A Molecular Dynamics Study of Carboxypeptidase A: Effect of Protonation of Glu 270

Lucia Banci, Ivano Bertini,* and Giovanni La Penna

Department of Chemistry, University of Florence, 50121 Florence, Italy

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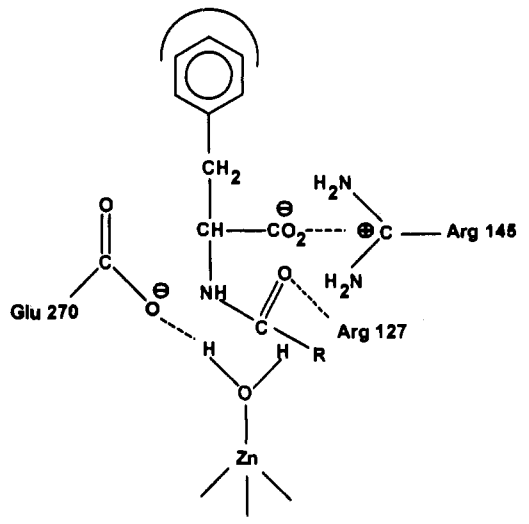
Introduction

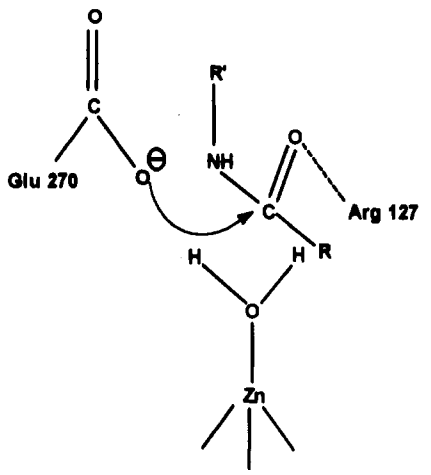
Carboxypeptidase A (CPA) catalyzes the hydrolysis of peptides with aromatic residues (Tyr and Phe) as C-terminations.[1-5] The protein contains an essential catalytic zinc ion which is coordinated by two histidines, a Glu residue in a bidentate fashion, and a water molecule. The water molecule is strongly hydrogen-bonded with the carboxylate group of Glu 270[6] (Figure 1). The strong hydrogen-bond accounts for the inability of N_3^- to displace the coordinated water.[7]

The X-ray structure for this enzyme is available at high resolution (1.54 Å);[6] in addition, crystallographic characterization is available for a large variety of complexes of the enzyme with inhibitors[8,9] and substrate analogues.[10-13] The enzyme has been extensively characterized also through spectroscopic measurements which, together with the X-ray characterization, have shed light on the enzymatic mechanism.[6,14-16] There is agreement that the terminal carboxylate of the substrate interacts with Arg 145 (see I). There is debate whether the carbonyl group is activated by the interaction with the metal ion or with an Arg residue, namely Arg 127. Christianson and Lipscomb favor the latter hypothesis.[17] At this point the nucleophilic attack is believed to be performed by the zinc-coordinated water[3,18] which is activated by Glu 270 (see Scheme I).



I

An alternative mechanism has also gained support which is based on the nucleophilic attack by Glu 270 (see II), which would then provide an anhydride intermediate.[19-23]



II

The enzymatic activity (as k_{cat}) experiences a pK_a around 6[24] which is ascribed to the ionization of Glu 270.[25] Indeed, when such a residue is methylated[26] or substituted through site-directed mutagenesis,[27] the enzyme is inactive. Anions are capable of replacing the coordinated water in the latter cases and for the case where Glu 270 is protonated.[28]

The carboxylate group of Glu 270 can also assist the substrate during the catalytic cycle; e.g. it interacts with the amino group of the cleaved amino acid in the zwitterionic form as shown by the X-ray structure of CPA(D-Phe).[9,10,26,29]

The investigation of the effect of the protonation of Glu 270 on the structure–function relation of the protein is approached here through molecular dynamics (MD) calculations which provide a description of the nonbonding interactions between groups in the active site.

