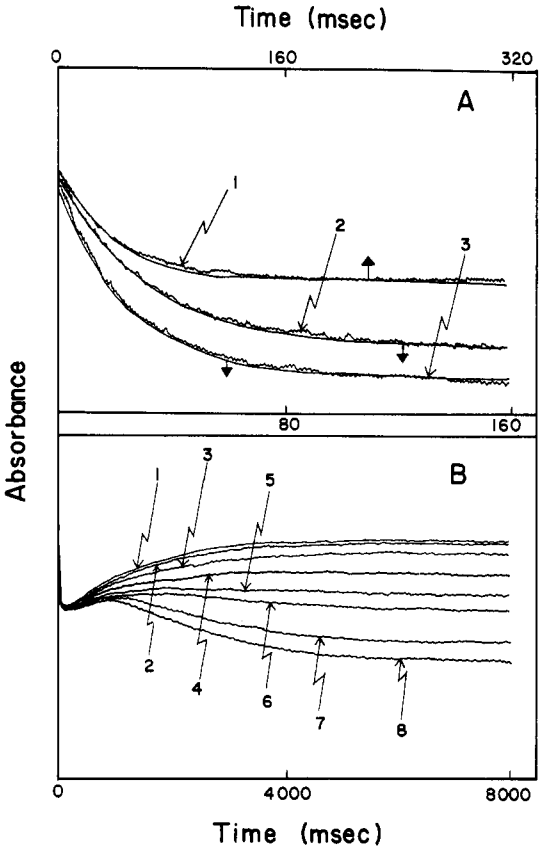


FIGURE 4: Stopped-flow traces of absorbance at 510 nm vs. time for [(Azo-CPD)Zn] color change by zinc ions in 0.05 M Tris-HCl buffer at pH 7.7 (0.5 M NaCl) at 25 °C. (A) Fast stage reaction. (1) Collection time 320 ms, absorbance (full scale) 0.02, [[(Azo-CPD)Zn]] = 1.5×10^{-5} M, and [Zn^{2+}] = 3×10^{-5} M; (2) collection time 160 ms, absorbance (full scale) 0.02, [[(Azo-CPD)Zn]] = 1.5×10^{-5} M, and [Zn^{2+}] = 6×10^{-5} M; (3) collection time 160 ms, absorbance (full scale) 0.02, [[(Azo-CPD)Zn]] = 1.5×10^{-5} M, and [Zn^{2+}] = 9×10^{-5} M. (B) Slow stage reaction. Conditions of these reactions are shown in Table I. Absorbance (full scale) in this reaction was 0.02, and collection time was 8000 ms.

Table II: Equilibrium and Kinetic Rate Constants at Various Stages

equilibrium and kinetic rate constants	pH 8.2	pH 7.7
K_A[a]	0.18	0.56
K_y'[a]	0.89	0.89
k_{1y} (s^{-1})		0.36
k_{-1y} (s^{-1})		0.40
K_{EM} (M^{-1})	3.9×10^5	7.1×10^4
k_1 ($M^{-1}\ s^{-1}$)	4.26×10^6	8.49×10^5
k_{-1}[b] (s^{-1})	11	12
K_{EM_2} (M^{-1})	1.8×10^3	7×10^2

[a] These values were calculated from the following equations, on the basis of Harrison et al. (1975): $K_A = [H^+]/K_1$ (K_1 is $10^{-7.45}$ in the literature): $K_y' = 1/K_2$ (K_2 is 1.1 in the literature). [b] These values were from Figure 5.

