Interaction of Cobalt(II) Complexes with Apoarsanilazotyrosine-248 Carboxypeptidase A†

Junzo Hirose‡ and Ralph G. Wilkins*

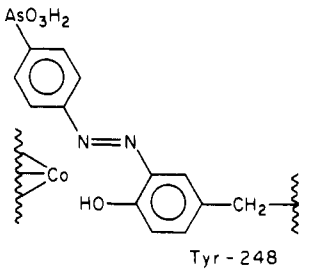
ABSTRACT: The kinetics of interaction of a variety of cobalt(II) and zinc(II) complexes with apoarsanilazotyrosine-248 carboxypeptidase A have been studied by stopped flow at pH 8.2, I = 1.0 M (NaCl), and 25 °C. The very rapid completion of the metal–azotyrosine-248 link at this pH is used to monitor spectrally the uptake of the metal by apoprotein. Only the 1:1 metal–ligand complexes were reactive. The second-order rate constants (M^{-1} s^{-1}) were measured for the following 1:1 cobalt–ligand complexes: 1,10-phenanthroline, 3×10^6; 1,10-phenanthroline-5-sulfonate, 2.4×10^6; 2,2′,2″-terpyridine, 1.2×10^6; water (aqua ion), 7.7×10^5; catechol-3,5-disulfonate, 3.2×10^5; 8-hydroxyquinoline-5-sulfonate, 9.5×10^4; pyridine-2,6-dicarboxylate, 9×10^4; bathophenanthrolinedisulfonate, $<10^5$. For zinc complexes, the values were as follow: 1,10-phenanthroline, 4×10^6; water, 5.0×10^6; 8-hydroxyquinoline-5-sulfonate, 7.0×10^5. The sequence is quite different from that previously measured for apocarbonic anhydrase.

Increasing interest is being displayed in the general system

$$P + ML \rightleftarrows PML \rightleftarrows PM + L \quad (1)$$

where P is a demetallated (apo) protein and ML is a metal–ligand complex. The forward direction probably represents the last step in the biosynthesis of the metalloprotein. A recent group of studies indicates that metallothioneins provide metal ions (Zn^{2+} and Cu^{2+}) to newly synthesized apoenzymes in tissues that are rapidly developing (Brady & Webb, 1981; Brady, 1982). In the reverse direction, ligand L removes metal ion M from the protein to produce the apo form. A sizable number of important metalloenzymes have been thus converted into apo forms that can be used to produce metallo derivatives, the metal constituent chosen so as to allow spectral, electron paramagnetic resonance (EPR) and other characterization (Vallee & Holmquist, 1980). An understanding of process 1 can lead to improved methods for producing apoprotein (Kidani et al., 1976; Hunt et al., 1977). Reaction 1 can be investigated from either direction depending on the concentration of reactants and pH, and information on both directions may be thus obtainable. The occurrence of the ternary species PML has been usually deduced from the observation of "saturation" kinetics (Kidani et al., 1976; Romans et al., 1978; Rogers & Billo, 1980) although occasionally the rate of its formation has been measured (Harrington & Wilkins, 1980). Configurational changes may occur in PM after the metal ion has been placed in the metal site (Rigo et al., 1978; Marks & Miller, 1979; Morpurgo et al., 1979; Harrington et al., 1981, and references cited therein; Schneider & Zeppezauer, 1983).

We have previously studied the kinetics of formation and dissociation of metallocarboxypeptidases (Billo et al., 1978). Carboxypeptidase is one of the most studied metalloenzymes. It can function as both an esterase and a peptidase. The native enzyme contains one zinc atom per molecule coordinated to His-69, Glu-72, and His-196. A water molecule completes a distorted tetrahedral coordination of the zinc. We now report on the interaction of a variety of cobalt(II) complexes with bovine apoarsanilazotyrosine-248 carboxypeptidase A (I minus Co). The use of this azo derivative of carboxypeptidase allows



Tyr-248

the ready visualization of process 1. Coupling of carboxypeptidase A with diazotized *p*-arsanilic acid specifically labels Tyr-248 without significant catalytic changes. The modified enzyme is intensely chromophoric and has been effectively used by Vallee and his co-workers to probe the characteristics of the enzyme with a variety of properties (Johansen & Vallee, 1971, 1973; Johansen et al., 1972; Harrison & Vallee, 1978; Schuele et al., 1981; Bachovchin et al., 1982).

