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On the Mechanism of Peptide Cleavage by Carboxypeptidase A and Related Enzymes

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The evidence so far available indicates that carboxypeptidase A uses the general base mechanism for peptide hydrolysis. Based on model studies, the Glu-270 carboxylate probably performs three different proton transfers.

Carboxypeptidase A is a member of a class of zinc-containing enzymes of considerable importance. They are much more effective than are serine peptidases such as α-chymotrypsin, and some play a key role in the production of physiologically active peptide hormones. Since the design of inhibitors for these zinc peptidases is an area of current pharmaceutical research, the enzyme mechanisms have practical as well as scientific interest.

The principal choice, for carboxypeptidase A, has involved selection between the nucleophilic "anhydride" mechanism and the direct general base mechanism.[1] In the nucleophilic mechanism, the carboxyl of Glu-270 attacks the peptide carbonyl group to form a tetrahedral intermediate stabilized by Zn^{2+}, and this then decomposes with protonation of the leaving group nitrogen. In a second step the anhydride is cleaved, possibly using the zinc-catalyzed mechanism we have demonstrated[1] in a model system.

There seems to be no solid evidence in favor of this mechanism with peptide substrates. Recently some evidence has been advanced[2] that certain esters may form anhydride intermediates during carboxypeptidase hydrolysis, and this may well be the case. However, there is strong evidence that esters and peptides do not use the same catalytic mechanism.[3] We have pointed out a simple geometric relationship that could rationalize the use of an anhydride mechanism for esters but not for peptides.[4] We also showed that ester substrates and peptide substrates with common acyl groups do not have the same O-18 isotope effect in H_2O-18/H_2O-16 hydrolysis.[1] If they both had formed the (common) anhydride, one might have expected the same isotope effect in the hydrolysis step.

Proposed anhydride mechanisms for peptide cleavage have used Tyr-248 as the acid catalytic group to protonate nitrogen (Scheme 1). However, recently it has been shown by genetic engineering experiments that Tyr-248 can be replaced by phenylalanine, with no loss in enzyme activity toward peptides or esters.[5] This makes the anhydride mechanism impossible for peptides. Unless some other acid

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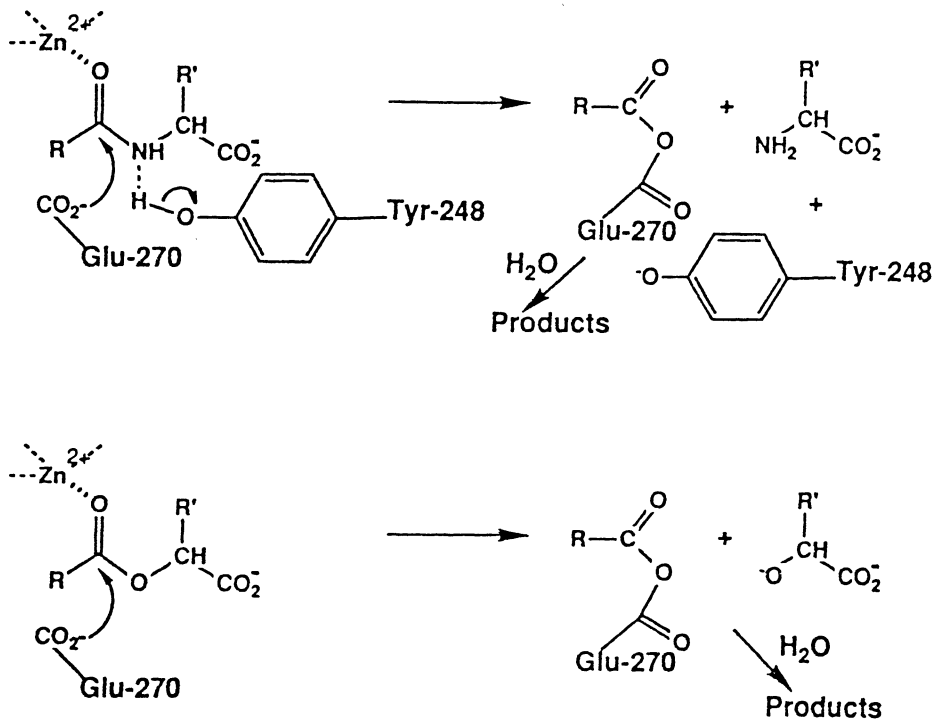
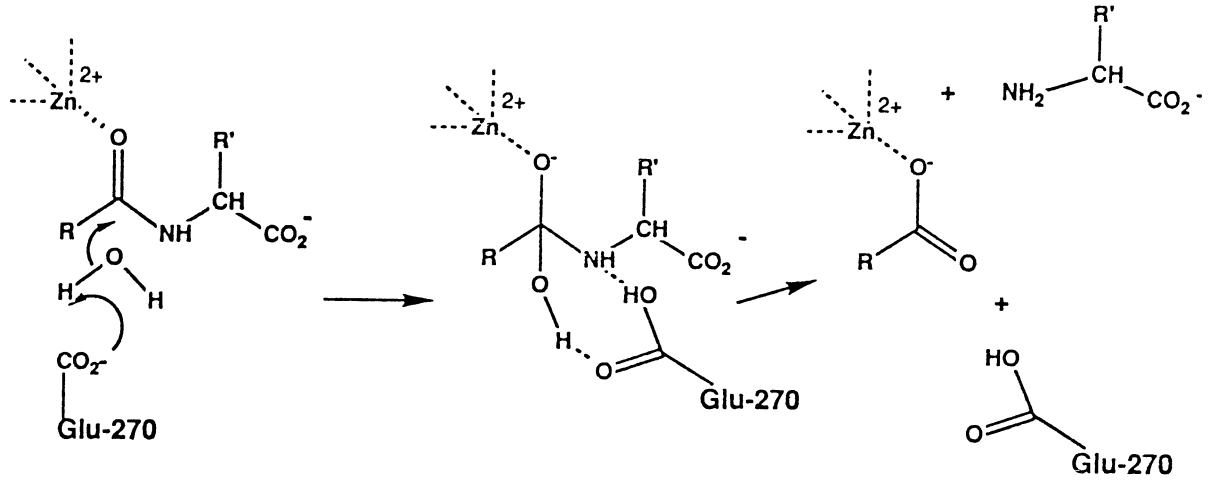
Dedicated to Professor Teruaki Mukaiyama on the occasion of his 60th birthday.



