Theoretical study of the role of arginine 127 in the water-promoted mechanism of peptide cleavage by carboxypeptidase A

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The water-promoted mechanism of peptide cleavage by carboxypeptidase A (CPA) has been studied by means of molecular dynamics simulations and AM1 quantum mechanical calculations. A representative molecular dynamics structure has been used to design a realistic quantum mechanical model involving 106 atoms, which includes for the first time the Arg-127 residue (simulated by a guanidinium group) among others. In turn, the accessibility of the conformations that are required for the quantum mechanical mechanism has been assessed from molecular dynamics simulations involving 8274 atoms. Our results show that proton transfer to Glu-270 from the water molecule attached to the Zn ion is required as a previous step to correct substrate anchoring. Arg-127 turns out to be important for initial binding of the substrate and stabilizing the nascent negative charge appearing on the carbonyl oxygen atom during formation of the tetrahedral intermediate when the activated water attacks the scissile peptide bond. After a suitable rotation of the substrate, the zinc ion is already able to coordinate the negative carbonylic oxygen atom, this way reinforcing the effect of Arg-127 and leading to a more stable tetrahedral intermediate. As a consequence, the proton transfer from Glu-270 to the nitrogen atom of the breaking bond becomes the step associated with the energetically highest transition state of the complete process. Finally, we feel that although quantitative values of enthalpy barriers could be somewhat overestimated by the AM1 Hamiltonian, the qualitative picture of the CPA catalytic mechanism described in this work is likely good enough and already includes the main key groups of the real system.

Carboxypeptidase A (CPA) is a metalloenzyme that hydrolyzes C-terminal amino acids from peptide or ester substrates, exhibiting a preference toward those containing hydrophobic (aromatic or aliphatic) residues.[1–4] The structure of the enzyme, isolated and bound to different inhibitors, has been determined by X-ray crystallography at high resolution.[5–8] At the active site, CPA contains a zinc ion coordinated by residues His-69, Glu-72 and His-196. Residues Glu-270 and Arg-127 and a water molecule bound to the zinc ion are important for catalysis, while other residues such as Arg-145, Asn-144, Arg-71 and Tyr-248 seem to be important for conferring the proper position, distortion and environment to the substrate.[3,9,10]

For the catalytic mechanism, two alternative pathways have been proposed. One is a nucleophilic pathway in which Glu-270 (acting as a base) attacks the scissile carbonyl to form a water-labile intermediate.[11] The other is the most commonly accepted mechanism, called the water-promoted pathway, proposed by Lipscomb's group.[3,12–14] In this latter mechanism, the water molecule bound to zinc is promoted to form a hydroxyl, assisted by Glu-270. Then, the hydroxyl oxygen attacks the carbon atom of the scissile peptide bond, forming a tetrahedral intermediate.

Experimental studies have contributed to support both mechanisms.[4,15] Some experiments on ester substrates at low temperature seem to be in favour of the nucleophilic pathway, showing accumulation of an acyl enzyme intermediate.[16] Contrary to this, experiments on peptide substrates are generally more in favour of the water-promoted pathway.[17,18] Recently, crystallographic and site-directed mutagenesis studies[8,19,20] have supported the important role played by Arg-127 in peptide catalysis, first suggested by Christianson and Lipscomb.[3] The guanidinium group of Arg-127 is hypothesized to stabilize, through electrostatic interactions, the transition state formed by the attack of water on the scissile peptide bond.

On the other hand, several theoretical studies have been performed in order to clarify the catalytic mechanism of CPA. Nakagawa and co-workers[21] demonstrated that the Zn-coordinated water molecule acts as a proton donor for Glu-270, simulating some zinc ligands with point fractional charges. They concluded that zinc ion plays a significant role in lowering the barrier height of that proton transfer. Alex and Clark[22] constructed a simplified model of CPA in which His-69 and His-196 were represented by two imidazoles; Glu-72 and Glu-270 were represented by an acetate and a formiate, respectively; and a formamide molecule was used to simulate a substrate. With that model, containing 39 atoms, they performed AM1 calculations on the different steps suggested by the water-promoted pathway proposed by Lipscomb's group.[3] The concerted addition of the $ZnOH^+$ moiety to the carbonyl bond of the formamide (substrate) was found to be the rate-determining step in the entire reaction sequence. Our group followed a related approach and in a previous paper[23] we designed a model of the active site of CPA plus a substrate, in which His-69 and His-196 were represented by imidazoles; Glu-72 and Glu-270 were both represented by acetate molecules; and *N*-ethylacetamide was used as a substrate. To ensure that this model, which contained 51 atoms, was able to mimic the natural system, it was validated by comparison with the structure of the tetrapeptide $(Gly)_3$—L—Tyr + water + CPA complex resulting from several energy minimization/molecular dynamics simulations. The reported AM1 results on the water-promoted pathway of peptide cleavage by CPA in this model showed that the attack of the hydroxyl oxygen on the carbon atom of the scissile peptide bond was the rate-determining step, with a high enthalpy barrier of 37.9 kcal mol^{-1}. This enthalpy barrier was dramatically decreased when a positive charge, attempting to