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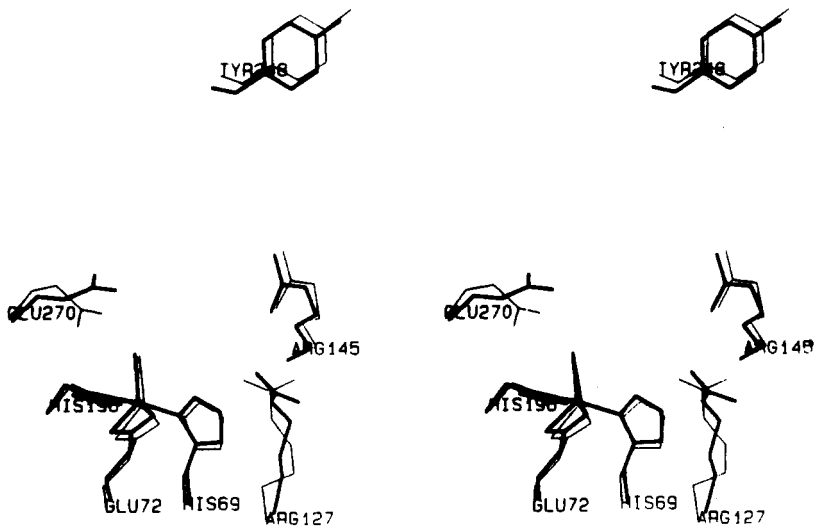
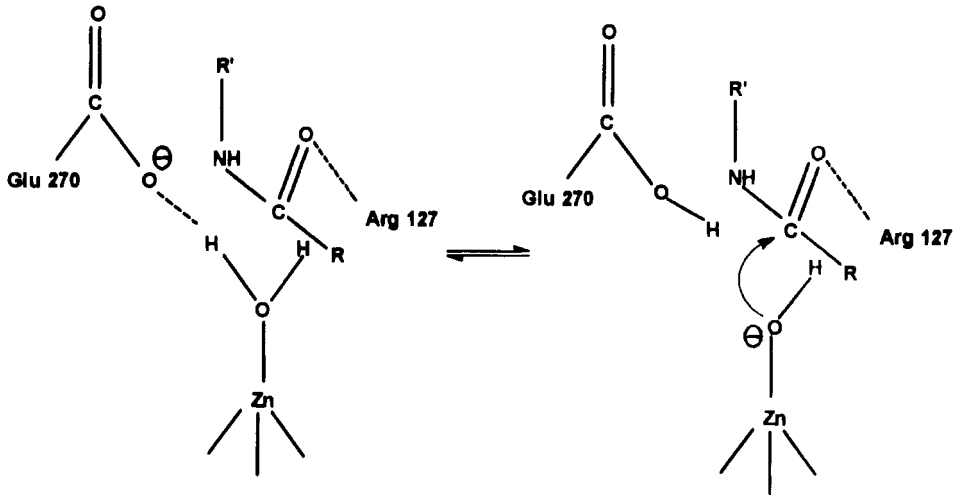


Figure 1. Comparison between stereoviews of the active site of CPA in the MD average structure (bold line) and in the X-ray structure (thin line).

Scheme I



MD calculations are a suitable tool for studying the structural and dynamical properties of proteins[30] and have been successfully applied to systems containing closed-shell metal ions.[31-34] Indeed, a MD study of the native enzyme and of some of its inhibitor adducts has been already reported.[35] Those calculations reproduced fairly well the structural features of the various adducts and showed that structural variations are responsible of the inhibition of the reaction mechanism. The same approach and the same parameters were used in the present study.

The present calculations are significant, for any proposed enzymatic mechanism,[3,20,24] in the absence of a low-pH structure of CPA due to the instability of the protein crystals at pH values lower than 7.[36]

Experimental Section

The molecular mechanics and dynamics calculations were performed with the AMBER 4.0 MD package,[37] on an IBM RS6000, Model 550.

We used the X-ray structure of bovine CPA[6] (307 residues corresponding to 2438 heavy atoms) as the starting point for building the system. Hydrogen atoms were added with the EDIT module of AMBER. Three systems were considered in the present work: (i) the native CPA (CPA hereafter) for which MD calculations were already available[35] but on which new calculations are now performed with an improved setup (increased number of water molecules, larger cutoff for nonbonded interactions, and slower heating of the system (see later)); (ii) the protein with the Glu 270 residue protonated on the Oε1 atom (CPAH1 hereafter); (iii) the protein with Glu 270 protonated on Oε2 (CPAH2 hereafter).

