Catalytic Role of the Metal Ion of Carboxypeptidase A in Ester Hydrolysis*

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The mechanism of action of bovine pancreatic carboxypeptidase A, (peptidyl-L-amino acid hydrolase; EC 3.4.12.2) has been investigated by application of cryoenzymologic methods. Kinetic studies of the hydrolysis of the specific ester substrate O-(trans-p-chlorocinnamoyl)-L-\beta-phenyllactate have been carried out with both the native and the Co\(^{2+}\)-substituted enzyme in the 25 to \(-45^\circ\)C temperature range. In the -25 to \(-45^\circ\)C temperature range with enzyme in excess, a biphasic reaction is observed for substrate hydrolysis characterized by rate constants for the fast (\(k_f\)) and the slow (\(k_s\)) processes. In Arrhenius plots, \(k_f\) extrapolates to \(k_{cat}\) at 25 \(^\circ\)C for both enzymes in aqueous solution, indicating that the same catalytic rate-limiting step is observed. The slow process is analyzed for both metal enzymes, as previously reported (Makinen, M. W., Yamamura, K., and Kaiser, E. T. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 3882-3886), to involve the deacylation of a mixed anhydride acyl-enzyme intermediate. Near \(-60^\circ\)C the acyl-enzyme intermediate of both metal enzymes can be stabilized for spectral characterization. The pH and temperature dependence of \(k_s\) reveals a catalytic ionizing group with a metal ion-dependent shift in \(pK_a\) and an enthalpy of ionization of 7.2 kcal/mol for the native enzyme and 6.2 kcal/mol for the Co\(^{2+}\) enzyme. These parameters identify the ionizing catalytic group as the metal-bound water molecule. Extrapolation of the \(pK_a\) data to 25 \(^\circ\)C indicates that this ionization coincides with that observed in the acidic limb of the pH profile of log(\(k_{cat}/k_{app}\)) for substrate hydrolysis under steady state conditions. The results indicate that in the esterolytic reaction of carboxypeptidase A deaclylation of the mixed anhydride intermediate is catalyzed by a metal-bound hydroxide group.

The mechanism of action of carboxypeptidase A (peptidyl-L-amino acid hydrolase; EC 3.4.12.2) has been conjectural despite a wide variety of thoroug kinetic, structural, and chemical studies (1-4). The roles of catalytic residues of the enzyme are not unambiguously defined for both peptide and ester hydrolysis, and kinetic studies have not revealed the nature and number of intermediates subsequent to substrate binding. Structural requirements for substrate specificity and rapid hydrolysis are a free carboxyl group and an aromatic or branched aliphatic side chain of the COOH-terminal residue. The binding relationships of substrates to carboxypeptidase A, determined by x-ray crystallographic methods, indicate that the terminal, free carboxyl group is salt-linked to arginine 145 and the aromatic side chain is enclosed in a deep hydrophobic pocket of the enzyme (1, 2). Once this binding mode is assumed, the only residues close enough to the substrate to enter into catalysis are the side chains of glutamate 270 and tyrosine 248, the tetrahedrally coordinated Zn\(^{2+}\) ion, and the metal ion-bound water molecule. Atomic model building studies, however, do not differentiate between the possible roles of glutamate 270 in direct nucleophilic attack on the carboxyl carbon of the substrate or in hydrolysis by a general base-catalyzed mechanism. Suggestions have been made that the binding of ester and peptide substrates may involve partially overlapping sites on the enzyme (5) and that their hydrolysis may occur by different mechanisms (6). While L phenylalanine and L-\beta-phenyllactate, frequently employed as the COOH-terminal residue in synthetic peptide and ester substrates, bind in similar configurations (7), x-ray studies have been carried out only for peptide substrates. No esterolytic substrate has been sufficiently sluggish for data collection by x-ray diffraction methods. In addition, difference Fourier studies (1, 2, 7) indicate that the structure of the enzyme in binding sluggish peptide substrates in the crystal is not compatible with hydrolysis catalyzed by a metal-hydroxide species, as demonstrated for small molecule metal ion complexes (8-10).

An approach developed in recent years to detect and characterize intermediates of enzyme reactions is the application of organic-aqueous cosolvent mixtures under conditions of subzero temperatures (11-13). By this method, reaction intermediates under nonturnover conditions can be accumulated in approximate stoichiometric concentrations in the 0 to 100 \(^\circ\)C range. We have demonstrated in a preliminary study (14) that temporal resolution of the reaction catalyzed by carboxypeptidase A can be achieved in the subzero temperature range and that hydrolysis of the specific ester substrate O-(trans-p-chlorocinnamoyl)-L-\beta-phenyllactate proceeds via formation of the covalent acyl-enzyme intermediate illustrated schematically in Structure I.

This initial study has served as an incisive approach in determining new details of the mechanism of carboxypepti-
dase A since discrete intermediates of the enzyme reaction had not been previously detected. In this communication, we demonstrate that the steps of the esterolytic reaction involving the chemical transformation of the substrate into products are identical in both cosolvent mixtures and in aqueous medium. In contrast to the interpretations of structural and chemical studies, our results indicate that the active species in ester hydrolysis catalyzed by carboxypeptidase A is a metal-hydroxide complex.

EXPERIMENTAL PROCEDURES

Materials—The specific ester substrate ClCPL was synthesized as described by others (15, 16). The tightly bound inhibitor t-benzylsuccinate (17) was a gift from Professor E. T. Kaiser of the Department of Chemistry at The University of Chicago. The crystalline α-isozyme of bovine pancreatic carboxypeptidase A obtained from Sigma was used as previously described (14). Preparation of CoCPA was carried out as described by Latt and Vallee (19) with use of deionized distilled water. The extent of metal substitution was evaluated by atomic absorption methods. Identical results in kinetic studies were obtained for all preparations of CoCPA with 88 to 98% metal replacement.

All chemicals were of analytical reagent grade. CoCl₂ was obtained by treatment of Specrep Co bar (Johnson Matthey Chemicals, Ltd.) with Ultrex HCl (Baker). Deuterium oxide (99% D₂O) was obtained from Stohler Isotopic Chemicals.