In the first stage, the relationship between the observed pseudo-first-order rate constants ($k_{obsd,1}$) and the concentration of EDTA is shown in Figure 7A. In Figure 7A, $k_{obsd,1}$ increases slightly with increasing EDTA concentration, and the intercepts are 12 (pH 7.7) and 11 s^{-1} (pH 8.2). These values are almost the same as k_{-1} (Table II), which were obtained in Figure 5A. From this result, the first stage of zinc removal by EDTA corresponds to $E_yZn \rightarrow E_y + Zn^{2+}$ in eq 4. In this

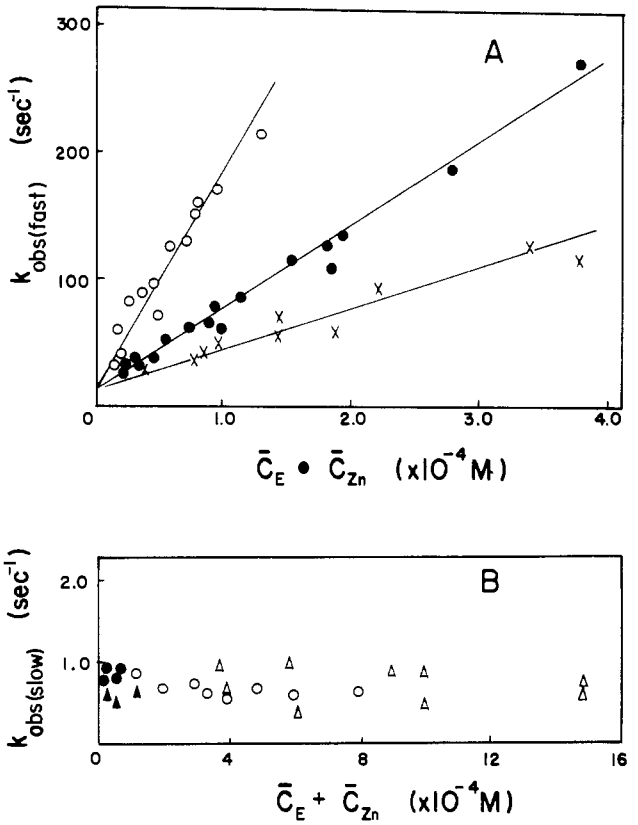


FIGURE 5: Relationship between pseudo-first-order rate constant (k_{obsd}) in the fast or slow stage and zinc concentrations. (A) Fast stage reaction: (×) pH 7.7, (●) 8.2, and (○) 9.0. (B) Slow stage reaction: (△, ▲) pH 7.7 and (○, ●) 8.2.

reaction, EDTA directly reacts somewhat with the [(Azo-CPD)Zn]·Zn^{2+} adduct, so that $k_{obsd,1}$ slightly increases with increasing EDTA concentration, but the main reaction is the self-dissociation of zinc ions from the E_yZn adduct.

In the second stage, the $k_{obsd,2}$ values were independent of EDTA (in Figure 7B) and zinc concentration (Table III). However, as Table III indicates, ΔA for the second stage increased with increasing zinc concentration. On the other hand, the ΔA for the first stage decreased with increasing zinc concentration. If it is assumed that one more zinc binds to a weak zinc binding site in E_yZn and E_yZn becomes E_yZn_2 with increasing zinc ions, the ΔA change in the first and second stages would be easily interpreted and is consistent with eq 4. The binding constant of the weak zinc binding site was calculated from the relationship between the ΔA of the second phase and zinc concentrations. The values of the binding constants of the second site (K_{EM_2}) are shown in Table III. $k_{obsd,2}$ is very small and independent of EDTA concentration. Therefore, the second stage reaction corresponds to (in eq 4)

$$E_yZn_2 \overset{k_{-1c}}{\rightarrow} E_yZn + Zn^{2+}$$

In the third stage, a decrease in absorbance at 510 nm was observed at pH 7.7 and 8.2; $k_{obsd,3}$ was almost constant with increasing EDTA concentration (Figure 7C), and ΔA was constant (Table III). Unfortunately, $k_{obsd,3}$ at pH 8.2 was not obtained because K_A at pH 8.2 was smaller than that of pH 7.7. When zinc ions are removed from E_yZn and E_yZn_2 by EDTA, both E_y and E_r result because the equilibrium between E_y and E_r is very fast. For this reason, ΔA increases were observed in the first and second stage. In the third stage, the E_y' species participates, so that some E_r changes to E_y'. Therefore, an absorbance decrease is expected. If the third stage is considered as a shift in the equilibria among E_r, E_y, and E_y', the decrease of the absorbance change in the third stage can be easily interpreted and is consistent with the mechanism of eq 4. $k_{obsd,3}$ is represented by

