Electronic Spectroscopy of Cobalt Angiotensin Converting Enzyme and Its Inhibitor Complexes†

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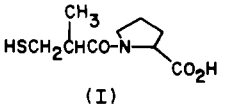
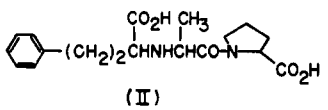
ABSTRACT: Zinc, the catalytically essential metal of angiotensin converting enzyme (ACE), has been replaced by cobalt(II) to give an active, chromophoric enzyme that is spectroscopically responsive to inhibitor binding. Visible absorption spectroscopy and magnetic circular dichroic spectropolarimetry have been used to characterize the catalytic metal binding site in both the cobalt enzyme and in several enzyme–inhibitor complexes. The visible absorption spectrum of cobalt ACE exhibits a single broad maximum (525 nm) of relatively low absorptivity (ϵ = 75 M^{-1} cm^{-1}). In contrast, the spectra of enzyme–inhibitor complexes display more clearly defined maxima at longer wavelengths (525–637 nm) and of markedly higher absorptivities (130–560 M^{-1} cm^{-1}). The large spectral response indicates that changes in the cobalt ion coordination sphere occur on inhibitor binding. Magnetic circular dichroic spectropolarimetry has shown that the metal coordination geometry in the inhibitor complexes is tetrahedral and of higher symmetry than in cobalt ACE alone. The presence of sulfur → cobalt charge-transfer bands in both the visible absorption and magnetic circular dichroic spectra of the cobalt ACE–Captopril complex confirm direct ligation of the thiol group of the inhibitor to the active-site metal.

Angiotensin converting enzyme (ACE,[1] dipeptidyl carboxypeptidase, EC 3.4.15.1) is a large (M_r 140 000), heavily glycosylated, membrane-bound enzyme that contains one catalytically essential mole of zinc per mole (Bünning & Riordan, 1985; Kleeman et al., 1986). ACE is present on the lumenal surface of the endothelial cell (Ryan et al., 1975) where it cleaves the circulating peptides angiotensin I to the potent vasoconstrictor angiotensin II (Skeggs et al., 1954) and the vasodilator bradykinin to inactive hydrolysis products (Yang et al., 1970).

Tight-binding inhibitors of ACE have been characterized (Patchett & Cordes, 1985, and references cited therein) that are now effectively employed in the clinical treatment of hypertension. Successful inhibitor design has, in part, relied on the fact that ACE contains an active-site zinc ion. Thus, two of the inhibitors currently in clinical use, Captopril (I) and Enalaprilat (II), contain putative anionic groups, a mercaptide



(I)



(II)

and a carboxylate, respectively, that have the potential for metal ion ligation (Cushman et al., 1977; Patchett et al., 1980). However, no direct evidence has yet been published to confirm their binding to the active-site metal in ACE. Indeed, the design of ACE inhibitors has been based on analogy with the active-site structure of other zinc proteases, especially carboxypeptidase A and thermolysin, even though the environment of the metal ion in ACE seems to differ from that in the latter two proteases. For example, the active-site zinc in ACE is bound 100- and 1000-fold more weakly than in carboxypeptidase A and thermolysin, respectively (Kleeman et al., 1986). In addition, whereas Zn(II), Co(II), Mn(II), Cd(II), Ni(II), Pb(II), and Cu(II) all restore significant activity to apocarboxypeptidase A (Vallee et al., 1983; Schäffer & Auld, 1986), only Zn(II), Co(II), and Mn(II) restore activity to apo-ACE (Cushman & Cheung, 1971; Bünning & Riordan, 1985).

To determine the effect of inhibitors on the environment of the active-site metal, chromophoric cobalt has been substituted for the naturally occurring zinc. Cobalt substitution at the active site of a zinc metalloenzyme together with examination of the electronic absorption and MCD spectra is often instructive in elucidating the geometry and coordination number of the metal binding site (Vallee & Holmquist, 1980; Holmquist, 1986). MCD, in particular, has proved to be diagnostic of overall coordination geometry and has been applied successfully to other cobalt-substituted zinc metalloenzymes (Holmquist et al., 1975; Vallee & Holmquist, 1980; Bicknell et al., 1986a,b). Due to the low molar absorptivities of cobalt-substituted enzymes, including ACE, large quantities of the enzyme are required. The recent development in this laboratory of an affinity purification procedure (Pantoliano et al., 1984) has permitted the isolation of high-purity ACE in the required quantities.

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[1] Abbreviations: ACE, angiotensin converting enzyme; CPD, carboxypeptidase A; TL, thermolysin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FaFGG, 2-furanacryloyl-Phe-Gly-Gly; FaGLA, 2-furanacryloyl-Gly-Leu-Ala; Sepharose–28–CA-Phe-Gly, *N*-[1(*S*)-carboxy-5-aminopentyl]-Phe-Gly immobilized on Sepharose with a 28-Å spacer; Enalaprilat, *N*-[1(*S*)-carboxy-3-phenylpropyl]-Ala-Pro; Captopril, D-(3-mercapto-2-methylpropanoyl)-Pro; HSAc, mercaptoacetyl; CD, circular dichroism; MCD, magnetic circular dichroism; TLC, thin-layer chromatography; SDS, sodium dodecyl sulfate; M, metal.

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The results presented here delineate the active-site metal ion geometry for both ACE and its inhibitor complexes and provide insights into the mechanism of ACE inhibition by Captopril and Enalaprilat. A preliminary account of this work has been reported (Riordan et al., 1986).