Methods—Initial velocity data were collected spectrophotometrically at 340 nm, specific for the hydrolysis of ClCPL. For ZnCPA, the reaction mixture was supplemented (19) with 10⁻³ M Zn²⁺ below pH 6, and for CoCPA, with 10⁻⁴ M Co²⁺ below pH 7.

For studies with cryosolvent mixtures, solutions were prepared as prescribed by Douzou and co-workers (11, 18). The pan of cryosolvent mixtures was confirmed spectrophotometrically with indicator dyes. For thermostating the spectrophotometer cuvette in kinetic and spectrophotometric studies, a cryostat assembly developed in this laboratory for use in a Cary 15 spectrophotometer was employed as described previously (20). The preparation of enzyme solutions in ternary solvent mixtures in the subzero temperature range was carried out below −20°C by injection of small aliquots of MeOH into an EG/H₂O mixture, coordinated with temperature regulation (21) to prevent precipitation of the enzyme. The addition of MeOH is associated with a marked decrease in the solubility of dissolved gases in the EG/H₂O mixture, observed by the appearance of small gas bubbles. Therefore, MeOH addition is best carried out only in the −25 to −20°C range where the escape of gas bubbles proceeds readily.

Initial velocity data were evaluated with use of the computer program ENZKIN provided by Professor J. L. Weston of the Department of Biochemistry at The University of Chicago. The steady state kinetic parameters kₐ and Kₗ of ZnCPA were calculated with this algorithm by an iterative nonlinear least squares fit to a rectangular hyperbolic function. Rate constants for the separated kinetic data for ZnCPA studies with enzyme in excess collected under subzero temperature conditions were calculated with use of a modified version of the nonlinear least squares algorithm of Marquardt (22), and, for CoCPA studies, by nonlinear regression analysis of the separated rate data with programmable calculators or by graphical analysis.

RESULTS

Ester Hydrolysis under Steady State Conditions

Influence of Cryosolvents—The influence of EG and MeOH on the hydrolysis of ClCPL has been determined on the basis of initial velocity data. The results are compared in Fig. 1 and in Table I. MeOH exhibits noncompetitive inhibition of ZnCPA but competitive inhibition of CoCPA. The inhibitory influence of EG on both ZnCPA and CoCPA is noncompetitive, in contrast to that of MeOH. For CoCPA in binary mixtures containing more than 30% EG, the value of Kₗ of ZnCPA is increased so that sufficiently high substrate concentrations cannot be achieved for accurate estimation of the kinetic parameters. In ternary solvent mixtures, the overall inhibitory influence of both cryosolvents appears to remain noncompetitive. In contrast to ZnCPA, steady state kinetic parameters for CoCPA in mixed solvents are complicated by substrate inhibition that becomes more pronounced with decreasing temperature.

Influence of Temperature—The variation of kₐ with temperature is compared in Fig. 2 for aqueous and cryosolvent mixtures. The Arrhenius plots yield values of Eₜ of 16.5 ± 0.8 kcal/mol for ZnCPA and 14.6 ± 0.9 kcal/mol for CoCPA in aqueous solution. With binary solvents containing MeOH, a change in the temperature dependence of kₐ is observed only for ZnCPA. For CoCPA the data for kₐ overlap with those observed in aqueous medium. This behavior is expected since MeOH acts noncompetitively toward ZnCPA but competitively toward CoCPA. On the other hand, the temperature dependence of Kₗ is altered for both ZnCPA and CoCPA in EG-containing solvent mixtures. The change in the temperature dependence of Kₗ indicates that above 0°C the enzyme reaction does not follow the same rate law under conditions of noncompetitive inhibition by mixed solvents as in aqueous solution. This interpretation is further supported by data in Table II. A deuterium isotope effect is observed for both ZnCPA and CoCPA in aqueous solution identical with that reported for the hydrolysis of O-(trans-cinnamoyl)-L-β-phenyllactate by the y-isozyme (23). This effect is not observed with noncompetitive inhibition by cryosolvents at room temperature.

Ester Hydrolysis under Conditions of Enzyme in Excess

Temporal Resolution of the Enzyme Reaction in the Subzero Temperature Range—With enzyme in excess, the hydrolysis of ClCPL in the subzero temperature range is characterized by a biphasic decrease in absorbance. In Fig. 3, the
Mechanism of Carboxypeptidase A

TABLE I

Comparison of kinetic parameters for hydrolysis of ClCPL in cryosolvent mixtures by ZnCPA and CoCPA

<table>
<thead>
<tr>
<th>Solvent mixture</th>
<th>ZnCPA</th>
<th>CoCPA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{\text{cat}}$</td>
<td>$K_{\text{m(app)}}$</td>
</tr>
<tr>
<td>0% EG</td>
<td>59.01 ± 3.54</td>
<td>1.01 ± 0.31</td>
</tr>
<tr>
<td>50% EG</td>
<td>3.12 ± 0.76</td>
<td>0.98 ± 0.53</td>
</tr>
<tr>
<td>10% EG</td>
<td>96.5 ± 28.1</td>
<td>0.72 ± 0.32</td>
</tr>
<tr>
<td>30% EG</td>
<td>53.6 ± 2.3</td>
<td>1.07 ± 0.08</td>
</tr>
<tr>
<td>EG/MeOH/H$_2$O (25:25:50)</td>
<td>9.29 ± 3.84</td>
<td>2.64 ± 1.67</td>
</tr>
<tr>
<td>EG/MeOH/D$_2$O (25:25:50)</td>
<td>10.9 ± 3.2</td>
<td>5.71 ± 0.29</td>
</tr>
</tbody>
</table>

a Solvent conditions: 24°C, 0.25 M NaCl, buffered to pH 7.5 with 0.01 M sodium cacodylate.

* Dissociation inhibitor constants ($K_a$) of EG are estimated as $-0.6$ M and $-2.9$ M for ZnCPA and CoCPA, respectively.

