(57 mg) was oxidized to sodium acetate (26 mg, 92%), and a portion of the sodium acetate was converted to *N*-acetyl-α-naphthylamine (**8**) by previously described procedures.[5]

C-4′ by Schmidt Reaction on Acetic Acid.[50] Sodium acetate (18 mg) was dissolved in concentrated sulfuric acid (1 mL). Sodium azide (60 mg) was added and the mixture was heated on a steam bath in a flask attached to a system of three gas traps. The carbon dioxide that evolved was passed by means of a stream of nitrogen into potassium hydroxide solution (15%, w/v). When gas evolution had ceased (approximately 4 h), the potassium hydroxide solutions in the traps were replaced by hydrochloric acid (1 M, 1 mL per trap). The acidic reaction mixture was cooled to near its freezing point in a dry ice/acetone bath, basified (to pH >12) by addition of potassium hydroxide solution (15%, w/v), and then heated 1.5 h on the steam bath while the system was flushed with nitrogen.

***N*-Methylphthalimide (9).**[60] *N*-Carbethoxyphthalimide (32 mg) was added to the hydrochloric acid solutions contained in the first two traps. The mixture was stirred, solid sodium carbonate was added to bring the mixture to about pH 9, and the mixture was stirred at room temperature for 1 h. The precipitate was collected by filtration and then sublimed at 75 °C (2×10^{-2} mmHg) (2.7 Pa). Recrystallization from methanol yielded pure *N*-methylphthalimide (9 mg, 42%). Thin-layer chromatography (silica gel, 1:4 ethyl acetate/cyclohexane, v/v) was used to monitor the presence of unchanged *N*-carbethoxyphthalimide (R_f 0.15) in the *N*-methylphthalimide (R_f 0.27): mp 134–135 °C (lit.[61] mp 134 °C); ^{1}H NMR ($CDCl_3$) δ 3.17 (s, 3 H), 7.61–7.94 (m, 4 H); MS, *m/e* 161 (M^+). Potassium phthalimide and methyl iodide were stirred in dimethylformamide for 3 h at room temperature. *N*-Methylphthalimide so formed was identical with the above sample.

C-5,6,7 by Permanganate Oxidation.[62] 4-Methyl-5-(β-phthalimidoethyl)thiazole (**6**) (21 mg) was suspended in dilute sulfuric acid (0.5 M, 1.5 mL) at room temperature. Potassium permanganate (80 mg) was added in portions over a 1-h period, and the mixture was stirred for an additional 2 h. It was decolorized by adding sodium hydrogen sulfite and extracted with ether (4 × 5 mL). The ether extracts were combined, washed with water (1 × 5 mL), dried over anhydrous magnesium sulfate, and evaporated in vacuo to dryness. The residue was sublimed at 105 °C (2×10^{-2} mmHg) (2.7 Pa) and recrystallized from water to yield pure *N*-phthaloyl-β-alanine (**7**) (9 mg, 53%): mp 150–151 °C (lit.[63] mp 150–151 °C); ^{1}H NMR (CD_3COCD_3) δ 2.74 (t, 2 H), 3.94 (t, 2 H), 7.83 (s, 4 H); MS, *m/e* 219 (M^+). This sample was identical with one obtained[64] by fusion of β-alanine with phthalic anhydride at 170 °C for 15 min.

Degradation of D-[6-^{14}C,6-^{3}H]Glucose (Experiment 18). Carrier D-glucose (500 mg) was added to a small portion (approximately 3 μCi) of the D-[6-^{14}C,6-^{3}H]glucose feeding solution, and this mixture was diluted to 100 mL with water.

β-D-Glucose Pentaacetate.[65] A portion (approximately 10 mL) of the above D-[6-^{14}C,6-^{3}H]glucose solution was evaporated to dryness in vacuo. Anhydrous sodium acetate (47 mg) and acetic anhydride (1 mL) were added to this noncrystalline residue (56 mg). The mixture was heated on a steam bath for 2.5 h, and ice water (5 mL) was added. An oil separated from solution at this point, but when it stood at 4 °C for 2 days, it became crystalline. The crystals were filtered off and sublimed at 120 °C (2×10^{-2} mmHg) (2.7 Pa) to yield pure β-D-glucose pentaacetate (42 mg, 34%), mp 131–132 °C (lit.[65] mp 131–132 °C).