For the two last systems we constructed an acidic Glu residue (GluH) using the PREP module of AMBER with the same topology as for the negative Glu residue and adding an H atom to Oε1 in CPAH1 and to Oε2 in CPAH2. MD calculations on the latter two systems were performed with two sets of charges. The first set of charges, calculated ab initio with a STO-3G basis set, had already been reported in literature for the

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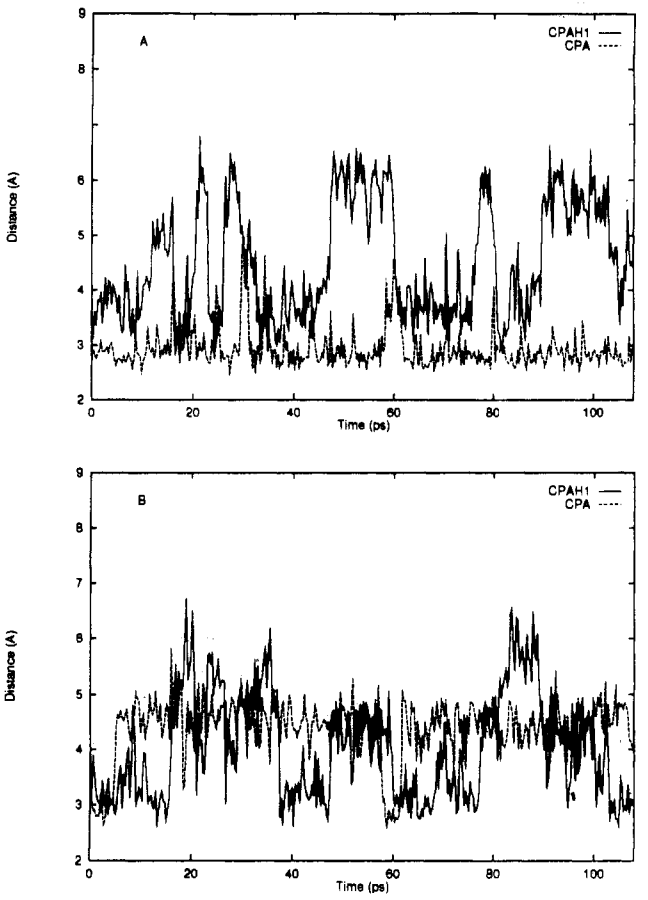

Figure 2. (A) Fluctuation of the distance between Oε1 of Glu 270 and O of the Zn-bound water (WAT) in CPA (dashed line) and between the same atoms in CPAH1 (solid line). (B) Fluctuation of the distance Oε2(Glu 270)–O(WAT) in CPA (dashed line) and CPAH1 (solid line).