Materials and Methods

Materials. ACE was purified from young rabbit lungs (Pel-Freez Biologicals, Inc.) by affinity chromatography on Sepharose–28–CA-Phe-Gly as previously described (Pantoliano et al., 1984). The affinity ligand was prepared by the improved procedure described below, based on the method of Escher and Bünning (1986). Cobalt chloride was of "SpecPure" grade from Johnson Matthey Co. Ltd., England. FaFGG and FaGLA were prepared and assayed as previously described (Bünning et al., 1983).[2] Captopril and 3-mercaptopropanoyl-Arg were gifts of Dr. S. J. Lucania, Squibb Institute for Medical Research, Princeton, NJ, and Enalaprilat was a gift of Dr. A. A. Patchett, Merck, Sharp & Dohme Research Laboratories, Division of Merck and Co., Inc., Rahway, NJ. Other inhibitors were prepared in an earlier study (Holmquist & Vallee, 1979).

Synthesis of CA-Phe-Gly. D-Phenyllactate benzyl ester was prepared by saturating a benzyl alcohol suspension of D-phenyllactic acid with dry HCl gas, allowing the cleared solution to stand 16 h, adding methylene chloride, extracting with saturated $NaHCO_3$, drying over $MgSO_4$, and distilling under vacuum. The ester was collected as a clear oil: bp 185 °C (2.5 mmHg); IR (Nujol) 3480, 1780, 1490, and 1450 cm^{-1}. To a stirred solution of the ester (5.1 g, 20 mmol) and pyridine (1.6 mL) in 30 mL of CH_2Cl_2 at −8 °C was added 8.5 g (30 mmol) of trifluoromethanesulfonic acid anhydride over a period of 30 min. The oil obtained upon evaporation of the CH_2Cl_2 under vacuum was taken up in 1:1 ethyl acetate/cyclohexane and applied to a 2 × 14 cm column of silica gel (Baker flash chromatography grade) and eluted with 1:4 ethyl acetate/cyclohexane. The product eluted with the front and was evaporated to an oil (6.5 g). The oil (4.95 g) dissolved in 20 mL of CH_2Cl_2 was added dropwise to a solution of triethylamine (1.15 g, 11 mmol) and 3.2 g (11 mmol) of N^{ϵ}-*t*-Boc-L-lysine methyl ester (Bachem) (prepared from the hydrochloride by treatment with 1 M K_2CO_3 and extraction into ethyl acetate in 50 mL of CH_2Cl_2). After 10 min at 0 °C and 16 h at room temperature, the solution was washed twice with water, dried with $MgSO_4$, evaporated to an oil that was taken up in a minimum of ethyl acetate/cyclohexane (1:2), and chromatographed with this solvent as above. The product, identified by TLC (silica gel, 1:2 ethyl acetate/cyclohexane, $R_f = 0.7$, ninhydrin spray), eluted after 150 mL of eluent. The combined fractions were evaporated to an oil and hydrogenated (10% Pd on C) at atmospheric pressure in methanol for 2 h. Upon removal of most of the methanol the white product (1.7 g) of *N*-(1-carbomethoxy-5-*t*-Boc-aminopentyl)-L-phenylalanine crystallized: mp 141–142 °C; IR (Nujol) 3400, 1750, and 1690 cm^{-1}. This material (1 g, 2.5 mmol) was suspended in 40 mL of CH_2Cl_2 with glycine methyl ester hydrochloride (1 g, 8 mmol), triethylamine (2.3 g, 23 mmol) at −12 °C, and propylphosphoric anhydride (Riedel de Haen, Hanover, Germany) (1.6 mL of a 50% solution in CH_2Cl_2) was added dropwise over 5 min and stirred at −12 °C for 2 h. The mixture was extracted with water, with 1 M KH_2PO_4 saturated with NaCl, with saturated $NaHCO_3$, and with 1 M NaCl and then dried over $MgSO_4$. The residue remaining after evaporation of solvent was taken up in 5 mL of CH_2Cl_2/methanol (12:1) and chromatographed in this solvent as above. The product eluted with the front and upon evaporation of the solvent gave an oil (0.8 g) that resisted crystallization: R_f = 0.82 (silica gel, CH_2Cl_2/methanol, 4:1); IR (neat) 3350, 2950, 1730, 1710, 1680, and 1510 cm^{-1}. The blocked ligand was stored as the oil and deblocked immediately prior to coupling to the resin (Pantoliano et al., 1984) by treatment of 0.5 g with 2 mL of H_2O and 10 mL of trifluoroacetic acid for 5 h at room temperature followed by evaporation in vacuo and then addition of 5 mL of methanol and 5 mL of 1 M NaOH. After 1 h at room temperature, the solution was neutralized with HCl. TLC on silica gel (ethanol/25% aqueous NH_3, 2:1) gave a single ninhydrin positive spot, $R_f = 0.71$, that comigrated with CA-Phe-Gly prepared by the earlier procedure (Pantoliano et al., 1984). Its K_i toward ACE (0.2 μM) was also identical.

Instrumentation. Absorption spectra were recorded with a Varian Cary 219 spectrophotometer. Spectra were recorded on 200-μL samples with a 1-cm path length. Apo-ACE was used as reference. An Apple IIe computer system with a Varian Master Scan program was utilized to store spectra directly onto floppy disks.

