2.11: Peptide Hydrolysis

At neutral pH the uncatalyzed hydrolysis of amides or peptides

$$R-CO-NH-R'+H_2O \rightleftharpoons R-COO^- + R'-NH_3^+ \quad (2.18)$$

is a slow process, with rate constants as low as $10^{-11}s^{-1}$. Peptide hydrolysis catalyzed by carboxypeptidase or thermolysin can attain k_{cat} values of $10^4\ s^{-1}$. Organic chemistry teaches us that amide hydrolysis is relatively efficiently catalyzed by acids and bases. The general mechanisms involve protonation of the carbonyl oxygen (or amide nitrogen), and addition of OH^- (or of a general nucleophile) to the carbonyl carbon atom. Several organic and inorganic bases have been found to be reasonably efficient catalysts. On the other hand, transition metal aqua-ions or small metal-ion complexes also display catalytic efficiency (Table 2.8).[107-114] A metal ion is a Lewis acid, capable of effectively polarizing the carbonyl bond by metal-oxygen coordination. Furthermore, the metal ion can coordinate a hydroxide group in such a way that there is a high OH^- concentration at neutral or slightly alkaline pH. It is thus conceivable that a metalloenzyme may combine some or all of these features and provide a very efficient catalyst.

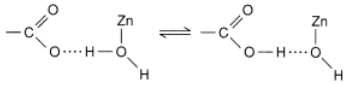
Table 2.8 - Rate constants for amide and ester hydrolysis catalyzed by acids, bases, or metal ions. * autohydrolysis

Compound	Catalyst and Conditions	Rate Constant	Reference
Glycine amide	pH 9.35 Cu^{2+}, pH 9.35	$1.9 \times 10^{-5}\ s^{-1}$ $2.6 \times 10^{-3}\ M^{-1}s^{-1}$	107 107
$[Co(en)_2(\text{glycine amide})]^{3+}$	pH 9.0[a]	$2.6 \times 10^{-4}\ s^{-1}$	108
D,L-phenylalanine ethylester	pH 7.3 Cu^{2+}, pH 7.3	$5.8 \times 10^{-9}\ s^{-1}$ $3.4 \times 10^{-2}\ M^{-1}s^{-1}$	109 109
$(tn)_2O_3PO—C_6H_4NO_2$	OH^-	$5.1\ M^{-1}s^{-1}$	110
$Co^{III}—(tn)_2O_3PO—C_6H_4NO_2$	—[a]	$7 \times 10^{-5}\ s^{-1}$	110
Ethyl-β-phenylpropionate	H^+ OH^-	$5 \times 10^{-3}\ M^{-1}\ s^{--1}$ $1.3 \times 10^{-6}\ M^{-1}s^{-1}$	111 111
Adenosine triphosphate	pH 5.3 Cu^{2+}, pH 5.3	$5.6 \times 10^{-6}\ s^{-1}$ $1.1 \times 10^{-2}\ M^{-1}s^{-1}$	112 112
Glycine methylester	Co^{2+}(1:1), pH 7.9 Cu^{2+}(1:1), pH 7.3	$1.6 \times 10^{-2}\ s^{-1}$ $4.2 \times 10^{-2}\ s^{-1}$	113 113
Glycine propylester	Co^{3+}(1:1), pH 0 Co^{3+}(1:1), pH 8.5	$1.1 \times 10^{-3}\ s^{-1}$ $>1 \times 10^{-2}\ s^{-1}$	114 114

Much experimental work has been done on mimicking ester and especially peptide hydrolysis with model coordination compounds. Most of the work carried out has involved[108,110,115,116] cobalt(III), Although such an ion may not be the best conceivable model for zinc-promoted hydrolytic reactions (see Section IV.G), it has the great advantage of being substitutionally inert, thus removing mechanistic ambiguities due to equilibration among isomeric structures in the course of the reaction, Interesting amide hydrolysis reactions also have been described using complexes with other metal ions, such as copper(Il)[117] and zinc(Il)[118] itself. In recent years efforts have focused on the construction of bifunctional catalysts to better mimic or test the enzymatic function. For instance, phenolic and carboxylic groups can be placed within reach of Co(III)-chelated amides in peptidase models.[116] The presence of the phenolic group clearly accelerates amide hydrolysis, but carboxyl groups are ineffective. This model chemistry is too simple to provide insights into the actual enzymatic mechanism, which must start with recognizing the substrate through several steps, orienting it, activating it, performing the reaction, and finally releasing the products. See the more specialized reviews dealing with nonenzymatic reactivity.[119-121]

