A Peptidase-inactive Derivative of Carboxypeptidase A Modified Specifically at Tyrosine 248

COBALT(III)(ETHYLENEDIAMINE-N,N'-DIACETATO)(ARSANILAZOTYROSINATO 248 CARBOXYPEPTIDASE A)∗

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The first derivative of carboxypeptidase A, cobalt(III)(ethylenediamine-N,N'-diacetato)(arsanilazotyrosinato 248 (Co(III)(EDDA)(AA-CPA-Zn)), in which only the active site residue tyrosine 248 is blocked, has been shown to be completely peptidase-inactive while retaining esterase activity. Peptides are excellent inhibitors of esterase activity showing $K_i$ values somewhat below the corresponding $K_m$ values of the native enzyme. Short peptides are noncompetitive inhibitors of both short and long ester hydrolysis by Co(III)(EDDA)(AA-CPA-Zn), implying that the binding positions for esters and peptides are not the same. This behavior is consistent with the differential effects of various inhibitors of peptide and ester hydrolysis in the native enzyme, indicating that substrate binding to Co(III)(EDDA)(AA-CPA-Zn) reflects the binding in the native enzyme. In contrast, long peptides are competitive inhibitors of both short and long ester hydrolysis. The results are consistent with nonidentical but overlapping binding sites for peptides and esters prior to their respective rate-determining steps.

Acetylation of two active site tyrosines in the native enzyme (Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1983) Biochemistry 2, 616–622) results in a peptidase-inactive derivative that is distinctly different from Co(III)(EDDA)(AA-CPA-Zn). In contrast to the native and Co(III)(EDDA) enzymes, peptides and esters appear to occupy a common binding position in the acetylated derivative. Peptides also bind much more poorly to the acetylated enzyme than to the native or Co(III)(EDDA) enzymes. Similar effects on peptide binding can be brought about by acetylation of Co(III)(EDDA)(AA-CPA-Zn).

The mammalian zinc neutral protease, carboxypeptidase A (CPA-Zn) catalyzes the hydrolysis of COOH-terminal amide and ester bonds. A considerable amount of evidence suggests that ester and amide cleavage proceed by differing mechanisms (Refs. 3 and 4 and references therein).

On the basis of chemical modifications of CPA-Zn, most notably acetylation, functional tyrosines have been implicated in the peptidase but not esterase activity (9). Acetylation of the enzyme modifies some 5 tyrosine residues, 2 of which can be protected from modification by the inhibitor, β-phenylpropionate (βPP). The unprotected enzyme loses peptidase activity upon acetylation while the apparent esterase activity increases under standard assay conditions. Modification in the presence of βPP yields a derivative with essentially unaltered peptidase and esterase activities.

X-ray crystallographic studies suggest the most likely sites of modification are tyrosines 248 and 198, both of which lie in the vicinity of substrate binding (6). Structural analysis of the enzyme-Gly-Tyr complex implicates tyrosine 248 as a proton donor in peptidase activity (6). Tyrosine 248 is apparently not required for specific ester hydrolysis (7).

If, as is most likely, acetylation occurs at Tyr-248 and Tyr-198, the loss of peptidase activity needs to be attributed to the modification of 1 or both tyrosine residues. Diazoo-tetrazole modification of CPA-Zn yields a derivative with 85% peptidase activity and 185% esterase activity (8). Upon nitration of the tetrazolylazo enzyme with tetranitromethane, the peptidase activity decreases to about 9%. By peptide analysis, the sites of modification have been shown to be primarily Tyr-248 for the diazotization and Tyr-198 for the nitration. Therefore, it appears that modification of Tyr-198 affects esterase activity and could account for the loss of peptide hydrolysis upon acetylation (8).

