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REGULAR ARTICLE

Theoretical investigation of the catalytic mechanism of the protein arginine deiminase 4 enzyme

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Abstract The mechanism of the transcriptional coregulator protein arginine deiminase 4 (PAD4) in catalyzing the calcium-dependent conversion of specific arginine residues to citrulline and ammonia, was studied in the framework of density functional theory using the hybrid B3LYP exchange correlation potential and a medium-large basis set. Active site of enzyme was modeled by four key catalytic residues: Asp350, His471, Asp473 and Cys645. A guanidinium-containing derivative was considered as substrate model. Results of the present investigation support the experimental hypothesis concerning the occurrence of a tetrahedral intermediate having ammonium as leaving group, during the catalytic process. Besides, in the transition state leading to this intermediate the nucleophilic activity of the thiolate anion of Cys645 and the ability of the imidazolinium cation of His471 in giving the proton to the substrate, appear mutually enhanced. The rate determining step of the whole process occurring in gas phase was recognized in the ammonia release step. Solvation effects on the catalytic mechanism determine significant modifications as far as the energetics is concerned. The data obtained in the protein medium indicate that the kinetics of the process could be controlled by the nucleophilic attack of cysteine sulfur to the substrate carbon atom concerted with the proton shift from imidazolium ion to one of guanidinium amino groups.

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1 Introduction

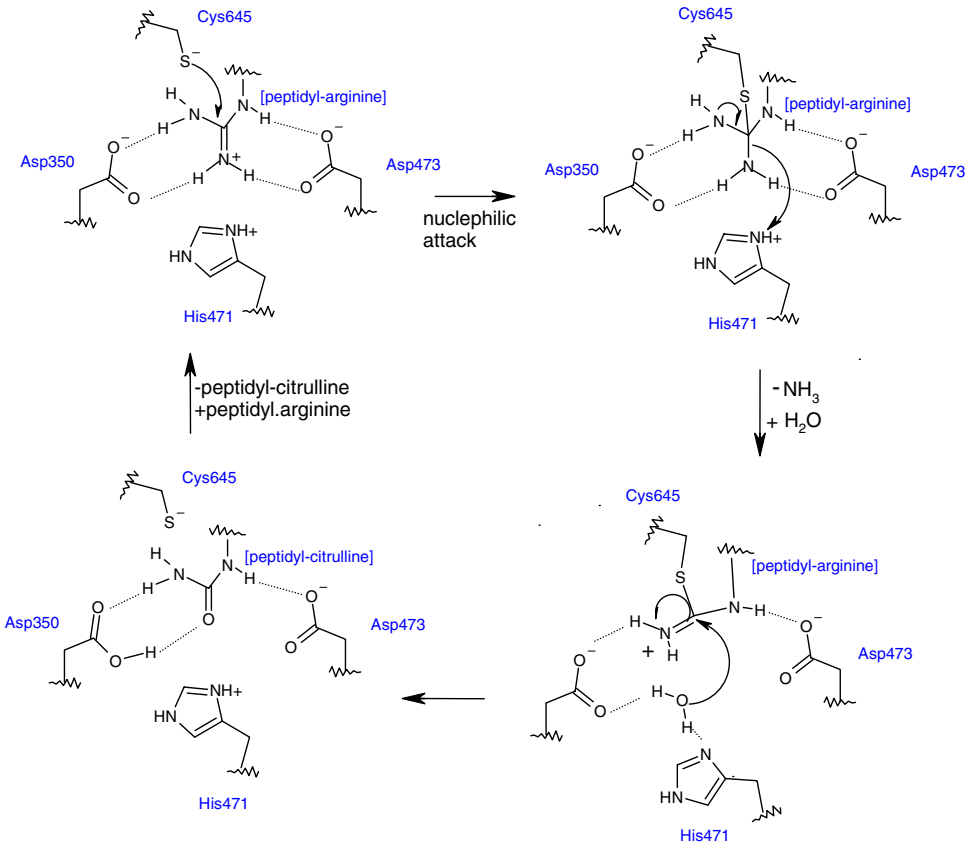
The guanidinium-modifying arginine deiminase 4 (PAD4) human protein, a 663-amino acid, 74 kDa, belongs to the amidinotransferase enzymatic family [1–3]. With respect to the other members of this family, regardless of shared active site structure, it shows fundamental differences in specific active site residues and therefore different kinetic properties [4–8].