TABLE II

Comparison of steady state kinetic parameters for hydrolysis of ClCPL in H$_2$O- and D$_2$O-containing solvents

<table>
<thead>
<tr>
<th>Solvent*</th>
<th>ZnCPA</th>
<th>CoCPA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{\text{cat}}$</td>
<td>$K_{\text{m(app)}}$</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>73.2 ± 5.3</td>
<td>0.98 ± 0.14</td>
</tr>
<tr>
<td>D$_2$O</td>
<td>36.5 ± 5.2</td>
<td>0.74 ± 0.17</td>
</tr>
<tr>
<td>EG/MeOH/H$_2$O (25:25:50)</td>
<td>9.3 ± 3.7</td>
<td>7.11 ± 0.29</td>
</tr>
<tr>
<td>EG/MeOH/D$_2$O (25:25:50)</td>
<td>10.9 ± 3.2</td>
<td>5.71 ± 0.29</td>
</tr>
</tbody>
</table>

a All solvents contained 0.25 M sodium chloride, buffered to pH 7.5 or pD 7.9 with 0.01 M sodium cacodylate at 25°C.

b Determined from Lineweaver-Burk plot of initial velocity data.

small deviation from the linear portion of pseudo-first order plots shows two kinetically discrete processes. The biphasic reaction is characterized by rate constants for the fast ($k_f$) and slow ($k_s$) processes which correspond to formation and breakdown of a covalent acyl-enzyme intermediate (14). For CoCPA, the deviation from the linear portion of the plot is observed most readily only at lower pH, as shown in Fig. 4. Here the deviation from the linear portion of the slow process is too small for accurate estimation of $k_s$ above -40°C. Below -45°C, on the other hand, the high viscosity of the cryosolvent mixtures results in long mixing times so that the fast process is not observed.

There is an additional difference in the nature of the decrease in absorbance for the ZnCPA and CoCPA reactions. For the ZnCPA reaction, the linear portion of the slow process characteristically extrapolates to the value of $(A_0 - A_a)$ predicted by $(S_0)$. For the CoCPA reaction, the linear portion of the slow process extrapolates to values of $(A_0 - A_a)$ that are greater by a value of $\Delta A$ of $-4000$ M$^{-1}$ cm$^{-1}$ than that predicted by $(S_0)$. The higher extrapolated value of $(A_0 - A_a)$ is of interpretative significance and is discussed later.

The temperature dependence of the kinetic data in the -25 to -45°C range is compared in Fig. 5. For both ZnCPA and CoCPA, $k_{\text{cat}}$ extrapolates to $k_{\text{cat}}$ measured in aqueous solution at 25°C. Similarly, as previously noted (14), $k_f$ extrapolates to the value of $k_{\text{cat}}$ at 25°C determined for ZnCPA in the identical...
cosolvent mixture. Comparable results are obtained for the three cryosolvent mixtures EG/H2O (50:50), EG/MeOH/H2O (25:25:50), and EG/MeOH/H2O (40:20:40) with both ZnCPA and CoCPA.

For conditions of enzyme in excess, $k_s$ is equivalent to the observed pseudo-first order rate constant $k_{obs}$, where $k_{obs} = k_{cat}([E_0]/(K_{m(app)} + [E_0])$; and may be, therefore, influenced by $k_{cat}$ and $K_{m(app)}$ if saturating levels of enzyme are not achieved. In connection with our preliminary study (14), we have estimated the limiting value of $k_s$ under conditions of very high ZnCPA concentrations. At ZnCPA concentrations of $\sim 8 \times 10^{-5}$ M, the limiting value of $k_s$ at $-40^\circ C$ was $\sim 9 \times 10^{-7}$ s$^{-1}$. In Fig. 5, this coincides precisely with the value obtained by extrapolation to $-40^\circ C$ of the least squares-determined slope of the $k_{cat}$ data in aqueous solution. The experimental conditions employed thus permit reasonable estimates of the observed first order rate constants, and the results indicate that the enzyme reaction is characterized by the same rate-limiting step in the subzero temperature range as in aqueous solution.

**pH Dependence of Hydrolysis of CICPL in Cryosolvent Mixtures**—The pH dependence of $k_s$ in cryosolvent mixtures is illustrated in Fig. 6. The data provide evidence for ionization of a group associated with the catalytic rate-limiting step. The pH dependent change in $k_s$ for CoCPA parallels that for ZnCPA but is shifted to a lower pH range. The pH dependence of $k_s$ has been plotted according to Equation 1

$$k_s = \frac{(k_{s,\text{lim}}) K_s}{K_s + [H^+]}$$

where $(k_{s,\text{lim}})$ represents the graphically estimated limiting value of $k_s$ at high pH, and $K_s$ the ionization constant. The temperature dependence of the $pK_s$ values yields a $\Delta H_{\text{lim}}$ of 7.2 $\pm$ 0.1 kcal/mol for ZnCPA and 6.2 $\pm$ 0.1 kcal/mol for CoCPA for the ionizing group.

In Tables III and IV, a comparison is made of the pH dependencies of the activation parameters of the slow process. Possible interpretations of the large positive entropy of activation have been discussed previously (14). By deriving the relationship for $d(\ln k_s)/d(1/T)$ on the basis of Equation 1, the observed enthalpy of activation can be separated into a pH independent $\Delta H^\circ$ term associated with $(k_{s,\text{lim}})$ and the $\Delta H_{\text{lim}}$ associated with the $pK_s$ of the ionizing group. An estimate of the $\Delta H_{\text{lim}}$ can be made thus from the maximum difference

**Fig. 3.** First order plot of the decrease in absorbance at 310 nm during hydrolysis of CICPL by ZnCPA at $-40^\circ C$, illustrating graphically two kinetically discrete processes. The enzyme solution was prepared in the ternary mixture containing EG/MeOH/H2O (40:20:40) buffered to pH 7.5 at $-40^\circ C$ with 0.01 M sodium cacodylate at an ionic strength of 0.26 M sodium chloride. The enzyme concentration was $1.2 \times 10^{-4}$ M, and the initial substrate concentration was $4.8 \times 10^{-5}$ M.