represent the Arg-127 residue, was included.

Recently, Abashkin *et al.*[24] have performed density functional theory calculations on the mechanism. The increase in calculation level forced them to reduce the size of the model, to the extent of constructing a model even more simplified than the model designed by Alex and Clark,[22] now including only 26 atoms. Their results[24] revealed that the rate-determining step is formation of a tetrahedral intermediate arising from the attack of a Zn-coordinated OH group to the carbonyl of the substrate. Owing to model simplifications, the authors state that their energy profile for the reaction can be only qualitatively correct.

Molecular dynamics has also been employed to study the enzymatic mechanism of CPA. Banci *et al.*[10] showed the structural variations induced by different inhibitors and the effect of these interactions on the catalytic mechanism and on the binding of substrate. The most remarkable is the mobility of Arg-127 and Tyr-248. Posterior molecular dynamics simulations performed by Banci *et al.*[25] suggested that both "nucleophilic" and "water-promoted" pathways are structurally feasible, although the water-promoted mechanism is energetically favoured.

At this point, several considerations about the theoretical treatment of an enzymatic reaction should be made. Because some bonds are broken and formed along the reaction, a quantum mechanical study of the evolution of the active center is required for understanding the catalytic mechanism. Given the size of real systems, important simplifications have to be made. However, the active center has to be modelled very carefully. The catalytic ability of the enzyme is due to the stabilizing interactions of the substrate with several key residues, particularly at the transition states. The main residues that have been experimentally proven to play an important role in the process should be included, in order to reproduce how the enzyme works. On the other hand, even with a good design of the active center and the substrate, quantum mechanical calculations on a reduced model could lead to a fair mechanism in the quantum model, but be completely unfeasible within the real entire enzyme–substrate system. For instance, in a recent theoretical study of the mechanism of CPA inhibition by zinc ions,[26] we have shown that the peptide cleavage seems to be even favoured by the action of the zinc inhibitor when a quantum mechanical reduced model of 57 atoms is considered. However, it would occur through a mechanism that is not allowed in the complete system as seen by means of molecular dynamics simulations on a system including more than 8000 atoms. Thus, in general, the accessibility of the conformations that are required for the quantum mechanical mechanism should be assessed from molecular dynamics simulations on more complete systems.

All the above considerations taken into account, the purpose of the present paper is to study the entire process of peptide cleavage by CPA *via* the water-promoted mechanism, using a more realistic model that includes for the first time residue Arg-127, simulated by a guanidinium group. This model includes 106 atoms and is much larger than the previous ones. We have combined molecular dynamics simulations and quantum mechanical calculations better to understand the enzymatic process. Our results clarify and confirm the structural and energetic role of Arg-127 in such a mechanism, and define in detail the main steps of the latter.

Calculation method

Quantum mechanical calculations

The calculations were carried out by using the standard AM1 procedure,[27] as implemented in the MOPAC[28] package of computer programs.

All geometry minima were obtained by minimizing the energy with respect to all geometrical variables by using the Davidon–Fletcher–Powell method.[29,30] The location of transition states was obtained with the McIver–Komornicki gradient minimization technique.[31] The characterization of both kinds of stationary points, minima and transition states, was carried out by diagonalizing their Hessian (force constant) matrices and looking for zero or one negative eigenvalue, respectively