C-6 of D-[6-^{14}C,6-^{3}H]Glucose as Formaldehyde Dimethone.[66] A small amount (approximately 6 mL) of the above D-[6-^{14}C,6-^{3}H]glucose solution was evaporated to dryness in vacuo. The noncrystalline residue (33 mg) was redissolved in water (2 mL), sodium bicarbonate solution (1 M, 2 mL) and sodium metaperiodate (200 mg) in water (2 mL) were added, and the mixture was kept at room temperature for 1 h. 5,5-Dimethylcyclohexane-1,3-dione (dimedone) (100 mg) in 95% ethanol (1 mL) was added, and after 10 min at room temperature, the white product was collected by filtration. Sublimation at 110 °C (2×10^{-2} mmHg) (2.7 Pa) yielded pure formaldehyde dimethone (53 mg, 98%), mp 190–192 °C (lit.[66] mp 189–190 °C).

Radioactivity Measurements. Triplicate samples of each compound were counted by liquid scintillation counting (Mark I liquid scintillation computer, Model 6860, Nuclear Chicago Corp.). Samples were dissolved in either water, aqueous ammonia (1%), *N*,*N*-dimethylformamide, or dimethyl sulfoxide and dispersed in Aquasol (New England Nuclear) (10 mL). The efficiency of counting was determined by external standardization with ^{133}Ba. The confidence limits shown in Tables I–VI are standard deviations from the mean.

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Registry No. 1, 67-03-8; **2**, 2908-73-8; **3**, 137-00-8; **4**, 82294-70-0; **4** semicarbazole, 67162-02-1; **5**, 533-45-9; **6**, 36956-91-9; **7**, 3339-73-9; **8**, 575-36-0; **9**, 550-44-7.

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^{1}H NMR Study of Cobalt(II)-Substituted Carboxypeptidase A

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Abstract: The water ^{1}H NMR relaxation rate has been measured for solutions containing cobalt(II)-substituted carboxypeptidase A in the Larmor frequency range 5–60 MHz. The values, corrected for the diamagnetic zinc enzyme, have been interpreted within the frame of the Solomon–Bloembergen theory and provide evidence for water coordinated at the metal. When the inhibitor β-phenylpropionate is added to the solution, the above type of measurements are consistent with the substitution of a water molecule by the inhibitor whereas a second water molecule remains coordinated. The correlation time is consistent with the five-coordination of the chromophore in both cases. The ^{1}H NMR spectra of the histidines bound to the metal have also been recorded. These data have been compared with those of a very refined X-ray structure.

Carboxypeptidase A (CPA hereafter) is a zinc enzyme of molecular weight 34 500 containing one zinc ion per molecule.[1-3] Its biological role is the hydrolysis of the C-terminal amino acid from a polypeptide substrate. X-ray data at 2-Å resolution have

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shown that the metal ion is bound to two histidine nitrogens and to a Glu residue.[4] In a very recent structural report at 1.75-Å resolution,[5] the latter residue is proposed to be bidentate with essentially equivalent Co–O distances. In the vicinity of the metal ion, there are Glu and Tyr residues: both of them play an important role in the catalytic mechanism; the latter group is flexible enough to have the possibility of binding to the metal for a fraction of the time,[6] although no evidence of such interaction is mentioned in the structural report. It is proposed that the coordination sphere is completed by one water molecule.[5]

As for other zinc enzymes, the native zinc(II) ion can be substituted by other dipositive metal ions, like Co^{2+}, Ni^{2+}, Mn^{2+}, Cu^{2+}, etc. Whereas nickel and manganese derivatives restore in part the peptidic activity, the cobalt ion is even able to enhance the catalytic activity of the enzyme.[7] Electronic and ESR studies performed on copper(II),[8,9] cobalt(II),[10-12] and nickel(II)[12] carboxypeptidases have shown that the active-site cavity may arrange metal ions in different stereochemistries. Interestingly, both the nickel and cobalt derivatives display enzymatic activity although they have different coordination geometries, six-coordinated in the former[12] and four[10,13] or five-coordinated[12,14] in the latter.

