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Chemistry

Unified picture of mechanisms of catalysis by carboxypeptidase A

(enzyme/oxygen-18 exchange/nucleophilic versus general base mechanisms)

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ABSTRACT **We have reported evidence that an anhydride intermediate is not involved in the hydrolysis of typical peptide substrates by carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.12.2), and we describe further evidence here. Recently an anhydride intermediate has been detected in the hydrolysis of an ester substrate by this enzyme. Other evidence also suggests that esters and peptides may not be cleaved by the same type of mechanism. A possible explanation is that the substrate carbonyl and a water molecule are always aligned between glutamate-270 and the zinc atom of the enzyme, but not always in the same sequence. With peptides the carbonyl is coordinated to zinc, and the water is delivered by glutamate acting as a general base. Esters are weaker ligands, and in some cases the ester carbonyl may not displace water from zinc. This would lead to a nucleophilic mechanism, with glutamate-270 forming an anhydride while zinc-aquo serves as a Brönsted acid. This picture is consistent with other evidence on ester cleavage, and resolves the otherwise baffling discrepant data on peptide as compared to ester substrates.**

Pancreatic carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.12.2) is one of the most extensively studied enzymes, but its mechanism is not yet clear (1). The x-ray structure work of Lipscomb and coworkers (2, 3) shows that the active site contains Zn^{2+} coordinated to three enzymatic groups, bearing a water molecule as the fourth ligand. The γ-carboxylate of glutamate-270 is located some distance away from the Zn^{2+}.

When a pseudosubstrate (glycyltyrosine, Gly-Tyr) is bound, the tyrosine carboxylate is coordinated to the guanidinium group of arginine-145; the phenyl ring is in a hydrophobic pocket, and the amide carbonyl oxygen of substrate is now bound to zinc. Binding of this peptide pseudosubstrate thus causes displacement of the water from Zn^{2+}; it also causes a conformational change that brings tyrosine-248 into the pocket to hydrogen-bond to the substrate.

The x-ray structure work has only been possible on a peptide pseudosubstrate that is not cleaved by the enzyme. This of course raises some question about the relevance of this structure to the binding of typical reactive substrates. The enzyme can also cleave esters of α-hydroxy acids, such as α-phenyllactic acid; no x-ray work on carboxypeptidase A with a bound ester substrate or pseudosubstrate has been done.

Extensive studies of this enzyme have emphasized the comparison of ester and peptide substrates (4, 5), particularly with modified enzyme. One modification is the substitution of other metal ions for Zn^{2+} (6); the effects on catalysis are *not* parallel for ester and peptide substrates. A particularly striking example is the report (7) that replacement of Zn^{2+} by Co^{3+}, which is inert to substitution in its first coordination sphere, leads to an enzyme that has lost all peptidase activity but still retains esterase activity. Other studies (8) also show that the phenolic hydroxyl of tyrosine-248 may be needed for peptide substrates but is not needed for the hydrolysis of all esters.

Two obvious classes of mechanisms can be suggested based on the x-ray and enzyme modification work. In one, the glutamate-270 carboxylate acts as a nucleophile at the scissile carbonyl of substrate, while Zn^{2+} and tyrosine play other catalytic roles. This would lead to an anhydride acyl-enzyme intermediate, with the acyl group temporarily attached to glutamate-270. In the other type of mechanism, the glutamate-270 carboxylate acts as a general base, delivering a water molecule to the scissile carbonyl. By this mechanism there is no acyl-enzyme intermediate. Alternate schemes have also been suggested (3,9).

We had reported evidence (10), from oxygen-18 exchange studies, that an acyl-enzyme intermediate is *not* involved in the hydrolysis of typical peptide substrates by carboxypeptidase A. On the other hand, Makinen *et al.* have recently shown that an anhydride intermediate is formed during the hydrolysis of a particular ester substrate, *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate (11). We now wish to describe further evidence against an anhydride intermediate in peptide hydrolysis. We will also propose a simple picture of the mechanisms used by this enzyme that accommodates all the disparate evidence so far available for both peptide and ester substrates.