Molecular dynamics calculations

Molecular dynamics (MD) and energy minimizations (EM) were performed with the GROMOS program package.[32] The recently modified GROMOS force field for solvated systems, 37C4-PSW,[33] was used for the MD simulations and energy minimizations. His-69 and His-196 have their exchanging imidazole proton not at the Nδ but rather at the Nε position (HisB in the GROMOS topology file definitions); thus the coordination to the Zn ion is done by the pair of free electrons of the nitrogen sp^2 orbital. The protein model, including a $(Gly)_3$—L—Tyr tetrapeptide substrate, was constructed from the X-ray crystallographic coordinates as described in a previous paper.[23] It is worth recalling here that the standard parameters of Zn within the GROMOS force field give charges of either 0 or +2, depending on the absence or presence of solvent. We have considered these charges unsuitable for the real system and therefore the charges of Zn and their coordinated atoms were substituted by those obtained with AM1 calculations. This protein model was solvated with PROBOX (GROMOS package) and the system was reduced to a sphere of 30 Å, containing 8274 atoms, centered on the zinc ion of the active site. The positions of the atoms in a 13 Å shell were constrained. Also, the zinc ion and those atoms directly coordinated with it were constrained. The restraining potential of these constraints was defined as an harmonic potential with $k = 900$ kJ mol^{-1} nm^{-2} fixing the positions at the X-ray coordinates.

Electrostatic forces were evaluated with a clean twin-range cutoff technique. All nonbonded forces within 8 Å were evaluated at each simulation step. Electrostatic forces at distances between 8 and 13 Å were reevaluated every 10 steps. A 2 fs time step was used in the integration, with the bond lengths being constrained by SHAKE.[34] The temperature of the MD simulations analyzed was set at 293 K, and a temperature relaxation time of 0.1 ps was used to maintain the system and the waters in a weak coupling bath.[35]

Results and Discussion

As mentioned in the introduction, in this work we shall study the entire process of peptide cleavage by CPA according to the water-promoted pathway. To this aim the first point is to analyze the ease of water entering into the active center. Starting from the enzyme with no substrate, surrounded by many water molecules as solvent in a sphere of 30 Å centered on the zinc atom, and without any water molecules inside the active site, an MD simulation shows a track of solvent water molecules approaching the active center. After 30 ps a water molecule appears already attached to the zinc ion at a distance of 2.21 Å, a value comparable to the crystallographic water coordination distance (2.05 Å). The simulation also indicates the presence of a second water molecule at 3.4 Å from the zinc ion.

Once the water molecule is bound to the zinc ion, the next point is whether the substrate reaches the proper position to undergo catalytic action before or after water activation by Glu-270. In this respect, two EM + MD simulations have been performed on the system containing 8274 atoms, that is, now including the substrate. The first simulation was initiated with the carbon atom of the scissile peptide bond in the substrate positioned at about 6 Å from the oxygen atom of the water attached to the zinc ion. After 50 ps of MD simulation

the substrate does not approach the active site in the correct orientation. The second simulation showed a different behaviour. In this case, we have first modified the topology and some parameters of the force field of the active center in order to represent a new situation in which Glu-270 has already abstracted a proton of the catalytic water (which becomes a hydroxide anion). Now, 50 ps along the simulation the substrate approaches the zinc atom to a distance of 2.98 Å between the carbon atom of the peptide bond and the hydroxide oxygen atom and is well-oriented to be attacked by that hydroxide. The final fate of this latter simulation is displayed in Fig. 1.

Starting from this last conformation but reconstituting the original water molecule, a MD simulation reshuffles the conformation of the system to an ill-oriented position of the water molecule to attack the substrate. Therefore, it seems likely that proton transfer to Glu-270 is required as a previous step for the correct substrate anchoring involving Arg-127. Assuming this the next steps should involve bond breaking and bond formation, a part of the mechanism that requires quantum mechanical calculations to yield a precise view. As a consequence, a reduced quantum mechanical model of the active center has to be designed. For this purpose we have selected the most relevant residues of the active center (Fig. 2*a*) whose geometrical disposition (Fig. 2*b*) has been taken from the conformation reached (see Fig. 1) by the previous MD simulation. To build up the CPA quantum mechanical model, the chains of histidines, arginines, Glu-72 and Glu-270 (in Fig. 2) have been cut in such a way that these residues become imidazoles, guanidinium cations, acetate anion and acetic acid, respectively, whereas the tyrosine of the substrate has been substituted by alanine. Even with these simplifications a sizable system containing 106 atoms is obtained, which justifies that an AM1 Hamiltonian be used instead of higher level approaches that demand currently unfeasible computational resources.