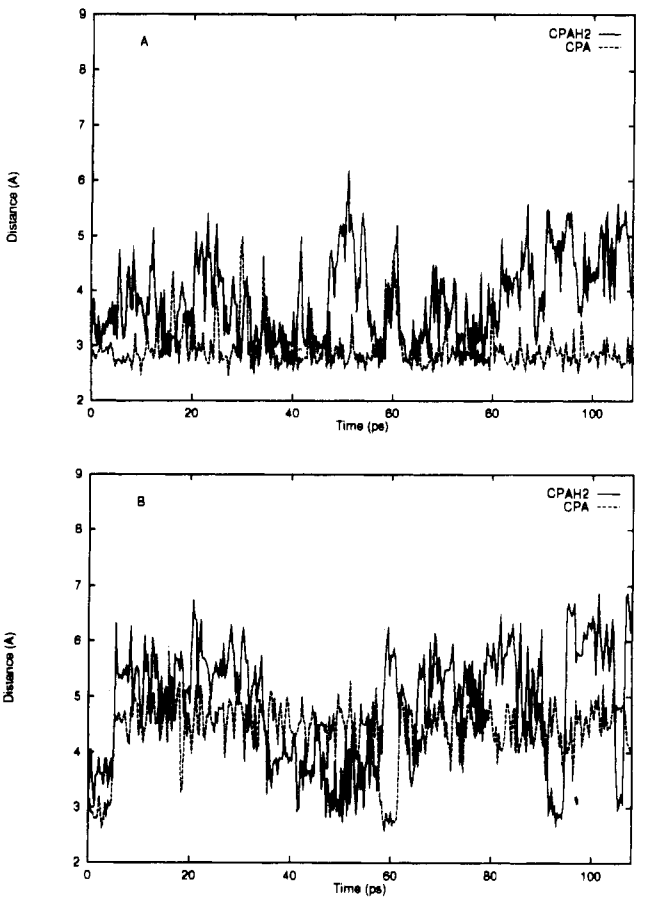

Figure 3. (A) Fluctuation of the distance between Oε1 of Glu 270 and O of the Zn-bound water (WAT) in CPA (dashed line) and between the same atoms in CPAH2 (solid line). (B) Fluctuation of the distance Oε2(Glu 270)–O(WAT) in CPA (dashed line) and CPAH2 (solid line).

Table I. Charges on Glu 270 in CPA and on GluH 270 in CPAH

atom	Glu	GluH	atom	Glu	GluH
N	–0.463		Hβ	0.092	0.128
H	0.252		Cγ	–0.398	–0.355
C	0.616		Hγ	0.071	0.103
O	–0.504		Cδ	0.714	0.654
Cα	0.035		Oε1	–0.721	–0.523
Hα	0.048		HOε1		0.340
Cβ	–0.184	–0.148	Oε2	–0.721	–0.414

GluH–GluH system.[38] For the second set, the charges for Cγ, Hγ, Cδ, Oε1, Oε2, and HOε1 or HOε2 of GluH 270 were computed through the MNDO semiempirical method using the MOPAC 5.0 package,[39] with geometry optimization, for the model system *N*-methyl-GluH-*N'*-acetamide, while for Cα, Hα, Cβ, and Hβ's the standard charges of AMBER were used, with small adjustments on Cβ and Hβ in order to have a unit charge on the residue. The point charges were determined by the fitting procedure available through the ESP option. The charges used for Glu 270 in the two systems here investigated (standard for CPA and MNDO-ESP for CPAH) are shown in Table I. In the system with a protonated Oε2 atom the charges of Oε1 and Oε2 were exchanged.

For the active site residues we used the charges and the force field parameters already successfully developed for CPA.[35] Standard all-atom force-field parameters were used for the residues within 8 Å from the zinc ion, while united-atom parameters were used for all other residues.[40]

The 314 crystallographic water molecules were taken into account, and the molecule was further solvated by adding a 6-Å thick shell of TIP3P[41] water molecules around the macromolecule with the BLOB option, discarding the molecules with oxygen atoms closer than 2.8 Å and hydrogens closer than 2.0 Å to any protein atom. This results in the addition of 1953 water molecules to the protein molecule. Three negative counterions (Cl^-) were placed close to the positively charged residues on the surface (Lys 51, Lys 239, and Arg 276) of CPA in order to have an overall charge of zero on the protein; one further counterion was added to CPAH1 and CPAH2 near the protonated His 13.

After equilibration of the water molecules, the entire systems were minimized. Then MD calculations followed, with a residue cutoff of 9 Å for the evaluation of nonbonded interactions. The pairlist was updated every 20 steps. The MD calculations were performed with a time step of 1.5 fs using the SHAKE[42] algorithm on all the bonds. We started equilibrating the system at 10 K coupled with a bath at 100 K for 3 ps, followed by other 3-ps simulations coupled with a bath at 200 K and finally at 300 K. MD calculations were performed on the residues in a sphere of 15 Å centered on the zinc ion. In addition, the complete secondary structure motifs, i.e. α-helices and β-sheets, were completely included in the dynamics even if outside the sphere of 15 Å. The coordinates and energies were collected every 200 steps for further analysis. The trajectories were performed for a total of 108 ps. For the following analysis we used the final 90-ps data, discarding those of the first 18 ps during which the equilibration was achieved.