Owing to the similarities in the coordination chemistry between zinc(II) and cobalt(II), the latter CPA derivative has been a matter of extensive investigations.

Recently we have shown that water proton NMR longitudinal relaxation rates at different frequencies of solutions containing high-spin cobalt(II) proteins provide useful information on the electronic properties of the cobalt(II) ion and on the structural properties of the environment of the metal ion.[15,16] Indeed, such measurements provide the value of the electronic correlation time, which is bound to the electron spin relaxation time and to the order and symmetry of the electronic energy levels. In an external magnetic field of 1 T, pseudotetrahedral complexes display electronic correlation times of the order of 10^{-11} s and five-coordinated complexes of the order of 10^{-12} s.[15-17] Furthermore, the above measurements provide information on the number of protons feeling the paramagnetic center.

In order to obtain further information on the coordination polyhedron of the metal ion in the active site of CoCPA as well as on the binding of inhibitors, we have performed ^1H T_1^{-1} measurements of the water protons of CoCPA solutions in the frequency range 5–60 MHz. The ^1H NMR spectra of water and D_2O solutions of the protein part of the enzyme derivative have also been recorded with the aim of detecting the protons of residues bound to the metal ion.

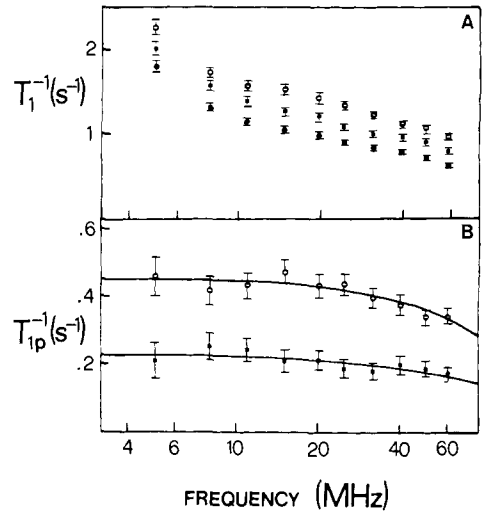

Figure 1. (A) Water ^1H T_1^{-1} values at 18 °C and pH 7.5 as a function of the Larmor frequency for (i) a solution of 2.2×10^{-3} M cobalt(II) carboxypeptidase, 1 M NaCl, 0.05 M Tris·HCl (○); (ii) a solution of 2.2×10^{-3} M cobalt(II) carboxypeptidase, 2×10^{-2} M β-phenylpropionate, 1 M NaCl, 0.05 M Tris·HCl (■); a solution of 2.2×10^{-3} M zinc(II) carboxypeptidase, 1 M NaCl, 0.05 M Tris·HCl (●). (B) ^1H T_{1p}^{-1} values obtained from the above data for water interacting with cobalt(II) carboxypeptidase (○) and its adduct with β-phenylpropionate (■).

Experimental Section

Carboxypeptidase A, prepared with the method of Cox,[18] was purchased by Sigma as an aqueous suspension and used without further purification. Apocarboxypeptidase and the cobalt(II) derivative were prepared as previously described.[10] Enzyme concentrations were determined from the electronic spectra in the UV region (ϵ_{278} 6.42×10^4 M^{-1} cm^{-1}).[19] The electronic spectra in the visible region satisfactorily compared with those previously reported.[10] The NMR spectra were run with an instrument based on a Bruker CXP console and a low-resolution Varian DA60 1.41 T electromagnet, equipped with an external lock circuit granting ±1-Hz long-term stability. The frequency dependence of the water proton longitudinal relaxation rates was investigated in the magnetic field range 0.094–1.41 T. The longitudinal relaxation rates, T_1^{-1}, were measured by the inversion–recovery method, using a nonlinear least-squares fitting program. The ^1H NMR spectra of the cobalt-coordinated histidine residues, in D_2O or H_2O solutions, were recorded with "modified DEFT"[20] or "Redfield"[21] pulse sequences in order to suppress solvent and diamagnetic signals. Instrumental details are given elsewhere.[22] T_1 measurements on the histidine signals were performed with a saturation–recovery type of experiment,[20] using the "modified DEFT" sequence and an appropriate nonlinear least-squares fitting method.