Materials and Methods

Carboxypeptidase Aα was obtained from Sigma as a crystalline suspension and used without further purification. Arsanilazotyrosine-248 carboxypeptidase A was prepared by treatment of carboxypeptidase crystals with diazotized *p*-arsanilic acid (Johansen & Vallee, 1971).[1] Apoarsanilazotyrosine-248 carboxypeptidase was prepared by suspending crystals of the azoenzyme (5 mg/mL) in 10^{-2} M 1,10-phenanthroline and 0.01 M Mes[2] 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). Apoazoenzyme was stored as a suspension in water at 4 °C. Immediately before use, it was dissolved in the buffer solution. Cobalt arsanilazotyrosine-248 carboxypeptidase was obtained by addition of the stoichiometric amount of Co^{2+} ion to apoazoenzyme. Protein concentration was determined spectrally [$\epsilon_{278} = 7.32 \times 10^4$ M^{-1}

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[1] Carboxypeptidase Aα (1 mg) catalyzed the hydrolysis of 28 μmol of Cbz-Gly-Phe/min from 1 mM Cbz-Gly-Phe at 25 °C and pH 7.0. The azoenzyme (1 mg) catalyzed the hydrolysis of 36 μmol of Cbz-Gly-Phe with the same conditions.

[2] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Cbz-Gly-Phe, *N*-carbobenzoxyglycyl-L-phenylalanine; EDTA, ethylenediaminetetraacetic acid.

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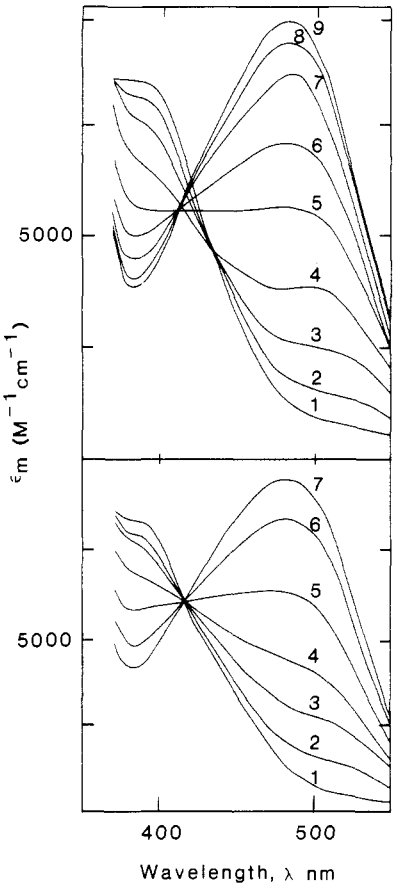


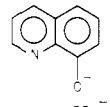
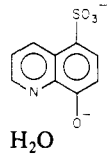
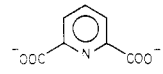
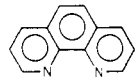
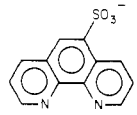
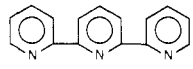
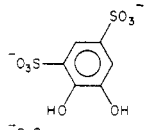
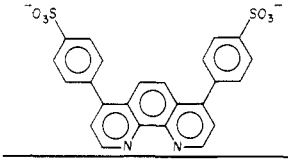
FIGURE 1: Effect of pH on the absorption spectrum of (lower) apoarsanilazotyrosine-248 carboxypeptidase and (upper) cobalt arsanilazotyrosine-248 carboxypeptidase, both in 50 mM Tris-HCl–1.0 M NaCl and at 25 °C. For the apo form, the pHs were (1) 6.8, (2) 7.5, (3) 8.0, (4) 8.5, (5) 9.0, (6) 9.6, and (7) 10.1. For the cobalt protein, the pHs were (1) 6.5, (2) 7.0, (3) 7.5, (4) 7.9, (5) 8.5, (6) 9.0, (7) 9.5, (8) 10.2, and (9) 10.7. Concentrations of protein used were (lower) 22 and (upper) 28 μM. The equivalent amount of Co^{2+} was added to the apoprotein and incubated to produce the cobalt(II) form.