EXPERIMENTAL

Bovine pancreatic carboxypeptidase A was purchased as an aqueous suspension with toluene preservative from Worthington Biochemical Corp. (lot no: 3AA, 34 H 808, and 34 M 667). It was centrifuged, washed with water, and dissolved in 10 mM *N*-ethylmorpholine/acetic acid buffer at pH 7.5, which was 2.0 M in LiCl, then diluted with an equal volume of 170 mM buffer and centrifuged. Activity was assayed with benzoylglycylphenylalanine (12). Oxygen-18-enriched substrates were prepared by hydrolysis of the corresponding methyl esters with $H_2{}^{18}O$ and sodium methoxide (*N*-acetyl-L-phenylalanine 25% ^{18}O in one carboxyl oxygen, *N*-acetylglycine 16% enriched, and *N*-benzoylglycine 75% enriched) or by hydrolysis of the acid chloride in $H_2{}^{18}O$ (*p*-chloro-*trans*-cinnamic acid 6.8% unlabeled, 92.7% singly labeled, 0.46% doubly labeled).

Oxygen-18 exchange studies were performed in pH 7.50 170 mM *N*-ethylmorpholine/acetic acid buffer ($\mu = 0.1$). Identical samples containing substrate, enzyme, and cosubstrate, if any, were made up. One was immediately freeze-dried while the other was incubated for the desired time, assayed for residual enzyme activity, then freeze-dried (the buffer is volatile). Isotopic abundance was determined with a chemical ionization mass spectrometer under a multiple ion detection program averaged over 50 scans. In the case of acetylglycine, the products were esterified with ethereal diazomethane prior to analysis by gas chromatography–chemical ionization mass spectrometry to avoid interfering peaks from other reaction components.

Unlabeled substrates were prepared in standard ways, and the novel ones were fully characterized: *N*-acetyl-L-phenylalanine methyl ester, mp 86–88°; *p*-chloro-*trans*-cinnamoyl-L-phenylalanine, mp 211–212°. *O*-[^{3}H]Acetyl-L-mandelic acid was prepared as described for the untritiated compound (13)

Table 1. Carboxypeptidase-A-catalyzed oxygen-18 exchange from carboxy-labeled substrates

Substrate (mM)	Added component (mM)	V/E (min^{-1})
N-Benzoylglycine (7.6)	None	<0.01
N-Benzoylglycine (10)	L-Phenylalanine (10)	8
N-Benzoylglycine (7.6)	L-β-Phenyllactic acid (10)	≤0.02
N-Benzoylglycine (10)	3-*p*-Hydroxyphenylpropionic acid (10)	<0.2
N-Benzoylglycine (8.3)	Leucine (10)	0.6
N-Benzoylglycine (7.6)	Proline (10)	<0.01
N-Benzoylglycine (7.6)	Glycine (10)	<0.02
N-Benzoylglycine (7.6)	Alanine (10)	<0.06
N-Benzoylglycine (7.6)	Threonine (10)	<0.09
N-Benzoylglycine (8.4)	*N*-Methylleucine (8)	≤0.02
N-Acetylglycine (10)	None	<0.008
	Phenylalanine (10)	0.6
	L-β-Phenyllactic acid (10)	<0.004
	Leucine (10)	0.02
N-Acetylphenylalanine (11)	None	2
	Phenylalanine (10)	4
	L-β-Phenyllactic acid (10)	<0.1
p-Chlorocinnamic acid (10)	None, Phe, Leu, L-β-phenyllactic acid (10)	<0.001

but with tritiated acetic anhydride; its specific activity was 2.73×10^{10} dpm/mol. It was incubated at 1.00 mM with 0.607 μM carboxypeptidase A in 5.00 M aqueous methanol at pH 7.5, 25.0°, for 13.2 min (0.2 M Tris·HCl, $\mu = 0.5$, NaCl). Unlabeled methyl acetate was added, the solution was frozen, and the solvent was collected by lyophilization. Methyl acetate was isolated by toluene extraction, then distillation.