CD and MCD measurements were performed at 25 °C on 1-mL samples with a 1-cm path length with a Cary 61 spectropolarimeter equipped with a Varian 4145 superconducting magnet and a Varian 4106 superconducting power supply. MCD spectra were recorded with a magnetic field strength of 4 T. The Cary 61 is interfaced to an Apple IIe microcomputer, allowing signal averaging and data manipulation procedures to be performed routinely. MCD spectra are corrected for CD, which in the 400–700-nm wavelength range was very weak for cobalt ACE and its derivatives.

Atomic absorption analysis was performed with a Perkin-Elmer Model 5000 graphite furnace spectrophotometer.

Methods. ACE was routinely assayed with FaFGG (Holmquist et al., 1979). Standard assay conditions were FaFGG (100 μM) in 0.05 M Hepes, 0.3 M NaCl, and 1 μM zinc acetate at pH 7.5 and 25 °C. One unit of activity is that amount of ACE which gives an initial velocity (ΔA_{334nm}/min) of 1. Enzyme purified by affinity chromatography had a specific activity of 82 units/mg and was >95% homogeneous as judged by SDS gel electrophoresis. ACE concentrations were determined on the basis of $\epsilon_{280nm} = 2.25 \times 10^5$ M^{-1} cm^{-1} (Shapiro & Riordan, 1984). ACE was concentrated as required by ultrafiltration using an Amicon concentrator with a YM 10 membrane. Standard precautions were taken to avoid adventitious metal ion contamination (Thiers, 1957), including metal extraction of all buffers with dithizone (0.001%) in carbon tetrachloride. Glass- and plasticware were placed in 6 M nitric acid for several hours and then thoroughly rinsed with deionized water before use.

Kinetic parameters were determined from complete progress curves by the half-time method of Wharton and Szawelski (1982). The dependence of ACE activity on cobalt concentration was determined by assays in which hydrolysis was first order in substrate ([S] ≪ K_m). Assays were initiated by addition of metal ion to the apo-ACE-containing substrate solution. On completion, each assay solution was analyzed by atomic absorption spectroscopy to determine the zinc and cobalt content. Activities expressed as k_{cat}/K_m, in units of M^{-1} min^{-1}, were obtained from half-lives after full hydrolysis according to

$$k_{cat}/K_m = k_{obsd}/[E]$$

where k_{obsd} is the first-order rate constant of the reaction and

[2] All amino acids are of the L configuration unless otherwise indicated.

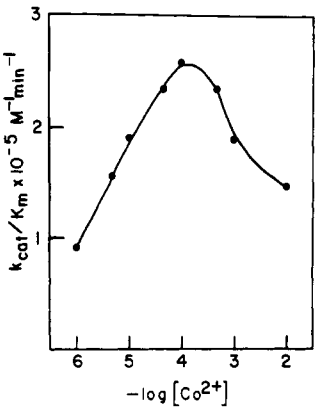


FIGURE 1: Restoration of enzymatic activity to apo-ACE (2 μM) by cobalt(II) chloride. Activities were measured as described under Methods. Conditions: 100 μM FaGLA, 0.05 M Hepes, 0.3 M NaCl, pH 7.5, 25 °C.

[E] is the enzyme concentration in the reaction mixture.

Preparation of Cobalt ACE. The zinc-free enzyme was prepared by dialysis of concentrated enzyme against a 200-fold volume excess of 1,10-phenanthroline (10 mM) dissolved in 0.05 M Hepes and 0.3 M NaCl at pH 7 and 4 °C for 12 h. The chelating agent was removed by dialysis against three changes of a 200-fold volume excess of metal-free 0.05 M Hepes and 0.3 M NaCl at pH 7.5 and 4 °C for 12 h. Cobalt ACE was prepared by addition of cobalt chloride as required. Spectra of cobalt ACE–inhibitor complexes were obtained by first adding the inhibitor followed by cobalt chloride. At pH 7.5 weak binding of cobalt to the apoenzyme necessitated addition of excess cobalt chloride to ensure full active-site occupancy by the metal (see below).

Spectral Determination of Cobalt Binding to Apo-ACE at pH 7.5. Apo-ACE (191 μM) in 0.05 M Hepes and 0.3 M NaCl, pH 7.5, was titrated with cobalt(II) chloride and the change in absorbance monitored at 520 nm. Subtraction of the enzyme concentration from the total cobalt concentration gave the excess free cobalt concentration from which the appropriate absorbance correction for free cobalt (ϵ_{520nm} = 4.6 M^{-1} cm^{-1}) was made. The correction assumes stoichiometric binding of cobalt to the enzyme; free cobalt that was present because of nonstoichiometric binding gave rise to a negligible absorbance. A plot of $1/A_{520nm}$ against $1/[Co^{2+}]$ was then made to obtain K_D.