**Fig. 4.** First order plot of the decrease in absorbance at 310 nm during hydrolysis of CICPL by CoCPA at $-40^\circ C$. The enzyme solution was prepared as in Fig. 3 but buffered to pH 5.0 at $-40^\circ C$ with 0.01 M sodium acetate. A, the total enzyme concentration was $1.2 \times 10^{-4}$ M and the initial substrate concentration was $0.40 \times 10^{-4}$ M. B, the total enzyme concentration was $0.60 \times 10^{-4}$ M and the initial substrate concentration was $0.20 \times 10^{-4}$ M.
observed in the values of $\Delta H_f$ at high and low pH. This treatment yields values of 9.4 and 6.6 kcal/mol for ZnCPA and CoCPA, in good agreement with those derived from the temperature dependence of the pK values. The similar behavior of the two metal enzymes in substrate hydrolysis in aqueous solutions and in cryosolvent mixtures indicates that identical catalytic processes obtain for both ZnCPA and CoCPA.

Metal-substituted derivatives of carboxypeptidase A, are less stable in the acidic pH range, requiring addition of excess metal ion for maximum activity (19, 24). Addition of 10$^{-4}$ M metal ion to cosolvent mixtures for low temperature studies caused a slow rate of background hydrolysis of CICPL not related to the active site, detected by the addition of excess L-benzylsuccinate prior to the addition of substrate. Subtraction of this background rate gave results comparable to those observed without added metal ion. This background hydrolytic activity is possibly related to extra metal ion-binding...
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The ternary solvent EG/MeOH/H2O (40:20:40) was buffered to pH 7.5 with 0.01 M sodium cacodylate and contained 0.26 M sodium chloride. The total enzyme concentration was 1.1 × 10^{-3} M with an equimolar quantity of CICPL. Further details of the procedure for recording the difference spectra have been provided in Ref. 14. ——, corresponds to the spectrum for ZnCPA; ---, corresponds to the spectrum for CoCPA. Extinction coefficients at 310 nm estimated for both covalent intermediates employing the extinction coefficient of 12,000 M^{-1} cm^{-1} for the substrate (15, 16) are ~22,000 and 19,000 M^{-1} cm^{-1} for ZnCPA and CoCPA, respectively, assuming complete conversion of the enzyme to the covalent intermediate.

**Spectral Characterization of the Acyl-enzyme Intermediate**

The ultraviolet difference spectrum of carboxypeptidase A at subzero temperatures versus versus carboxypeptidase A at ambient room temperature remains invariant to at least -60°C. Addition of CICPL causes a change in the difference spectrum attributable to accumulation of a covalent intermediate of the reaction (14). The difference spectra observed under these conditions for ZnCPA and CoCPA are illustrated in Fig. 7. The spectrum is not altered when L-benzylsuccinate is added after substrate, and addition of the inhibitor prior to addition of substrate prevents formation of the spectrally identified species. Over the pH range 6.0 to 9.0, the difference spectrum is invariant for both ZnCPA and CoCPA and cannot be accounted for by the difference spectrum of products versus the substrate. Furthermore, under conditions of enzyme in excess near -40°C, the slow phase of the biphasic decrease in absorbance due to hydrolysis of CICPL is not retarded by addition of L-benzylsuccinate. These observations indicate that a complex of the substrate with the enzyme is formed which must have a dissociation equilibrium constant less than the K1 ~ 10^{-7} M of L-benzylsuccinate (17). Noncovalent interactions of the substrate with carboxypeptidase A are unlikely to account for these observations. The spectrally identified species for ZnCPA and CoCPA, therefore, are best interpreted as resulting from accumulation of a covalent intermediate of the enzyme reaction formed prior to the rate-limiting step governed by k3. We have previously outlined the evidence indicating that the covalent intermediate is formed by acylation of the γ-carboxylate oxygen of glutamate 270, yielding the highly reactive mixed anhydride acyl-enzyme intermediate illustrated in Structure I (14). The chemical bonding structure of the intermediate has been confirmed by nucleophilic trapping studies with the use of hydroxylamine.2

As indicated above, extrapolation of the slow process of kinetic data for CoCPA, as in Fig. 4, yields a value of (A0 - A∞) consistently higher than that predicted by the initial substrate concentration. This is equivalent to approximately 80% of the extinction coefficient of the covalent intermediate of CoCPA at 310 nm. Similarly, for ZnCPA an extinction coefficient of the covalent intermediate of approximately 70% of that predicted by the difference spectrum could be estimated from kinetic data (14). We conclude that in kinetic studies with CoCPA at subzero temperatures, the initial fast process has occurred largely within the mixing time and only the breakdown of the intermediate species identified in Fig. 7 is observed.

**DISCUSSION**

The Reaction Scheme of Carboxypeptidase A in Ester Hydrolysis—Data presented in Figs. 3-5 and 7 indicate that two kinetically discrete reactions with observed rate constants k1 and k2, are correlated with formation and breakdown of a covalent acyl-enzyme intermediate in the subzero temperature range. The basis for the analysis of the hydrolysis of CICPL catalyzed by carboxypeptidase A as two consecutive first order reactions has been outlined (14). The analysis leads to a straightforward mathematical relationship between the time-dependent change in absorbance, the observed rate constants, and the concentrations of the substrate, intermediate, and products of the reaction, as given in Equation 2

\[
A - A_\infty = (S_0) \left[ \frac{\epsilon_{xT} - \epsilon_{xI} + \left( \frac{k_1 \epsilon_{xI} - k_2 \epsilon_{xT}}{k_3 - k_1} \right) \exp(-k_1 t)}{k_3 - k_1} \right] \exp(-k_2 t)
\]

or Equation 3

\[
A - A_\infty = a \exp(-k_1 t) + b \exp(-k_2 t)
\]

In Equation 2, (S0)εSTR represents the initial absorbance at t0, and the various ε values refer to the molar extinction coefficients of the chemical species of the reaction3 (defined below). Kinetic data such as those in Figs. 3 and 4 can be described by Equation 3 only under the condition that b > 0. This condition assigns the rate-limiting step governed by k2, to deacylation of the covalent acyl-enzyme intermediate (14).