The reactivity of *N*-benzoylglycine methyl ester toward phenylalanine, as catalyzed by carboxypeptidase A, was evaluated both directly and by competition with oxygen-18 exchange from labeled *N*-benzoylglycine. The equilibrium constant for hydrolysis of this ester was determined in acid by direct equilibration in 0.5 M HCl in 56% aqueous methanol by nuclear magnetic resonance integration. The result, K (hydrolysis) in acid, was 19 M (H_2O assigned unit activity) from either direction, and was corrected to the operating pH using the potentiometric pK_a of *N*-benzoylglycine.

RESULTS

In contrast to the report of Ginodman *et al.* (14), we find that carboxypeptidase does not catalyze oxygen-18 exchange from acetylglycine unless an amino acid such as phenylalanine is added (Table 1). Similarly, *N*-benzoylglycine undergoes such exchange only in the presence of an added amino acid. Acetylphenylalanine does undergo some exchange without added phenylalanine, but it is being cleaved by the enzyme to produce its own phenylalanine part way through the reaction. The data in Table 1 show that not all amino acids are equally effective as cocatalysts of this exchange, and that β-phenyllactic acid or 3-*p*-hydroxyphenylpropionic acid is not effective.

Any hydrolytic enzyme must be able to catalyze not only the hydrolysis of a substrate, but also its resynthesis, and the two processes are connected by the normal relationship between the equilibrium constant and the ratio of rate constants. If we assume that the cocatalyst is present to permit enzyme-catalyzed resynthesis of a peptide bond in the overall exchange process, we can calculate the expected rate of such an exchange in benzoylglycine in the presence of phenylalanine and/or β-phenyllactic acid. The relevant expression, Eq. **1**, has terms for the binding and inhibition constants of various components (K_M is for *N*-benzoylglycylphenylalanine, K_M' for *N*-benzoylglycyl-β-phenyllactate) and the rate and equilibrium constants (15) for hydrolysis of the peptide and ester.

$$V/E = \frac{k_{cat}[BzGly][Phe]/K_{eq}}{K_M + \frac{[BzGly][Phe]}{K_{eq}} + \frac{K_M[BzGly]}{K_{BzGly}} + \frac{K_M[Phe]}{K_{Phe}} + \frac{K_M[HOPhe]}{K_{HOPhe}}} + \frac{k'_{cat}[BzGly][HOPhe]/K_{eq}'}{K_M' + \frac{[BzGly][HOPhe]}{K_{eq}'} + \frac{K_M'[BzGly]}{K_{BzGly}} + \frac{K_M'[Phe]}{K_{Phe}} + \frac{K_M'[HOPhe]}{K_{HOPhe}}} \quad [1]$$

The results of the calculation are listed in Table 2, along with the observed results. The agreement is very good; in particular, the observed rate of oxygen-18 exchange does not greatly exceed that which must occur by peptide synthesis.

There is no detectable enzyme-catalyzed methanolysis of

Table 2. Comparison of observed rates of oxygen-18 exchange with those calculated by Eq. 1, assuming enzyme-catalyzed synthesis of peptide or ester substrates

Substrate (mM)	Cocatalyst (mM)	$(V/E)_{calc}$ (min^{-1})	$(V/E)_{observed}$ (min^{-1})
N-Benzoylglycine (10)	Phenylalanine (10)	5.8	8
N-Benzoylglycine (10)	Phenylalanine (1)	1.4	2
N-Benzoylglycine (4.1)	Phenylalanine (1)	0.57	0.8
N-Benzoylglycine (7.6)	β-Phenyllactic acid (10)	0.0021	≤0.02
N-Benzoylglycine (7.1)	β-Phenyllactic acid (10) + phenylalanine (5)	0.035	0.03
N-Acetylglycine (10)	β-Phenyllactic acid (10)	0.00071	<0.004
p-Chlorocinnamic acid (10)	Phenylalanine (10)	0.0032	<0.001
	β-Phenyllactic acid (10)	0.00036	≤0.001