The PAD4 was previously recognized as a dependent enzyme able to convert arginine residues to citrulline in histones and also monomethylated arginine residues to citrulline, both in vivo and in vitro [2].

So far, few types of human PADs were characterized by cDNA cloning [9] and some of them were associated with human diseases [10,11].

In particular, over the last years, various biochemical studies related the PAD4 activity to the onset and progression of rheumatoid arthritis (RA) [10,11], a chronic and progressive autoimmune disorder affecting about 1% of the adult US population and causing a mean reduction in life expectancy of 5–10 years [12,13].

More recently, PAD4 deiminating action was proven to be overactive in RA. In fact, autoantibodies recognizing citrullinated proteins, such as RA-associated mutations in the PAD4 gene [14], are specifically found at early phases of the manifestation of the disease in the majority of RA patients [15–17]. It was suggested that the deiminating activity of PAD4 is upregulated in RA patients making PAD4 an important therapeutic drug target. However, all the physiological roles played by PAD4 are not yet fully known, rather they are only beginning to be interpreted.



Scheme 1 Catalytic mechanism of protein arginine deiminase 4 (PAD4)

The knowledge of catalytic mechanism of PAD4 makes possible the identification of important mechanistic features that can be exploited for inhibitor development. In fact, PAD4 inhibitors could be used to treat an underlying cause of the disease rather than merely its symptoms.

In the work of Knuckley et al. [18], a series of specific mutations in the PAD4 gene at sites involved in substrate binding is seen as the confirmation of the essential role of Asp350 and Asp473 residues as well as that of Cys645 and His471 that exhibit nucleophilic and acid/base properties, respectively [18]. The same work reinforces the hypothesis that Cys645 exists as thiolate and His471 is protonated in the active form of the free enzyme suggesting that, as a consequence of pH values, this enzyme utilizes a reverse protonation mechanism [18] and not a substrate-assisted mechanism of deprotonation [19–21]. Such conclusions were inferred from taking into account the pH dependence of the kinetic parameters in order to identify and characterize the roles of active site residues. These studies ascertained the presence of two key ionizable groups with pKa values of 7.3 and 8.2 that should correspond to the protonation states of Cys645 and His471 before the substrate has bound to the enzyme. The assignment of these values yielded to the indication that the pKa of Cys645 and His471 are 8.2 and 7.3, respectively, that is the reverse of the standard assumption. So these residues should exist in a state of reverse protonation in the active enzyme [18]. Besides, it is worth noting that such a type of mechanism was hypothesized in a series of other investigation concerning different enzymes [19,22,23].

On the basis of the data present in literature [4,18] for PAD4 enzyme, it was possible to draw the reaction mechanism (depicted in the Scheme 1).

The working model proposes that the thiolate of Cys645 acts as nucleophile on the substrate and this species is stabilized via the formation of an ion pair with His471 similar to the thiolate–imidazolium ion pair observed in papain [24].

In this work, we present for the first time a detailed density functional study of the hydrolysis of a guanidinium-ion containing substrate by arginine deiminase 4 (PAD4), according to the mechanism suggested in literature for this enzyme. The aim of investigation was that to elucidate the catalytic function of amino acid residues and to give better insight in the kinetic and thermodynamic aspects of the reaction applying a computational protocol that was proven to be realistic enough in the description of other enzymatic mechanisms [25–29].

2 Computational details

All the computations reported here were carried out with the Gaussian03 code [30].

The geometries of the stationary points involved in the PAD4's deiminating reaction path were optimized using the hybrid Becke's [31] three parameter exchange–Lee-Yang-Parr [32,33] correlation potential (B3LYP) and using the double-z quality 6-31+G(d,p) basis set for all atoms.

Frequency calculations were performed at the same level of theory on each system with the aim to evaluate their character of minima and saddle points. Zero point energy corrections, obtained from the vibrational frequencies, were then included in all the relative energy values.