The data presented in Fig. 5 demonstrate that the reaction catalyzed by ZnCPA and CoCPA is characterized by the same rate-limiting step in aqueous solution as in cryosolvent mixtures at subzero temperatures. Thus, the subzero temperature data permit deduction of the nature of the rate-limiting step of the enzyme-catalyzed reaction in aqueous solution. For the enzyme reaction, hydrolysis of CICPL must involve first noncovalent binding to form the Michaelis complex prior to formation of the acyl-enzyme intermediate (14).

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The reaction in aqueous solution can be described on this basis by the minimal scheme in Equation 4.

\[
E + S \overset{k_1}{\rightarrow} ES \overset{k_2}{\rightarrow} ESY \overset{k_3}{\rightarrow} E + P_1 + P_2 + \frac{K}{P_1}
\]

\[
A - A_\infty = \left[ \frac{(S_0)\epsilon_{xI} + \left( \frac{k_1 \epsilon_{xI} - k_2 \epsilon_{xT}}{k_3 - k_1} \right) \exp(-k_1 t)}{k_3 - k_1} \right] \exp(-k_2 t)
\]

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where $EY$ is the covalent intermediate of the reaction, $K_p$ is the dissociation constant of the enzyme complexed with the first product of hydrolysis, L-β-phenyllactate, and $k_d$ governs the rate-limiting step and, therefore, is to be associated with $k_i$ in cryosolvent mixtures at subzero temperatures.

The results in Fig. 2 and in Table II indicate an alteration in the rate law for the enzyme reaction in the presence of noncompetitive cryosolvent inhibitors. To account for the overall noncompetitive inhibition of the cryosolvent mixtures employed, we modify the reaction scheme in Equation 4. The appropriate series of consecutive reactions is illustrated in Scheme I, and the corresponding rate equation is provided under “Appendix.”

In Fig. 5, data for $k_f$ for the reaction catalyzed by ZnCPA in the subzero temperature range extrapolate to the value of $k_{-1}$ observed at 25°C in the same cryosolvent mixture. We have previously suggested (14) that the fast process represents rate-limiting acylation above $\sim -10^\circ C$ both in cryosolvent mixtures and in aqueous solution, but that at lower temperatures deacylation becomes rate-determining. This suggestion must be modified in view of the more complete data presented here which define the interaction of cryosolvents with the enzyme during substrate hydrolysis. In aqueous solution, deacylation governed by $k_3$ is rate-limiting. The rate law describing the enzyme reaction in cryosolvent mixtures in the 25-0°C range is altered from that in aqueous solution and contains a composite rate constant which includes the equilibrium governed by $K_{OQ}$. Under conditions of noncompetitive inhibition, enzyme with bound cryosolvent represented as $EQ$ can further bind substrate to form the noncovalent complex $ESQ$. This complex is in equilibrium with the Michaelis complex $ES$, and further catalytic steps of the reaction proceed only along the pathway normally observed in the absence of noncompetitive inhibition. The change in the rate law simply arises as a result of binding of noncompetitive cryosolvent inhibitor, altering the availability of $ES$ for further reaction.

A comparable reaction scheme must also obtain in the subzero temperature range with respect to the interaction of cryosolvent inhibitors with the enzyme. However, to account for the pH dependence of $k_a$, an equilibrium involving ionization of a residue on the mixed anhydride intermediate prior to rate-limiting deacylation must be included in the reaction scheme. The corresponding sequence of reaction is illustrated in Scheme II, and the appropriate rate equation is again provided under “Appendix.”

Evaluation of the interaction of cryosolvents with carboxypeptidase A, during the hydrolysis of CICPL is comparable to the analysis (26) of the effect of inhibitors on the kinetic parameters of an enzyme reaction that proceeds via an acyl-enzyme intermediate. In the presence of cryosolvent inhibitors, the enzyme reaction is altered only by formation of dead-end complexes. In the case of the hydrolysis of CICPL by carboxypeptidase A, complexing of the Michaelis complex with cryosolvents only alters its availability in the reaction pathway. Therefore, the important result of this analysis is that the catalytic steps of the reaction of CICPL with carboxypeptidase A, responsible for the chemical transformation of substrate to products are identical in aqueous solution and in cryosolvent mixtures.

Identification of the Ionizing Group in the Acyl-enzyme Intermediate—The pH dependence of $k_a$ provides evidence for ionization of a group associated with deacylation of the covalent acyl-enzyme intermediate in the subzero temperature range. Douzou and co-workers (27, 28) have previously observed that the enthalpies of ionization of amino acid residues in cryosolvent mixtures are dependent upon solvent composition and that the compensation temperature of an ionizing residue remains the only identifiable criterion for assignment. Therefore, the parallel behavior of the pH dependence of $k_a$ and the $\Delta H_{ion}$ for both ZnCPA and CoCPA independent of solvent composition indicates that the environment of the ionizing group must be very nearly identical for both enzymes. This behavior could occur only for a group buried in the interior region of the acyl-enzyme intermediate inaccessible to bulk solvent.

The metal ion-dependent shift in $pK_a$ indicates that this group must be in the immediate environment of the metal ion. The decrease in $pK_a$ upon substitution of the open shell Co$^{2+}$ cation for the closed shell Zn$^{2+}$ cation is consistent with the change expected for ionization of a ligand directly coordinated to the metal. The amino acid side chains of histidine 196, histidine 69, and glutamate 72 that coordinate the metal ion are sterically inaccessible for direct participation in the catalytic process (1, 2). Also, the $pK_a$ of ionization of the pyrrole hydrogen of a metal-coordinated imidazole group is too high (29) to account for the pH dependence of $k_a$. On this basis, the only residue that could be responsible for the pH dependence of $k_a$ is the metal-coordinated water molecule. The estimates for $\Delta H_{ion}$ of 6.2 ± 0.1 and 7.9 ± 0.1 kcal/mol are in good agreement with values of 6.0 to 8.3 kcal/mol observed for ionization of the water ligand in model five-coordinate Co$^{2+}$ and Zn$^{2+}$ complexes (30). In contrast, a $\Delta H_{ion}$ of 0 to 1 kcal/mol would be expected for the catalytic amino acid side chain of glutamate 270 (31). We also note that an estimate of the compensation temperature of the ionizing group based on the combined data in Tables III and IV yields a value of $\sim 345 K$. This is consistent only with that expected for ionization of a cationic acid (27, 28). The only cationic acid group in the active site region that can be directly involved in the catalytic process is the solvent molecule coordinated to the divalent metal ion with an overall charge of +1.