cm^{-1} (Johansen & Vallee, 1971)]. All other chemicals were reagent grade and used without further purification. The complex Co(terpy)Cl_2 was prepared by the method of Hogg & Wilkins (1962). Buffer solutions were treated with 0.1% dithizone in CCl_4 to remove traces of metal ions and deaerated with argon to avoid any oxidation of cobalt(II) complexes by air. Stock solutions of Co^{2+} and Zn^{2+} ions were standardized by EDTA. The interaction of cobalt(II) and zinc(II) complexes with apoarsanilazotyrosine-248 carboxypeptidase was measured on a Gibson-Durrum stopped-flow apparatus interfaced with an OLIS data-collecting system (On-Line Instrument Systems, Jefferson, GA) for data acquisition and manipulation. The formation of the zinc and cobalt azoenzymes was monitored at 510 and 500 nm, respectively. All work was carried out at pH 8.2, I = 1.0 M, and 25 °C.

Results

The effect of pH on the spectra of apoarsanilazotyrosine-248 carboxypeptidase and the cobalt derivative is shown in Figure 1. The apo form titrates at 480 nm with a single pK = 8.9. There is an isosbestic point at 415 nm, and the basic form has a maximum at 480 nm ($\epsilon = 9.7 \times 10^3$ M^{-1} cm^{-1}). With cobalt arsanilazotyrosine-248 carboxypeptidase at low pH (6.5–7.9), there is an isosbestic point at 430 nm and a peak at ~510 nm. At higher pH (8.5–10.2), the isosbestic point moves to 410 nm, and the peak intensifies and shifts to shorter wavelengths ($\epsilon_{480nm} = 1.0 \times 10^4$ M^{-1} cm^{-1}). The maximum absorbance difference between apo and cobalt forms is at 500 nm and pH 8.2–8.5, and these conditions were chosen to investigate the kinetics. The titration of apoarsanilazotyrosine-248 carboxypeptidase with Co^{2+} and Zn^{2+} ions at pH 8.2 is shown in Figure 2. Absorbance changes and activity measurements gave consistent data, and the metalloprotein was completely formed at a 1:1 mole ratio of metal ion and apoprotein. The activity of the cobalt derivative toward Cbz-Gly-Phe was 6.1 times that of the zinc form at pH 8.2.

Table I: Interaction of Co(II) Complexes (CoL^{n+}) with Apocarbonic Anhydrase[a] and Apocarboxypeptidase[b]

L	apocarbonic anhydrase[a] (M^{-1} s^{-1})	apoazocarboxypeptidase[b] (M^{-1} s^{-1})
	$\sim 4 \times 10^3$	
	1.9×10^2	9.5×10^4 (7.0×10^5)[d]
H_2O	81	7.7×10^5 (5.0×10^6)[d]
SCN^-	strong acceleration[c]	no effect
	71	9×10^4
	30	3×10^6 (4.0×10^6)[d]
		2.4×10^6
	3	1.2×10^6
		3.2×10^5
		$<1.0 \times 10^5$

[a] Using bovine carbonic anhydrase B, pH 7.5, I = 0.1 M (Gerber et al., 1975). [b] Using bovine carboxypeptidase Aα (Billo et al., 1978). [c] Harrington & Wilkins, 1980. [d] For zinc complexes.