$$k_{obsd,3} = k_{1y}/(1 + 1/K_A) + k_{-1y} \qquad (13)$$

K_y' and K_A are known, so that k_{1y} and k_{-1y} were calculated by eq 13. k_{-1y} at pH 7.7 was 0.40 s^{-1}. Therefore, on the basis of eq 12 and k_{-1y} (0.40 s^{-1} at pH 7.7), $k_{obsd(slow)}$ in eq 12 is calculated as 0.52–0.40 s^{-1}. This value is consistent with those of Table I. Harrison et al. (1975) measured the rate constant

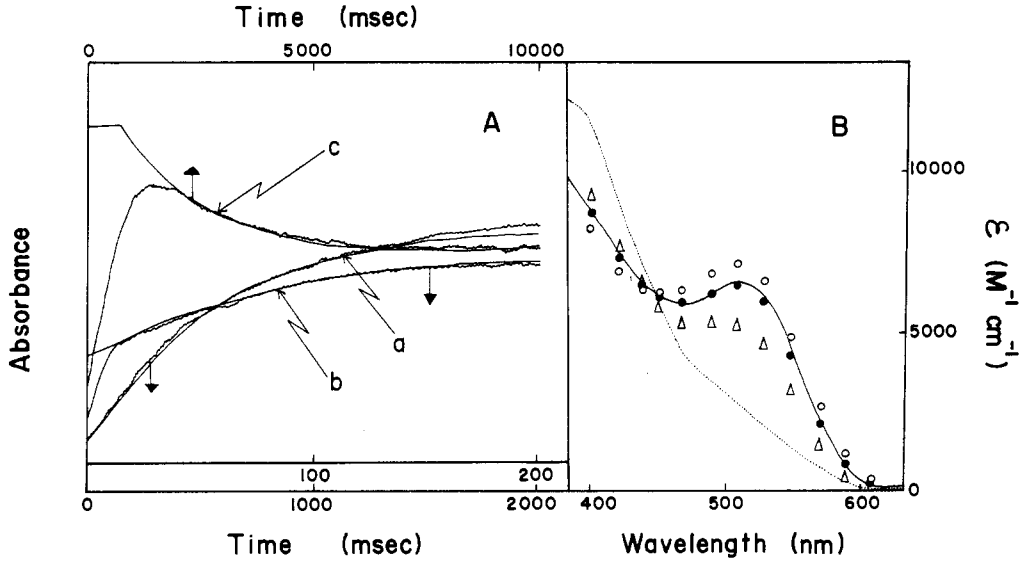


FIGURE 6: Stopped-flow traces for the zinc removal from [(Azo-CPD)Zn]·Zn^{2+} adduct by EDTA. (A) Stopped-flow traces of absorbance at 510 nm vs. time for [(Azo-CPD)Zn]·Zn^{2+} color change of fast, middle slow, and very slow phase by EDTA in 0.05 M Tris-HCl at pH 7.7. The solution containing [(Azo-CPD)Zn] (2.8×10^{-5} M) and Zn^{2+} (10^{-3} M) was mixed with EDTA (1.2×10^{-2} M) solution. (a) First stage reaction: collection time 200 ms and absorbance (full scale) 0.02. (b) Second stage reaction: collection time 2000 ms and absorbance (full scale) 0.05. (c) Third stage reaction: collection time 10 000 ms and absorbance (full scale) 0.02. Temperature 25 °C. (B) Spectra of the products of zinc removal from the [(Azo-CPD)Zn]·Zn^{2+} adduct by EDTA, reconstructed from stopped-flow experiments [same condition as in (A)] at 20 nm intervals. The spectrum at zero time (···), obtained from the spectrophotometric method, and changes by 200 ms of the spectrum (△) followed by the second stage (○) and third stage (●) to finally reach the equilibrium spectrum (—).