Single point refinements of B3LYP energies were obtained using the triple zeta 6-311+G(2d,2p) basis set including one diffuse function on heavy atoms and one polarization function on all atoms. The model of the active site used in our calculations was extracted from the high-resolution crystal structure deposited in Protein Data Bank (PDB entry 1WDA) [1]. Following a consolidate procedure [25–29,34,35], the coordinates of one atom of each residue were kept frozen to their crystallographic position, to avoid an unrealistic expansion of the protein. This practice can generate few small imaginary frequencies, typically on the order of $10i\ \mathrm{cm}^{-1}$ that, however, do not invalidate the ZPE.

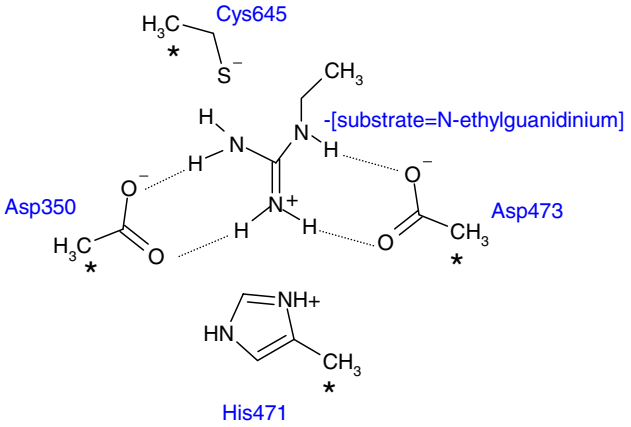
The substrate was left without any constraint during the optimization.

Solvent effects were computed at the same theory level used for optimizations as single point calculations on 6-311+G(2d,2p) equilibrium geometries, through the conductor-like polarizable continuum model (CPCM) method [36–39]. The dielectric constant value $\varepsilon = 4$ was chosen for describing the protein environment of the active site, according to previous suggestions for proteins [29,34].

3 Results and discussion

On the basis of the crystallographic structure of PAD4 [1] and following other mechanistic details present in literature [2,4,18,40], we have built up a model that includes the four key catalytic residues: Asp350, His471, Asp473 and Cys645. Arginine substrate was simulated by a guanidinium ion derivative (see Scheme 2).

Cys645 residue acts as a nucleophile in the thiolate form to give rise to a covalent *S*-alkylthiouronium intermediate that is a species encountered in other cysteine hydrolases [18]. The better candidate for the catalytically active form of the enzyme was recognized as that in which the His471 is the only positively charged residue with a charge quenched by the presence of thiolate in a sort of ion pair [24]. Actually, the PAD4 active site is decidedly anionic owing to the presence of the Asp350 and Asp473 deprotonated residues, thus, thiolate presence can be assured only by a protonated His471 on the basis of a phenomenon defined as "reverse protonation mechanism" [19,20]. However, at physiological pH, only 15% of the enzyme is present in the catalytic active form proposed [18].



Scheme 2 Model used for the simulation of PAD4 active site. *Stars* indicate atoms whose coordinates were kept fixed during optimizations

The B3LYP energetic profile of the work mechanism of PAD4 was reported in Fig. 1.

In the protein arginine deiminase 4, reaction starts with the formation of the enzyme-substrate complex (**ES**) (see Fig. 1a).

In this complex, all amino acid residues interact with the substrate giving rise to a network of hydrogen bonds whose values are reported in Fig. 2. The structural arrangement of **ES** species evidences the imidazolium ion acid character in the presence of an opportune orientation of thiolate that lies still far enough from its proton and substrate carbon atom (C_{sub}–S_{Cys} bond length is 3.948 Å). Asp350 and Asp473 residues act both as acceptors of guanidinium amino hydrogens.

The transition state **TS1** proposes the simultaneous proton shift from imidazolium ion to one of $-NH_2$ groups of substrate and the nucleophilic attack of Cys645 sulfur to the carbon atom of the substrate, after a rotation. The visualization of the vibrational normal modes suggests that this concerted action occurs at the frequency of $88i\ \mathrm{cm}^{-1}$. In **TS1**, the bond C_{sub}–S_{Cys} (2.847 Å) is forming while, the substrate $-NH_2$ group nearer to the His471 is practically already protonated as can be argued by the C_{sub}–N_{sub} distance value (1.439 Å) that appeared longer than in the **ES** complex (1.363 Å).

