oxide)$_6$(ClO$_4$)$_2$, 18533-53-4; ZnSO$_4$, 7733-02-0; Mg(acetate)$_2$, 142-72-3; Co(Me$_2$SO)$_6$(ClO$_4$)$_2$, 15553-90-9; Co(2-picoline *N*-oxide)$_5$(ClO$_4$)$_2$, 21460-56-0; Co(MePh$_2$AsO)$_4$(NO$_3$)$_2$, 97150-41-9; Cs$_3$CoCl$_5$, 13774-14-6; Cs$_3$CoBr$_5$, 13826-97-6; [(CH$_3$)$_4$N]$_2$ZnCl$_4$, 14240-97-2; Cs$_2$CoCl$_4$, 15007-14-4; ZnO, 1314-13-2; CdS, 1306-23-6; ZnS, 1314-98-3; PbWO$_4$, 12737-98-3; Zn[H$_2$B(pz)$_2$]$_2$, 18131-19-6; Co(Et$_4$dien)Br$_2$, 20506-81-4; Co[(Me$_6$tren)Cl]Cl, 97150-42-0; Co(terpyridyl)Cl$_2$, 14854-50-3; Co(MePh$_2$AsO)$_4$(ClO$_4$)$_2$, 18712-83-9; ZnSO$_4$, 7733-02-0; Al$_2$O$_3$, 1344-28-1; MgF$_2$, 7783-40-6; MgO, 1309-48-4; CoCl$_2$, 7646-79-9; Co, 7440-48-4; yttrium gallium garnet, 12024-41-8.

Ground Term Splitting of High-Spin Co^{2+} as a Probe of Coordination Structure. 2. The Ligand Environment of the Active Site Metal Ion of Carboxypeptidase A in Ester Hydrolysis[1a]

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Abstract: The value of the zero-field splitting of Co^{2+}-substituted carboxypeptidase A is employed to assign the coordination structure of the active-site metal ion. The zero-field splitting of the Co^{2+} ion is 8.3 cm^{-1} in the free enzyme, 51 cm^{-1} in the enzyme–L-benzylsuccinate inhibitor complex, and 39 cm^{-1} for the mixed anhydride reaction intermediate formed with *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate and stabilized by cryoenzymologic techniques. The value of 8.3 cm^{-1} is compatible only with a distorted tetraliganded complex while the value of 39 cm^{-1} for the acylenzyme reaction intermediate can be accounted for only by a pentaliganded active-site metal ion. Together with previous results of electron paramagnetic resonance experiments with use of ^{17}O-enriched substrate or solvent (Kuo, L. C.; Makinen, M. W. *J. Biol. Chem.* **1982**, *257*, 24–27], the results demonstrate that the coordination number of the metal ion in the free enzyme is increased in the mixed anhydride intermediate to accommodate both a water molecule and the carbonyl oxygen of the scissile bond of the substrate, in addition to the three ligands from protein residues. The mechanism of carboxypeptidase A in esterolysis is reevaluated in light of these findings, and criteria for accumulation of the reaction intermediate by cryoenzymologic methods for structural characterization are discussed.

Enzymes catalyze reactions via the sequential formation of a series of reaction intermediates. An important objective to understand the basis of enzyme function is to determine the structures of reaction intermediates. One means to achieve this goal is through application of an integrated, multidisciplinary approach employing cryoenzymologic techniques[2-4] with kinetic and structural methods. By use of cryoenzymologic methods, we have demonstrated that in the esterolytic reaction catalyzed by carboxypeptidase A an acylenzyme (mixed anhydride) reaction intermediate is formed with the specific ester substrate *O*-(*p*-chlorocinnamoyl)-L-β-phenyllactate.[5-9] A salient result of these investigations, in contrast to the implications of structural studies of inhibitor complexes,[10-13] is that the water molecule coordinated to the metal ion is not displaced by the substrate in the mixed anhydride intermediate.[6] These results imply that the tetraligand coordination environment of the active-site metal ion in the free enzyme[10-13] is altered to accommodate a fifth ligand in the course of the reaction. An important objective in structural studies of this enzyme, therefore, is to assign directly the coordination environment of the active-site metal ion in the acylenzyme (mixed anhydride) reaction intermediate.

Our kinetic and cryoenzymologic studies reveal that the mechanism of ester hydrolysis is identical for both native Zn^{2+}-containing and Co^{2+}-reconstituted carboxypeptidase A and that on this basis the paramagnetic Co^{2+} ion may serve as a direct spectroscopic probe of catalytically required structural relationships in the active site. To determine the ligand environment of the active-site metal ion in the acylenzyme reaction intermediate, we have carried out a detailed evaluation of the spectroscopic properties of high-spin Co^{2+}.[14-16] On the basis of these results, we

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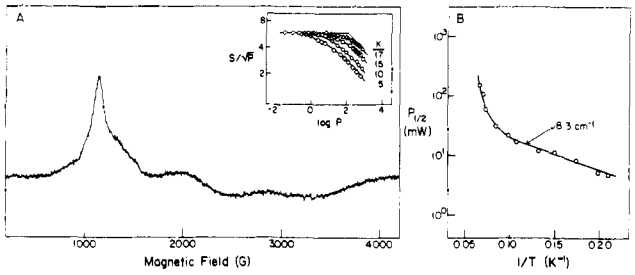

Figure 1. First-derivative EPR absorption and cw microwave power saturation behavior of Co^{2+}-reconstituted carboxypeptidase A. The crystalline enzyme was dissolved to a concentration of 3.7×10^{-4} M in a solution of 0.25 M NaCl buffered with 0.02 M sodium cacodylate to pH 7.5 at 0 °C. The spectrum in (A) was recorded with the sample at 8 K with 2-mW microwave irradiation and 25-G modulation amplitude. The inset to (A) illustrates the general cw microwave power saturation behavior of the peak-to-peak resonance absorption centered at ~1530 G. The data illustrate that T_2 remains temperature invariant over the 5–17 K range. The intersection of the asymptotes defines the value of $P_{1/2}$. (B) illustrates the temperature dependence of $P_{1/2}$. The linear portion in the 5–10 K region yields an estimate of 8.3 cm^{-1} for the value of Δ for the high-spin Co^{2+} ion in the metal-substituted enzyme. We have previously pointed out that the spectroscopic properties of CoCPA are not altered by introduction of cryosolvents employed for stabilization of reaction intermediates.[6]

demonstrate that the coordination number of the metal ion in carboxypeptidase A is increased to accommodate both the carbonyl oxygen of the scissile bond of the substrate and a solvent molecule in the acylenzyme reaction intermediate.