In developing a general method for the incorporation of substitution-inert metal ions into peptide hormones and proteins (9, 10), we have produced a derivative of carboxypeptidase A modified specifically at tyrosine 248 with Co(III). The approach involves diazotization of the enzyme to produce the amsanilazotyrosine 248 derivative (AA-CPA-Zn) as described by Johansen and Vallee (11), followed by in situ oxidation of Co(II)(EDDA) to yield Co(III)(EDDA)(AA-CPA-Zn) (10).
arsenalazotyrosine 248 derivative (prior to Co(III)EDDA in corporation) has activity comparable to that of the native enzyme. In the Co(III)(EDDA)(AA-CPA-Zn) derivative, the phenolic oxygen of tyrosine 248 is coordinated to exchange inert Co(III) eliminating this residue as a potential proton donor for the hydrolysis of substrates. It should be stressed that Co(III) is bound only to arsonalazotyrosine 248 and the essential active site Zn(II) remains in the active site (10).

To date, a modification of CPA-Zn which eliminates the phenolic oxygen of only Tyr-248 as a proton donor has not been reported. Co(III)(EDDA)(AA-CPA-Zn) is the first such modification (10). In the present investigation, the capacity of this specifically modified enzyme to act as a peptidase and esterase with peptides and their structurally analogous depsipeptides is reported. The possible role of Tyr-248 and the influence of Tyr-198 in the enzymatic hydrolysis of peptides are discussed.

MATERIALS AND METHODS

Carboxypeptidase A Derivatives—All studies were conducted with bovine pancreatic CPA-Zn isolated by the method of Cox (Sigma, lot No. 56C-8100 and 16C-8205). The commercial enzyme was purified as previously described (10). Dialysis of the native enzyme with diacetyl imidazole (Aldrich) according to Simpson et al. (5). Enzyme Concentrations—Enzyme concentrations were determined spectrophotometrically utilizing the following molar absorptivities: CPA-Zn, 6.42 × 10$^4$ M$^{-1}$ cm$^{-1}$ at 278 nm (5); Co(III)EDDA(arsenalazotyrosine 248 CPA-Zn) was carried out according to Urdea and Legg (10). Ac-CPA-Zn and Ac(III)(EDDA)(AA-CPA-Zn) were prepared with a 48-fold excess of acetyl imidazole (Aldrich) according to Johansen and Vallee (11). Co(III)EDDA incorporation to zincfree enzyme with diazotized arsanilic acid was performed according to Legg (10). Ac-CPA-Zn and Ac(III)(EDDA)(AA-CPA-Zn) were prepared with a 48-fold excess of acetyl imidazole (Aldrich) according to Simpson et al. (5).

Enzyme Concentrations—Enzyme concentrations were determined spectrophotometrically utilizing the following molar absorptivities: CPA-Zn, 6.42 × 10$^4$ M$^{-1}$ cm$^{-1}$ at 278 nm (5); Ac-CPA-Zn, 7.32 × 10$^4$ M$^{-1}$ cm$^{-1}$ at 278 nm (12); Co(III)-CPA-Zn, 5.92 × 10$^4$ M$^{-1}$ cm$^{-1}$ at 278 nm (6), and Co(III)(EDDA)(CPA-Zn), 9.04 × 10$^4$ M$^{-1}$ cm$^{-1}$ at 274 nm (calculated from amino acid analysis; Ref. 10). The concentration of Co(III)(EDDA)(AA-CPA-Zn) was determined by the Bio-Rad Technical Bulletin No. 1051. For kinetic studies, all enzymes were stored as 10-g M. stock solutions in 0.5 M NaCl, 0.01 mM Tris, pH 7.5. For actual kinetic runs, these solutions were diluted daily to make up 1 × 10$^{-2}$ to 1 × 10$^{-1}$ M stock solutions in 0.5 M NaCl, 0.01 mM Tris, pH 7.5. Typically, these solutions were used within 2 h of dilution.