An energy optimization of the quantum mechanical model leads to two minima, R1 and R2, in which the C4—O1 distances between the carbonyl carbon atom of the scissile peptide bond of the substrate and the hydroxide oxygen (see Fig. 2*a* for atom numbering) are, respectively, 4.05 Å and 2.83 Å. Thus the substrate in R2 is better positioned to be attacked by the hydroxide oxygen atom O1 and is very near to the situation obtained by the MD simulation (Fig. 1). Although the minimum R2 is energetically 4.8 kcal mol^{-1} above R1 in our quantum mechanical model, we think that it better represents the actual position of the substrate in the entire system when the catalytic reaction is to begin. The structure of R2 is depicted in Fig. 3.

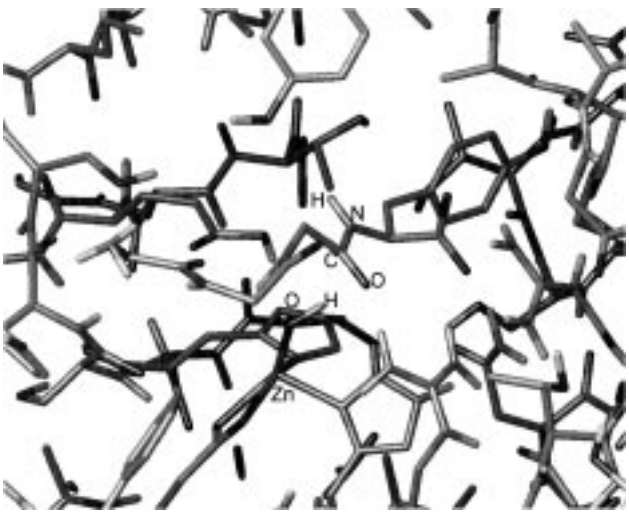

Fig. 1 Final fate of a molecular dynamics simulation of a CPA/substrate complex after Glu-270 has already abstracted a proton from the catalytic water. Scissile peptide bond atoms, hydroxide atoms and zinc ion are marked

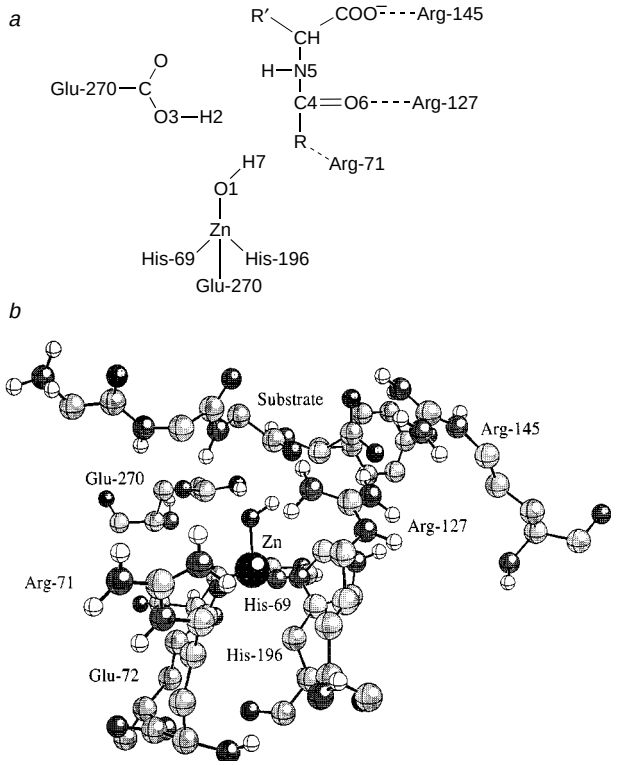

Fig. 2 (*a*) Schematic of the quantum mechanical model of the active center of the CPA/substrate complex. In our model, R and R′ stand for $CH_2NHCOCH_2NHCOCH_3$ and CH_3, respectively. (*b*) Geometrical disposition of the residues schematically shown in Fig. 2*a*

Next, we will present the different steps of the mechanism calculated with our quantum mechanical model, starting from the R2 structure. The first step is the hydroxide oxygen (O1) attack on the carbonyl carbon atom (C4) of the scissile peptide bond of the substrate. This step is schematically shown in Fig. 4. The enthalpy barrier for this attack is 19.9 kcal mol^{-1}; a value that is 18 kcal mol^{-1} lower than the enthalpy barrier obtained in our previous study[23] performed with a more reduced model. This enthalpy barrier decrease can be attributed mainly to the presence of Arg-127 that stabilizes the increase of the negative charge on O6. In the transition state