The results of this study require that breakdown of the acylenzyme intermediate occurs via nucleophilic attack by the metal-hydroxide group on the carbonyl carbon of the substrate. Therefore, we review the results of X-ray, chemical, and kinetic studies in which different assignments of the mechanism of action of carboxypeptidase A have been proposed. Also, since cryoenzymologic methods have been employed in this investigation to stabilize the mixed anhydride reaction intermediate for structural characterization, we review in the Appendix the requirements for application of these techniques, in view of recent criticisms[17] of the results of our earlier studies.

Experimental Procedures

Materials. Crystalline bovine pancreatic ZnCPA[18] obtained from Sigma Chemical Co. and prepared according to the method of Cox et al.[19] was used as previously described.[5] CoCPA was prepared and characterized according to the method of Latt and Vallee.[20] The extent of metal substitution evaluated by atomic absorption methods was not less than 88%. The preparation of all other materials has been described.[5-9] Organic–aqueous cosolvent mixtures[21] for preparation of the mixed anhydride, acylenzyme reaction intermediate of CoCPA were employed as previously described.[5,6] We have discussed further criteria for preparation of the mixed anhydride reaction intermediate in the Appendix.

Methods. EPR spectra were recorded with an X-band Bruker ER200D spectrometer at 100-kHz frequency modulation in the TE_{102} mode as described in the accompanying publication.[16] The cw microwave power saturation technique developed in this laboratory[16,22] was employed for estimating the splitting (Δ) between the two lowest Kramers doublets of high-spin Co^{2+} in the metal-substituted enzyme. We have previously shown[6,16,22] that in applying this technique the reproducibility of the parameter log $P_{1/2}$ determined from progressive saturation curves and of the splitting of the two lowest Kramers doublets determined from the temperature dependence of $P_{1/2}$ is to within ±10%. Our previous publications[16,22] should be consulted for the mathematical relationships that relate $P_{1/2}$ to T_1 and T_2, the spin–lattice and spin–spin relaxation times, and determination of Δ on the basis of the temperature dependence of $P_{1/2}$ for spin–lattice relaxation via an Orbach process.[23,24] For progressive microwave saturation studies, at least three independently prepared samples of each derivative of the enzyme were used.

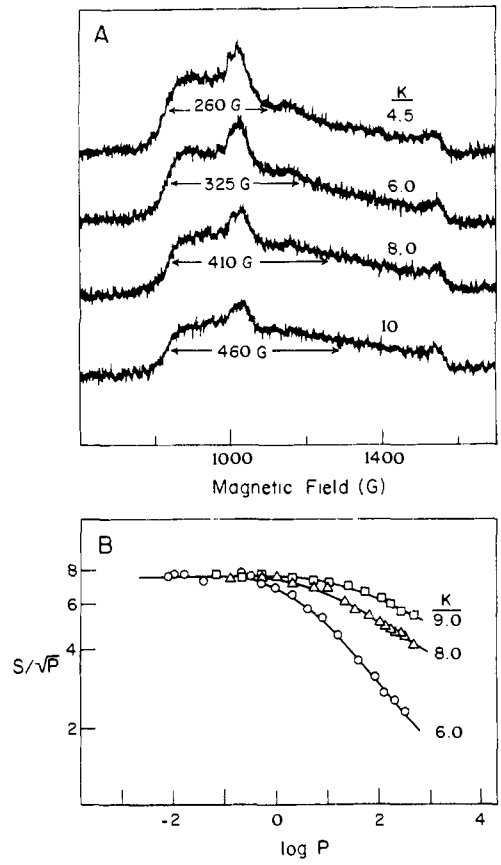

Figure 2. Low-field (first-derivative) EPR absorption spectra and cw microwave power saturation behavior of the complex of CoCPA with gly-L-tyr. The enzyme–inhibitor complex was prepared at 0 °C by the addition of the dipeptide to a solution of CoCPA. The final enzyme solution contained 5.9×10^{-4} M CoCPA and 3×10^{-3} M gly-L-tyr in 0.25 M NaCl buffered to pH 7.5 at 0 °C with 0.02 M sodium cacodylate. After thorough mixing for 30 s, the samples were frozen in liquid nitrogen. (A) shows spectra in the low-field region recorded at 1-mW incident microwave power and 25-G modulation amplitude. With increasing temperature, the line width at half-maximal signal amplitude is seen to increase from 260 to 460 G. The weak signal at 1540 G arises from paramagnetic impurities in the quartz sample tube. (B) illustrates that the microwave power saturation behavior of the gly-L-tyr inhibitor complex of CoCPA as a function of temperature. The data show that the slope at high values of P is not invariant with temperature.