The reaction of excess Co^{2+} ion with apoarsanilazotyrosine-248 carboxypeptidase was studied at 500 nm by stopped flow, and the first-order rate constant was directly proportional to the concentration of Co^{2+} ion (Figure 3). The second-order rate constant, $k_{Co^{2+}}$ (from the slope), is shown in Table I. The second-order rate constant was also measured at 15 °C ($k_f = 4.3 \times 10^5$ M^{-1} s^{-1}) as was the dissociation rate constant with EDTA (Billo et al., 1978), $k_d = 4.8 \times 10^{-3}$ s^{-1} (independent of EDTA concentration ranging from 0.6 to 5.0 mM). Combination of k_f and k_d gives the formation constant of the cobalt(II) azoprotein ($K = k_f/k_d = 9 \times 10^7$ M^{-1} at pH 8.2, I = 1.0 M, and 15 °C). The addition of Cbz-Gly-Phe (40 mM) to apoazocarboxypeptidase (37 μM) prior to reaction with Co^{2+} (200 μM) produced only a slow production of Co azoenzyme.

The determination of the rate constants for reaction of cobalt(II) complex species with apoazoenzyme was carried out

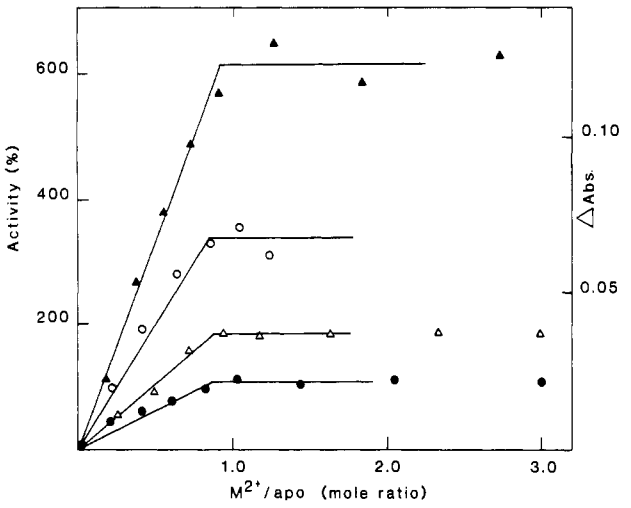


FIGURE 2: Titration of apoarsanilazotyrosine-248 carboxypeptidase with Co^{2+} and Zn^{2+} ions at pH 8.2, in 50 mM Tris-HCl–1.0 M NaCl, and at 25 °C. Activity of zinc arsanilazotyrosine-248 carboxypeptidase is assigned 100%. ΔAbs is at 500 nm for Co^{2+} and 505 nm for Zn^{2+}, and spectra were run at 13 °C. Symbols: (△) absorbance of Co^{2+} form; (○) absorbance of Zn^{2+} form; (▲) activity of Co^{2+} form; (●) activity of Zn^{2+} form. Concentration of protein used is 20 μM.