Results and Discussion

Figure 2 shows the distance between the oxygen of the zinc-bound water and the two oxygen atoms of Glu 270 (in CPA and CPAH1) as a function of the trajectory time, by using the charges reported in the second column (MNDO-ESP calculations) of

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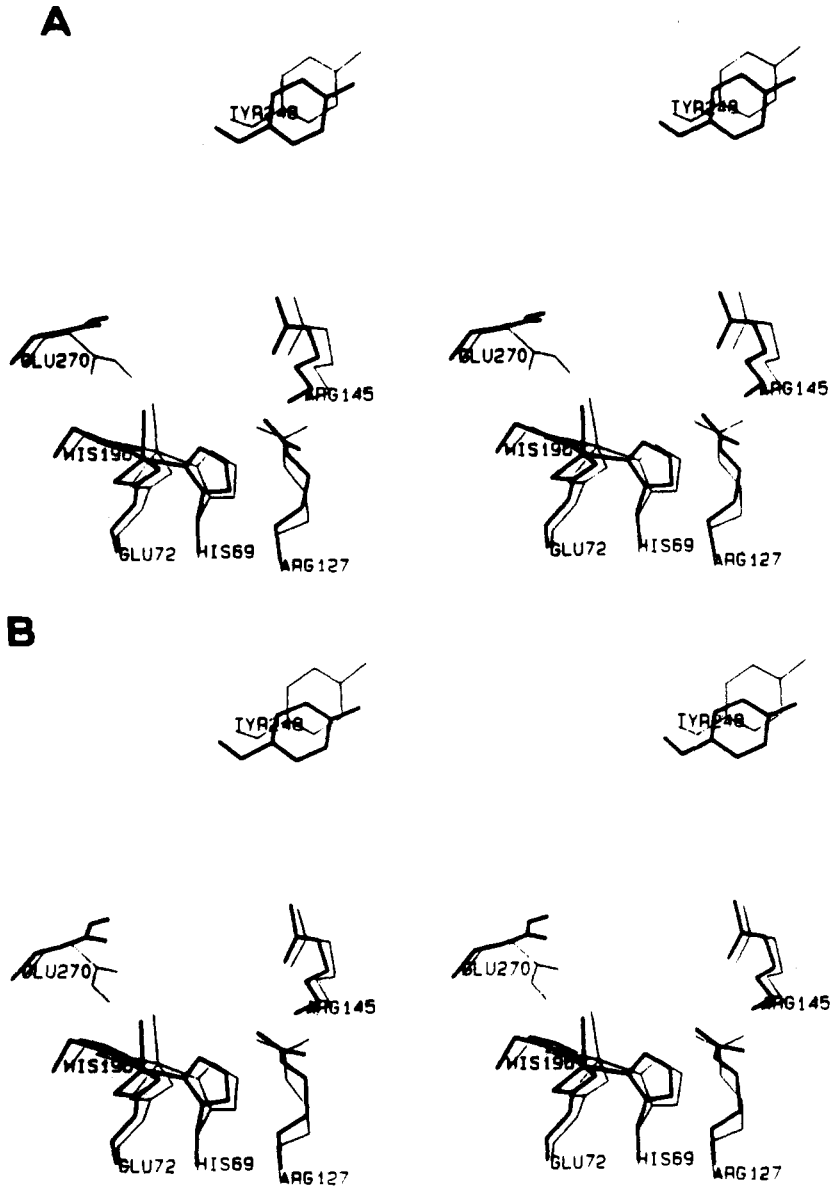

Figure 4. Comparison between the CPAH1 MD average structure (bold) and the starting X-ray structures (thin) for CPAH1 (A) and CPAH2 (B).

Table II. Some Relevant Distances (Å) in the Cavities of CPA and CPAH

		CPA		CPAH1		CPAH2	
	X-ray structure	minimized	MD av	minimized	MD av	minimized	MD av
Cδ(Glu 270)–O(WAT)	3.2	3.2	3.6	3.3	4.4	3.6	4.3
Oε1(Glu 270)–O(WAT)	2.5	2.8	2.9	2.9	4.5	3.1	3.8
Oε2(Glu 270)–O(WAT)	3.2	2.9	4.4	2.9	4.0	3.2	4.7
O(WAT)–Cζ(Arg 127)	6.1	6.0	6.5	6.0	6.5	5.8	6.3
Cδ(Glu 270)–Cζ(Arg 127)	9.1	8.8	9.3	9.0	10.1	9.1	9.9

Table I. By comparing the evolution of the distances in the cases of deprotonated and protonated enzyme (CPA and CPAH1), we can see an increase in distance between Glu 270 and the zinc-bound water molecule (WAT) upon protonation of the former group. Figure 3 shows the comparison of the behavior of the CPAH2 system with that of CPA. Also in CPAH2 a movement of Glu 270 away from the zinc-bound water is observed.