Results

The first-derivative EPR absorption spectrum of CoCPA shown in Figure 1 is characterized by broad resonance features. Essentially identical EPR properties were observed also for the enzyme in cryosolvent mixtures. The inset to Figure 1A illustrates representative cw microwave power saturation curves of CoCPA evaluated on the basis of the change in the peak-to-peak amplitude

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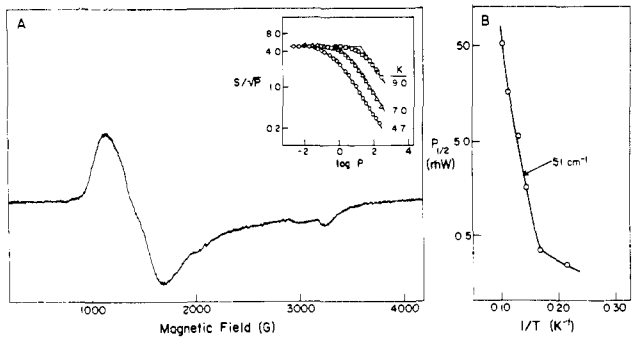

Figure 3. First-derivative EPR absorption spectrum and cw microwave saturation behavior of the complex of CoCPA with L-benzylsuccinate. The complex was prepared with a 2-fold excess of the inhibitor in 0.25 M NaCl buffered to pH 7.5 with 0.01 M sodium cacodylate. The enzyme concentration was 5×10^{-4} M. In (A) is illustrated the spectrum recorded with the sample at 4.7 K under conditions of 1-mW microwave power irradiation and 16-G amplitude modulation. The inset to (A) illustrates the cw power saturation behavior of the resonance band centered at 1200 G, demonstrating that the slope remains invariant with temperature. (B) illustrates the temperature dependence of $P_{1/2}$.

centered at 1530 G, as in our previous study employing ^{17}O-enriched materials.[6] There is no change in the slope of the saturation curves with temperatures, and no change is seen in the total linewidth within the 4–12 K region. On this basis, the temperature dependence of $P_{1/2}$ remains a function of only the spin–lattice relaxation rate.[16,22] In Figure 1B a rapidly rising portion is seen at $T > 10$ K, indicating the onset of spin–lattice relaxation via the Raman process. The temperature dependence of $P_{1/2}$ in the 5–10 K range is, thus, readily identified as corresponding to spin–lattice relaxation of the Co^{2+} ion via the Orbach mechanism.[23,24] According to our previous investigations of the saturation behavior of high-spin paramagnetic ions,[16,22] this linear portion of the plot yields an estimate of 8.3 cm^{-1} for the splitting between the two lowest Kramers doublets of high-spin Co^{2+} ion in the metal-substituted enzyme.

In the complex of ZnCPA formed with the dipeptide inhibitor gly-L-tyr, the active-site metal ion is coordinated by the carbonyl oxygen of the scissile amide bond.[10,11] The water molecule that ligates the metal ion in the free enzyme is displaced in the inhibitor complex since the terminal amine group of the inhibitor occupies a site that is sterically overlapping with that occupied by the water molecule in the free enzyme.[11a,25] We have previously reported[6] the EPR spectrum of the gly-L-tyr inhibitor complex formed with CoCPA. In Figure 2 we have illustrated the variation in the spectrum and in the slope of the cw power saturation curve with increasing temperature. In contrast to all other complexes of high-spin Co^{2+} investigated hitherto,[6,14–16] the change in these two parameters with temperature prevents a direct estimate of Δ. We have previously demonstrated that ^{17}O-enriched water does not influence the electronic spin–lattice relaxation of Co^{2+} in the inhibitor complex of CoCPA formed with gly-L-tyr, in contrast to that observed in the free enzyme.[6] This observation is consistent with displacement of the metal-bound water molecule upon inhibitor binding.

The synthetic inhibitor L-benzylsuccinate binds tightly to ZnCPA ($K_I \sim 10^{-8}$ M) and is thought to mimic the structure of hydrolysis products retained in the active site of the enzyme after bond cleavage.[28,29] The EPR absorption spectrum of the complex

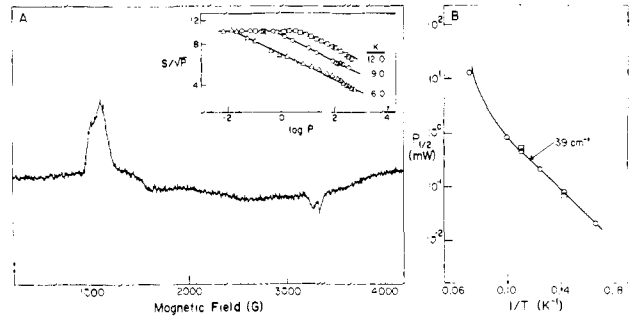

Figure 4. First-derivative EPR absorption spectrum and cw microwave saturation behavior of the acylenzyme intermediate of CoCPA formed with ClCPL. The reaction intermediate was prepared directly in EPR sample tubes. Samples containing CoCPA in ethylene glycol/H_2O mixtures (50:50 v/v) were immersed into a mixture of dry ice in CCl_4 (−30 °C) for the addition of methanol. The final enzyme solution in the ethylene glycol/methanol/H_2O (40:20:40) ternary solvent mixture was then cooled to −70 °C in a bath of dry ice and $CHCl_3$ for the addition of a 3-fold molar excess of ClCPL. Upon substrate addition, the solution was thoroughly mixed before being frozen in liquid nitrogen. The sample contained 2.9×10^{-4} M CoCPA in 0.25 M sodium chloride buffered with 0.02 M sodium cacodylate to pH 7.5 at −70 °C. The spectrum in (A) was recorded at 7 K at 2-mW microwave power and 25-G modulation amplitude. The inset illustrates the saturation behavior of the strong resonance feature centered at 1100 G. In (B) the temperature dependence of $P_{1/2}$ of the acylenzyme intermediate in the 4–12 K range yields a value of 39 cm^{-1} for the splitting between the two lowest Kramers doublets of the Co^{2+} ion. We have previously pointed out[6] that the EPR absorption spectrum and CW power saturation properties observed after mixing of the substrate and enzyme under these conditions are distinguishably different from those of the enzyme in the presence of hydrolysis products. It is also readily seen that the spectroscopic characteristics cannot be accounted for by those of the free enzyme illustrated in Figure 1. In (B) the circles represent data collected in this investigation. The open squares indicate the $P_{1/2}$ values for the intermediate prepared in the presence of ^{17}O-enriched substrate from previous studies.[6] The latter two values are frame shifted to demonstrate that their relative change with temperature adheres to that defined by the open circles.