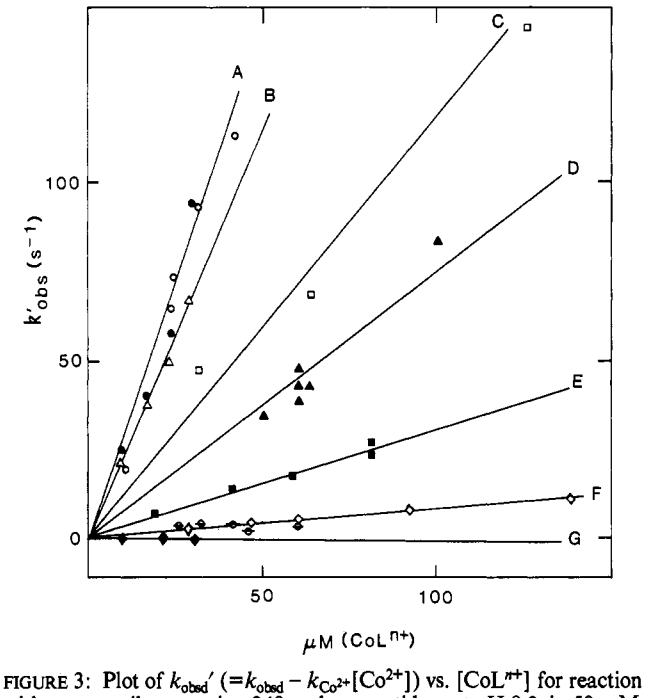


FIGURE 3: Plot of k_{obsd}' ($=k_{obsd} - k_{Co^{2+}}[Co^{2+}]$) vs. $[CoL^{n+}]$ for reaction with apoarsanilazotyrosine-248 carboxypeptidase at pH 8.2, in 50 mM Tris-HCl–1.0 M NaCl, and at 25 °C: (A) 1,10-phenanthroline [(○) excess ligand; (●) excess Co^{2+} ion]; (B) 1,10-phenanthroline-5-sulfonate ion; (C) 2,2′,2″-terpyridine; (D) aquated ion; (E) catechol-3,5-disulfonate ion; (F) 8-hydroxyquinoline-5-sulfonate ion (◇) and 2,6-pyridinedicarboxylate (⊖); (G) bathophenanthrolinedisulfonate.

as previously described for apocarbonic anhydrase (Gerber et al., 1975; Romans et al., 1978). Cobalt(II)–ligand mixtures were reacted with apoazoenzyme in a stopped-flow apparatus. First-order formation of the azoenzyme was observed (rate constant k_{obsd}). All reactions proceeded to completion as shown by similar absorbance changes (for a constant apo concentration) independent of the concentration and nature of the ligand. The raw kinetic data for varying concentrations of total cobalt and ligand are shown in the table in the supplementary material (see paragraph at end of paper regarding supplementary material). Also included are the concentrations of the various cobalt(II) species present in the mixture, calculated (Hirose et al., 1982) with assessed equilibrium constants in conditions most resembling ours (Smith & Martell, 1975; Perrin, 1979). Since the cobalt species are in labile equilibrium with each other, the observed rate constant k_{obsd} is given by (2). Using relatively large ratios of L to Co^{2+} slows down

$$\text{rate}/[\text{apo}] = k_{obsd} = k_{Co^{2+}}[Co^{2+}] + \sum_{m=1}^{m=2\text{or}3} k_{CoL_m{}^{n+}}[CoL_m{}^{n+}] \quad (2)$$

the rate of incorporation of the metal into the protein, showing that the higher species ($m > 2$) contribute insignificantly to the rate. The contribution of the term $k_{Co^{2+}}[Co^{2+}]$ can be assessed with each condition. The plot of k_{obsd}' ($=k_{obsd} - k_{Co^{2+}}[Co^{2+}]$) against CoL^{n+} is linear (Figure 3), and the slope is equal to the second-order rate constant for reaction of CoL^{n+} (Table I). Values for the rate constant for reaction of Co(terpyr)$(H_2O)_3{}^{2+}$ were obtained directly by using freshly dissolved solid Co(terpyr)Cl_2, which equilibrates to a metal ion–mono–bis mixture quite slowly (Hogg & Wilkins, 1962; Huchital & Kiel, 1981).

The interaction of Zn^{2+} ion with apoarsanilazotyrosine-248 carboxypeptidase was triphasic. The first phase was shown from the absorbance changes to produce zinc arsanilazotyrosine-248 carboxypeptidase. The subsequent changes relate to the interaction of Zn^{2+} ion with the azoprotein, as could be shown by separate study (J. Hirose, work in progress). Two other zinc complexes were also studied in a similar manner to the cobalt complexes (table in supplementary material). With these, only uniphasic processes were observed. The second-order interaction rate constants for all three species with apoprotein are shown in Table I.

Discussion

Preliminary data indicated that the rate of reaction of Zn^{2+} ion with the apoazo protein is very rapid, barely within the stopped-flow range, and that, therefore, the ligand effects on the metal ion entry would be best studied with the slower reacting cobalt(II) ion and complexes. In addition, the uptake of Zn^{2+} is triphasic, the last two stages representing second-order interaction of Zn^{2+} with arsanilazotyrosine-248 carboxypeptidase ($k = 6.7 \times 10^5\ M^{-1}\ s^{-1}$) followed by a first-order change (J. Hirose, work in progress). Spectral examination shows that in the last two stages, the zinc bond with the azo-modified tyrosine-248 is ruptured and that there is also a loss of enzyme activity. An inhibition of native enzyme activity by Zn^{2+} (in millimolar concentrations) has been reported (Vallee et al., 1960) and is presumably due to zinc binding to the protein with concomitant changes at the active site.