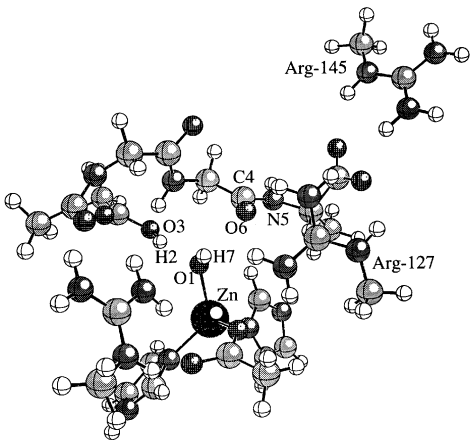

Fig. 3 Structure of R2, which represents the quantum mechanical model situation when the catalytic reaction is about to begin

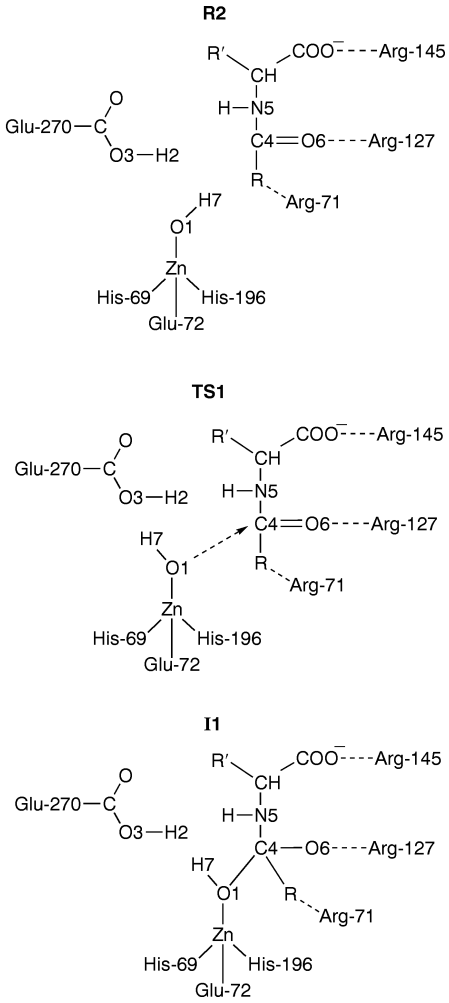

Fig. 4 Schematic structures of R2, TS1 and I1 corresponding to the hydroxide oxygen (O1) attack on the carbonyl carbon atom (C4) of the scissile peptide bond. In our model, R and R′ stand for $CH_2NHCOCH_2NHCOCH_3$ and CH_3, respectively

structure (TS1), the C4—O1 distance is 1.74 Å and the system adopts the conformation shown in Fig. 5. As a result of this step (see Fig. 4), a tetrahedral intermediate is obtained (I1) in which the C4—O1 distance is 1.54 Å. Notice that O6 maintains an electrostatic interaction with Arg-127 from R2 to I1, but does not interact with the zinc ion, in contrast to previous studies where Arg-127 was not included,[22,23] and in which the zinc ion was responsible for that electrostatic stabilization.

To ensure that the conformation of the quantum transition state of this step (TS1) is not unrealistic, we have compared it with the final MD structure presented in Fig. 1. Superposition of the coordinates of both structures (quantum mechanical TS1 and the MD structure in Fig. 1), trying to fit the coordinates of the two Zn ions and their ligands, has been performed. We have found that the substrate has moved only slightly, approaching the scissile peptide bond to the hydroxide group. Interactions of the substrate with the residues that have been simulated in our quantum mechanical model are comparable to those present in the MD structure of the complete system. However, in the quantum mechanical model some residues (for instance, Arg-145) adopt a position that is unallowed in the real system; this is unimportant because the function of Arg-145, which is to anchor the terminal carboxylate of the substrate, is maintained in both the quantum mechanical and MD models. We think that these unfeasible movements are due to the lack of inclusion of a more complete set of residues of the enzyme environment in the quantum mechanical model. However, the adopted conformation of the substrate in the reduced model fits well into the active site of the MD structure. This means that the quantum mechanical substrate could also interact with the key residues positioned as in the final MD structure conformation.

According to the water-promoted mechanism, the next step consists in H2 proton transfer from Glu-270 to N5. The enthalpy barrier of this proton transfer from I1 is 20.7 kcal mol^{-1}, which means that the corresponding transition state (TS2a) is about 40 kcal mol^{-1} above R2. The magnitude of this enthalpy barrier from R2 exceeds the error that could be attributed to the AM1 methodology, invalidating this reaction path for a biological process.