Table 3. Competition of methanol with water in enzymatic hydrolysis reactions

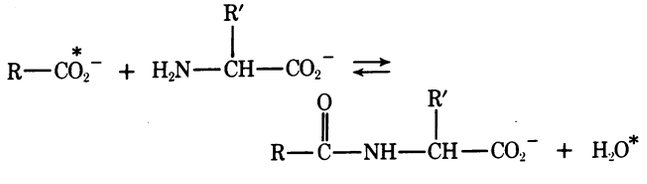
Reaction	k_{MeOH}/k_{H_2O}
Bz-Gly-Phe cleavage by carboxypeptidase A	<0.0003
O-Acetyl-L-mandelate cleavage by carboxypeptidase A	<0.06
α-Chymotrypsin-catalyzed cleavage reactions	39–584*
Trypsin-catalyzed cleavage	82†

* Ref. 16.
† Ref. 17.

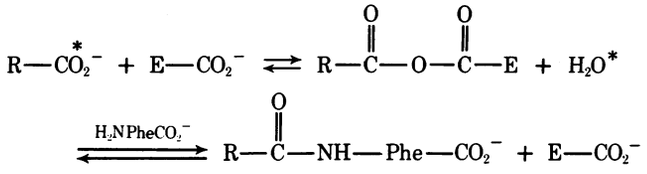
O-acetylmandelic acid. Similarly, there is no detectable enzyme-catalyzed hydrolysis of *N*-benzoylglycine methyl ester in the presence of phenylalanine. This latter result, that the hypothetical methanolysis of *N*-benzoylglycylphenylalanine cannot be reversed by the enzyme, can be combined with the equilibrium constant for such a process to yield an upper limit to the rate of the forward reaction. This is listed in Table 3, along with some comparison values (16, 17). We also find no detectable inactivation of the enzyme or trapping in the products if the enzymatic hydrolysis of *N*-benzoylglycylphenylalanine is performed in the presence of hydroxylamine or sodium borohydride. The enzyme does not hydrolyze *N*-benzoylglycylphenylalanine amide in the presence of phenylalanine. All these results show that in the hydrolysis direction water cannot be replaced by methanol, hydroxylamine, ammonia, or borohydride anion.

DISCUSSION

Our results show that carboxypeptidase A can catalyze oxygen-18 exchange from a substrate such as *N*-benzoylglycine, but only in the presence of a cocatalyst such as phenylalanine. Of course, such a reaction must be observed. Any enzyme must be able to catalyze not only hydrolysis, but also resynthesis of a substrate such as *N*-benzoylglycylphenylalanine. Synthesis followed by hydrolysis is a mechanism for oxygen exchange, and Table 2 shows that the expected rate of such a mechanism agrees well with our observed rate. If the enzyme uses a direct hydrolysis mechanism, without any acyl-enzyme intermediate, such a sequence is indeed the only likely mechanism for exchange.



If, however, the enzyme uses an acyl-enzyme pathway, such as the formation of an intermediate anhydride, then another exchange process is in principle possible. Here the oxygen exchange could occur in the absence of a second component such as phenylalanine, since this component is formally involved only in the second step of the resynthesis sequence (first step of the hydrolysis sequence). Our results exclude the simplest version of such a mechanism. Anhydride formation in the absence of phenylalanine would lead to oxygen exchange in the substrate benzoylglycine [it is known (18) not to exchange into the enzyme], and this is not observed.



More complex possibilities must be considered. One possibility is that there is an anhydride intermediate, but its formation and hydrolysis are catalyzed by the amino acid such as phenylalanine. A conformational or catalytic effect of bound phenylalanine is certainly possible, but our results make this very unlikely.

There is an excellent parallel between the rate of oxygen exchange and the rate expected if the second component is directly involved in peptide resynthesis, not simply catalytic. Thus, amino acids such as glycine, alanine, proline, or threonine are not effective cocatalysts. Benzoylglycylalanine, for instance, should be synthesized slowly because it is hydrolyzed slowly by carboxypeptidase. The amino acids that are ineffective cocatalysts are all known to be poor COOH-terminal residues in the hydrolytic direction. The most striking case is the contrast between leucine, a good COOH-terminal residue and *N*-methylleucine, a poor one.