Table I. Summary of Spectroscopic Parameters of Complexes of Co^{2+}-Substituted Carboxypeptidase A

enzyme derivative	g_1	g_2	g_3	Δ^a (cm^{-1})
CoCPA	5.97	2.65	1.99	8.3
CoCPA (+ gly-L-tyr)	6.76	*b*	*b*	
CoCPA (+ L-benzylsuccinate)	5.63	4.31	2.11	51
(*p*-chlorocinnamoyl)-CoCPA	6.32	2.77	1.97	39

[a] Represents the splitting between the two lowest Kramers doublets of the high-spin Co^{2+} ion as defined in ref 16. [b] Resonance features are too broad to assign the value of the g factor.

of CoCPA formed with L-benzylsuccinate is shown in Figure 3. The spectrum characterized by broad resonance features is suggestive of near-axial symmetry of the metal ion environment. The saturation behavior of the feature at $g \sim 5.63$ shows that the value of T_2 is invariant with temperature over the 4–12 K range. On this basis, the linear portion of the temperature dependence of $P_{1/2}$ in Figure 3B yields a value of 51 cm^{-1} for the splitting between the two lowest Kramers doublets of the Co^{2+} ion.

The EPR absorption spectrum of the acylenzyme (mixed anhydride) reaction intermediate[6,8] of CoCPA formed with ClCPL is illustrated in Figure 4. We have shown that the shape of the saturation curve and the total line width are temperature invariant in the 4–12 K range.[6] The temperature dependence of $P_{1/2}$ is illustrated in Figure 4B. The slope of the graph yields an estimate of 39 cm^{-1} for the value of Δ. In the Appendix we discuss the criteria employed in our cryoenzymologic studies to ensure accumulation and stabilization of the mixed anhydride intermediate at temperatures of −70 °C for spectroscopic characterization.

(25) Earlier interpretations of the difference Fourier synthesis of this inhibitor complex calculated with unrefined phases suggested that the carbonyl oxygen atom directly displaced the metal-bound water molecule.[26,27] More recent results using refined data at 1.75-Å resolution have shown that the coordination site occupied by the carbonyl oxygen atom is not overlapping with that of the metal-bound water molecule in the free enzyme.[11a]

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The spectroscopic properties of the complexes of Co^{2+}-substituted carboxypeptidase A and of the acylenzyme reaction intermediate are summarized in Table I.

Discussion

A. Ligand Environment of the Active-Site Metal Ion of Carboxypeptidase A. In Table I we have compared the spectroscopic parameters of derivatives of CoCPA, including the values of Δ determined by the cw power saturation method. In the accompanying publication[16] we have shown that the value of Δ serves as a diagnostic signature of coordination structure (≤ 13 cm^{-1} in tetracoordinate sites, 20–25 cm^{-1} in pentacoordinate sites, and ≥ 50 cm^{-1} in hexacoordinate sites). On this basis, the value for Δ of 8.3 cm^{-1} obtained for free CoCPA is compatible only with a tetracoordinate site. This interpretation is consistent with the results of a recent, high-resolution X-ray diffraction study of CoCPA at 1.75-Å resolution.[30] In this study, the CoCPA-minus-ZnCPA difference electron density map shows that the positions of the Co^{2+} ion and its coordinated ligands from the side chains of His-69, His-196, and Glu-72 remain identical to within ±0.10 Å with those in the native enzyme, and the difference map outlines electron density features that can be accounted for by only one inner-sphere coordinated water molecule as the nonprotein ligand.

With respect to the recent X-ray studies carried out at 1.54-Å resolution with refined phases, it is of interest to note that the carboxylate oxygen atoms of Glu-72 are equidistant from the metal ion.[11,31] On this basis, Lipscomb and co-workers[11,30] have designated the coordination number as 5 for the native enzyme. This designation is consistent with the definition of coordination number generally employed[32] as the number of atoms within the inner coordination sphere of the metal ion. The ligand field effect of a coordinated carboxylic acid group is the mean of the two oxygens, each contribution weighted by its respective metal–oxygen distance,[33] and the ligand field stabilization energy of a carboxylate group is equivalent only to that of other monooxygen donor ligands.[34] Since the magnitude of the zfs is a function of the strength of the crystal field (cf. ref 16), our observation of a value of 8.3 cm^{-1} for Δ in the Co^{2+}-substituted enzyme indicates that the carboxylate group of Glu-72 behaves spectroscopically like a classical unidentate ligand.[35]

Recently Bertini and co-workers have applied nuclear magnetic resonance techniques to study the (water) proton relaxation enhancement of Co^{2+}-substituted enzymes to determine coordination number.[36-39] They have detected[39] a metal-bound water molecule in fast chemical exchange in the inhibitor complex formed with L-β-phenylpropionate and have observed what the electronic spin–lattice relaxation rate is identical with that in the free enzyme. They conclude that the metal ion in the free enzyme has two coordinated water molecules, one of which is displaced by the inhibitor. Hartsuck and Lipscomb[10b] have pointed out that an outer-sphere bound water molecule may obtain in this enzyme–inhibitor complex. The spin–lattice relaxation of paramagnetic metal ions is sensitive to both inner- and outer-sphere coordinated ligands,[40] and no relaxation data as a function of temperature or frequency are presented by Bertini and co-workers[39] that could distinguish between these possible alternative interpretations. In the crystalline state, CoCPA has only one metal-bound water molecule, as in native ZnCPA.[30] The addition of a second inner-sphere coordinated water molecule as a fifth ligand would have significantly increased the value of Δ, as we have observed in structurally defined, small molecule complexes of pentacoordinate Co^{2+} [16] and in pentacoordinate inhibitor complexes of Co^{2+}-reconstituted liver alcohol dehydrogenase.[14,41]