RESULTS

Activation and Inhibition of ACE by Cobalt. Apo-ACE was assayed with FaGLA over a range of cobalt ion concentrations (10^{-6}–10^{-2} M) to determine the cobalt ion dependence of catalysis. The slow turnover of this substrate [for Zn ACE: k_{cat} = 410 min^{-1}, K_m = 2.4 mM (Bünning et al., 1983)] permitted a high enzyme concentration (2 μM) to be used in an assay, thus minimizing activity due to adventitious metal ion contamination. Assays were initiated by addition of metal ion to a solution containing apo-ACE and substrate. This procedure allowed initial determination of the "apparent" apoenzyme activity, which was less than 3% of that of the zinc enzyme and which corresponded to that expected from the determined zinc contamination (160 nM) of the reaction solution. Figure 1 shows that addition of cobalt to apo-ACE generates activity that peaks at 10^{-4} M cobalt and is inhibited by higher metal concentrations (Bünning & Riordan, 1985). The peak cobalt activity corresponded to 120% of the zinc enzyme activity. The absence of a well-defined plateau of maximal activity indicates that the cobalt binding constants for the catalytic and inhibitory sites are similar and that partial

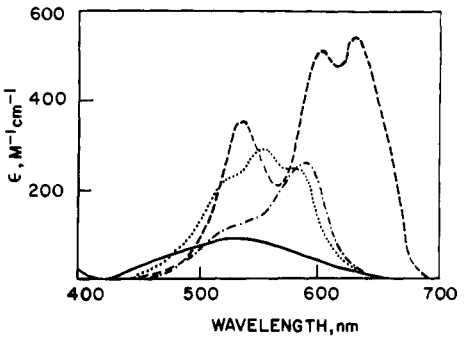


FIGURE 2: Visible absorption spectra of cobalt(II) ACE (—) and the enzyme–inhibitor complexes with Captopril (---), HSAc-Phe-Ala (–·–), and Enalaprilat (···). Conditions: ACE (111 μM), 0.3 M NaCl, 0.05 M Hepes, pH 7.5, and (i) 1.5 mM cobalt(II) chloride, (ii) Captopril (200 μM) and cobalt(II) chloride (111 μM), (iii) HSAc-Phe-Ala (1 mM) and cobalt(II) chloride (200 μM), and (iv) Enalaprilat (111 μM) and cobalt(II) chloride (111 μM). The spectrum of cobalt(II) ACE is corrected for absorption of excess cobalt(II) chloride. The spectrum of the ACE–HSAc-Phe-Ala complex is corrected for absorption due to the presence of a cobalt–inhibitor complex formed by the excess cobalt. Addition of Captopril (200 μM) to cobalt(II) chloride (111 μM) did not change the absorption spectrum within the range 255–700 nm at this pH.

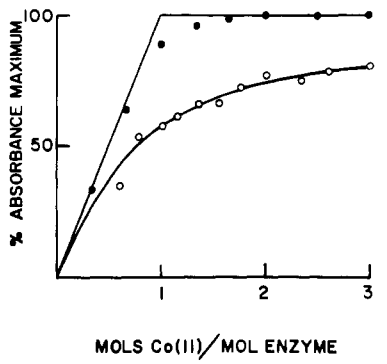


FIGURE 3: Spectral titration of apo-ACE with cobalt(II) chloride in the absence of (○) and presence (●) of 1 equiv of Enalaprilat. In the absence of Enalaprilat the titration was carried out at 520 nm, in its presence at 550 nm. The curve through the points obtained in the absence of Enalaprilat is theoretical and was calculated by assuming a K_D for Co(II) of 133 μM and an ϵ_{520} for Co(II) ACE of 75 M^{-1} cm^{-1} (see Figure 2). Conditions: ACE [either 191 (○) or 111 μM (●)], 0.3 M NaCl, 0.05 M Hepes, pH 7.5.

occupancy of the inhibitory site occurs before full occupancy of the tighter catalytic site. Cobalt(II) is, however, more tightly bound by the catalytic site in ES complex(es). Thus, assay under zero-order conditions (saturating substrate) showed that k_{cat} was invariant with cobalt(II) concentrations between 5 and 300 μM. The determined kinetic parameters (k_{cat} = 600 min^{-1}, K_m = 2.2 mM) confirmed that FaGLA is a slow substrate not only for the zinc but also for the cobalt(II)-substituted enzyme.

Titration of Apo-ACE with Cobalt and Visible Absorption Spectrum of the Cobalt Enzyme. Addition of cobalt to apo-ACE also generates visible absorption in the 450–600-nm spectral region. The absorption spectrum of fully formed cobalt ACE (Figure 2) is comprised of a broad band of low absorptivity with a maximum at 525 nm (ϵ = 75 M^{-1} cm^{-1}) and minor shoulders to either side. Spectral titration of apo-ACE with cobalt monitored at 520 nm indicates weak association (Figure 3). Thus, with 191 μM ACE more than 5 mol of cobalt/mol of enzyme is required to achieve greater than 90% saturation. A double-reciprocal plot of $1/A_{520nm}$ against $1/[Co^{2+}]$ is linear and gives an estimated K_D of 133 μM for cobalt binding to the catalytic metal site. The absence of significant change in the absorption spectrum with cobalt

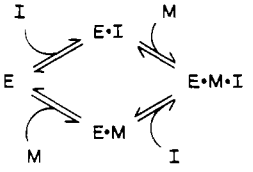
Table I: Inhibition Constants for ACE and Absorption Bands of Cobalt ACE and Cobalt ACE–Inhibitor Complexes[a]

inhibitor	$K_{i(app)}$[b] (nm)	absorption bands, λ_{max} (nm) [ϵ (M^{-1} cm^{-1})]
none		525 (75)
Captopril	0.33[c]	540 (350), 618 (520), 637 (560)
3-mercaptopropanoyl-Arg	65.0[d]	542 (350), 610 (520), 634 (520)
HSAc-Phe	75.0[e]	552 (350), 604 (470), 623 (480)
HSAc-Phe-Ala	510.0[e]	525 (130), 587 (310)
Enalaprilat	0.05[c]	524 (240), 552 (300), 580 (260)

[a] Conditions for recording of spectra: cobalt ACE (111 μM), 0.05 M Hepes, 0.3 M NaCl, pH 7.5, 25 °C. The spectra of the Captopril and Enalaprilat complexes were recorded in the presence of 1 enzyme equiv of cobalt(II) chloride. The spectrum of cobalt ACE was recorded in the presence of 1.5 mM cobalt(II) chloride and is corrected for absorption by excess free cobalt. The remaining complexes were recorded in the presence of 1 mM cobalt and were corrected for absorption due to formation of a cobalt–inhibitor complex by the excess cobalt and inhibitor present. [b] For inhibition of zinc ACE. [c] Shapiro and Riordan (1984). [d] Cushman et al. (1977). [e] Holmquist and Vallee (1979).

concentrations above 0.5 mM indicates that cobalt binding to the inhibitory site is spectrally weak.