Some atom–atom distances for residues relevant to the enzymatic process are reported in Table II. The Glu 270–WAT distances reported in Table II and Figures 2 and 3 clearly indicate that in CPAH the hydrogen-bond between Glu 270 and WAT is broken along all the dynamics.

In CPA we observe, after a few picoseconds, a rotation of the carboxylate group of Glu 270 around the Cβ–Cδ bond that moves Oε2 farther from the oxygen of WAT; Oε1 remains close to the oxygen of WAT with an average distance of 2.9 Å (Table II). In any step of the trajectory, however, an oxygen atom of Glu 270 is always at the correct distance to interact with the zinc-bound water.

In the case of CPAH1, on the contrary, the two oxygen atoms of the carboxylate of Glu 270 are farther from O(WAT), with average distances of 4.0 and 4.4 Å (Table II). The oxygen bearing the proton moves farther from O(WAT) than the other. Also the distance between Cδ(Glu 270) and O(WAT) increases during the trajectory time, indicating that the overall distance between the whole carboxylate group of Glu 270 and WAT increases. The oxygen (Glu 270)–oxygen (WAT) distances are too large for any interaction to occur between Glu 270 and WAT. The carboxylate group of Glu 270 experiences large fluctuations (Figure 2) which

are consistent with the lack of any hydrogen-bond with the zinc-coordinated water molecule.

For CPAH2 we observe a behavior similar to that of CPAH1. Also in this system the oxygen of Glu 270 bearing the proton (Oε2) moves farther from WAT than the other, the average Oε2(Glu 270)–O(WAT) distance being 4.7 Å. Also the deprotonated oxygen (Oε1) remains, on average, far from WAT, with an oxygen–oxygen distance of 3.8 Å, which rules out the presence of any hydrogen-bond. This is further supported, as in the case of CPAH1, by the large fluctuations experienced by Glu 270, as shown in Figure 3, indicating that no hydrogen-bond can exist with the zinc-coordinated water molecule.

An even larger movement is observed when the MD calculations are performed with the charges of GluH reported by Merz,[43] resulting in a deprotonated Oε2(GluH 270)–O(WAT) distance of 5.0 Å in the average structure.

Consistent with this dynamic behavior, the COOH group of Glu 270 in CPAH has a much smaller energy of interaction with the zinc-bound water molecule, with respect to what is found in the case of CPA.

Protonation of Glu 270 induces also a minor solvation of the active site, the water molecules present in the cavity being less in CPAH than in CPA. GluH 270 still hydrogen-bonds with some water molecules during the dynamics, but these water molecules are highly mobile and move rapidly in and out during the trajectory. GluH 270, in the new position far from the zinc ion, is not interacting significantly with other residues of the protein; its movement should be ascribed essentially to electrostatic factors. The extra positive charge present on the GluH 270 residue strongly reduces the energy of interaction between the negative deprotonated Glu 270 and the positive active-site zinc ion in native CPA.

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Figure 1 shows the comparison between the average structure of CPA and the crystallographic one, while parts A and B of Figure 4 show the comparison between the average structures of CPAH1 and CPAH2, respectively, and the starting structures which are the X-ray structures with the addition of a proton to Glu 270. These figures show the movement of GluH 270 and the fairly maintained positions of the zinc ligands. The other groups in the active cavity undergo only minor changes. Arg 127, which is proposed to interact with the scissile carbonyl group,[3] results, in CPAH1 and CPAH2, in a conformation similar to that in CPA.

The present investigation has therefore proposed a reliable picture of the enzyme in the inactive acidic form. The movement of Glu 270 in CPAH is similar to that found when an amino acid binds the enzyme with the carboxylate group interacting with Arg 145.[3,9,35] The X-ray data of CPA(D-Phe)[9] show that the Glu 270–WAT interaction is broken and a new interaction occurs between Glu 270 and the amino group of D-Phe. Upon breaking of the carboxylate–water bond, the zinc-bound water becomes easily displaced by anions.[44,45] The inactivity of the acidic form of the enzyme is easily consistent with II since a protonated Glu is disfavored in the formation of an anhydride. However, it is also completely consistent with the mechanism of Scheme I: the protonation of Glu 270 breaks a catalytically important hydrogen-bond with the zinc-bound water and accounts for the enzyme becoming inactive at low pH.

The present picture is a sound model for further investigations of the action of CPA.

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