Scheme 1. The anhydride mechanism for peptides and for esters.

catalytic group is found a mechanism can be excluded that has no source of acidic proton for nitrogen. However reaction of an ester with a carboxylate forms an anhydride by loss of an alkoxide group; no acid catalytic group is needed. Thus if the phenolic OH plays no catalytic role we can see even more clearly that ester substrates might use the anhydride mechanism while peptides do not.

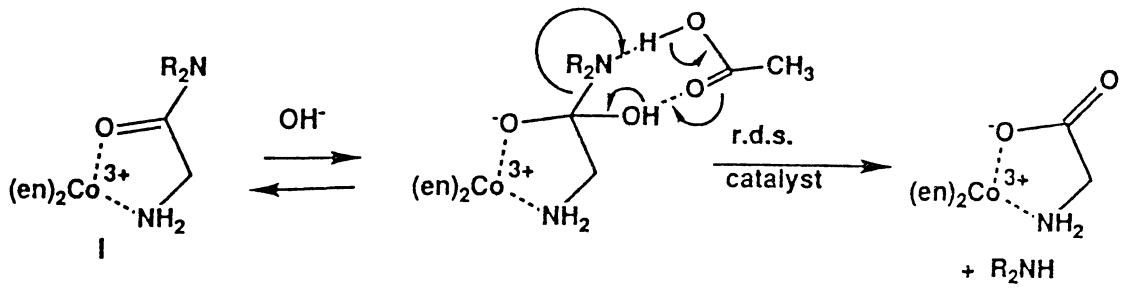
The general base mechanism has no such problem. As we originally formulated it[4] the Tyr-248 OH was used to protonate nitrogen, but there is another attractive possibility. When the Glu-270 carboxylate delivers H_2O to the carbonyl group the Glu picks up a proton. The resulting Glu-CO_2H can act as an acid to protonate the nitrogen, as Scheme 2 shows. This was first pointed out by Matthews,[6] who also concluded that in thermolysin, a related enzyme, such a protonation is geometrically possible. It is mechanistically related to the role of His-57 in chymotrypsin.[7] That enzyme also has no acid group at the start. His-57 acts as a general base to promote attack of Ser-195. Then the resulting protonated His transfers its new proton to the substrate nitrogen, to permit its departure. The general base mechanism generates its acid group part way through the reaction; if no other acid group is present, it must be the operative mechanism for peptide cleavage.



Scheme 2. Our preferred mechanism for peptide hydrolysis.

Recent studies on a model system[8] indicate that the Glu-CO_2^- may have an additional catalytic role. We prepared the Co(III) complex (I), to study the cleavage of a fixed model [Co (III) is substitution inert] for the carboxypeptidase-substrate complex. We found that the hydrolysis involved formation of the tetrahedral intermediate by reversible addition of OH^-, and that the further reaction of this was catalyzed by acetic acid. One might think that the AcOH simply protonates the nitrogen, but this is not the case. Simple proton donors such as ImH^+ or $PhNH_3^+$ are <u>not</u> catalysts, while the bifunctional AcOH and $H_2PO_4^-$ are; $H_2PO_4^-$ is the best catalyst we have found.

In the model system this means that a bifunctional mechanism operates: AcOH or $H_2PO_4^-$ can do a double proton transfer, forming the product carboxylate ion directly (Scheme 3). We propose that Glu-270 does this in the enzyme, as shown in



Scheme 3. Hydrolysis of the model by OH^- and acetic acid.

Scheme 2. We have previously pointed out[1,4] the desirability of such a double proton transfer in the enzymatic hydrolysis of peptides, but used the now-discredited Tyr-248 OH to do it. Our new proposal also makes it clear why the zinc enzymes use a carboxylate as general base, while the serine peptidases use imidazole. In the serine peptidases such a double proton transfer to form a carboxylate is not needed (no metal coordination) or even reasonable (the H_2O attacks an intermediate ester, not a peptide). Thus imidazole is a sufficient catalyst. In carboxypeptidase A and related enzymes the carboxylate is needed [raised to a pK_a near 6 in the enzyme, and thus resembling $H_2PO_4^-$] to play this special multifunctional role: base in the first step, double proton transfer catalyst in the second.

In summary, the data so far available on carboxypeptidase A suggest that it uses the general base mechanism (Scheme 2) for peptide cleavage but may well use the nucleophilic mechanism (Scheme 1) for some ester substrates. In peptide cleavage, the Glu-CO_2^- catalyzes three different proton transfers. Our model studies show that the mechanism of Scheme 2 can indeed operate as suggested.

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