Visible Spectra of Cobalt ACE–Inhibitor Complexes and Titration of Apo-ACE with Cobalt in the Presence of Inhibitors. The response of the cobalt ACE spectrum to inhibitors is both dramatic and varied (Figure 2 and Table I). Changes occur in both the maxima and intensities of the cobalt d → d transitions. The cobalt ACE–Enalaprilat spectrum displays bands at lower wavelengths with a central band at 552 nm (300 M^{-1} cm^{-1}) and side bands at 524 (240 M^{-1} cm^{-1}) and 580 nm (260 M^{-1} cm^{-1}) (Figure 2). The spectrum of the cobalt ACE–Captopril complex is characterized by two intense bands at 618 (520 M^{-1} cm^{-1}) and 637 nm (560 M^{-1} cm^{-1}) and a less intense absorption at 540 nm (350 M^{-1} cm^{-1}) (Figure 2). The 2-mercaptoacetyl dipeptide HSAc-Phe-Ala induces an intense absorption band at 587 nm (310 M^{-1} cm^{-1}) and two shoulders at 525 (130 M^{-1} cm^{-1}) and 545 nm (160 M^{-1} cm^{-1}), respectively (Figure 2). In general, the 525-nm absorption band of cobalt ACE shifts to higher wavelength when an inhibitor complex is formed (for example, with Captopril a band is observed at 637 nm), and the molar absorptivities increase concurrently from 75 M^{-1} cm^{-1} to values as large as 560 M^{-1} cm^{-1}. Cobalt binding to ACE is much tighter in the presence of inhibitors such as Enalaprilat. Enalaprilat binds weakly to the apoenzyme (Bull et al., 1985) but tightly to metal-containing ACE and shifts the equilibrium to the form E·M·I:



Thus, titration with cobalt is stoichiometric in the presence of 1 or more equiv of Enalaprilat, with an enzyme concentraion as low as 111 μM (Figure 3).

Spectra of the cobalt ACE–Captopril and cobalt ACE–Enalaprilat complexes were also recorded in the 250–400-nm region. The difference spectrum of the cobalt ACE–Captopril complex minus those of both (i) cobalt ACE alone and (ii) Captopril exhibits a band at 286 nm (Figure 4) indicative of a sulfur → cobalt(II) charge-transfer transition. In contrast, no absorption changes in this region were detected upon Enalaprilat binding to cobalt ACE.

MCD Spectra of Cobalt ACE and the Inhibitor Complexes with Captopril and Enalaprilat. The MCD spectrum of cobalt ACE is unusual for a cobalt(II) species in that it exhibits a broad, weak negative ellipticity between 480 and 600 nm (Figure 5A, Table II). The spectra of the inhibitor complexes are both more structured and more intense than those of the uncomplexed enzyme. In particular, that of the cobalt ACE–Captopril complex has an intense negative extremum at an unusually high wavelength (640 nm). The CD and MCD spectra of the cobalt ACE–Captopril complex were also recorded at lower wavelength, 250–400 nm (Figure 5B). A sulfur → cobalt(II) charge-transfer absorption is apparent at 336 nm with $\Delta\epsilon_M$ = 0.23 M^{-1} cm^{-1} T^{-1}. The wavelength extremum and intensity of this band are similar to those assigned to sulfur → cobalt(II) charge-transfer transitions in several other enzymes and enzyme–inhibitor complexes (Table III). This band is not resolved from tailing protein absorption in the electronic absorption spectrum. In addition, a more intense band is observed as a derivative spectrum at lower wavelength (inflection at 281 nm, $\lambda_{extrema}$ = 272.5, 287 nm, $\Delta\epsilon_M$ = +9.15, −6.5 M^{-1} cm^{-1} T^{-1}) (Figure 5B) corresponding to the 286-nm sulfur → cobalt(II) charge-transfer transition seen in the absorption spectrum (Figure 4). In the absence of enzyme, cobalt(II) and Captopril when mixed at the same concentration and at the same pH give no signal between 250 and 400 nm.

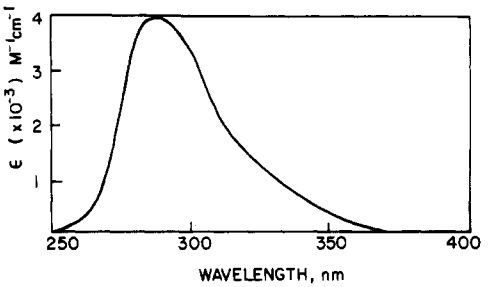


FIGURE 4: Near-UV absorption difference spectrum of the cobalt ACE–Captopril complex against cobalt(II) ACE alone. Conditions: ACE (8.9 μM) in 0.05 M Hepes, 0.3 M NaCl, Captopril (88 μM), pH 7.5. Captopril does not absorb within this wavelength range.