The energy requested to clear the barrier and to proceed with the reaction is of 6.7 kcal/mol.

This relatively value low is not surprising since, despite the involvement of a proton shift that is known as a energetic wasteful process, there is, in **TS1**, a series of hydrogen stabilizing interactions and a new incoming C_{sub}–S stable bond. Furthermore, the nucleophilic attack on the substrate carbon enhances strongly the imidazolium ion acidity making the proton transfer quite easy.

The next intermediate (**INT1**) is a tetrahedral adduct as invoked by experimental studies [4,18,36]. Ammonia leaving

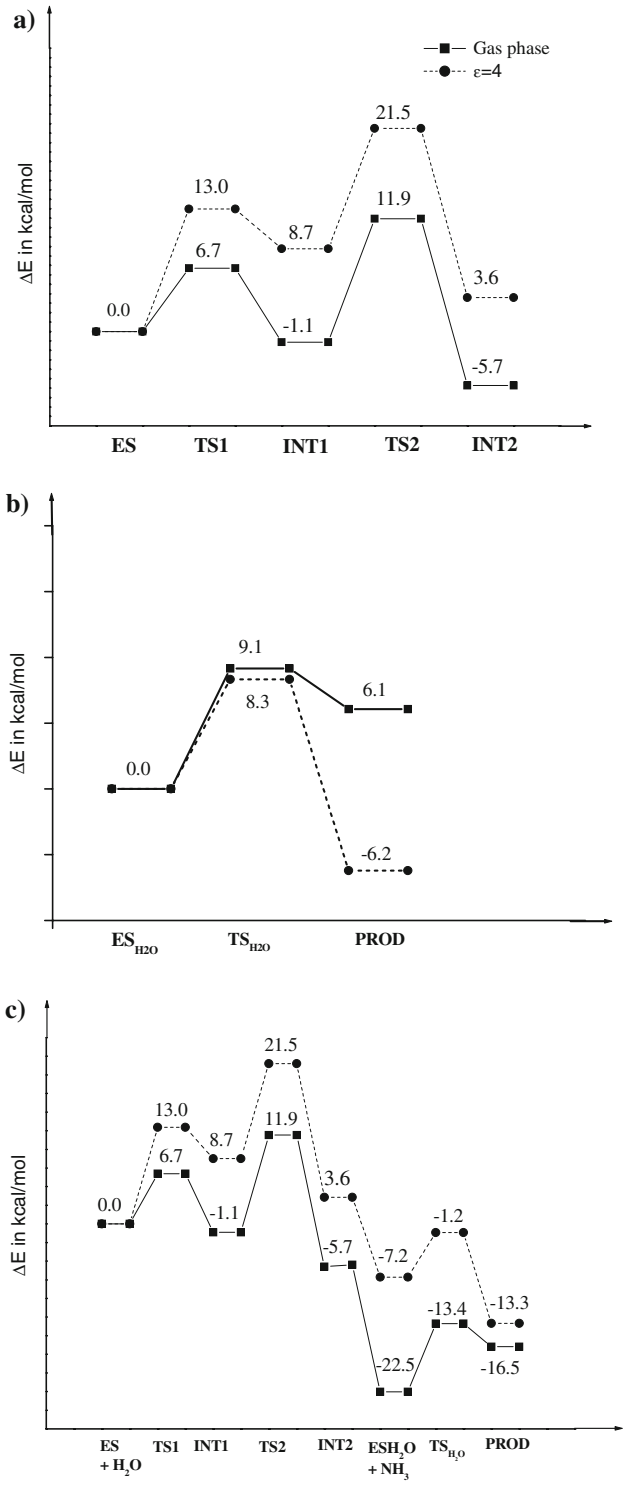


Fig. 1 **a**, **b** Energetic profiles for the two steps of the catalytic mechanism of PAD4. **c** Global energetic path

group is still bound to substrate. C_{sub}–S_{Cys} and C_{sub}–N_{sub} lengths are slightly shorter and longer than in **TS1**, respectively (see Fig. 2). **INT1** lies at 1.1 kcal/mol below the **ES** complex.