The temperature dependence of the $pK_a$ for ZnCPA in Fig. 6 with an estimated $\Delta H_{ion}$ of 7.2 ± 0.1 kcal/mol extrapolates to a $pK_a$ of 6.1 at 25°C for the catalytic residue. Corresponding considerations place the $pK_a$ for CoCPA at 4.9. These $pK_a$ values and the $\Delta H_{ion}$ of 7.2 kcal/mol are characteristic of a water ligand.
values for the ionization of a metal-coordinated water molecule are low in comparison to that of ~10 generally associated with hexaquo complexes of Zn\(^{2+}\) and Co\(^{3+}\) ions (39). There are, however, several factors suggesting that they are reasonably expected for the acyl-enzyme intermediate. In complexes of transition metal ions, the redistribution of bonding capacity upon a decrease in coordination number results in stronger metal-ligand bonding interactions. In fact, the infrared \(v(\text{OH})\)stretching frequency of hydrates of transition metals, particularly of Zn\(^{2+}\) complexes of low coordination number, indicate enhanced O—H bond polarization and, therefore, an increased ease of proton ionization (33). Furthermore, the electrostatic interactions between the positively charged metal cation and the bound water molecule polarizing the O—H bond will be increased in the acyl-enzyme intermediate. Loss of the negative charge associated with the carboxylate of glutamate 270 in the active site cavity upon formation of the covalent acyl-enzyme intermediate will enhance the electrostatic field of the metal cation on the bound water molecule. Conversion of the enzyme active site upon substrate binding from a water-filled to a hydrophobic region (1, 2) is associated with a change in the local environment of the metal ion to one with a low dielectric constant. The decrease in the dielectric constant will aid the ionization of the metal-bound water molecule through the electrostatic energy to be gained (34) supporting these arguments, we note that the \(pK_a\) of -8.0 and -10.2 observed for the hexaaquo-coordinated Zn\(^{2+}\) complex (15, 16). Comparable \(pK_a\) values are observed in the hydrolysis of peptide substrates (24, 38). The ionization with \(pK_a > 9\) has been attributed to a tyrosine residue (16, 24, 38) or the ionization of the metal-coordinated water molecule (3, 16, 39). The only basis for the latter interpretation appears to be the \(pK_a\) of ~10.2 observed for the hexaaquo-coordinated Zn\(^{2+}\) complex (32). The estimate of \(\Delta H_m\) of ~5 kcal/mol for this ionization (38) favors the tyrosine assignment but does not adequately differentiate between the two possible interpretations. We note, however, that is ionization is not sensitive to metal ion substitution in the hydrolysis of peptide and ester substrates (24).

The \(pK_a\) of ~6.4 on the acidic side of the pH profile of log \(k_{\text{cat}}/K_{\text{M(app)}}\) has been assigned (1–3, 16, 24, 39) to glutamate 270 on the basis that the hydrophobic environment of the active site cavity would tend to raise the \(pK_a\) of a carboxylic acid group. However, the estimate of \(\Delta H_m\) of ~6.5 kcal/mol obtained from kinetic studies of peptide hydrolysis (38) clearly rules against the assignment of a carboxylic acid group for this ionization. It is noteworthy that for peptide hydrolysis the \(pK_a\) detected on the acidic side of the pH profile of log \(k_{\text{cat}}/K_{\text{M(app)}}\) is sensitive to metal ion substitution and is shifted from 6.2 in ZnCPA to ~5.0 in CoCPA (24). As indicated above, \(pK_a\) values of ~6.1 for ZnCPA and ~4.9 for CoCPA at 25°C are predicted for the ionizing group from low temperature data. The metal ion-dependent shift in \(pK_a\) associated with the rate-limiting process in the subzero temperature range together with the \(\Delta H_m\) serve as firm diagnostic criteria for assignment of the ionization to the metal-coordinated water molecule. We, therefore, suggest that the \(pK_a\) observed on the acidic side of the pH profile of log \(k_{\text{cat}}/K_{\text{M(app)}}\) of carboxypeptidase A\(\text{a}\) in substrate hydrolysis under steady state conditions is due to the ionization of the metal-bound water molecule in the catalytic rate-determining step.

The Mechanism of Action of Carboxypeptidase A in Ester Hydrolysis—Results of atomic model building studies suggest that in productive binding the carbonyl oxygen of the susceptible peptide bond of the substrate molecule is coordinated to the metal ion as the fourth ligand by displacing the water molecule (1, 2). This configuration is generally considered as characteristic of the mechanism of action of carboxypeptidase A\(\text{a}\) for both peptide and ester hydrolysis (1–3, 40). The pH dependence of \(k_s\), indicating ionization of the metal bound water molecule cannot be rationalized by simple displacement of the solvent molecule by the substrate, and an alternative explanation of the chemical processes responsible for deacylation of the acyl-enzyme intermediate must be made. To this end we have illustrated in Fig. 8 a schematic outline of the chemical pathway for the hydrolysis of the specific ester substrate CICP by carbamyl phosphate A. The scheme differs from other formulations (1–3, 6, 40, 42) of the mechanism of action of carboxypeptidase A\(\text{a}\) with respect to the catalytic role of the metal ion and water molecule, based previously only on the results of atomic model building studies. However, the structural basis for specific binding of the terminal carboxyl group of the substrate by arginine 145 and the aromatic side chain in the hydrophobic cavity established by x-ray diffraction studies (1, 2, 7) remains unaltered.