A hydroxyacid, L-β-phenyllactic acid, is not effective. Here the hydrolysis rate constant is good (for hydrolysis of *O*-acyl esters of this acid), but the equilibrium constant for ester hydrolysis is large so the rate of resynthesis of such an ester must be small. If the second component were serving the function only of catalyzing the formation and hydrolysis of the anhydride, it would be a remarkable coincidence that this would run parallel to its expected ability to condense with the substrate, which, with hydroxyacids, leads to a weaker bond.

p-Chlorocinnamoyl-L-phenylalanine is a poor peptide substrate for carboxypeptidase A; we find K_m 0.57 mM and k_{cat} 1.3 min^{-1}. However, an ester of this acid is a good substrate, and Makinen *et al.* have shown (11) that this ester forms an anhydride intermediate with the enzyme. We see in Table 1 that phenylalanine does not catalyze oxygen exchange with this acid. This is expected if the exchange mechanism is direct peptide formation, but it is not clear otherwise why the putative catalysis of anhydride formation is not seen.

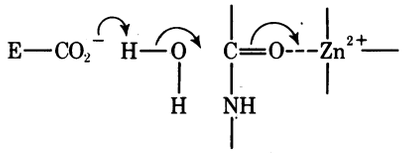
A further argument against the idea that the second component simply catalyzes anhydride formation is seen in Table 2. The observed rate of exchange is close to that expected from the resynthesis of peptide, which *must* occur. If there is a prior anhydride in the synthesis direction that can hydrolyze rapidly compared with acylation to form peptide, the exchange rate should have been higher than calculated ignoring such a possibility.

Thus, our data are really not consistent either with simple or with amino-acid-catalyzed anhydride formation. One final possibility remains by which an acyl-enzyme intermediate might be allowed. The overall exchange reaction might not occur simply on formation and hydrolysis of the anhydride because there may be a binding site for water on the enzyme. If so, the water formed when the substrate carboxylic acid condenses with the enzyme carboxyl group in the synthesis direction would be used again in the hydrolysis, and no exchange would be observed. By this hypothesis, reaction of the anhydride with bound amino acid to form a peptide would be required so as to form a substrate stable enough that water could exchange before hydrolysis.

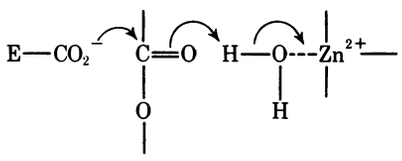
This formal possibility cannot be excluded, but it raises other problems. For instance, the hypothetical water binding site could be the Zn^{2+}, so that anhydride hydrolysis involved attack by such a bound water as in one of our model systems (19). In the synthetic direction this water could then be displaced only after formation of the peptide, and in the hydrolytic direction the observed structure of the glycyltyrosine complex with the enzyme (1, 3) would thus have to be irrelevant.

Although we cannot yet close the bound-water loophole, we strongly favor the simpler interpretation: peptides are hydrolyzed directly, not through an acyl-enzyme anhydride intermediate. If this is true, it contrasts with the finding by Makinen *et al.* (11) that a particular ester, *p*-chlorocinnamoyl-β-phenyllactate, hydrolyzes via an anhydride mechanism. This need in principle pose no problem, in view of all the evidence suggesting differences in mechanism for ester and peptide substrates. However, we wish to suggest a specific interpretation.

(*i*) Peptide substrates bind so as to displace water from Zn^{2+}. Then a water molecule is delivered by the glutamate carboxylate.



(*ii*) Ester substrates *may* bind without displacing the water from Zn^{2+}. This then puts them in position for a nucleophilic attack by the glutamate.