We have previously shown with use of ClCPL or H_2O selectively enriched with ^{17}O that the carbonyl oxygen of the scissile bond of the substrate and a water molecule are coordinated to the metal ion when the (mixed anhydride) acylenzyme reaction intermediate is stabilized at −70 °C.[6] The results of this earlier study do not directly negate the interpretation that a mixture of species is stabilized under cryoenzymologic conditions in which either the substrate carbonyl oxygen or the H_2O molecule is coordinated to a tetracoordinate metal ion. This argument can be ruled out by the results of this investigation. The value of 39 cm^{-1} for the zfs of the Co^{2+} ion in the acylenzyme reaction intermediate is compatible only with coordination by five ligands. Therefore, both the carbonyl oxygen of the scissile bond of the substrate and a water molecule are retained in the inner coordination sphere of the metal ion, in addition to the three donor ligands from protein residues. We conclude that the environment of the metal ion in the active site of carboxypeptidase A is expanded from that in the free enzyme to accommodate the carbonyl oxygen of the acyl moiety of the substrate in the mixed anhydride reaction intermediate and that the metal-bound water is not displaced.

Recent X-ray studies at 1.75-Å resolution of the inhibitor complex of ZnCPA with gly-L-tyr show that the site occupied by the metal-bound water in the free enzyme overlaps with that of the terminal amine group and that the carbonyl oxygen of the scissile amide bond is within the inner coordination sphere of the metal ion.[11a] We are unable to provide a direct estimate of the value of Δ for the Co^{2+} ion in this enzyme–inhibitor complex. However, the absence of a metal-coordinated water molecule in this inhibitor complex has been confirmed through our earlier EPR studies[6] of the gly-L-tyr inhibitor complex of CoCPA prepared in the presence of ^{17}O-enriched water.

X-ray crystallographic studies have not been extended to the inhibitor complex of ZnCPA with L-benzylsuccinate. Since there are three ligands to the metal ion from amino acid side chains of protein residues, the splitting of 51 cm^{-1} indicates that the metal ion is coordinated by at least five ligands and suggests that the carboxylate group of the inhibitor is coordinated together with a solvent molecule.[42] This latter configuration is not sterically incompatible with the structure of the active site, in view of the difference Fourier analysis[11a] of the gly-L-tyr inhibitor complex. Also, in thermolysin, with similar active-site residues to those in ZnCPA, binding of the inhibitor L-benzylsuccinate occurs through coordination of only one carboxylic oxygen atom to the Zn^{2+} ion.[43] A water molecule is localized ~3 Å from the Zn^{2+} ion in the active site.[44] On the basis of the similar structural and catalytic

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(31) In defining the positions of the carboxylate oxygens of Glu-72, there is correlation in the covariance of the positions calculated from electron density data. Although these errors have not been explicitly discussed,[11,42] the distances of the two carboxylic oxygen of Glu-72 from the metal ion may be expected to differ by no more than 0.2-0.3 Å.

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(35) It is possible that for the free enzyme in solution, small structural changes occur resulting in inequivalent interactions of the metal ion with the carboxylate oxygens of Glu-72. Such configurations also remain compatible with tetracoordinate geometries. At present we have no evidence to support this interpretation in favor of that provided above.

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(42) We cannot rule out the possibility that there is more than one water molecule coordinated in this case, making an essentially hexaliganded species. In this case, the splitting of 51 cm^{-1} designates the separation of the two lowest doublets that derive from the orbitally degenerate $^4T_1$ ground state of a Co^{2+} ion in O_h symmetry. We believe that this configuration is less likely in view of the difference Fourier study of the thermolysin–L-benzylsuccinate inhibitor complex.[43]

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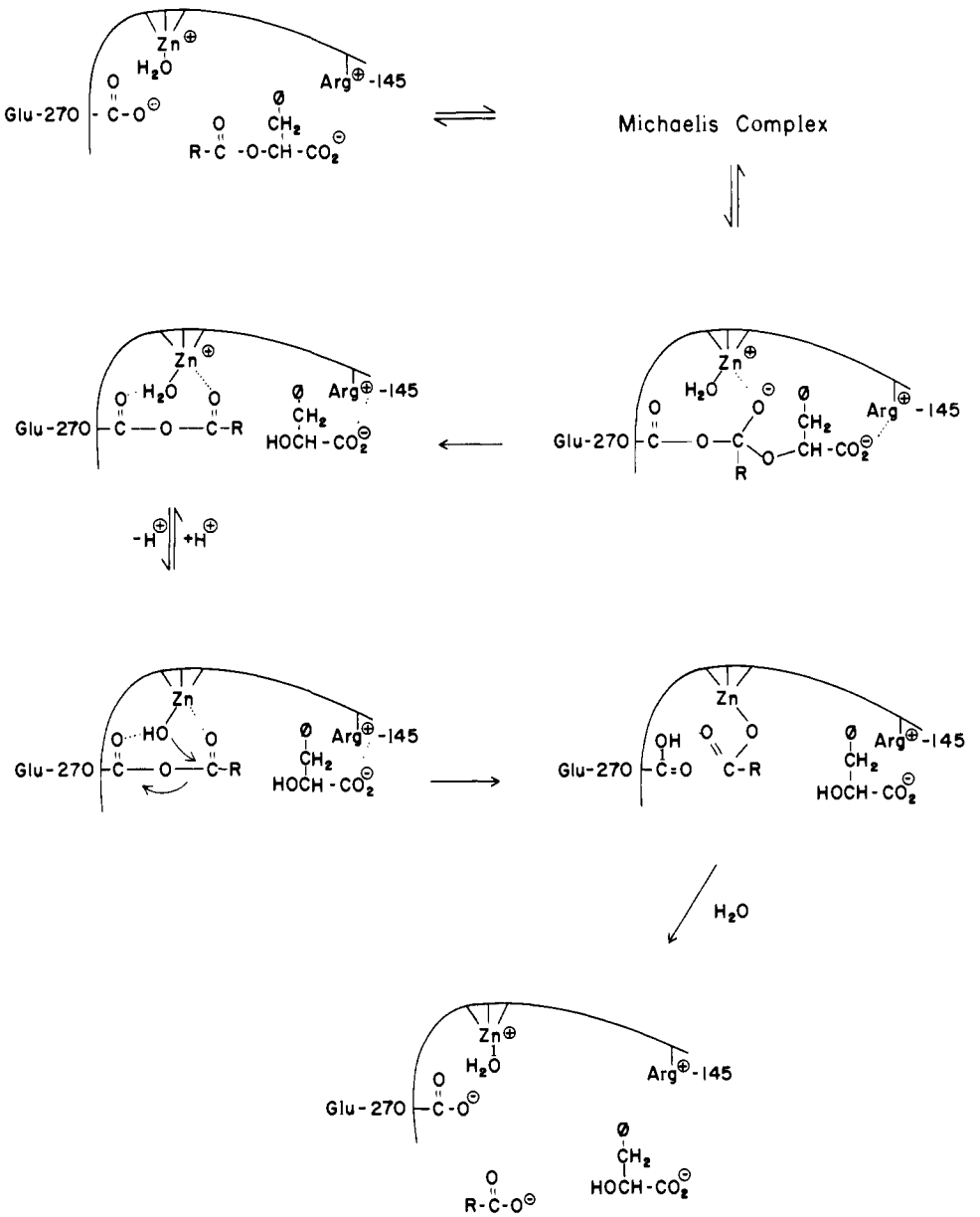