At this point, we consider the possibility that the zinc ion plays a role in stabilizing the negative charge on the O6 atom, as observed in previous studies.[22–24] The only way that zinc could coordinate the O6 atom is by means of a substrate rotation around the C4 atom. This motion takes place through a transition state (TSrot) 3.3 kcal mol^{-1} above I1, which finally leads to a new intermediate I1′ (Fig. 6). In this structure the negative charge on the O6 atom is stabilized by both the Zn ion and Arg-127.

Starting from I1′, we consider again the H2 proton transfer from Glu-270 to N5. The transition state (TS2) of this proton transfer is clearly energetically favoured in comparison to the above discarded TS2a, involving an enthalpy barrier of 27.1 kcal mol^{-1} from R2 and leading to the I2 intermediate. In

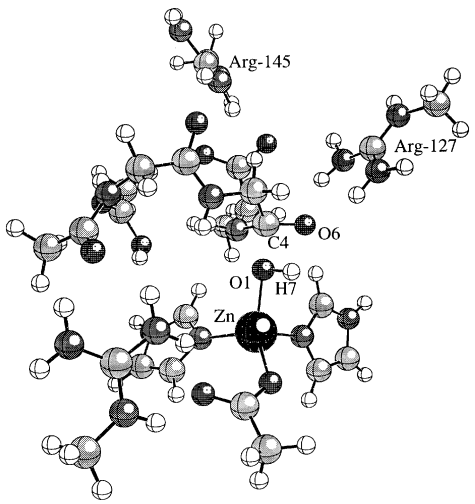

Fig. 5 Structure of the transition state TS1

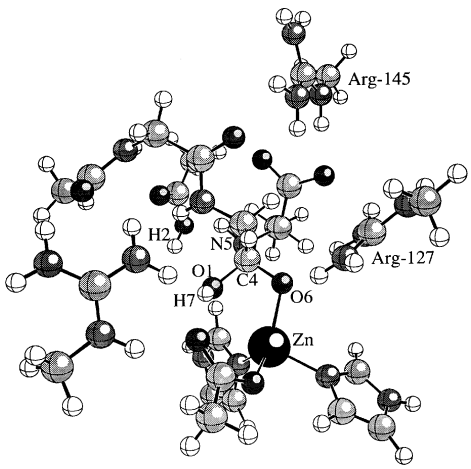

Fig. 6 Structure of the intermediate I1′

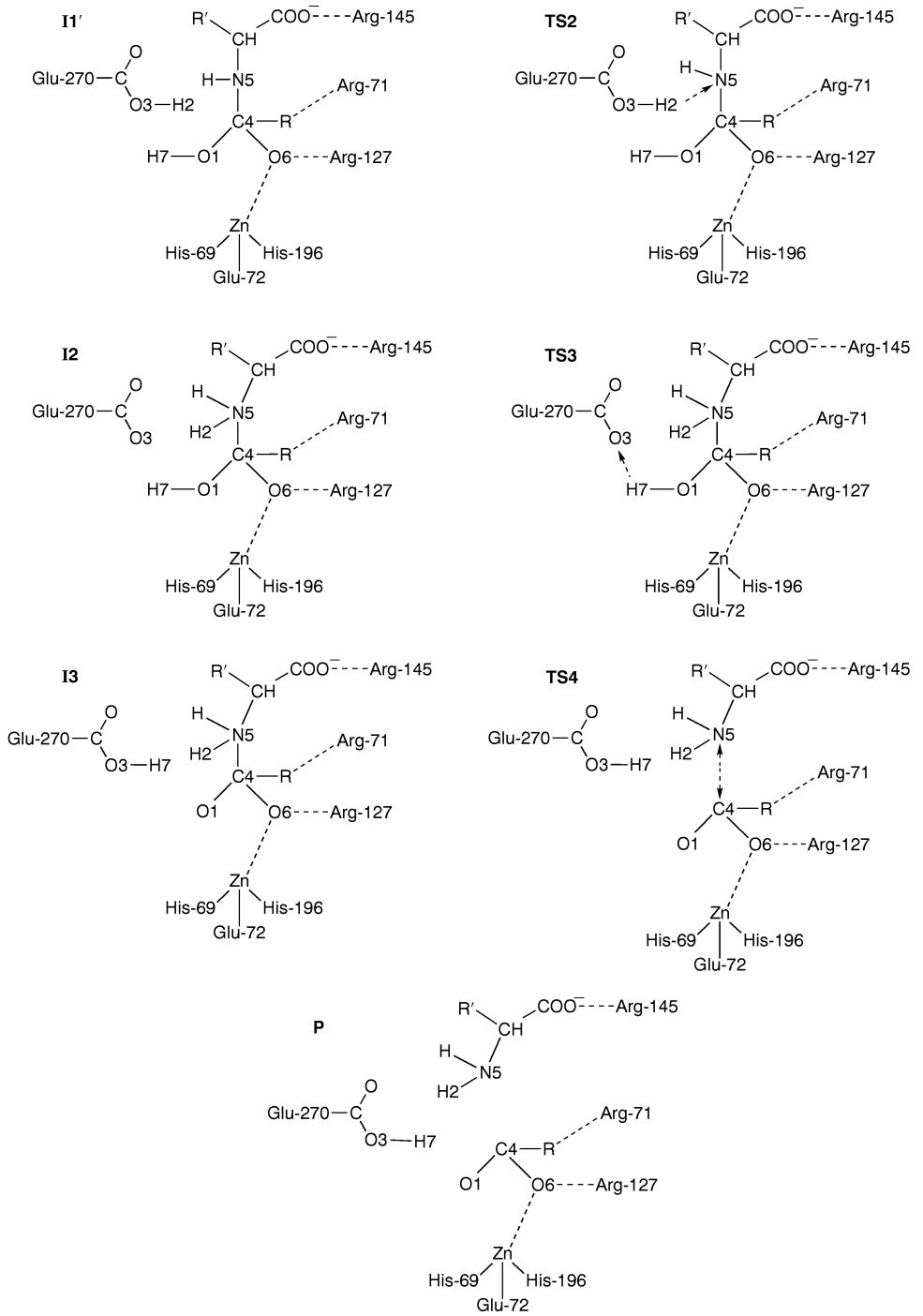

Fig. 7 Schematic structures of I1′, TS2, I2, TS3, I3, TS4 and P, which, in this order, represent the evolution of the process from I1′ to final products. In our model, R and R′ stand for $CH_2NHCOCH_2NHCOCH_3$ and CH_3, respectively