Ammonia release begins in a next step **(TS2)** in which it is possible to forecast the incipient formation of an amidino-Cys intermediate. In fact, the most significant change described by the **TS2** is the lengthening of C_{sub}–N_{sub} bond of about 0.5 Å with respect to **INT1**. Consequently, the C_{sub}–S_{Cys} length becomes shorter (1.817 versus 1.942 Å). The vibrational frequency associated essentially to the stretching of the C_{sub}–N_{sub} bond was computed to be $254i$ cm^{-1}.

The covalent nature of C_{sub}–S_{Cys} bond was confirmed by the natural bond order (NBO) analysis that indicates an overlap between two *sp* orbitals belonging to carbon and sulfur atoms.

From the energetic point of view, the ammonia release entails an expense of 10.8 kcal/mol.

In the amidino-Cys intermediate (**INT2**) obtained after the clearing of the previous barrier, ammonia is once and for all removed. In fact, its nitrogen atom lies at 4.448 Å from substrate carbon. C_{sub}–S_{cys} bond measures now 1.783 Å. The two C_{sub}–N_{sub} distances are practically equal suggesting clearly the presence of an electronic delocalization. **INT2** appears to be 5.7 kcal/mol more stable than **ES** species.

Considering that the experiment does not give information about the exact modalities with which the ammonia molecule leaves the active site, in order to allow the entrance of a water molecule, we have considered the event as if this has already happened.

The entrance of a water molecule in the active site of enzyme completes the process giving rise to the second reaction product (citrulline). The mechanism by which this occurs is illustrated in Fig. 1b. The first stationary point of the energetic profile is a new enzyme–substrate complex (**ES$_{H2O}$**) (see Fig. 3 for its geometry). The most stable **ES$_{H2O}$** adduct, obtained after exploring many other possible arrangements, proposes the water molecule placed in the middle of the two Asp350 and His471 residues. Hydrogen bonds formed between hydrogens of water and donors atoms of the above mentioned amino acids, whose length is about 2 Å, fix the water oxygen at 4.818 Å from the carbon atom of substrate. In the next transition state **TS$_{H2O}$**, the water molecule is activated. Two simultaneous proton shifts snatch hydrogens from water allowing to its oxygen atom to link quite firmly the substrate carbon (C_{sub}–O_w = 1.337 Å). At same time, the bond C_{sub}–S_{sub} becomes longer changing from a value of 1.786 Å in the **ES$_{H2O}$** to another of 2.130 Å in the **TS$_{H2O}$**.

Imaginary vibrational frequency was found to be $261i$ cm^{-1} and associated to the stretching of all incoming and outgoing bonds.

The predominant role in activating water should be ascribed to the imidazole ring of His471. In fact, a careful glance to the structural features of this transition state indicates that the hydrogen atom of water, results completely transferred to the nitrogen of imidazole as confirmed by their