The suggested pathway for the hydrolysis of the ester substrate in Fig. 8 is consistent with (i) the minimal reaction scheme in Equation 4; (ii) the temperature and metal ion dependence of the \(pK_a\) in Equation 1; and (iii) the evidence for a covalent acyl-enzyme mixed anhydride intermediate (14). Nucleophilic attack on the carbonyl carbon of the substrate to form a mixed anhydride intermediate most likely proceeds via formation and collapse of a transient tetrahedral intermediate. Decacylation of the mixed anhydride intermediate with a metal-bound hydroxide group in the active site may then occur by two possible pathways, as suggested by model studies of the metal ion-catalyzed hydrolysis of esters and peptides (8–10). Direct nucleophilic attack by the metal-bound hydroxide group on the carbonyl carbon atom of the substrate may occur with solvolysis of the resultant complex of the metal ion. Alternatively, decacylation may occur via indirect general base catalysis by an intervening water molecule hydrogen bonded to the metal-bound hydroxide group. Our data do not distinguish between the two possibilities. Direct nucleophilic attack of the metal-bound hydroxide group is the kinetically preferred pathway in model systems (9). While the deuterium isotope effect on \(k_{\text{cat}}\) supports the role of glutamate 270 as a nucleophile (23, 43), the influence of deuterium on \(k_{\text{cat}}\) as shown in Table II also remains compatible with the general base catalyzed process of an intervening water molecule. For either pathway cleavage of the C—O bond must occur between the substrate carbonyl carbon atom and the oxygen of the \(gamma\)-carboxyl group of glutamate 270, for studies (44) of the incorporation of \(^{18}O\) into active site residues of carboxypeptidase A\(\text{a}\) show that the oxygens of the carboxyl group of glutamate 270 are not exchangeable. Model building studies indicate that binding of the acyl moiety of the sub-
FIG. 8. Schematic illustration of the pathway for the hydrolysis of the specific ester substrate ClCPL catalyzed by carboxypeptidase A, showing the structural relationships of the catalytic residues of the enzyme to the substrate molecule. See text for discussion. Since formation of a metal-hydroxide complex is required for deacylation of the mixed anhydride intermediate, we have indicated for purposes of simplification that the metal-bound water molecule is also retained during formation of the preceding intermediates of the reaction. Our data do not define the role of the metal-bound water molecule during formation of noncovalently bound enzyme-substrate complexes. The metal ion requirement for the binding of ester substrates (41) may be compatible also with outer sphere coordination although displacement of the metal-bound water molecule by the carbonyl oxygen atom of the substrate is generally assumed (1-3, 6, 39, 40, 42) on the basis of model building studies (1, 2). For purposes of simplification, the reaction scheme is not balanced with respect to protons.

The primary advantage of the application of cryoenzymologic methods (11-13) to the study of enzyme reactions derives from the potential to accumulate, in stoichiometric accounts, and to stabilize, for long periods of time, an intermediate of the reaction for physical characterization. The results of this investigation thus provide firm support for the conclusions that hydrolysis of substrates by carboxypeptidase A, involves at least one covalent enzyme-substrate intermediate formed after the Michaelis complex and, most importantly, that hydrolysis is sterically compatible with peptide hydrolysis (1, 2), a metal-bound water molecule could also account for the apparent inconsistencies (6) of the isotope exchange studies. Moreover, difference Fourier studies of sluggishly reactive peptides indicate that not all of the solvent molecules in the active site cleft are displaced upon substrate binding (1, 2, 7). The similarity in the pH profiles of log $k_{cat}/K_{mapp}$ for peptide and ester hydrolysis (16, 24, 38, 46) and the estimate of $\Delta H_{mo}$ of $\sim 6.5$ kcal/mol for the $pK_a$ of 6.2 in peptide hydrolysis (38), closely overlapping with that estimated in this study for the hydrolysis of CICPL, indicate that a metal-hydroxide species is also formed in peptide hydrolysis.
The application of such criteria in cryoenzymologic studies cannot be overemphasized. Temperature changes will influence equilibria governing the formation of dead-end complexes or may result in formation of intermediates of the enzyme reaction (11). Such temperature changes correspond to formation of true productive intermediates of the catalytic pathway consequently rests on utilization of cryosolvent methods. For instance, the vent was noted to act as an inhibitor of the enzyme reaction equilibria involving formation of complexes of catalytic intermediates with cryosolvent molecules also may obtain for those in the subzero temperature range exhibit minimal reaction schemes comparable to that in Equation 4 with at least one covalent intermediate (11-13). In numerous cases the cryosolvent catalyzed by carboxypeptidase A proceeds via a metal-hydroxide species, we conclude that interaction of the metal ion coordinating directly the carbonyl oxygen cannot be a catalytically productive configuration in ester hydrolysis.

Cryosolvents as Inhibitors in Low Temperature Studies—In the application of cryoenzymologic methods, it is necessary to demonstrate that the catalytic pathway in cryosolvent mixtures remains unchanged from that in aqueous medium. This requirement has been implicit since the introduction of cryoenzymology (46). However, except for the obvious case of alcoholic cryosolvents competing for water in the enzyme reaction, the mechanistic implications of the influence of cryosolvents by complexing to intermediates of enzyme reactions have not been assessed quantitatively. Thus, the results of these studies outlining the differential action of two cryosolvents as inhibitors of carboxypeptidase A underlying emphatically the importance of evaluating solvent effects.

A variety of hydrolytic enzymes that have been investigated in the subzero temperature range exhibit minimal reaction schemes comparable to that in Equation 4 with at least one covalent intermediate (11-13). In numerous cases the cryosolvent was noted to act as an inhibitor of the enzyme reaction (12). Therefore, the analysis presented here suggests that equilibria involving formation of complexes of catalytic intermediates with cryosolvent molecules also may obtain for those enzymes. Our observations on the influence of cryosolvents on carboxypeptidase A, therefore, introduce a note of caution into the application of cryosolvent methods. For instance, heating-cooling cycles are often applied to effect transformation of intermediates of the enzyme reaction (11). Such temperature changes will influence equilibria governing the formation of dead-end complexes or may result in formation of complexes with bound cryosolvent that enter into parallel catalytic reaction pathways. The assessment that spectral changes correspond to formation of true productive intermediates of the natural catalytic pathway consequently rests on the rigorous application of kinetic criteria. The importance of application of such criteria in cryoenzymologic studies cannot be overemphasized.