From basic knowledge of the chemistry of hydrolytic reactions, the x-ray structures of carboxypeptidase A and a variety of its derivatives with inhibitors as substrate analogues, product analogues, and transition-state analogues have revealed several features of the active site that are potentially relevant for the catalytic mechanism (Figures 2.26-2.28 See color plate section, pages C4, C5.).[122] The metal ion is coordinated to two histidine residues (His-69 and His-196), to a glutamate residue that acts as a bidentate ligand (Glu-72), and to a water molecule. The metal is thus solvent-accessible and, as such, can activate the deprotonation of a

water molecule to form a hydroxide ion, or polarize the carbonyl oxygen of the substrate by coordinating it in the place of the solvent molecule, or both, if some flexibility of the coordination sphere is allowed. Another glutamic-acid residue (Glu-270) is in close proximity to the metal center. If the role of the metal were mainly to polarize the carbonyl carbon, Glu-270 in its deprotonated form could be positioned to perform a nucleophilic attack on the carbonyl carbon, yielding an anhydride intermediate. Alternatively, the metal could mainly serve to provide a coordinated hydroxide ion that, in turn, could attack the carbonyl carbon; here Glu-270 would help form ZnOH by transferring the proton to the carboxylate group:



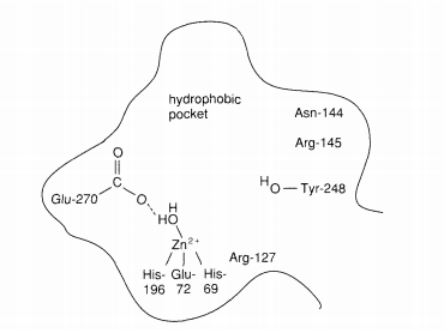


Figure 2.26 - Schematic drawing of the active-site cavity of carboxypeptidase A.[122] Only the residues believed to playa role in the catalytic mechanism are shown.

On the opposite side of the cavity is a tyrosine residue that has been shown to be quite mobile and therefore able to approach the site where the catalytic events occur. The cavity has a hydrophobic pocket that can accommodate the residue, R, of nonpolar C-terminal amino acids of the peptide undergoing hydrolysis (Figures 2.26 and 2.28), thereby accounting for the higher efficiency with which hydrophobic C-terminal peptides are cleaved. Finally, an Asn and three Arg residues are distributed in the peptide-binding domain; Asn-144 and Arg-145 can interact via hydrogen bonds with the terminal carboxyl group. Arg-127 can hydrogen-bond the carbonyl oxygen of the substrate.

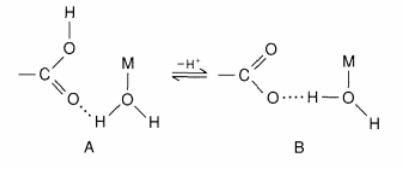
All these features have enabled detailed interpretation of many chemical and physico-chemical data at the molecular level. The essential data are as follows:

1. *Metal substitution*. Table 2.9 lists the divalent metals that have been substituted for zinc(II) in CPA, together with their relative peptidase (and esterase) activities.[22] For some of them, the available x-ray data show[123] that the active-site structure is essentially maintained. Even the copper derivative is slightly active. The apoenzyme is completely inactive, however.