Peptidase Activities—Below 1 mM concentrations, Bz-GP and Bz-GP hydrolases were followed spectrophotometrically as reported by Folk and Schirmer (1) on a Cary model 14 spectrophotometer thermostated at 26 ± 0.1°C ($\Delta_{275}$ = 1390 for Bz-GP and $\Delta_{278}$ = 286 for Bz-GP). Assays were performed in 0.5 M NaCl, 0.05 M Tris, pH 7.5. All peptides were assayed by a method employing the quantitative detection with ninhydrin of terminal amino acids released by the method of Stewart and Young (14) fashioned after Enzler and Neurath (2). For 2 to 6 ml complex ($10^2$ to $10^3$ M$^{-1}$), 0.5 M NaCl, and 0.05 M Mes, Tris, or Ches with appropriate peptide concentrations), fractions of 0.2 to 1.0 ml were removed. A typical example of n-hexahydrate peptide substrate was analyzed directly to the reaction mixture and color developed at 100°C for 15 min. The tubes were then cooled immediately, made up to 3.0 ml with 50% ethanol, shaken well, and the absorbance was read at 570 nm. Standards of leucine (100 µg/ml) and phenylalanine (100 µg/ml) gave absorbances of 0.89 and 0.75, respectively. Typically, hydrolases were carried out to about 15 min (0 to 20% hydrolysis for the native enzyme). Compared to the CPA-Zn controls, the Co(III)(EDDA) derivative showed no more than 0.1% hydrolysis for any of the peptide substrates. Peptides were tested at 0.1 and 1.0 mM in pH 6.5, 7.5, and 9.5 buffers.

Esterase Activities—Esterase activity was determined by pH titrations of the proteases released upon hydrolysis using a pH-stat constructed in this laboratory. The instrument, which is more versatile and sensitive than commercially available instruments, can accurately deliver from 0.20 to 500 µl of base/min with a 1-ml Hamilton syringe. All titrations were carried out with 10 to 400 mM base under a nitrogen atmosphere and were thermostated at 25 ± 0.1°C. Unless otherwise indicated, all assays were carried out in 0.5 M NaCl, pH 7.5, with 0.1 to 0.01 mM Tris. Between 40 and 100 µl of enzyme stock solutions were added to 2-ml mixtures of substrates. Dilutions were accounted for in all subsequent calculations. At low Bz-GOP concentrations (below 1 mM), assays were also performed spectrophotometrically (15) as described for the peptidase activities ($\Delta_{275}$ = 598). All Bz-GOP activities are expressed in terms of the l isomers (S0 = S0 and S0/2).

Initial Velocities and Kinetic Parameters—Primary experimental data were fit to the integrated rate equation with the plotting procedure of Foster and Nieman (16).

\[ V_{max} = \frac{1}{K_I + K_I} \sqrt{S_0} \]

where $K_I$ is the Michaelis constant, $S_0$ is the initial substrate concentration, $S_0$ is the substrate concentration at time t, and $V_{max}$ and $K_I$ have their usual meanings. The pH-stat records $S_0 - S_0$ versus $t$ directly.

An experiment performed at a specific $S_0$ will yield a linear ($S_0 - S_0$)/t versus 1/t plot with a slope equal to $K_I$ and an ordinate intercept of $V_{max}$. Lines drawn through the origin having slopes equal to $S_0$ values intersect with lines defined by the experimental data at a point corresponding to the initial velocity, $V_{max}$, with respect to the ordinate. A straight line drawn through this series of intersections ($V_{max}$) from a series of experiments run with different $S_0$ values at a given enzyme concentration, yields a line with a slope equal to $K_I$ and an ordinate intercept of $V_{max}$ and an abcissa intercept of $V_{max}/K_I$ (Fig. 1).

The kinetic constants can be derived in a unified procedure from primary experimental data that can be displayed in full on a single plot. The method described is minimalized since initial velocities need not to be evaluated separately and reactions can be studied in their final, intermediate, and initial stages. In situations where it is difficult to follow the initial stages, data from 10 to 80% hydrolysis can be utilized reliably to determine the initial velocities. Typically, assays were followed from 15 to 70% of completion.