Figure 5. Schematic illustration of the chemical pathway of carboxypeptidase A in ester hydrolysis. The pathway is constructed on the basis of experimental evidence discussed in the text. To emphasize the deprotonation step of the metal-bound water molecule and the role of the metal-hydroxide species as a nucleophile, the scheme is not balanced with respect to protons. The structural environment of the metal ion in the Michaelis complex is not illustrated although it is probable (cf. text) that the metal-bound water molecule is sequestered throughout the reaction until attack on the carbonyl carbon by the metal-hydroxide nucleophile.

properties of these two enzymes, it is probable that the binding configuration of L-benzylsuccinate in carboxypeptidase A is comparable.

B. Catalytic Role of the Metal–Water Complex in Carboxypeptidase A. There are three distinct regions of enzyme–substrate interactions in the active site of carboxypeptidase A:[7,10-12,26,27] (i) the hydrophobic pocket with Arg-145 for binding the COOH-terminal residue of the substrate; (ii) the bond cleavage site, consisting of the Zn^{2+} ion and the γ-carboxylate group of Glu-270, at which splitting of the scissile amide or ester bond occurs; (iii) four subsites[10,45] near the entrance into the active site termed the sites of secondary substrate recognition. The substrate is stabilized by binding to the hydrophobic pocket and Arg-145. Bond cleavage requires coordination of the carbonyl oxygen of the scissile bond to the metal ion.[6,26,46] The carboxylate group of Glu-270 acts as a nucleophile to form the acylenzyme (mixed anhydride) reaction intermediate, and breakdown of the the mixed anhydride formed with ClCPL is governed by ionization of the metal-bound water molecule.[5] Designation of both a water molecule and the carbonyl oxygen of the scissile bond as donor–ligand groups to the metal ion in the acylenzyme (mixed anhydride) reaction intermediate, as determined in this investigation, requires reevaluation of the mechanism of ester hydrolysis by carboxypeptidase A.

In Figure 5, we have outlined the chemical pathway for the hydrolysis of the specific ester substrate ClCPL. The pathway is based on (i) the minimal reaction scheme for ClCPL hydrolysis determined through previous kinetic and cryoenzymologic studies;[5,8,9] (ii) the evidence[5] for ionization of the metal-bound water molecule governing rate-limiting deacylation of the reaction intermediate; (iii) the evidence presented here for five ligands coordinated to the active-site metal ion in the mixed anhydride

(44) For the thermolysin–L-benzylsuccinate complex, the X-ray diffraction data that define the metal ion coordination environment have not been refined as in the case of the inhibitor complex of ZnCPA with gly-L-tyr.[11a] For this reason, the water molecule may actually be closer to the metal ion than implied by the difference Fourier map calculated with unrefined phases.

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intermediates; and (iv) the evidence from our previous EPR studies[6] that both a water molecule and the carbonyl oxygen of the substrate are coordinated to the metal ion. The mechanism explicitly indicates the role of Glu-270 in forming a mixed anhydride intermediate, as suggested first by Lipscomb and coworkers[47] and shown through our previous cryoenzymologic studies.[5,8,9] We assign the mechanism of breakdown of the acyl-enzyme to metal-hydroxide nucleophilic attack on the carbonyl carbon of the substrate's scissile bond. This pathway, in our estimation, is the mechanism that is most consistent with all of the structural, chemical, kinetic, and spectroscopic data.

With reference to the mixed anhydride pathway, Lipscomb[12,13] has suggested that the solvent molecule may be initially displaced by the substrate with a new water molecule binding after anhydride bond formation and that the carbonyl oxygen of the acyl group may dissociate from the metal ion for attack by the incoming metal-hydroxide nucleophile. The combined structural, chemical, and kinetic data suggest that the metal-bound water molecule is sequestered upon substrate binding. The observation[48] that ^{18}O is exchanged from peptide substrates at the same rate that resynthesis of the peptide bond is catalyzed by carboxypeptidase A requires, for the anhydride mechanism, sequestering of a water molecule in the active site in the course of the reaction. In the binding of β-phenylpropionate, a water molecule is retained in close proximity to the metal ion,[10b,39] and; similarly to thermolysin,[43] we have suggested in this investigation that a metal-bound water molecule is retained in the inhibitor complex formed with L-benzylsuccinate. Although high-resolution difference Fourier studies of the gly-L-tyr inhibitor complex show that the terminal *free* amine group of the inhibitor displaces the metal-bound water molecule,[11a,25] oligopeptide or ester substrates that exhibit high reactivity toward hydrolysis catalyzed by carboxypeptidase A do not have a free amine group at the same position that would sterically overlap with the metal-bound water. The suggestion[12,13] of displacement of the metal-bound water molecule, therefore, has been made primarily on the basis of an inhibitor complex formed with the dipeptide gly-L-tyr. The stereochemical relationships of gly-L-tyr to the enzyme with respect to the displacement of the metal-bound water molecule may not be sufficiently similar to those required in active catalysis for evaluation of this aspect of the reaction pathway.