spite of this hydrogen transfer, the C4—N5 peptide bond does not break at this stage, although the C4—N5 distance increases up to 1.55 Å. In order to break the C4—N5 peptide bond, a second proton transfer is needed. The hydrogen H7 jumps from O1 to O3, leading first to an intermediate (I3) that evolves almost barrierless towards the peptide cleavage products. Evolution from I1' to products is schematically shown in Fig. 7. In Fig. 8, the energy profile of the entire cleavage mechanism from R2 to products is depicted.

We can observe that TS2, which corresponds to the H2 proton transfer from Glu-270 to N5, is the highest point along the energy profile of the reaction. As a consequence, the global reaction rate constant corresponding to the entire process (from R2 to P) would be dominated by this TS2 structure. However, if the catalytic process is considered as a set of suc-

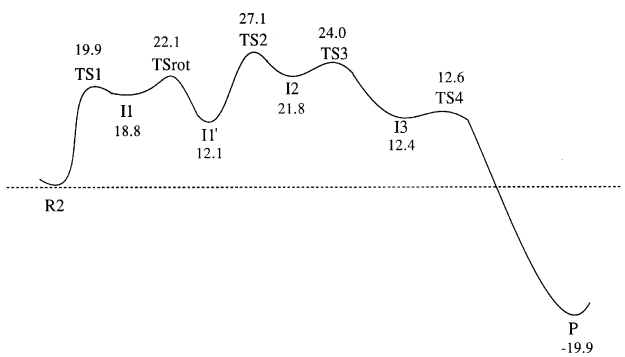

Fig. 8 Energy profile of the entire cleavage mechanism from R2 to products. Enthalpy values are given in kcal/mol and are relative to R2

cesive chemical steps, the rate-determining step would be the hydroxide oxygen (O1) attack on the carbonyl carbon atom (C4) of the scissile peptide bond of the substrate, which leads to a tetrahedral intermediate (I1). This conclusion agrees with all previous theoretical studies.[22–24] Nevertheless, we have analyzed if an alternative proton transfer leading to final products could exist, involving less energy than the TS2 structure. However, the only alternative proton shift, H7 proton transfer from O1 to N5, would take place through a transition state 24.6 kcal mol^{-1} above TS2. Therefore, it is not feasible.