The behavior of the apoprotein on changing the pH resembles closely that reported by Vallee and his co-workers (Johansen & Vallee, 1973, 1975). In different conditions (0.5 M NaCl), a single pK at 9.4, an isosbestic point at 416 nm, and a maximum for the basic form at 485 nm were reported. These data are consistent with two interconvertable species, the two ionized forms of the azophenol (I minus Co; OH and O^-, respectively). Similarly, the spectral profile of cobalt arsanilazotyrosine-248 carboxypeptidase with pH has marked similarities to that of the zinc form. Thus the species present in the pH range examined can be considered, in analogy to the zinc system, to be the protonated azotyrosine-248 (pH 6.5) (I), the intramolecular azotyrosine-248–Co complex (pH 8.2–8.5) (I, O^- and azo N coordinated to Co), and the azotyrosine-248 phenolate ion (pH 10.2) (I, O^- not coordinated). These are related by two pK values of ~7.6 and ~9.1 to compare with values of 7.7 and 9.5 deduced for the zinc system

(Johansen & Vallee, 1973, 1975). At pH 8.2, cobalt arsanilazotyrosine-248 carboxypeptidase A has about 6 times the activity of its zinc analogue. With carboxypeptide A, the activity ratio is only ~2 (Coleman & Vallee, 1960). There have been no previous reports on the cobalt(II) form, but cadmium(II), nickel(II), zinc(II), and manganese(II) derivatives of the azoprotein have been described. These have absorption maxima at 490–550 nm and pK's in the ranges 6.5–8.1 and 9.4–10.1 (Johansen & Vallee, 1975). At pH 8.2 therefore, addition of Co^{2+} ion to the apoazocarboxypeptidase is attended by strong absorbance changes. Since the zinc–azotyrosine bond is extremely labile [relaxation time <35 μs (Harrison & Vallee, 1978)] and the cobalt–azotyrosine bond would be expected to be also, formation of the azotyrosine–Co^{2+} bond will rapidly follow the entry of Co^{2+} into the apo pocket and therefore act as a genuine monitor of the latter event. For the zinc analogue, it has been estimated as ~55% complexing of the metal by the azo arm at pH 8.8 from NMR experiments (Bachovchin et al., 1982). The extent of complexing for the cobalt analogue is unknown but is not a consideration when it acts simply as a chromophoric monitor.

In the kinetic experiments, prior attack of the metal ion species on the unattached arsanilazotyrosine–248 arm in the apo form and subsequent transfer of the whole moiety to the apo pocket is not considered likely for the following reasons. The absorbance change is markedly slowed in the presence of Cbz-Gly-Phe, which is known to bind to *apo*carboxypeptidase at or near the metal site with a formation constant $> 2 \times 10^2$ M^{-1} (Coleman & Vallee, 1962; Felber et al., 1962). The blocking of the metal site would not be expected to prevent metal ion interaction with the arsanilazotyrosine-248 arm, however, and *provided* this is attended by an absorbance change, the rapid process would still have been observed even in the presence of Cbz-Gly-Phe. In addition, the second-order rate constants for reaction of Co^{2+} and Zn^{2+} with apoazoprotein are quite close to those for reaction with apocarboxypeptidase itself, where no such ambiguity exists [k = 2×10^5 M^{-1} s^{-1} and 1.4×10^6 M^{-1} s^{-1}, respectively, at pH 8.2, 1.0 M NaCl, and 25 °C (Billo et al., 1978)].

From simple considerations, it can be concluded that the 1:1 cobalt(II) complexes with bi- and terdentate ligands must interact in toto with the protein and not via dissociated Co^{2+} ion. This has previously been shown for the reaction of cobalt(II) complexes with apo carbonic anhydrase (Table I) (Wilkins, 1973; Gerber et al., 1975) and is the current thinking for the mechanism of transfer of metal from zinc and copper thionein to a number of apoproteins (Li et al., 1980; Brady, 1982; Winge and Miklossy, 1982). Two-step processes have been previously noted with metal ion binding to apo liver alcohol dehydrogenase (Schneider & Zeppezauer, 1983) and with Cu^{2+} ion binding to apostellacyanin (Morpurgo et al., 1979), apoazurin (Marks & Miller, 1979), and apo superoxide dismutase (Rigo et al., 1978). These steps were interpreted as fast binding of metal ion, followed by a slower conformational change leading to native protein (L = H_2O or other ligands):

$$P + ML \underset{}{\overset{K_3}{\rightleftharpoons}} \text{intermediate} \xrightarrow{k_3} PM + L \qquad (3)$$