For competitive inhibition, the line through the $V_{max}$ points has a slope of $-K_I$ (1/t) (16). For noncompetitive inhibition (both $K_I$ values identical), the ordinate intercept equals

\[ V_{max} = \frac{1}{1 + K_I} \]

RESULTS

For all $S_0$ values employed in the inhibitor studies, the experimental data fit the integrated rate equation well as evidenced by the linear ($S_0 - S_0$)/t versus 1/t (ln $S_0$) plots (Fig. 1).

The apparent activity of Co(III)(EDDA)(AA-CPA-Zn) toward Bz-GOP under standard assay conditions (0.0, 0.5 M NaCl, pH 7.5) is increased over a CPA-Zn control by 250% (as compared to a 450% increase for Ac-CPA-Zn). A plot of $S_0$ versus $V_{max}$ demonstrates the anomalous behavior of the native and modified enzymes toward the short ester (Fig. 2). The kinetic parameters for the different enzyme forms, under various conditions, are given in Table I. At low substrate concentrations (0.01 to 0.30 mM), the apparent $K_I$ of the Co(III)(EDDA) derivative is lower than either the native or arsenilazo enzyme. The $V_{max}$ is also lower than either CPA-Zn or AA-CPA-Zn. The $V_{max}$ of the acetylated enzyme is below that of the native enzyme in this range of substrate concentra-

a B. D. Warner, G. Boehme, M. S. Urdea, K. H. Poul, and J. I. Legg, manuscript submitted for publication.
crease. This appears to indicate a substrate activation at this
zymes change dramatically. In both cases, $K_m$ and $V_{\text{max}}$, in-
kineatic parameters for the acetylated and Co(III)EDDA en-
ordinate). The $V_{\text{max}}$, defined by experimental points with a slope equal to
higher substrate concentrations (1.0 to 10.0 mM), the activity at 0.06 mM substrate should be about 200 as
compared to the observed activity of 3200. The presence of unmodi-
cations used: A, 0.01 to 0.30 mM; B, 1.0 to 10.0 mM.

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>$K_m$</th>
<th>$V_{\text{max}}$</th>
<th>$K_m$</th>
<th>$V_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPA-Zn</td>
<td>0.047</td>
<td>35,000</td>
<td>0.033</td>
<td>3,200</td>
</tr>
<tr>
<td>AA-CPA-Zn</td>
<td>0.042</td>
<td>8,400</td>
<td>4.5</td>
<td>31,000</td>
</tr>
<tr>
<td>Co(III) (EDDA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(AA-CPA-Zn)</td>
<td>0.20</td>
<td>14,600</td>
<td>3.7</td>
<td>50,000</td>
</tr>
</tbody>
</table>

As has been reported previously, dipeptides (e.g. Bz-GP and
and structurally analogous esters (e.g. Bz-GOP) (15) show complex kinetic behavior characterized by multiple
substrate binding modes as evidenced by the nonlinearity of
behavior at higher concentrations.

Since the longer esters do not display the kinetic anom-
ies of the shorter esters, the kinetic parameters of the various
ezyme derivatives can be more confidently compared (Table II).
The $K_m$ values of all four enzyme forms (not including
$Ac(Co(III)(EDDA)(AA-CPA-Zn))$ are nearly the same for
Bz-GGGOP, but $V_{\text{max}}$ decreases dramatically in the order
CPA-Zn > AA-CPA-Zn > Co(III) (EDDA) (AA-CPA-Zn) >
Ac-CPA-Zn.