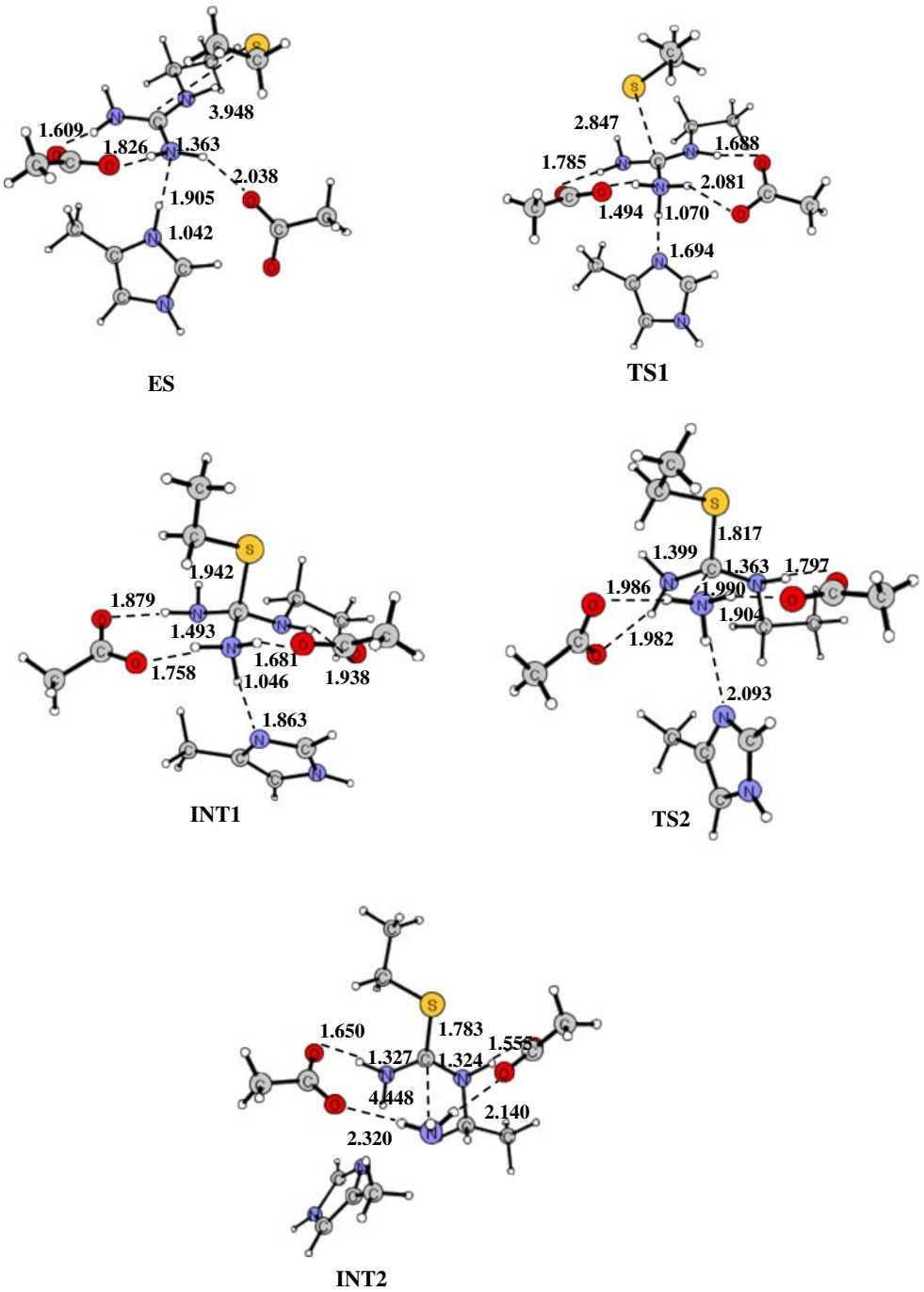


Fig. 2 Equilibrium geometries of the stationary points **ES**, **TS1**, **INT1**, **TS2** and **INT2** belonging to the portion of the path reported in Fig. 1a. Distances are in Å

reciprocal distance of 1.041 Å, while the second hydrogen is still shared between the oxygens of both Asp350 and water (O_{Asp}–H_w = 1.182 and O_w–H_w = 1.216 Å).

This finding is partially in agreement with previous literature data [1,18], where the activation of water is thought to occur in two consecutive steps involving firstly an hydrogen shift towards His471 imidazole ring, and then a further hydrogen shift towards one of Asp350 oxygens. However, we think that our result agrees well with the presence in the Asp350 residue of two negative oxygen atoms that can participate in the dehydrogenation of water at least with the same potentiality than the His471 nitrogen. On the other hand, the experimental information comes from studies concerning other enzymes belonging to the same amidinotransferase family [1–3] where often a glutammate residue, lying below and interacting with His471, serves for making imidazole nitrogen more negative than in the PAD4.

TS_{H2O} was located at 9.15 kcal/mol above the **ES_{H2O}** complex so that the corresponding activation barrier could be widely convenient for a good catalytic performance.