Table II: Major Wavelength Extrema in the MCD Spectra of Cobalt(II) ACE and Its E·I Complexes with Enalaprilat and Captopril

	$\lambda_{extremum}$ (nm)	$10^2\Delta\epsilon_M$ (M^{-1} cm^{-1} T^{-1})
Co(II) ACE	525 (br)	−12
	550 (br)	−12
Co(II) ACE + Enalaprilat	518	+4.5
	545	−11.5
	561	−16.5
	578	−23
	591 (s)	−16.5
Co(II) ACE + Captopril	336	+23
	565	+11.5
	645	−43
	666 (s)	−18

Table III: Comparison of Sulfur → Cobalt(II) Charge-Transfer Transitions Reported for Some Cobalt-Substituted Zinc Metalloenzymes and Enzyme–Inhibitor Complexes As Detected by MCD

Co enzyme	$\lambda_{extremum}$ (nm)	$\Delta\epsilon_M$ (M^{-1} cm^{-1} T^{-1})	ref
β-lactamase II	348	+0.1	Baldwin et al. (1980)
	350	+0.056	Bicknell et al. (1986)
CPD–HSAc-D-Phe	350	+0.362	Holmquist and Vallee (1979)
TL-HSAc-Phe-Ala	328	+0.298	Holmquist and Vallee (1979)
ACE–Captopril	336	+0.23	this paper
	281	inflection	this paper

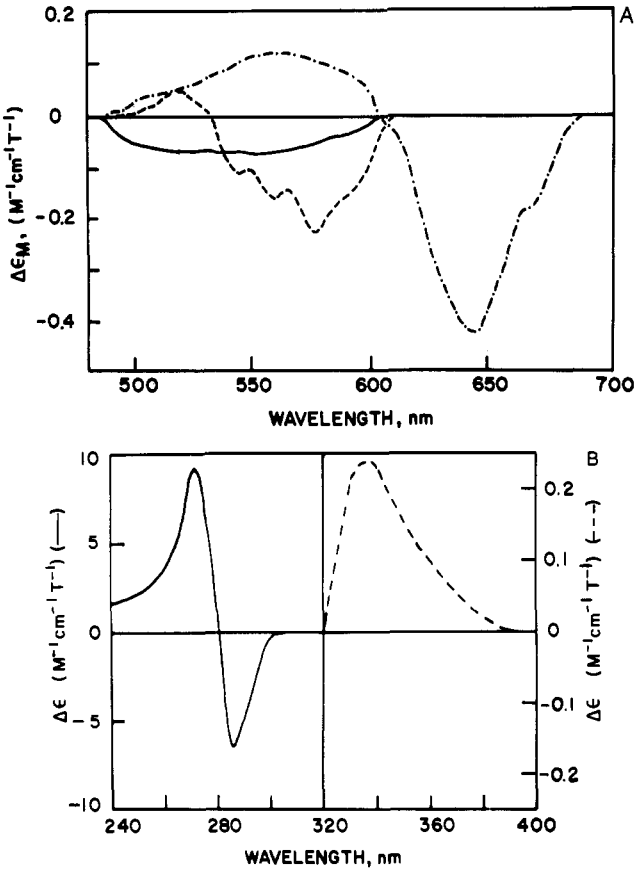


FIGURE 5: (A) MCD spectra of cobalt(II) ACE (72 μM) (—) and its complexes with Enalaprilat (---) and Captopril (-·-) in 0.3 M NaCl and 0.05 M Hepes, pH 7. Cobalt(II) chloride (1 mM) was present during recording of the cobalt(II) ACE spectrum, and in this case the MCD spectra were corrected for weak signals due to free cobalt(II). Excess cobalt(II) was not present in the inhibitor-containing samples. Twenty scans were computer averaged to obtain a final spectrum. One equivalent (72 μM) of either Enalaprilat or Captopril was added to obtain the E·M·I complex. (B) MCD difference spectrum of the cobalt(II) ACE–Captopril complex against cobalt(II) ACE alone, showing charge-transfer bands at 281.3 and 336 nm. The spectrum is corrected for natural CD. Five scans were computer averaged to obtain the final spectrum. Conditions: (left panel) 240–320 nm, ACE (8 μM), Captopril (8 μM), and cobalt(II) chloride (8 μM); (right panel) 320–400 nm, ACE (80 μM), Captopril (80 μM), and cobalt(II) chloride (80 μM). Both were in 0.05 M Hepes and 0.3 M NaCl, pH 7.5.

Discussion

Spectral studies of enzymatically active, cobalt-substituted zinc metalloenzymes have revealed a variety of active-site zinc coordination geometries, for example, four coordinate in carboxypeptidase A (Latt & Vallee, 1971) and thermolysin (Holmquist & Vallee, 1974), five coordinate in *Bacillus cereus* β-lactamase II (Bicknell et al., 1986b), and six coordinate in the regulatory site of *Aeromonas* aminopeptidase (Prescott et al., 1985) and in the structural and catalytic sites of *B. cereus* phospholipase C (Bicknell et al., 1986a). The spectral properties of the cobalt atom in these enzymes and its response to factors such as pH, inhibitors, and substrates at equilibrium and during various stages in the catalytic pathway have allowed deductions concerning the metal coordination geometry and the role of the metal in catalysis (Latt & Vallee, 1971; Auld et al., 1984, 1986; Geoghegan et al., 1986; Bicknell et al., 1986b).