Results and Discussion

The water ^1H T_1^{-1} values for solutions containing 2.2×10^{-3} M CoCPA, 1 M NaCl, and 0.05 M Tris·HCl at pH 7.5 and 18 °C have been measured as a function of magnetic field in the proton Larmor frequency range 5–60 MHz; the measurements have been repeated in the presence of the inhibitor β-phenylpropionate (2×10^{-2} M), i.e., under the conditions of binding saturation.[10,23] The experimental values are reported in Figure 1A together with the values relative to a solution of the reconstituted ZnCPA under the same conditions. Both the zinc and cobalt samples were obtained from the same apoprotein solution

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Table I. Geometrical Factor and τ_c Values for Cobalt(II) Carboxypeptidase and Its Derivative with β-Phenylpropionate

	G, pm^{-6}	τ_c, s
cobalt(II) carboxypeptidase	5.8×10^{-15} (±3%)	3.1×10^{-12} (±3%)
cobalt(II) carboxypeptidase + β-phenylpropionate	2.9×10^{-15} (±1%)	3.1×10^{-12} (±1%)

in order to minimize the errors. The difference in T_1^{-1} between the two sets of samples (Figure 1B) is relatively small, although reproducible within 10%. To a first approximation such differences may be ascribed to the presence of the unpaired electrons and to their paramagnetic effects. Therefore the difference in T_1^{-1} can be defined as follows:

$$T_{1p}^{-1} = (T_1^{-1})_{Co} - (T_1^{-1})_{Zn}$$

Measurements between 4 and 25 °C have shown that the T_{1p}^{-1} values are constant with temperature, thus indicating fast exchange between free and bound water molecules. Under these circumstances T_{1p}^{-1} is entirely due to the coupling term between resonating nuclei and unpaired electrons. Such coupling should be in principle factorized in a dipolar and a contact contribution. It has been shown[15,16,24] that for a high-spin cobalt(II) complex the contact contribution is completely quenched. Solomon and Bloembergen have derived the following equation[25] for a fully dipolar origin of the coupling between nuclei and unpaired electrons under the limit conditions that the electronic ground state is orbitally nondegenerate and the excited states are high in energy:

$$T_{1p}^{-1} = fG\frac{2}{15}S(S+1)\gamma_I^2 g^2\beta^2\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right) \quad (1)$$

where $G = \sum_i(1/r_i^6)$. Here f is the ratio between the enzyme concentration and the molarity of protons in water, r_i is the distance of the various exchanging protons from the metal ion, τ_c is the correlation time, and the other symbols have the usual meaning. Clearly such an equation is only approximate[26] for high-spin cobalt(II) systems in which several excited states are close to the ground state. As a consequence, the magnetic susceptibility is different from the spin-only value, and magnetic anisotropy arises. The G and τ_c values obtained through a two parameters fitting of the T_{1p}^{-1} data to eq 1 are reported in Table I. Although the absolute values may be somewhat affected by the inadequacy of the theory, their order of magnitude and the comparison between the two cobalt systems are still quite meaningful.

First of all, the τ_c values of about 3×10^{-12} s are 1 order of magnitude smaller than the values found for cobalt carbonic anhydrase, which is pseudotetrahedral,[27] and 1 order of magnitude larger than the octahedral $Co(CH_3OH)_6^{2+}$ complex.[28] They are of the same magnitude as five-coordinate inhibitor derivatives of cobalt carbonic anhydrase.[15] Therefore the present data are strongly in favor of five-coordination for both chromophores. The comparison of the G factors is also instructive: by addition of β-phenylpropionate the G factor decreases from 5.8×10^{-15} to 2.9×10^{-15} pm^{-6}, τ_c remaining the same.