Table III: Reaction between [(Azo-CPD)Zn] Solution Containing Various Concentrations of Zinc Ions and EDTA (pH 7.7, 0.05 M Tris-HCl and 0.5 M NaCl)[a]

	first stage		second stage		third stage	
Zn^{2+} concn ($\times 10^{-4}$ M)	k_{obsd} (s^{-1})	ΔA	k_{obsd} (s^{-1})	ΔA	k_{obsd} (s^{-1})	ΔA
10	13.3	+0.027	1.7	+0.018	0.40	−0.011
5	14.9	+0.033	2.0	+0.007	0.47	−0.011
2.5	15.0	+0.033	2.0	+0.002	0.45	−0.011

[a] [(Azo-CPD)Zn] solutions (2.7×10^{-5} M) containing various concentrations of zinc ions (2.5×10^{-4}–1.0×10^{-3} M) was mixed with EDTA solution (4×10^{-3} M). The absorbance was monitored at 510 nm.

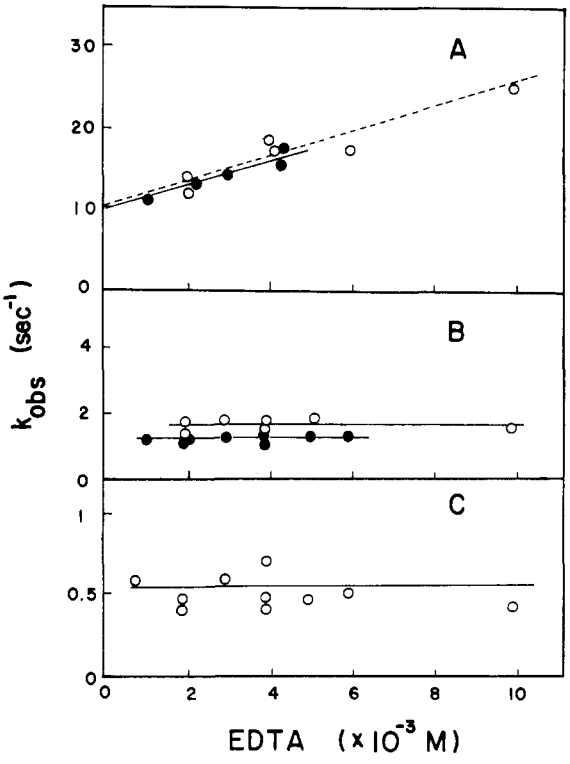


FIGURE 7: Relationship between k_{obsd} and EDTA concentrations. (A) First stage ($k_{obsd,1}$). (B) Second stage ($k_{obsd,2}$). (C) Third stage ($k_{obsd,3}$). (○) pH 7.7; (●) pH 8.2.

between E_y and E_y' by a pH-jump method [low pH → pH 8.0 or 8.5 (final pH)]. At the final pH of 8.0 and 8.5, k_{obsd} values are 2.1 and 5.0 s^{-1}, respectively. These values are larger than our k_{obsd} [0.5 ± 0.1 s^{-1} (Figure 7C)] at pH 7.7. But the k_{obsd} of the pH-jump method decreased with decreasing final pH, so that, at pH 7.7, the value may not be different from our rate constant at pH 7.7.

Comparison between the Data Obtained from the Equilibrium and Kinetic Methods. To compare the equilibrium data with the kinetic data, the theoretical concentration of red form ($[E_r]$) was calculated from the kinetic data. $[E_r^0]$, which is the red form concentration without zinc ions present, is calculated from the equation:

$$[E_r^0]/[E_T] = 1/(1 + K_A + K_AK_y') = [E_r^0]/([E_r^0] + [E_y^0] + [E_y']) \quad (14)$$

where $[E_T]$ is the total enzyme concentration and K_A and K_y' are the equilibrium constants in eq 4. When excess zinc ions are added to [(Azo-CPD)Zn], only E_r and E_y participate in the zinc binding reaction of the fast phase. Therefore, $[E_T^{fast}]$, which is the total enzyme concentration participating in the fast stage of zinc binding, is expressed by the equation:

$$[E_T^{fast}] = [E_r^0] + [E_y^0] = [E_r^0](1 + K_A) \quad (15)$$

When excess zinc ions are added to [(Azo-CPD)Zn], the red

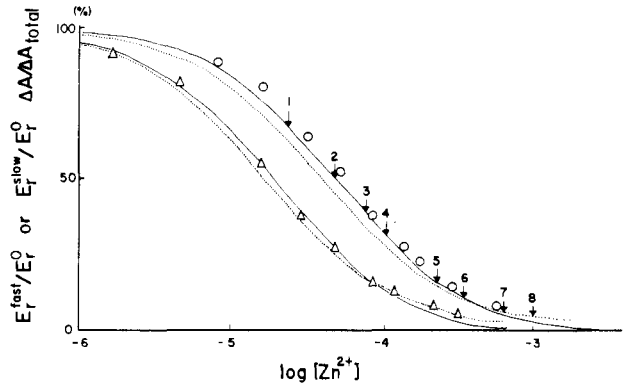


FIGURE 8: Relationship between the absorbance change ratio when zinc ions were added to [(Azo-CPD)Zn] and the theoretical red form conformer ratio of [(Azo-CPD)Zn] in the fast and slow reaction. The absorbance change ratio when zinc ions were added to [(Azo-CPD)Zn]: (○) pH 7.7; (△) pH 8.2. The red form ratios of [(Azo-CPD)Zn] which are calculated from eq 17 (fast equilibrium) (···) and from eq 19 (slow equilibrium) (—).

form concentration at the end of the fast stage ($[E_r^{fast}]$) was calculated by the equation:

$$[E_T^{fast}] = [E_r^{fast}] + [E_y^{fast}] + [E_yZn] = [E_r^{fast}](1 + K_A + K_AK_{EM}[Zn^{2+}]) \quad (16)$$

From eq 15 and 16

$$[E_r^{fast}]/[E_r^0] = (1 + K_A)/(1 + K_A + K_AK_{EM}[Zn^{2+}]) \quad (17)$$

On the basis of eq 17, the red form concentration at equilibrium in the fast stage of zinc binding to [(Azo-CPD)Zn] can be calculated.

In the slow stage of zinc binding to [(Azo-CPD)Zn], the final concentration of the red conformation is expressed by the equation:

$$[E_T] = [E_r^{slow}] + [E_y^{slow}] + [E_y'] + [E_yZn] + [E_yZn_2] = [E_r^{slow}](1 + K_A + K_AK_y' + K_AK_{EM}[Zn^{2+}] + K_AK_{EM}K_{EM_2}[Zn^{2+}]^2) \quad (18)$$

$[E_T]$ in eq 18 is substituted by eq 14.

$$[E_r^{slow}]/[E_r^0] = (1 + K_A + K_AK_y')/(1 + K_A + K_AK_y' + K_AK_{EM}[Zn^{2+}] + K_AK_{EM}K_{EM_2}[Zn^{2+}]^2) \quad (19)$$

From eq 19, the red form content in the final equilibrium condition can be calculated.

On the basis of eq 17–19, $[E_r^{fast}]/[E_r^0]$ and $[E_r^{slow}]/[E_r^0]$ were calculated by using the K_A, K_y', K_{EM}, and K_{EM_2} values which are shown in Table II and are given as theoretical curves in Figure 8. $[E_r^{slow}]/[E_r^0]$ is theoretically equal to the absorbance change ratios that were obtained in the final equilibrium condition ($\Delta A/\Delta A_T$) in Figure 2. In Figure 8, the solid curve is reasonably consistent with the absorbance change ratios when zinc ions were added to [(Azo-CPD)Zn] at equilibrium. Therefore, the validity of the scheme in eq 4 is also supported by the equilibrium data. At low zinc concen-

trations, $[E_r^{fast}]/[E_r^0]$ is smaller than $[E_r^{slow}]/[E_r^0]$, which implies that the rearrangement of species in the slow stage is accompanied by an increase in absorbance at 510 nm. But, at high zinc concentration, $[E_r^{fast}]/[E_r^0]$ is larger than $[E_r^{slow}]/[E_r^0]$, so that now the slow stage shows a decrease in absorbance at 510 nm. When ΔA changes for the slow stage at various zinc concentrations in Table I (free zinc concentrations in Table I are shown as the arrows in Figure 8) are compared with the differences between $[E_r^{fast}]/[E_r^0]$ and $[E_r^{slow}]/[E_r^0]$ in Figure 8, there is a good correlation. Therefore, this result also supports the validity of the scheme in Figure 4 for zinc binding to [(Azo-CPD)Zn].