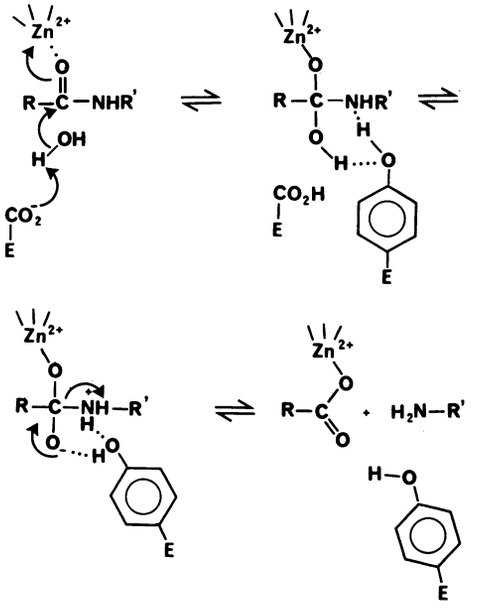


This simple picture, in which the same groups are arranged between the carboxylate and the zinc but in reverse order, is consistent with much other information. The ester group is a weaker ligand than is the amide, so it might well not displace water. This proposal explains the otherwise remarkable finding that the Co^{3+}-enzyme is an esterase, but not a peptidase. The water of Co^{3+} cannot be displaced, but Co^{3+}-H_2O could act as a proton acid with ester substrates as shown. Peptides could not use the second mechanism because conversion of a peptide to an anhydride is too endothermic. The peptide would also no longer be in position for catalysis by tyrosine-248. Ester conversion to anhydride is less endothermic, and the tyrosine interaction is not required.

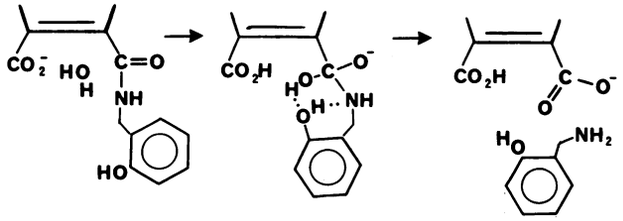
If an anhydride mechanism is allowed for some esters, one might wonder why we observed oxygen exchange only in the presence of amino acids. Direct synthesis of a peptide, without anhydride formation, is simply a faster process. This is the meaning of the statement that it is the preferred mechanism; anhydride formation is too slow to be detected by exchange.

The great preference of carboxypeptidase A for H_2O over any other lytic agent has been noted by us previously (10). For ester hydrolysis the interpretation of the hydrolysis/methanolysis ratio depends on whether *all* esters use the anhydride mechanism. For instance, Kaiser's ester (11) is special stereochemically, having a trigonal carbon next to the scissile carbonyl group. This feature could contribute to the binding geometry that promotes the anhydride mechanism.

For peptides, where we favor the direct hydrolysis mechanism, the large preference for H_2O over any other lytic species can be interpreted by a specific detailed sequence (see ref. 10). After delivery of hydroxyl to the carbonyl there must be an additional proton transfer to permit cleavage, so both protons of H_2O are required. With methanol the initial addition, if it occurred, would simply reverse.



The requirement for such a proton transfer in amide cleavage is well known (20), and indeed we found evidence for it in a model system (21) in which a carboxylate and a phenol cooperated in cleaving an amide. Strikingly, in that model system the amide cleavage at neutrality went by the general base, not nucleophilic, mechanism, while a related ester in this model system used the nucleophilic anhydride mechanism.



Our peptide hydrolysis mechanism for carboxypeptidase A leads to the formation of a carboxylate ion product bound to Zn^{2+}. This is consistent with other evidence (4) that substrates can bind to the Zn^{2+}, not just to arginine-145. Our interpretation is that such substrate binding is needed in the *synthesis* direction. It might be generally noted that an exopeptidase needs two alternate binding sites for the substrate carboxylate, so that binding can occur in both catalytic directions.

The role we suggest for tyrosine-248 in proton transfer would not be used in ester hydrolysis. Further, peptide cleavage would be blocked if the catalytic phenol were acetylated, as is known (8). However, simple titration of the phenol would only slow an otherwise rapid step in the sequence. This need not affect the overall rate until the proton transfer step became rate-determining, many pH units away from the phenolic pK_a.

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