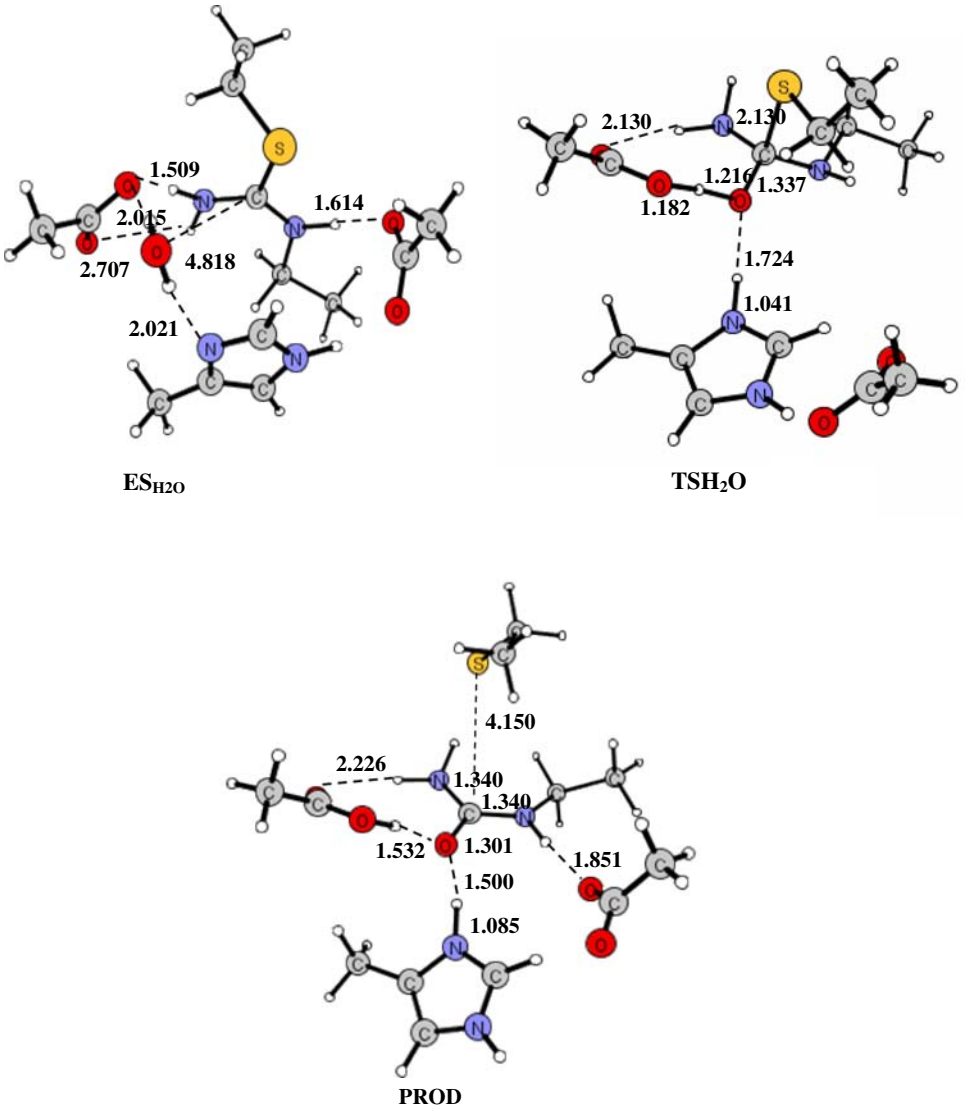


Fig. 3 Equilibrium geometries of the stationary points **ES$_{H2O}$**, **TS$_{H2O}$**, and **PROD** belonging to the portion of the path reported in Fig. 1b. Distances are in Å

The geometrical parameters of the next species (**PROD**) suggest that as a consequence of the C_{sub}–O_w bond shortening, the–SCH_2CH_3 fragment is pushed away from substrate (it lies at 4.150 Å from C_{sub}). Asp350 and His471 residues appear to be protonated and hydrogen bonded to the oxygen of C_{sub}–O_w bond. Other hydrogen stabilizing interactions involve citrulline and both aspartate residues (see Fig. 3).

The product **PROD**, that still retains the citrulline slightly anchored to active site, was found to be 6.0 kcal/mol less stable than reactants.

The energetic of the whole process can be better inferred from the combination of the in gas phase profiles referred to the previous separated steps (see Fig. 1c solid line).

As we can note, the rate limiting step in the whole path does correspond to the transition state **TS2** from which the amidino-Cys intermediate **INT2** originates. In any case, the barrier is low enough to allow the reaction's course. All the rest of the profile lies below the asymptote meaning that this part of the process is highly favored both from the kinetic and thermodynamic point of views.