Co(III) (EDDA) (AA-CPA-Zn) was tested as a peptidase at
pH 6.5, 7.5, and 9.5 and with 0.1 and 1.0 mM solutions of Bz-GP,
Co(III)EDDA, CPA-Zn, and Acetyl (C) carboxypepti-
dases toward Bz-GOP. Conditions for assays are given under "Ma-
terials and Methods."

tration, while the apparent $K_m$ is increased about 4-fold. At
higher substrate concentrations (1.0 to 10.0 mM), the apparent
kinetic parameters for the acetylated and Co(III)EDDA en-
zymes change dramatically. In both cases, $K_m$ and $V_{\text{max}}$
crease. This appears to indicate a substrate activation at this
intermediate substrate concentration range. For both Ac-

The activity between 0.01 and 0.30 mM substrate is certainly
above that which can be accounted for by the apparent $K_m$ and $V_{\text{max}}$
at intermediate substrate concentrations. Presuming that the $V_{\text{max}}$ and $K_m$ at low Bz-GOP levels are equal to the values obtained at 1.0
to 10.0 mM, the activity at 0.06 mM substrate should be about 200 as
compared to the observed activity of 3200. The presence of unmodi-
fied CPA-Zn cannot account for this activity since there is no pepti-
dase activity. In any case, these kinetic parameters must be viewed
with some caution.
tive enzyme were employed as inhibitors of ester hydrolysis. For Ac-CPA-Zn, it has been shown that Cbz-GP and Bz-GP do indeed inhibit the hydrolysis of Bz-GOP, but the $K_i$ is considerably greater than the respective $K_m$ values in the native enzyme (20) (see Table III). Likewise, Bz-GP and Bz-GGP are effective inhibitors of Bz-GOP and Bz-GGGOP hydrolysis by the Co(III)EDDA derivative, but the $K_i$ values are less than the respective $K_m$ values for hydrolysis by the native enzyme (Table III). Considering the slow turnover of peptide substrates by CPA-Zn, $K_m$ values evidently reflect well the $K_d$ ($K_m = k_i/k_o$ = $K_d$ if $k_i$ is small). Since $K_i$ reflects the $K_m$ of peptides as inhibitors, it appears as though peptides bind somewhat more tightly to the Co(III)EDDA derivative than to the native enzyme.

Most interestingly, the mode of inhibition of the Co(III)EDDA enzyme differs depending upon which combination of peptides and esters is studied. Bz-GGP is a competitive inhibitor of Bz-GOP and Bz-GGGOP hydrolysis (Fig. 3, A and B), while Bz-GP is a noncompetitive inhibitor of Bz-GOP and Bz-GGGOP (Fig. 4, A and B, Table III).

When the Co(III)EDDA enzyme was incubated with 0.05 mM Bz-GP for 2 min prior to the addition of enough Bz-GOP to achieve a 0.134 mM solution, the same pH-stat tracing was obtained as when enzyme was added to a 0.50 mM Bz-GP, 0.134 mM Bz-GOP solution (the standard procedure). This indicates that products of peptide hydrolysis by the enzyme do not account for the inhibition of esterase activity. This also suggests that the observed mode of inhibition by the peptide is not representative of a pre-equilibrium association of the enzymes and inhibitor.

When the Co(III)EDDA enzyme is acetylated to yield Ac(Co(III)(EDDA)(AA-CPA-Zn)), Bz-GGP hydrolysis proceeds less effectively. The $V_{max}$ is decreased by 56% while the $K_m$ is increased by 560% compared to Co(III)(EDDA)(AA-CPA-Zn) (Table II). In contrast to the Co(III)EDDA enzyme, Bz-GGGOP hydrolysis by Ac(Co(III)(EDDA)(AA-CPA-Zn)) is competitively inhibited by Bz-GP with a $K_i$ greater than the $K_m$ for the native enzyme (Table III).

\( \beta \)-Phenylpropionate (\( \beta \)PP) is a competitive inhibitor of Bz-GOP and Bz-GGGOP hydrolysis by Co(III)(EDDA)(AA-CPA-Zn), showing a $K_i$ below that which is obtained from similar studies on CPA-Zn (Table III). Neither Co(II)(EDDA)(H$_2$O)$_2$ nor Co(III)(EDDA)(H$_2$O)$_2$ were found to be inhibitory of either Co(III)(EDDA)(AA-CPA-Zn) or AA-CPA-Zn. The form of inhibition with various inhibitors and substrates is summarized in Table IV.