Removal of the active-site zinc from ACE gives an inactive apoenzyme. Addition of cobalt to apo-ACE restores activity comparable to that observed with the native zinc enzyme, suggesting that the cobalt ion is bound to the active site of the enzyme and serves the catalytic role usually performed by zinc. The spectral K_D for cobalt binding to apo-ACE is 133 μM at pH 7.5, indicating significantly weaker binding than to carboxypeptidase A (Coleman & Vallee, 1961) or thermolysin (Holmquist & Vallee, 1974). This is consistent with the finding that the dissociation constant for zinc from native ACE is also low, 2–3 orders of magnitude weaker than that determined for carboxypeptidase A and thermolysin (Kleeman et al., 1986). The binding of cobalt to apo-ACE is, however, much tighter in the presence of the inhibitor Enalaprilat (Figure 3). This reflects formation of a tight E·M·I ternary complex and is in accord with previous findings that 1 mol of zinc is bound per mole of ACE (Bünning & Riordan, 1985).

Although spectral changes identify only one ACE metal binding site, it is clear from studies on the metal ion dependence of catalysis (Bünning & Riordan, 1985; Figure 1) that it also has at least one inhibitory metal binding site. A comparison of the spectral K_D (133 μM) with the relative activity data of Figure 1 shows that under first-order assay conditions the inhibitory site is partially occupied by cobalt before full occupation of the tight catalytic site occurs. This contrasts with zinc, where binding at the catalytic site is much tighter (K_D = 6 nM; Kleemann et al., 1986) than binding at the inhibitory site (Bünning & Riordan, 1985), which leads to full activity in first-order assays with zinc concentrations between 10^{-7} and 10^{-4} M.

The absence of a detectable spectral change when cobalt binds to the inhibitory site suggests that the inhibitory metal must be in an octahedral coordination sphere [see Holmquist et al. (1975) and Vallee and Holmquist (1980)].

The absorption spectrum of cobalt ACE (Figure 2) is characterized by a maximum at 525 nm with a molar absorptivity (75 M^{-1} cm^{-1}) comparable to that of cobalt carboxypeptidase A [maxima at 555 (150 M^{-1} cm^{-1}) and 572 nm (150 M^{-1} cm^{-1}), shoulder at 500 nm] and cobalt thermolysin [maximum at 555 nm (90 M^{-1} cm^{-1}), shoulder at 500 nm]. In the latter two cases, the active-site metal ion is known to have an irregular tetrahedral geometry [see Vallee and Galdes (1984) and references cited therein]. In contrast to the MCD spectra of cobalt-substituted carboxypeptidase A or thermolysin, which show similarity to those of tetrahedral model complexes (Holmquist et al., 1975), that of cobalt ACE (Figure 5A) is unusual in showing no clear similarity to those of either four-, five-, or six-coordinate models. Symmetric octahedral cobalt(II) complexes have molar absorptivities in the region of 5–10 M^{-1} cm^{-1} (Banci et al., 1982), and although asymmetry may increase this parameter by up to a factor of 10 (Ballhausen, 1960), the value of 75 M^{-1} cm^{-1} for cobalt ACE would (in the absence of sulfur ligands; see below) be unusually large for octahedral cobalt(II).

The absence of characteristic sulfur → cobalt charge-transfer bands in the cobalt ACE spectrum indicates that cysteine is not a metal ligand in ACE.

Interaction of Inhibitors with Cobalt ACE. The cobalt ACE visible absorption spectrum is unusually sensitive to inhibitor binding (Figure 2), and the spectra of the resultant cobalt ACE–inhibitor complexes have several features in common. Relative to the spectrum of cobalt ACE they show maxima at longer wavelengths and absorptivities several fold higher (as high as 560 M^{-1} cm^{-1} in the case of the cobalt ACE–Captopril complex). In addition, they show a well-resolved structure; in most instances three bands can be clearly discerned. Taken together, these features suggest that inhibitor binding promotes significant changes in the coordination environment of the metal ion and that in the E·M·I complexes

the active-site metal acquires a more regular tetrahedral-like geometry. However, the spectra of the various inhibitor complexes do not correspond in detail to those of model tetrahedral cobalt(II) complexes, suggesting some degree of metal coordination sphere distortion.

The MCD spectra of the cobalt ACE–Enalaprilat and cobalt ACE–Captopril complexes clearly show the metal to occupy a tetrahedral coordination sphere. The major difference between the spectra lies in the band positions, those of cobalt ACE–Captopril are of substantially greater wavelength than those of cobalt ACE–Enalaprilat. This difference probably arises as a result of the different inhibitor ligands to the metal (carboxylate in Enalaprilat and sulfhydryl in Captopril) and inherent differences in the electron-donating properties of oxygen and sulfur atoms.

Direct Interactions of Captopril with the Metal Ion. Sulfur to cobalt(II) charge-transfer bands have been observed in the absorption, CD, and MCD spectra of several cobalt-substituted metalloenzymes, notably horse liver alcohol dehydrogenase (Sytkowski & Vallee, 1978) and *B. cereus* β-lactamase II (Davies & Abraham, 1974; Baldwin et al., 1980). In both these enzymes the sulfur ligands are cysteine residues of the protein. Similar charge-transfer bands have also been observed in cobalt-substituted metalloenzymes that lack an intrinsic sulfur ligand to the metal, but form complexes with sulfhydryl-containing inhibitors in which the sulfur ligates the metal, e.g., *N*-(2-mercaptoacetyl)-D-Phe and carboxypeptidase A or *N*-(2-mercaptoacetyl)-Phe-Ala and thermolysin (Holmquist & Vallee, 1979). The observation of a similar band (Figure 4, Table III) when Captopril was bound to cobalt ACE confirms that here also the inhibitor binds directly to the metal.