In the case of cobalt carbonic anhydrase, G values ranging from 2.4×10^{-15} to 5.5×10^{-15} pm^{-6} were taken as evidence of coordinated water,[15] while outer-sphere contributions were found to be 2–6 times smaller. We feel that the present data are indicative of exchanging protons interacting with the cobalt ion and in particular that a water molecule is still present in the coordination sphere when the inhibitor is bound to the metal. Since the τ_c values are typical of a five-coordinated chromophore while the electronic spectra rule out the possibility of six-coordination, it is proposed that in the cobalt derivative the Glu residue behaves as monodentate. Consistently the 2-fold increase in the G value of the noninhibited enzyme would indicate the presence of two water molecules in the coordination sphere. The possibility exists that Cl^-, which is present in large concentration in order to allow the solubilization of the protein and cannot therefore be present in the X-ray structure, may also compete, although to minor extent, to one of the two binding sites.

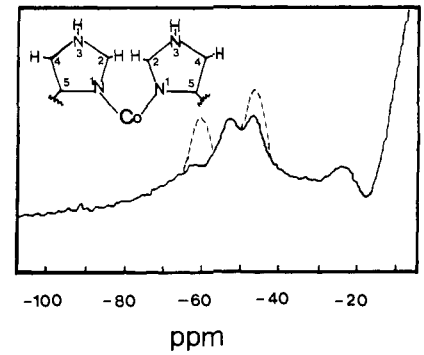

Figure 2. 60-MHz ^{1}H NMR spectrum of cobalt(II) carboxypeptidase at pH 7.5 (1 M NaCl, 0.05 M Tris·HCl). The dotted lines refer to the imino protons of the cobalt-bound histidines, which are observed in H_2O solution. The mode of binding of the two histidine residues is also shown.

The availability of two coordination positions to solvent and solute molecules agrees with several pieces of evidence obtained on different metal derivatives, such as the still large ^{1}H T_{1p}^{-1} values observed in the copper derivative after addition of β-phenylpropionate,[9] the finding that in the manganese derivative water and fluoride can be simultaneously bound to the metal,[29] and the report of an unusually close water molecule bound to manganese in the noninhibited enzyme,[30] which may indicate binding of more than one solvent molecule. Kinetic studies of inhibition by bidentate ligands have also pointed out this possibility in the cobalt derivative.[31] Finally, a partially bidentate behavior of the substrate glycyl-L-tyrosine has been found even in the recent X-ray investigation on the zinc enzyme;[5] owing to the bidentate behavior of Glu-72 reported there, zinc is proposed to be at least fractionally six-coordinated. Interestingly, the coordinated carbonyl oxygen of the Gly-Tyr substrate, equivalent to the carboxyl oxygen of the β-phenylpropionate used in the present study, does not overlap the coordination site of water in the pure enzyme.[5]

The small τ_c value of CoCPA stimulated us to record the ^{1}H NMR spectrum of the enzyme as a whole, since such a small value should not cause too severe a broadening of the resonances of protons close to the paramagnetic center. The spectra recorded at 60 MHz in D_2O show two well-resolved signals at −46 and −52 ppm downfield from Me_4Si (Figure 2); their T_1 values are 6.9 and 6.7 ms, respectively. A broad signal appearing as a shoulder in the downfield region may also be located at about −60 ppm. Another signal, again broad, is present at −26 ppm; its relative proximity to the diamagnetic protein signals, which fall between −10 and +2 ppm, has prevented us from measuring the relaxation rate. For the assignment of the signals, it should be recalled that the two histidines are bound through N1[4,5] (Figure 2). Therefore they should give rise to two signals from the H2 and two from the H4 protons. The shape of the signals at −52 and −46 ppm recalls that of the H4 proton of His-119 in cobalt carbonic anhydrase, which also binds the metal through N1. The two broader signals at −60 and −26 ppm may be assigned to the H2 protons,

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which are expected to relax very fast. A further support to the assignment and to the whole interpretation of the relaxation properties of the system comes from the evaluation of τ_c for these protons. In fact, assuming a cobalt-H4 distance of 530 pm as found in a model complex,[32] a τ_c of 3.5×10^{-12} is calculated from the Solomon-Bloembergen equation, which is in fair agreement with the value determined from the various magnetic field measurements on the water protons.