**DISCUSSION**

It is difficult to argue that peptides and esters do not lie in different binding clefs of CPA-Zn prior to their respective rate-determining steps. It is remarkable that the change of 1 atom at the scissile bond of peptides versus otherwise structurally identical esters can result in the observed differences in kinetic behavior. Although the binding sites of peptides and esters are distinctly different, they apparently overlap as the size of peptides is extended to tripeptides, as will be discussed.

An overall binding picture of various substrates and inhibitors is presented in Fig. 5 (also refer to Table IV). Site AB is the site in which productive binding of short esters (e.g. Bz-GOP) occurs, while site D is the binding cleft for short peptides (e.g. Bz-GP). The nonoverlapping nature of sites AB and D is suggested by the observation that Bz-GP is a noncompetitive inhibitor of Bz-GOP hydrolysis in Co(III)(EDDA)(AA-CPA-Zn). This mode of inhibition was predicted since, in the native enzyme, \( \beta \)PP, indole 3-acetate, and phenylacetate competitively inhibit ester hydrolysis but noncompetitively inhibit peptide hydrolysis (3). Site A is the locus for \( \beta \)PP in its main inhibitory binding mode as implied by the competitive inhibition of Bz-GOP hydrolysis (site AB). Longer ester hydrolysis is also inhibited competitively by \( \beta \)PP in the native and Co(III)EDDA enzymes (site ABC). On the other hand, hydrolysis of the peptide, Bz-GGP, by CPA-Zn is noncompetitively inhibited by \( \beta \)PP (3). Although \( \beta \)PP has multiple binding modes (22, 23), the binding position of \( \beta \)PP with respect to Bz-GGGP inhibition is apparently the same as the binding position during noncompetitive inhibition of Bz-GGP, since the $K_i$ values are identical (3). The binding cleft for long esters, ABC, necessarily coincides with the \( \beta \)PP binding site, but sites D and DE for peptides do not overlap site A.

The extension of peptides by one amino acid from Bz-GP (site D) to Bz-GGP (site DE) alters the mode of inhibition of esters from noncompetitive to competitive. Although the competitive inhibition of short and long esters by Bz-GGP suggests an overlapping of ester and long peptide binding sites, the of substrates used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM. Range of substrate concentrations used was from 0.01 to 1.0 mM.
sites are not identical since, in contrast to esters, short and long peptide hydrolysis in the native enzyme is noncompetitively inhibited by \( \beta \)PP. Since, in the native enzyme, \( \beta \)PP noncompetitively inhibits the hydrolysis of both Bz-GP and Bz-GGP with roughly equal \( K_i \) values (18), both short and long peptides most likely occupy a common binding position. Although shown as two distinct sites, E and C could be coincidental.

With the Co(III)EDDA derivative, it has been possible to examine in detail peptide inhibition of esterase activity. The results are consistent with the binding model of Vallee et al. (20) in that different binding sites are observed for ester and peptide substrates and strongly suggest that these are overlapping sites. Since kinetic studies on Co(III)EDDA(AA-CPA-Zn) corroborate the inhibitor studies on the native enzyme, it is likely that the binding modes observed in the present study reflect those of the native enzyme. This is also substantiated by the fact that the peptides bind as well to the Co(III)EDDA derivative as to the native enzyme. The model does not preclude other potential binding modes for the substrates or inhibitors, but is based on the apparent modes observed under the conditions of the studies described.