Our results provide detailed theoretical grounds for the interpretation of previous experimental studies, which led to the formulation of the water-promoted mechanism and of the role of Arg-127 in the stabilization of the transition state.[3,8,19,20] Thus, the dramatic effect of the substitution of Arg-127 by Lys, Met or Ala, through site-directed mutagenesis of carboxypeptidase A, on the k_{cat} for different substrates,[19] agrees with the capability of this cation to significantly lower the energy barriers of the here proposed mechanism.

Finally, we have to mention that in a recent DFT study[24] no energy barrier for the initial deprotonation of catalytic water to form the hydroxide was found, whereas previous AM1 studies[22,23] have predicted a barrier. We have also studied the water activation in our quantum mechanical model, obtaining an enthalpy barrier of 8.5 kcal mol^{-1}. The difference between the DFT and AM1 results could be due to the different simulation of the zinc ligands, which provokes changes in the acidity of the water coordinated to zinc. For instance, AM1 calculations show that the deprotonation of water bonded to a Zn ion coordinated to three NH_3 groups is 30 kcal mol^{-1} more favoured than the same process when the Zn ion is coordinated to three imidazoles. The selection of the zinc ligands for this kind of theoretical study is therefore crucial for the achievement of a simulation close to the natural one.

Conclusions

In this paper we have studied by theoretical approaches the entire process of peptide cleavage by CPA according to the water-promoted mechanism, deriving the different mechanistic steps and their values in the energy profile.

Energy minimizations and MD simulations on the complete system containing more than 8000 atoms indicate that proton transfer to Glu-270 from the water molecule attached to the Zn ion is required as a previous step to correct substrate anchoring. A representative MD structure has been used to design a quantum mechanical model involving 106 atoms, simulating for the first time the Arg-127 residue, among others.

AM1 calculations on that model permit us to understand in detail the peptide cleavage mechanism and particularly, the role of Arg-127. First of all, this residue is important for initial binding of the substrate, which becomes properly positioned into the active site by forming a hydrogen bond with the carbonyl group of the scissile peptide bond. This hydrogen bond also stabilizes the nascent negative charge appearing on the oxygen atom of that carbonyl during tetrahedral intermediate formation, lowering in this way the corresponding enthalpy barrier.

From the mechanistic point of view, a key step in the cleavage process is the rotation of the substrate, which promotes the migration of its carbonyl oxygen atom (the one that bears the negative charge) towards the zinc ion, in this way reinforcing the effect of Arg-127 and leading to a more stable tetrahedral intermediate. This rotation has not been explicitly taken into account in previous proposed mechanisms.[3,22–24] The combined action of the zinc ion and Arg-127 gives rise to a noticeable lowering of the energy of the reaction path in the region corresponding to the nucleophilic attack on the carbonyl group. As a consequence, the first proton transfer required to break the peptide bond (from Glu-270 to the nitrogen atom of the breaking bond) becomes the step associated with the energetically highest transition state of the complete process.

It is also worth mentioning that the mechanistic pathway that we have derived does not consider intermediates in which the zinc ion could be coordinated at the same time to O1 and O6, as originally suggested for the water-promoted mechanism.[3]

On the other hand, comparison of our results with previous calculations on CPA shows that a good representation of the substrate and active center of the enzyme in the quantum mechanical model is absolutely required in order to reproduce the actual catalytic mechanism. We will remark on three examples: (*a*) the use of NH_3 groups as zinc ligands significantly reduces the computational time but overestimates the acidity of the zinc-attached water; (*b*) introduction of residues that fix the position of the substrate is needed to allow only those conformations that are possible in the entire system. In this sense, molecular dynamics simulations are very useful to verify the feasibility of the motions suggested by quantum mechanical calculations and (*c*) the inclusion of Arg-127 in the calculations is essential because it plays a key role in stabilizing the nucleophilic attack of the hydroxide on the carbonyl group.

Finally, we feel that although quantitative values of enthalpy barriers could be somewhat overestimated by the AM1 Hamiltonian, the qualitative picture of the CPA catalytic mechanism described in this work is likely good enough and already includes the main key groups of the real system. In any case, additional work based on hybrid QM-MM techniques[36–41] would be helpful in order to confirm the results obtained in this paper.

Acknowledgements

Financial support from DGES through project PB95-0637 and the use of the computational facilities of the Centre de Computació i de Comunicacions de Catalunya are gratefully acknowledged.

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Received in Montpellier, France, 11*th August* 1997;
Paper 7/08751I