In the metal ion assisted hydrolysis of esters, amides, and anhydrides, M^{n+}–OH^- species are highly effective intramolecular catalysts in nucleophilic attack, to the almost total exclusion of intermolecular, general base catalyzed mechanisms.[49] In comparison, M^{n+}–OH_2 species are less effective. In Figure 5 we have indicated that ionization of the metal-bound water[5,6] may be assisted by hydrogen bonding to the carbonyl oxygen of Glu-270, in accord with the stereochemical relationships pointed out by Argos et al.[50] On the basis of a difference Fourier study of a pentaliganded hydroxamate inhibitor complex of thermolysin, Holmes and Matthews[51] have proposed a mechanism of hydrolysis whereby a metal-coordinated water molecule in the reaction intermediate is translocated toward the carboxylic acid group of Glu-143 to act as a nucleophile through an essentially general base catalyzed mechanism. Translocation of the metal-bound water molecule to a more distant hydrogen-bonded site would result in a less effective nucleophile in comparison to the most potent[49] nucleophilic species, i.e., the metal–OH^- species, already formed in the active site prior to water translocation. Ionization of a metal-bound water molecule has been assigned to the $pK_a \sim 6$ governing k_{cat} in thermolysin[52] on the basis of an enthalpy of ionization and pK_a value essentially identical with those observed for carboxypeptidase A. A plethora of small-molecule studies[49,53-55] shows that a metal-hydroxide species is a far more efficient catalyst as a nucleophile in hydrolytic reactions than a hydrogen bonded water molecule is in a general base catalyzed mechanism. We concur that the metal-hydroxide pathway is the more probable mechanism in carboxypeptidase A.

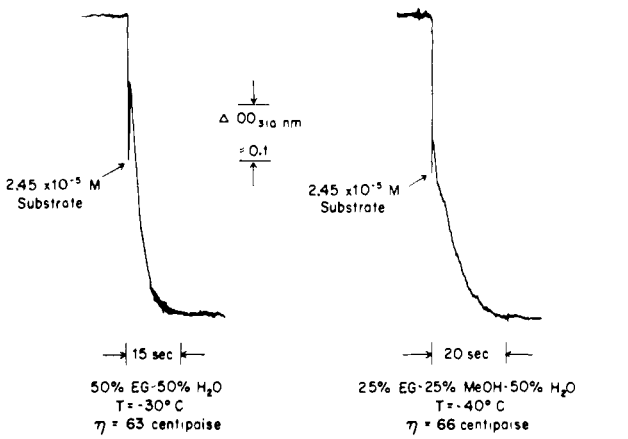

Figure 6. Comparison of the mixing time under conditions of addition of ClCPL to viscous, cryosolvent mixtures. In both cases, a 10-μL aliquot of a methanolic solution of ClCPL ($\sim 10^{-2}$ M) was added to a cuvet containing 2.5 mL of cryosolvent mixture. The cuvet containing the solvent was placed in the cryostat assembly[59] in a Cary 15 recording spectrophotometer. After temperature equilibration the aliquot of the solution was added under the separate conditions of temperature and cryosolvent composition indicated. The traces show the change in absorbance with time after substrate addition indicating the time required for disbursing the substrate uniformly throughout the cryosolvent. We have found that only if these conditions of mixing efficiency are maintained, as ascertained by testing before and after each kinetic experiment, are the kinetic data sufficiently free of mixing artifacts to warrant analysis.

With reference to Figure 5, we emphasize that it has not been directly determined whether metal-hydroxide nucleophilic attack on the carbonyl carbon proceeds after dissociation of the carbonyl oxygen or through a direct, intramolecular mechanism. Coordination of the carbonyl oxygen to the metal ion is likely to occur upon initial substrate binding, for polarization of the carbonyl bond to facilitate nucleophilic attack is an important aspect to catalysis by carboxypeptidase A. For instance, at neutral pH the rate acceleration due to bond polarization by complexing to a metal ion is matched only by unusually strong acid conditions (10^2–10^6 M; cf. ref 49), and the stronger influence of a metal ion in polarization of the carbonyl bond over that of hydrogen bonding is also simulated through molecular orbital calculations.[56,57] These considerations indicate that coordination of the carbonyl oxygen to the metal ion is an essential aspect to facilitate anhydride bond formation with the side chain of Glu-270. However, subsequent attack on the acyl moiety by the metal-hydroxide nucleophile may not require further coordination of the carbonyl oxygen to the metal ion since an anhydride bond is intrinsically less stable than the scissile ester or amide bond in the substrate. In this respect the recent difference Fourier study showing hydrogen bonding of the carbonyl oxygen of gly-L-tyr to the side chain of Arg-127

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in the metal-free enzyme provides support for this hypothesis.[58] On the other hand, intramolecular attack by a metal-coordinated water or hydroxide group is well established in coordination chemistry (cf. ref 49), and the sterochemical relationships of the metal-coordinated water molecule and the configuration of the substrate carbonyl group, as defined on the basis of the gly-L-tyr complex,[11a] are compatible with intramolecular attack without dissociation of the carbonyl oxygen.

Acknowledgment. We thank Professor W. N. Lipscomb and Dr. K. Hardman for communication of ref 30 prior to publcation.