Table 2.9 - Catalytic activities of metal-substituted carboxypeptidases.[a] [22] a) Activities are relative to the native enzyme, taken as 100%. b) Some activity toward both peptides and esters has recently been observed. [22]

	Peptidase	Esterase
Apo	0	0
Cobalt	200	110
Nickel	50	40
Manganese	30	160
Cadmium	5	140
Mercury	0	90
Rhodium	0	70
Lead	0	60
Copper	—[b]	—[b]

2. *Active-site modifications*. Chemical modification and site-directed mutagenesis experiments suggest that Glu-270 is essential for catalysis.[124,125] Tyr-248,[126] Tyr-198,[127] and one or more of the arginines[124] are involved but not essential.
3. *Kinetics*. k_{cat}/K_m pH profiles are bell-shaped, characterized by an acid pK_a, limb around 6 and an alkaline pK_a limb around 9: k_{cat} increases with the pK_a of 6 and then levels off, and K_m increases with a pK_a of 9. Several lines of evidence suggest that the $pK_a \approx 6$ corresponds to the ionization of the Glu-270-coordinated H_2O moiety:



(2.19)

   Site-directed mutagenesis has ruled out Tyr-248 as the group with the pK_a of 9 in the rat enzyme.[125,126] Unfortunately, in this enzyme the pK_a of 9 is observed in k_{cat} rather than K_m; so the situation for the most studied bovine enzyme is still unclear. Tyr-248 favors substrate binding three to five times more than the mutagenized Phe-248 derivative.[126] The three possible candidates for this pK_a, are the coordinated water, Tyr-248, and the metal-coordinated His-196, whose ring NH is not hydrogen-bonded to any protein residue.[128] The x-ray data at different pH values show a shortening of the Zn—O bond upon increasing pH.[129] This favors the ZnOH hypothesis.

4. *Anion binding*. The metal binds anionic ligands only below pH 6, i.e., when Glu-270 is protonated, when Glu-270 is chemically[130] or genetically[125] modified, or when aromatic amino acids or related molecules are bound in the C-terminal binding domain (Arg-145 + hydrophobic pocket).[131-134]
5. *Intermediates*. An anhydride intermediate involving Glu-270 for a slowly hydrolyzed substrate may have been identified.[135] Some other intermediates have been observed spectroscopically at subzero temperatures with the cobalt(II) derivative.[22,136] Peptides bind in a fast step without altering the spectroscopic properties of cobalt(II), following which a metal adduct forms and accumulates.[22] Thus, if an anhydride intermediate is formed, it is further along the catalytic path.

On the basis of these data, and many related experiments, a detailed mechanism can be formulated (Figure 2.29). The incoming peptide interacts with arginine residues through its terminal carboxylate group. The interaction could initially involve Arg-71 (not shown); then the peptide would smoothly slide to its final docking position at Arg-145, while the R residue, if hydrophobic, moves to the hydrophobic pocket (Figure 2.29B). The carbonyl oxygen forms a strong hydrogen bond with Arg-127. Additional stabilization could come from hydrogen bonding of Tyr-248 to the penultimate peptide NH. This adduct might be the first intermediate suggested by cryospectroscopy [22,136] (Figure 2.24).