Discussion

On the basis of kinetic and equilibrium data, the mechanism for zinc binding to [(Azo-CPD)Zn] is proposed by eq 4. In eq 4, excess zinc ions react with only the E_y form of the enzyme. E_y' and E_r forms which are different conformational states do not bind to zinc ions. Therefore, zinc binding to [(Azo-CPD)Zn] is a very selective reaction. The binding constants between E_y and zinc ions at pH 7.7 and 8.2 are 7.1×10^4 and 3.9×10^5 M^{-1}, respectively. These binding constants are much smaller than the binding constant of the zinc ion to the active site of apoenzyme [(Azo-CPD)]] ($\sim 10^{10}$ M^{-1} at pH 8.0) which was obtained by Billo et al. (1978). The binding rate constant of excess zinc ions to [(Azo-CPD)Zn] (4.2×10^6 M^{-1} s^{-1} at pH 8.2; 8.5×10^5 M^{-1} s^{-1} at pH 7.7) is almost the same as that to the active site or [(Azo-CPD)] (10^6 M^{-1} s^{-1} at pH 8.0), but the dissociation rate constant of zinc ions from the [(Azo-CPD)Zn]·Zn^{2+} adduct (11 s^{-1} at pH 8.2; 12 s^{-1} at pH 7.7) is much larger than that of the zinc ion from the active site of [(Azo-CPD)Zn] (10^{-4} s^{-1} at pH 8.0) (Billo et al., 1978). This large dissociation rate constant of zinc ions from the [(Azo-CPD)Zn]·Zn^{2+} adduct results in the small formation constant between excess zinc ions and [(Azo-CPD)Zn].

Where in [(Azo-CPD)Zn] do zinc ions bind? The arsanilazotyrosine residue has the ability for zinc binding, but the spectral changes of [(Azo-CPD)Zn]·Zn^{2+} with pH, in the presence of excess zinc ions, closely resemble those of apoenzyme [[(Azo-CPD)]]. This result indicates that zinc ions do not bind to the arsanilazotyrosine residue in the [(Azo-CPD)Zn]·Zn^{2+} adduct. The changes in the spectrum of [(Azo-CPD)Zn] induced by adding zinc ions are directly correlated to the inhibition of peptidase activity of the enzyme. This inhibition was also observed in native carboxypeptidase A (Vallee et al., 1960; Auld & Vallee, 1970), so the same zinc binding site is present in the native enzyme which of course does not have the arsanilazotyrosine residue. Therefore, it is considered that the zinc binding site is independent of the arsanilazotyrosine residue. The binding constant of zinc ions to [(Azo-CPD)Zn] is $\sim 10^6$ M^{-1} at pH 8.2. From this strength of binding, it is presumed that two or three amino acid residues are involved in the zinc binding (Hirose et al., 1981, 1982, 1983). There is the possibility that, in only the E_y conformation, there are two or three amino acid residues so arranged as to be suited to bind the zinc ion and that, in the other conformational species (E_r and E_y'), the arrangement of the amino acid residues is not conducive to binding zinc ion. On the basis of this assumption, the specificity of zinc ion binding to the E_y form is easily understood. The inhibition with zinc ions is similar in [(Azo-CPD)Zn] and the native enzyme. Therefore, in the native enzyme, there is the possibility that three conformational states are present as in [(Azo-CPD)Zn]. A more detailed study of the inhibition of the enzyme activity by zinc ions would be needed to clarify this assumption.

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Registry No. Zn, 7440-66-6.

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