Appendix

In a recent note Kaiser and collaborators[17] have stated that they have been unable to detect accumulation of the reaction intermediate formed with ClCPL or with *O*-[*p*-(dimethylamino)-cinnamoyl]-L-β-phenyllactate under cryoenzymologic conditions by resonance Raman spectroscopy. On this basis they suggest that our previous studies[5-8] to structurally characterize the mixed anhydride reaction intermediate of CPA are misleading. This criticism, if unanswered, would imply that the results of studies reported in this publication with respect to the mixed anhydride intermediate are, also, incorrect.

A critical requirement in kinetic studies to identify conditions under which enzyme reaction intermediates can be accumulated is high efficiency of mixing of substrate and enzyme in the viscous, cryosolvent mixtures[21] that obtain at subzero temperatures. For our kinetic studies[5,7] carried out in a variety of cryosolvent mixtures exhibiting a change in viscosity of over 4 orders of magnitude, we have designed a cryostat and efficient vibrating stirrer.[59] An example of the efficient stirring that can be obtained with this apparatus is illustrated in Figure 6. The shortest mixing time for these specific solvent conditions that we have been able to achieve is ~20 s, and requirements for achieving these conditions have been detailed.[59] At temperatures higher than −20 °C efficient mixing of substrate and enzyme is not a difficult task. However, such is not the case for the solvent conditions described in Figure 6. Therefore, in all of the studies carried out in this laboratory,[5,6,9] kinetic data collected in the subzero temperature range were analyzed only for those experiments in which it was demonstrated through consecutive trials that the mixing time had not changed and remained ≤20 s for cryosolvent mixtures with comparably high viscosities. Kaiser and co-workers[17] report no technical data to demonstrate that they have adequately achieved comparable experimental conditions, as carried out in our laboratory, and interestingly report kinetic data only in the 25 to −23 °C range, under which conditions solvent viscosities remain relatively small.[21,60] Furthermore, the apparent activation energy calculated from their Arrhenius plots of the two observed rate constants under conditions of enzyme in excess for the hydrolysis of *O*-[*p*-(dimethylamino)cinnamoyl]-L-β-phenyllactate is identically 13.9 kcal/mol for both the fast (k_f) and slow (k_s) phases. If the two temporally resolved phases for the hydrolysis of this substrate corresponded to those observed[5,7] for ClCPL, as is their claim, the activation energies should be of the order of ~8 kcal/mol for k_f and ~17 kcal/mol for k_s. The observed value of ~14 kcal/mol is suspiciously close to the average of these two, indicating inadequate mixing efficiency.

For preparation of the reaction intermediate in spectroscopic studies, we have developed additional criteria to ensure accumulation of the mixed anhydride, which have been documented in detail.[5-8] For instance, to characterize the intermediate formed with the Co^{2+}-reconstituted enzyme and the spin-label (nitroxide) ester substrate *O*-[3-(2,2,5,5-tetramethylpyrrolinyl-1-oxyl)-propen-2-oyl]-L-β-phenyllactate, we have employed the criterion that addition of the tightly bound inhibitor L-benzylsuccinate[28,29] ($K_I \sim 10^{-8}$ M) does not displace the substrate, conditions that would result in an EPR spectrum of the *free* spin-label substrate. Indeed, as we have shown, when the spin-labeled reaction intermediate is prepared at temperatures lower than −65 °C with efficient mixing, a change in the spectrum is observed upon addition of L-benzylsuccinate that can be accounted for only by binding of the inhibitor to the low-temperature stabilized enzyme–substrate species.[7] No evidence for displacement of the spin-label substrate is observed, and no change in the spectrum would have occurred if conditions of inefficient mixing of reagents had obtained.

Similarly, for the experiments reported here, we have demonstrated that the zfs of the Co^{2+} ion in the enzyme–L-benzylsuccinate complex is distinguishably different from that of the free enzyme and of the mixed anhydride species. We have ensured conditions of efficient mixing in preparing the reaction intermediate for EPR studies by similarly demonstrating in parallel experiments that subsequent addition of the inhibitor L-benzylsuccinate after mixing of the substrate and enzyme at temperatures near −70 °C does not result in the characteristic EPR spectrum of the enzyme–inhibitor complex. As evident in Figures 3 and 4, the two species are readily distinguished by their spectroscopic properties. Also, the same peak-to-peak intensities within experimental error limits are observed for the mixed anhydride intermediate, when formed, as described in Figure 4, under conditions of either stoichiometric (1:1) or excess molar quantities of the substrate. Furthermore, we have determined in parallel experiments that other molecular species such as free enzyme, enzyme–product complexes, etc. that could be postulated under conditions of inadequate mixing have spectroscopic and cw microwave power saturation characteristics substantially different from those of the mixing anhydride as to cause recognizable breaks and changes in the slope of the saturation curves if present in significant amounts. For the data presented in this publication, no such effects were observed, as is seen in Figures 1–4. We can conclude only that the failure of Kaiser, Carey, and co-workers[17] to detect accumulation of the mixed anhydride by resonance Raman methods for a mixture of ClCPL and ZnCPA in the highly viscous[21,60] cryosolvent mixture of 50:50 (v/v) ethylene glycol/H_2O at −40 °C is due to inefficient mixing.[61] For instance, we have frequently observed under conditions of inadequate mixing efficiency with highly viscous, cryosolvent mixtures that the aliquot of added substrate is not dispersed in the reaction chamber.

Technical details in mastering the techniques for cryoenzymologic experiments have been long well recognized and discussed.[2,60,62] For these reasons we have provided not only detailed descriptions of conditions and criteria for ensuring absence of artifacts but also physical evidence that binding of the substrate or inhibitors to the enzyme has occurred under the temperature and cryosolvent conditions employed.

Registry No. L-GlyTyr, 658-79-7; (*S*)-(*E*)-$PhCH_2CH(OH)C(O)OC(O)CH{=}CHC_6H_4Cl$-*p*, 69519-47-7; (*S*)-$HO_2CCH_2CH(CH_2Ph)CO_2H$, 3972-36-9.

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