The exothermicity was computed to be −16.5 kcal/mol.

3.1 Solvent effects

In order to simulate the protein environment around the active site, a dielectric constant $\varepsilon = 4$ was chosen as commonly suggested by literature [29,34].

The energetic profiles related to the first and second part of the enzymatic mechanism involving the CPCM corrections, are reported in Fig. 1a and b as dashed lines.

As it can be seen, the two parts of the energetic path are influenced in a different manner by the presence of surrounding medium. As far as the process yielding ammonia product is concerned (Fig. 1a), except the case of TS1, for which we found a destabilization of 6.3 kcal/mol, the whole profile is raised by about 10 kcal/mol with respect to that obtained

in gas phase making the reactive event slightly unfavored also by a thermodynamic point of view, the **INT2** intermediate being located at 3.6 kcal/mol above the **ES** complex. On the contrary, the second step from which citrulline is formed, occurs more quickly than in gas phase and the process becomes exothermic by 6.2 kcal/mol (see Fig. 1b). However, despite the above-mentioned raising of the path, the relative solvent effects between minima and TS appears to be quite small, in the order of few kcal/mol.

The most significant aspects related to these new data can be argued from Fig. 1c in which again we represent the whole path. Although by a small amount of energy, the nucleophilic attack of Cys645 sulfur to the substrate carbon atom concerted with the proton shift from imidazolium ion to one of $–NH_2$ groups of substrate, supersedes the ammonia's release in the role of rate determining step for the reaction. In fact, 13.0 kcal/mol and 12.8 are required to go from **ES** to **INT1** and from **INT1** to **INT2**, respectively. However, taking into account the fact that the energetic values in solvent are the result of single point calculations, we cannot exclude that the slight difference in the **TS1** and **TS2** barrier heights can derive from the lack of geometry optimization.

Overall, with respect to the gas phase and depending on the choice of the rate limiting step in solvent, further 3.4 or 3.2 kcal/mol need to ensure the reaction course in solvent. As the Arrhenius equation for the evaluation of kinetics suggests, this entails a reduction of the reaction velocity in the condensed phase. In fact, on the basis of this formula, a difference of few kcal/mol between two barriers could imply rates change by some orders of magnitude. However, the barriers remain still low enough for a catalytic mechanism. Similar to what occurs in the gas phase, in this case also the second part of the reaction is fast and energetically favorable (is exothermic by 13.3 kcal/mol) owing to the fact that all stationary points lie below the reactants asymptote.

4 Concluding remarks

The catalytic mechanism of guanidinium-modifying arginine deiminase 4 (PAD4) human protein by which citrulline and ammonia products are obtained, was examined according to the experimental proposal at B3LYP level of theory using a model to mimic the active site and the substrate.

Results can be summarized as follows:

The whole reaction is composed by two different steps. In the first one a proton shift from imidazolium ion to one of $–NH_2$ groups of guanidinium ion occurring simultaneously with the nucleophilic attack of Cys645 sulfur to the carbon atom of the substrate, generates the ammonia leaving species. Afterwards, the entrance of a water molecule activated simultaneously by both Asp350 and His471 residues, binds the substrate carbon moving away the thiolate group of Cys645 and forming citrulline.

As far as the gas phase is concerned, the process appears on the whole exothermic and the rate determining step was recognized in the **TS2** transition state belonging to the first part of the reaction and associated with the ammonia release.

Our findings are substantially in agreement with experimental hypotheses except for the fact that water molecule is activated in a single step by Asp350 and His471 amino acids through two concerted proton shifts rather than in two successive steps involving His471 before Asp350.

Computations taking into account the protein environment effects introduce significant novelties with regard to the energetics of the process. The slightly different destabilization of the species due to the solvent, determines the variation of the rate determining step with respect to the gas phase. The highest barrier appears to be associated with the nucleophilic attack and proton shift simultaneous processes occurring after the enzyme substrate complex formation but the barrier for the ammonia release is lower by only 0.2 kcal/mol. In the more drastic case, the energetic amount required to clear the hurdle was estimated equal to 13.0 kcal/mol. This value suggests that we have to expect a worse kinetic in solvent rather than in gas phase but this in all probability, should not prevent reaction from proceeding.

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