The peptidase activity of the arsanilazotyrosine 248 enzyme can be fully returned by reducing the Co(III)EDDA derivative.
### Table IV: Comparison of inhibition modes

<table>
<thead>
<tr>
<th>Substrates</th>
<th>βPP</th>
<th>Inhibitors</th>
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</thead>
<tbody>
<tr>
<td>Short peptides (Bz-GP)</td>
<td>N₁</td>
<td>C₁</td>
</tr>
<tr>
<td>Short esters (Bz-GOP)</td>
<td>C₁,₂,₃</td>
<td>N₁,₂,₃; C₃ C₂</td>
</tr>
<tr>
<td>Long peptides (Bz-GGP)</td>
<td>N₁</td>
<td>C₁</td>
</tr>
<tr>
<td>Long esters (BzGGGP, BzGGGOMP)</td>
<td>C₁,₂</td>
<td>N₁,₂; C₄ C₂</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>Position modified</th>
<th>Kᵢ of native enzyme</th>
<th>Position of peptide binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native CPA-Zn</td>
<td>Tyr-248</td>
<td>Kᵢ = Kᵢ</td>
<td>Site I</td>
</tr>
<tr>
<td>Co(III)(EDDA) (AA-CPA-Zn)</td>
<td>Tyr-248</td>
<td>Kᵢ &gt; Kᵢ</td>
<td>Site I</td>
</tr>
<tr>
<td>Ac-CPA-Zn</td>
<td>Tyr-248 and Tyr-198&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Kᵢ &lt; Kᵢ</td>
<td>Site II</td>
</tr>
<tr>
<td>Ac (Co(III) (EDDA) (AA-CPA-Zn))</td>
<td>Tyr-248 and Tyr-198&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Kᵢ &lt; Kᵢ</td>
<td>Site II</td>
</tr>
</tbody>
</table>

<sup>a</sup> Refer to Fig. 5
<sup>b</sup> Presumed sites of modification. See “Discussion.”

### Table V: Effect of modifications on peptide binding

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>Position modified</th>
<th>Kᵢ of native enzyme</th>
<th>Position of peptide binding</th>
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</thead>
<tbody>
<tr>
<td>Native CPA-Zn</td>
<td>Tyr-248</td>
<td>Kᵢ = Kᵢ</td>
<td>Site I</td>
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<tr>
<td>Co(III)(EDDA) (AA-CPA-Zn)</td>
<td>Tyr-248</td>
<td>Kᵢ &gt; Kᵢ</td>
<td>Site I</td>
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<tr>
<td>Ac-CPA-Zn</td>
<td>Tyr-248 and Tyr-198&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Kᵢ &lt; Kᵢ</td>
<td>Site II</td>
</tr>
<tr>
<td>Ac (Co(III) (EDDA) (AA-CPA-Zn))</td>
<td>Tyr-248 and Tyr-198&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Kᵢ &lt; Kᵢ</td>
<td>Site II</td>
</tr>
</tbody>
</table>

The βPP protection against acetylation of these particular tyrosine residues is consistent with this assignment, since diazotization of Tyr-248 (24) and nitration of Tyr-198 (8) are both protected against by βPP.

It appears that it is modification of Tyr-198 in the acetylated enzyme that results in the change in the binding position of peptides. Tyr-198 is close enough to the active site to influence the binding of dipeptides (6) and, therefore, its modification could change the ability of peptides to bind. This suggestion is substantiated by the acetylation of Co(III)(EDDA)(AA-CPA-Zn) which results in a derivative whose esterase activity is also competitively inhibited by Bz-GP with a K₁ 4.5 times greater than the noncompetitive inhibition of the unacetylated Co(III)EDDA enzyme by Bz-GP (Table V).

It is possible that the loss of peptidase activity in the acetylated native enzyme could be the result of Tyr-198 modification. Nitration of Tyr-198 nearly eliminates the peptidase activity of the tetrazoylazotyro sine 248 enzyme even though Tyr-248 is free to participate in catalysis prior to nitration (as evidenced by the nearly full peptidase activity of the tetrazoylazoc derivative (8)). Acetylation of the tetrazoylazotyro sine 248 derivative also abolishes the peptidase activity (24).

The Co(III)(EDDA)(AA-CPA-Zn) enzyme, in contrast, is modified exclusively at Tyr-248. Peptides are not hydrolyzed despite the fact that the substrates bind in the same position and as tightly as in the native enzyme. Therefore, Tyr-248 does apparently play a role in peptide hydrolysis.