For cobalt(II) in a site of distorted tetrahedral geometry and with a single sulfur ligand, four S → Co(II) ligand to metal change-transfer transitions are possible ($s_{\pi,\sigma} \rightarrow d_{x^2}$, $d_{x^2-y^2}$). In practice, two are usually observed within the 230–400-nm region (Holmquist, 1986), and indeed the Captopril–Co(II) ACE complex showed two bands, an intense derivative absorption with an inflection centered at 281 nm and a weaker absorption at 336 nm (Figure 5B, Table III), the latter apparent only in the MCD spectrum.

Three types of MCD transition, called A, B, and C terms, have been characterized (Serber, 1932). A terms arise when the transition is to a degenerate excited state [see Holmquist (1986)]. They are seen as symmetrical, biphasic signals crossing the zero axis at λ_{max}. While the Captopril–Co(II) ACE 281-nm transition is probably of this class, the absence of perfect symmetry points to multiple transitions and an apparent derivative absorption could arise from two overlapping simple transitions of opposite sign. The longer wavelength 336-nm transition is either a B (arising from magnetically induced mixing of states) or C (degenerate ground state) term. A knowledge of the temperature dependence of the transition intensity is required to distinguish between B and C terms [see Schatz and McCaffery (1970) and references cited therein].

The 286-nm band in the absorption difference spectrum and the 281-nm MCD band probably correspond to the same transition. The discrepancy in the λ_{max} and $\lambda_{extrema}$ likely results from an uncertainty in the λ_{max} of the absorption difference spectrum which arises because the charge-transfer absorption is such a small percent of the total absorption in this wavelength region. The 336-nm MCD band is unresolved in the absorption difference spectrum and illustrates the advantage of MCD over absorption spectroscopy in its ability to detect transitions hidden under an absorption envelope. Charge-transfer absorptions provide the first direct evidence that Captopril binds to the active-site metal ion in ACE.

Comparison of ACE with Other Zinc Metalloproteases. This investigation of cobalt-substituted ACE confirms earlier evidence (Kleeman et al., 1986) that the metal binding site of ACE differs markedly from those of the well-characterized metalloproteases carboxypeptidase A and thermolysin. Notably, (i) metal ions bind more weakly to ACE, (ii) spectra of the cobalt enzyme point to an exceptionally low symmetry metal ligand environment, and (iii) an unusually large spectral response accompanies inhibitor binding to the cobalt enzyme.

MCD spectra of cobalt ACE and its complexes with Captopril and Enalaprilat demonstrate that the metal ligand environment changes on binding inhibitor from one of low symmetry to a tetrahedral geometry. Such a change is in accord with the entatic state hypothesis of Vallee and Williams (1968), which states that relaxation of a low-symmetry center to one of high symmetry would be accompanied by an increase in ligand field stabilization energy, a factor that may play a part in the exceptionally tight binding of these inhibitors.

The physiological relevance of weak metal binding and a potential for reorganization of the metal ion environment are not clear, although physiological substrates of ACE such as angiotensin I and especially bradykinin are tightly bound by the enzyme (Bünning et al., 1983). Indeed, it may well be necessary for ACE to have such tight binding capability since many of its potential substrates are present in vivo at very low concentrations. Whether or not the ready dissociation of zinc from the enzyme under slightly acidic conditions has any significance in terms of regulation of activity remains to be established.

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Temperature Dependence and Mechanism of Local Anesthetic Effects on Mitochondrial Adenosinetriphosphatase

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ABSTRACT: Chloroform-released ATPase prepared from beef heart mitochondria is inhibited by tetracaine and dibucaine over the entire temperature range in which the enzyme is active. The temperature of maximal activity is at 60 °C in the absence of anesthetic and is shifted upward by 2–3 °C by the addition of 0.3 mM dibucaine. Local anesthetics protect ATPase from irreversible cold inactivation. The kinetics of this protective effect are analyzed by a thermodynamic model in which the associated/dissociated subunit equilibrium is shifted toward the associated state by the preferential binding of anesthetic to the associated state. The accessibility of buried sulhydryl groups to reaction with 5,5′-dithiobis(2-nitrobenzoic acid) is increased by local anesthetics; this is interpreted to mean that the anesthetics increase the conformational flexibility of the protein. It is proposed that the hydrophobic moieties of local anesthetics and related compounds bind to numerous hydrophobic sites or crevices on ATPase; this binding induces a perturbation of the protein conformation, which in turn causes a decrease of enzyme activity. This model is sufficiently general to encompass the diversity of molecules which have similar anesthetic-like effects, and since it relates to common fundamental features of protein structure, it may also be the mechanism of the nonspecific effects of these molecules on other proteins.

Local anesthetics, tricyclic antipsychotics, and related compounds inhibit mitochondrial ATPase in a partial and reversible manner (Penefsky et al., 1960; Vanderkooi et al., 1981; Palatini, 1982; Chazotte et al., 1982; Laikind et al., 1982; Saishu et al., 1983; Adade et al., 1984; Bullough et al., 1985). The membrane-bound ATPase and also the lipid-free preparations of F_1ATPase and chloroform-released ATPase are similarly inhibited by these compounds. The primary objective

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