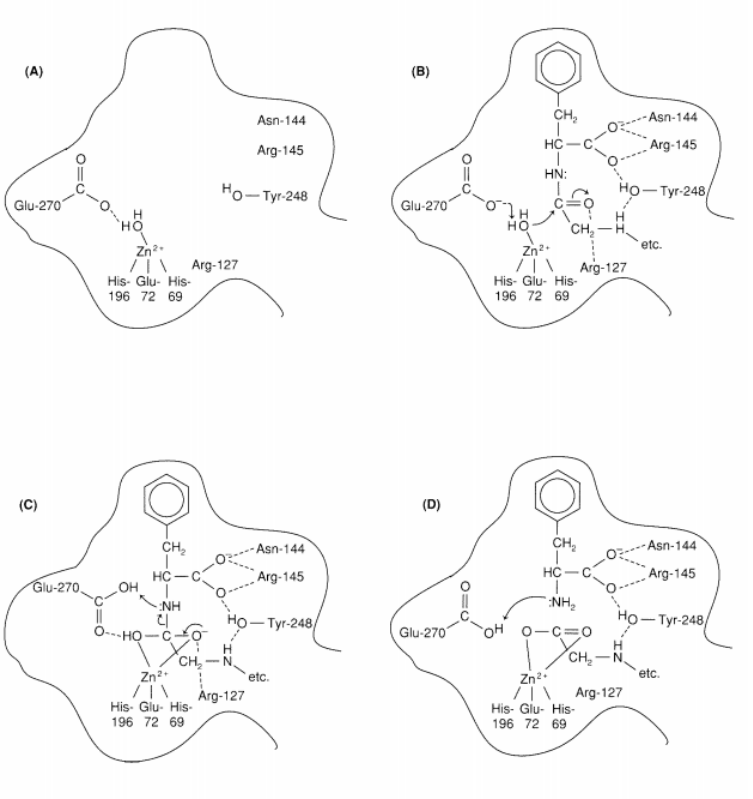


Figure 2.29 - Possible catalytic cycle of CPA.

At this point the metal-bound hydroxide, whose formation is assisted by Glu-270, could perform a nucleophilic attack on the carbonyl carbon activated by Arg-127 and possibly, but not necessarily, by a further electrostatic interaction of the carbonyl oxygen with the metal ion. The structure of the substrate analogue α-R-β-phenylpropionate shows that the carbonyl binds in a bidentate fashion:

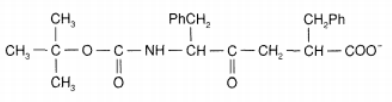


Figure 2.29C).

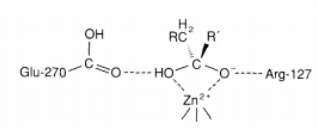


Figure 2.30 - Binding mode of α-R-β-phenylpropionate to the zinc(II) ion in CPA.[137]

The system then evolves toward breaking of the C—N bond, caused by addition of a proton to the amino nitrogen. This proton could come from Glu-270, which thereby returns to the ionized state. The breaking of the peptide bond could be the rate-limiting step.[22] The second proton required to transform the amino nitrogen into an NH_3^+ group could come from the coordinated carboxylic group of the substrate, which now bears one excess proton, again through Glu-270 (Figure 2.29D). The system shown in Figure 2.29D can, in fact, be seen as a ternary complex with a carboxylate ligand and an amino-acid zwitterion, bound synergistically.[131-134] Finally, the metal moves back to regain a bidentate Glu-72 ligand, and the cleaved peptide leaves, while a further water molecule adds to the metal ion and shares its proton with the free carboxylate group of Glu-270.

Once the hydrolysis has been performed, the cleaved amino acid still interacts with Arg-145 and with the hydrophobic pocket, whereas the amino group interacts with Glu-270. The carboxylate group of the new terminal amino acid interacts with zinc. This picture, which is a reasonable subsequent step in the catalytic mechanism, finds support from the interaction of L- and D-phenylalanine with carboxypeptidase.[131-134,138]

This mechanism, essentially based on the recent proposal by Christianson and Lipscomb,[137] underlines the role of the Zn—OH moiety in performing the nucleophilic attack much as carbonic anhydrase does. This mechanism can apply with slight changes to

thermolysin[139] and other proteases. Thermolysin cleaves peptidic bonds somewhere in the peptidic chain. The mechanism could be very similar, involving zinc bound to two histidines and Glu-166 (Figure 2.31).[139] Glu-166 is monodentate. The role of Glu-270 in CPA is played by Glu-143 and the role of Arg-127 is played by His-231.

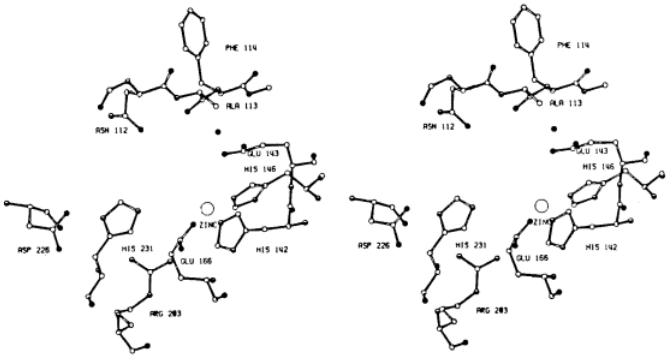


Figure 2.31 - Stereo view of the active site of thermolysin.[139]