The spectra recorded in H_2O show evidence of two further protons whose signals fall at -60 and -46 ppm (Figure 2), which can be safely assigned to the NH protons in position 3.[22] Upon comparison of the present spectra with those of five-coordinated derivatives of cobalt carbonic anhydrase,[22] it appears that the signals of the present system are broader, although the τ_c values of the two systems are rather close. Perhaps the high concentration of NaCl, necessary to dissolve CPA, contributes to the line width; furthermore, the large flexibility of the active cavity[33] may allow movements of the coordinated histidines with an exchange time of the order of the difference in chemical shift among the various positions. Such mechanism would account for the large line width observed. A similar justification was given for the failure to observe an NMR signal from ^{113}Cd in the cadmium(II) derivative.[34] In the case of the NH groups, the chemical exchange through hydrogen bonds with water in the cavity may be another source of line broadening.

In conclusion, measurements at various magnetic fields of the water ^1H T_1^{-1} of solutions containing CoCPA and the inhibitor β-phenylpropionate have provided an electronic correlation time supporting the hypothesis of a five-coordinated chromophore. The geometrical parameters deduced through the Solomon-Bloembergen equation suggest the presence of two water molecules, which would complete the coordination sphere together with the protein ligands, two histidines, and one glutamate residue. The inhibitor substitutes a water molecule, leaving the coordination number unaltered.

In the ^1H NMR spectra of the protein part a significantly larger line width with respect to what would have been expected from the T_1 values and from the comparison with analogous systems[22,35] has been attributed to some conformational interactions whose justification stems on the large flexibility of the system.

Acknowledgment. The research reported here has been sponsored in part by the United States Army through its European Research Office.

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Communications to the Editor

A Synthetic Isorhodopsin Formed with a Retinal Derivative Lacking an Intact Ring

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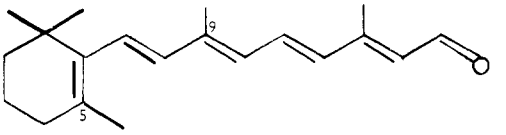
The photosensitive pigment of the vertebrate retina is rhodopsin, a lypoprotein consisting of the chromophore 11-*cis*-retinal linked to the protein via a protonated Shiff base linkage.[1] A number of derivatives of retinal **1** have now been synthesized that examine certain structural[2] and electronic[3] features of the binding site of the chromophore within the protein. As previous attempts to form pigment from retinal derivatives not containing a ring structure have been unsuccessful,[4] it has been assumed that the ring is essential for pigment formation. We report here the synthesis of the retinal analogue **2**, which does not contain an intact ring, and the formation of a stable pigment between bovine opsin and the cis isomer of this compound corresponding to the 9-cis isomer of retinal.

2-Ethylbutanal (Eastman) was combined with ethyl 4-(diethoxyphosphinyl)-3-methylcrotonate[5] in the presence of sodium



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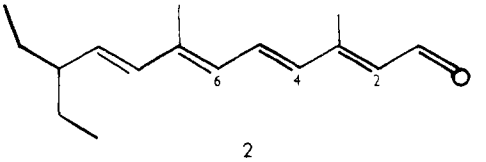
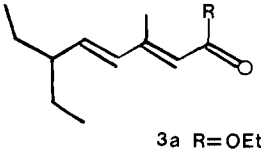


2



3a R=OEt
3b R=H

amide to yield the ester **3a** (82%). Lithium aluminum hydride reduction and manganese dioxide oxidation in ethyl ether afforded 6-ethyl-3-methyl-2,4-octadienal **3b** (45%), which was purified by preparative thin-layer chromatography (TLC). The aldehyde **3b** was condensed, reduced, and oxidized as above to yield the product 3,7-dimethyl-10-ethyl-2,4,6,8-dodecatetraenal **2** (32%). The

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