Uniphasic reactions only were observed in the present work, and this may indicate a simple bimolecular attack of ML on the apoprotein. Moreover, the absence of any leveling off of the k_{obsd}' vs. [CoL^{n+}] plots in Figure 3 up to 100 μM CoL^{n+} shows that K_3 in (3) must be $\leq 10^3$ M^{-1} and the fast step unaccompanied by substantial spectral change. Nevertheless, the reactions studied here may proceed in two (or more) steps. These may be envisaged in a number of ways. They could involve fast binding of metal complex, followed by slower conformational change, or, alternatively, very rapid attachment near the site and subsequent transfer (Schneider & Zeppezauer, 1983), or even "loose" complex formation transforming to "strong" complexation (Hunt & Ginsburg, 1981; Harrington et al., 1981, and references cited therein). In these events, $k_{obsd}' = K_3k_3$, and the composite quality of k_{obsd}' will compound the interpretation of ligand effects on metal ion entry. It is reasonable to assume that if the species PML is formed (say in the k_3 step), L is *very* rapidly released in a nonobservable process and PM and L result.

The second-order rate constant for interaction of Co^{2+} is about 10^3-fold larger with apocarboxypeptidase than with apo carbonic anhydrase. We have attributed this to a more flexible site in carboxypeptidase (Rosenberg et al., 1975a,b; Billo et al., 1978). This difference persists when cobalt(II) complexes also are compared, although there is not uniform behavior toward the two proteins. Thus, compared with the aquated ion, coordinated phenanthroline accelerates the entry of cobalt(II) into apoazocarboxypeptidase and decelerates that into apocarbonic anhydrase, so that the rate ratio is now 10^5. Coordinated 8-hydroxyquinoline-5-sulfonate has the opposite effect. Large coordinated ligands markedly slow the entry of the attached cobalt(II), although eventually complete incorporation of the metal takes place. This makes even more remarkable the reported similar rate constants for reaction of the bulky zinc thionein and zinc ion with apo carbonic anhydrase (Li et al., 1980). Although there are strong similarities in the active site geometries of carbonic anhydrase and carboxypeptidase (Argos et al., 1978; Lipscomb, 1980), subtle factors must determine the different effects of ligands (Table I). For example, one observes a rapid assimilation of the blue (tetrahedral?) cobalt(II)–thiocyanate complex by apo carbonic anhydrase (Harrington & Wilkins, 1980) but not by apoazocarboxypeptidase, in comparison with the aquated ion.

Finally, the formation constants of cobalt carboxypeptidase and cobalt azocarboxypeptidase as determined by kinetics are similar [6×10^7 M^{-1} at 25 °C (Billo et al., 1978) and 9×10^7 M^{-1} at 15 °C, respectively; both at pH 8.2 and I = 1.0 M]. In addition, they have similar rate constants for formation and dissociation (Billo et al., 1978). It is apparent then that the arsanilazotyrosine-248 arm has little influence on the stability of the cobalt protein.

A start has barely been made on the influence of ligand on the process generally illustrated in eq 1. Variable effects can already be seen quite difficult at this stage to rationalize. Although these studies are not directed per se to the elucidation of the mechanism of enzyme or protein function, ligand binding at the metal site can be a formidable probe of catalytic and inhibitive function (Pocker & Fong, 1983).

Acknowledgments

We appreciate helpful comments on the manuscript by Dr. B. L. Vallee.

Supplementary Material Available

Table showing raw kinetic data for varying concentrations of total cobalt and ligand and of total zinc and ligand (4 pages). Ordering information is given on any current masthead page.

Registry No. Co, 7440-48-4; Zn, 7440-66-6.

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