The necessity of Tyr-248 as a proton donor in peptidase activity was originally suggested by Reeke et al. (25). Consistent with this proposal, Co(III)(EDDA)(AA-CPA-Zn) has no detectable peptidase activity. This suggests that blocking of the phenolic oxygen by Co(III) coordination has eliminated the potential of Tyr-248 to participate in the proposed capacity.

Another role for Tyr-248 is possible that can be hindered by sterically interfering with the phenol's potential to interact with peptides. Mock (26) has proposed that peptide hydrolysis requires that a twisting of the C₆-N amide bond must take place in order to facilitate attack at the carbonyl. The twist could be brought about by interaction of the peptide with Tyr-248 (27) and presumably would be necessary for productive binding of peptides. The required interaction could be prevented by Co(III)EDDA incorporation resulting in loss of peptidase activity. Since esters rotate freely about the C₆-O bond, the ability to hydrolyze esters would not require the twisting and would not necessarily be affected by Tyr-248 modification.

Tyr-248 is certainly not required as a proton donor in ester cleavage, since, upon eliminating the phenolic hydroxyl as a proton source, the enzyme still acts as an esterase. At intermediate Bz-GOP concentrations, the Vₘₚ for the acetylated and Co(III)EDDA-modified enzymes actually increase signif-
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icantly. This is consistent with the similarity in the basic limits of the \( \frac{V_{\text{max}}}{K_m} \) versus pH profiles for the specific ester, O-(trans-p-chlorocinnamoyl)-L-\( \beta \)-phenyllactate in the native, arsanilazo-Tyr-248 (\( pK_a = 9.4 \)) and nitro-Tyr-248 (\( pK_a = 6.3 \)) enzymes, indicating that the loss of esterase activity above pH 8.5 is not due to deprotonation of Tyr-248 (7).

If Tyr-248 is truly a proton donor in peptidase activity, then the peptidase and esterase activities proceed by very different mechanisms (3, 29). Alternately, if the amide twisting role is operative, peptide and ester hydrolysis may actually proceed by very similar mechanisms (27, 29). This is consistent with the ostensibly identical mechanisms of peptide and ester hydrolysis in other zinc neutral proteases (30).

If esters and peptides are hydrolyzed by the same mechanisms, how can they be bound to different sites on the enzyme? Perhaps, as proposed by Cleland (97), the mechanisms of ester and peptide hydrolysis differ only in the respective rate-determining steps. If Site II (the ester binding site) is considered to be the site at which actual hydrolysis of both esters and peptides occurs, then the movement of peptides from Site I to Site II would be the rate-limiting process. This movement would presumably require Tyr-248 to twist and align the peptide productively in Site II. Esters could assume the proper productive binding mode without the assistance of Tyr-248, since no twisting would be required. In terms of this view, esters act as transition state analogs for peptide hydrolysis. Consistent with this suggestion, esters bind about 10 times more tightly to the enzyme than do peptides (see Tables II, III, and IV). As is evident from the peptide inhibition studies on the acetylated native and acetylated Co(III)EDDA enzymes, peptides apparently can bind in the ester binding site (Table V).

In summary, we have specifically blocked the phenolic oxygen of Tyr-248 of carboxypeptidase A by coordinating it to substitution-inert Co(III). Like the acetylated enzyme, peptidase activity is lost and the enzyme maintains its esterase activity. As opposed to the acetylated enzyme, peptides bind to the Co(III)(EDDA)(AA-CPA-Zn) enzyme as they do in the native protein. Although Tyr-248 is apparently required for peptidase activity as evidenced by this study, the role may be either to donate a proton to the scissile bond or to twist the amide bond permitting facile nucleophilic attack at the Co position.

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REFERENCES

17. Davies, R. C., Riordan, J. F., Auld, D. S., and Vallee, B. L. (1968) Biochemistry 7, 1090-1099

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