APPENDIX

The Rate Equation for Scheme I—For Scheme I the rate equation has the form

\[ \frac{1}{k_{cat}} = \frac{(k_{-1} + k_{3})(1 + QK_{Q})}{k_{1}k_{2}} \frac{1}{(1 + Q'K_{Q})} \]

(Al)

where \( Q \) and \( Q' \) represent the concentration of noncompetitive and competitive cryosolvent inhibitor molecules, \( k' \) 's represent microscopic rate constants, and \( K' \) 's the association equilibrium constants. The rate equation is derived for the condition \( Q' \gg (E_0) \) and independent binding of inhibitors \( Q \) and \( Q' \) to the enzyme. The catalytic rate constant for Scheme I is then given by the relation

\[ \frac{1}{k_{cat}} = \frac{(1 + QK_{Q})}{k_{2}} + \frac{1}{k_{3}}. \]

(A2)

Comparison of Equation A2 to the catalytic rate constant for the reaction in aqueous solution (Equation 4)

\[ \frac{1}{k_{cat}} = \frac{1}{k_{2}} + \frac{1}{k_{3}}, \]

for which \( k_{cat} = k_{3} \) under the condition \( k_{2} \gg k_{3} \), indicates that binding of the Michaelis complex ES by noncompetitive cryosolvent could alter the rate law for the reaction in the 25-0°C temperature range.

Since the pH dependence of \( k_{cat} \) has not been evaluated, ionizations associated with the rate constants \( k_{1} \) and \( k_{2} \) have been omitted for purposes of simplification. For Scheme I, a pH dependence of \( k_{2} \) is implicit from the discussion for the derivation of the rate equation for Scheme II given below.

The Rate Equation for Scheme II—For Scheme II the rate equation is given by Equation A3

\[ \frac{(E_0)}{v_0} = \left( \frac{(k_{-1} + k_{3})(1 + QK_{Q})}{k_{1}k_{2}} \frac{1}{(1 + Q'K_{Q})} \right) \]

\[ + \left( \frac{1 + QK_{Q}}{k_{2}} \right) \frac{1}{(1 + Q'K_{Q})} \left( \frac{k_{-1} + k_{3}}{k_{1}} + \frac{1}{k_{3}} \right) \]

\[ + \left( \frac{1 + QK_{Q}}{k_{2}} \right) \frac{1}{(1 + Q'K_{Q})} \left( \frac{k_{-1} + k_{3}}{k_{1}} + \frac{1}{k_{3}} \right) \]

(3)

(A3)

where the appropriate rate constants and association equilibrium constants are indicated respectively by \( k' \) 's and \( K' \) 's. The catalytic rate constant is then given by the relation

\[ \frac{1}{k_{cat}'} = \frac{(1 + QK_{Q})}{k_{2}} + \frac{1 + Q'K_{Q}}{k_{3}} + \frac{1}{k_{1}} \]

(4)

(A4)

Further simplification of Equation A4 is made by evaluating the relative magnitudes of the component terms. In Fig. 6, the dependence of \( k_{1} \) on pH indicates that \( k_{-1} \gg k_{3} \). Therefore, the predominant term in Equation A4 is the part containing \( 1/k_{3} \). Then

\[ k_{cat}' \approx k_{3} = \frac{k_{3}}{K_{c} + (H^{+})} \]

(A5)

The relationship of Equation A5 to Equation 1 is immediately recognizable. Scheme II thus accounts for the kinetic behavior of the reaction of CICPL with carboxypeptidase A, and the pH dependence of \( k_{1} \) in the subzero temperature range.

On the other hand, binding of noncompetitive cryosolvent inhibitor by EHY and EY to form the dead-end complexes EHYQ and EYQ (governed by the association equilibrium constants \( K_{Q}^{c} \) and \( K_{Q}^{y} \)) leads to the following relation for the catalytic rate constant

\[ k_{cat}^{c} = \frac{k_{3}}{(1 + QK_{Q})} \left( \frac{K_{c}}{K_{c} + (H^{+}) (1 + QK_{Q})} \right) \]

(A6)

The noncompetitive behavior of cryosolvents and the pH dependence of \( k_{2} \) were identical in all cryosolvent mixtures studied, indicating that differential binding affinities of cryosolvents to EHY and EY are unlikely. and, therefore, \( K_{Q}^{c} \) \( \approx K_{Q}^{y} \). Under these conditions, the term within the brackets in the above equation containing \( K_{3} \) becomes equivalent to that in Equation A5. Furthermore, under conditions of enzyme
in excess, the values of $k_\alpha$, estimated as observed pseudo-first order rate constants, are independent of solvent composition, as shown in Fig. 5. These data, thus, indicate that the denominator term $(1 + QK_0)$ is $1$ and that at most only very weak binding of cryosolvent inhibitor by EHY and EY can obtain. We conclude, therefore, that Scheme II is the simplest minimum scheme which outlines the sequence of reactions compatible with the kinetic data.

The analysis of Laidler and Bunting (26) suggests that binding of a competitive inhibitor could also occur with EY forming a complex which collapses to products by a reaction parallel to that governed by $k_1$ in Scheme II. This condition implies methanolation in the case of CoCPA. Transesterification would result in little change in the absorbance properties of the $p$-chlorocinnamoyl moiety at 310 nm from those of CICPL. Since comparable changes in absorbance at 310 nm are observed for substrate hydrolysis catalyzed by both ZnCPA and CoCPA in cryosolvent mixtures, we conclude that methanolation is not catalyzed by CoCPA. Moreover, methanolation has not been detected for ZnCPA (47).

To derive appropriate expressions for the rate equations of Schemes I and II, we have considered only irreversibility of which reaction catalyzed by carboxypeptidase A, (Equation 4), in ClCPL. Since comparable changes in absorbance at 310 nm when considered either as a double displacement or as a sequential mechanism.

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