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Promiscuous Ability of Human Carbonic Anhydrase: QM and QM/MM Investigation of Carbon Dioxide and Carbodiimide Hydration

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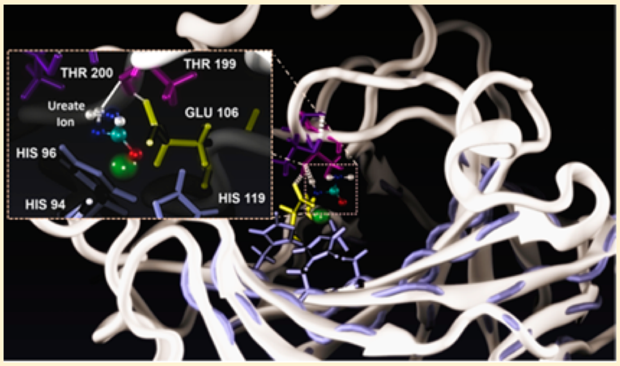
S *Supporting Information*



ABSTRACT: The hydration of carbodiimide, isoelectronic with carbon dioxide, mediated by human carbonic anhydrase (EC 4.2.1.1) was studied at theoretical level in comparison with the native substrate. Quantum-mechanical (QM) and combined quantum-mechanics/molecular-mechanics (QM/MM) approaches indicate that human carbonic anhydrase is able to catalyze also the hydration of carbodiimide to urea with an energy barrier higher than that required by the native CO_2. The rate-determining step for both substrates is represented by the nucleophilic addition to the double bond, showing the final product ureate more strongly anchored to the enzyme active site than the hydrogen carbonate. The reduced catalytic activity for the carbodiimide substrate can be ascribed not only to the highest barrier but also to the difficulty in releasing the product in favor of the water molecule, delaying the catalytic turnover as indicated by QM and QM/MM analyses.

INTRODUCTION

Carbonic anhydrase (CAs) enzymes (14 iso-enzymes) are one of the most ancient and interesting enzymatic families involved in different biological processes.[1,2] Between the CAs, the human Carbonic Anhydrase II (hCAII), which catalyzes a simple but very important physiological reaction, the reversible interconversion of CO_2 to HCO_3^-, is one of the most widely studied enzyme from both experimental and theoretical points of view. In fact, the presence in the literature of thousands of scientific papers devoted to this system accounts for its importance in chemistry, biology, and other fields.[2,3] Despite this impressive amount of interest, hCAII continues to reserve surprises to the researchers since this system can be considered one of the first examples of enzymes showing broad promiscuous catalytic activity.[4−7] In fact, besides the native CO_2, it is able to process substrates with different structures (substrate promiscuity), to catalyze distinct chemical reactions (catalytic promiscuity), and to be still efficient when the native Zn^{2+} metal center is replaced with other metal ions.[8−10] As mentioned above, whereas the mechanism of CO_2 transformation in bicarbonate was extensively studied, it is still not fully clear how the enzyme catalyzes the hydration of carbodiimide (and its tautomer cyanamide) to urea. Carbodiimide is a noncompetitive inhibitor toward the esterase activity of hCAII but, at the same time, can be catalyzed by hCAII, giving rise to the urea product.[11,12] It is interesting to note that at the end of this reaction the catalytic cycle is sensibly turned down. X-ray crystallographic and spectroscopic studies[13] reveal the presence of the reaction product (ureate) in the catalytic site and the binding mode of the carbodiimide within the hCAII active site. A qualitative reaction mechanism for the hydration reaction of carbodiimide was proposed,[13] but the energetic details are still lacking; thus, the reduction of the catalytic efficiency with respect to the native substrate remains an open problem. To the best of our knowledge, while the hydration of CO_2 by hCAII was studied at the quantum-chemical level by employing a cluster model,[14−17] theoretical studies on the cognate substrates do not exist in literature. Some previous works were devoted to the hydration of carbodiimide itself in the gas phase and in solution.[18,19]

In the present work, detailed potential energy surfaces (PESs) for the reaction catalyzed by hCAII toward carbodiimide were obtained by using both a quantum-mechanical (QM) cluster model[20−24] and quantum-mechanics/molecular-mechanics (QM/MM)[25−28] approaches. Because of the lack in the literature of a PES at the QM/MM level for the hydration of native CO_2 substrate, we have studied also this reaction.

COMPUTATIONAL METHODS

For both QM and QM/MM our own *n*-layered integrated molecular orbital and molecular mechanics (ONIOM) calculations, the Gaussian03 package was used.[29] Geometry optimizations were

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performed using the hybrid density functional B3LYP[30,31] associated with the 6-31+G (d,p) basis set. More accurate energies were then obtained by single-point 6-311+G(2d,2p) calculations carried out on the previously optimized geometries. The use of the B3LYP functional is largely established in the literature.[20−24,32] In QM calculations the effects of the protein surroundings were mediated by using the popular polarizable continuum model (PCM)[33,34] previously tested in a huge number of papers.[20−24,32,35] As requested by one of the referees, to further verify the reliability of the B3LYP functional, we redid the computation of the rate-determining barrier by using the PBE0 exchange-correlation one.[36] Results for both of the considered substrates give barriers of 4.5 and 17.7 kcal/mol for CO_2 and carbodiimide, respectively. For the rate-determining barrier universal solvation method density (SMD)[37] method was also used. Comparison shows very similar results (19.3 versus 20.8 kcal/mol at PCM and SMD levels, respectively). In both PCM and SMD, the suggested value of 4 was assumed for the dielectric constant of the protein environment.[32] In the case of ONIOM, the remaining portion of the protein, where the chemical events (formation and breaking of bonds) do not take place, is explicitly included in the MM region. To verify the nature of the minimum or maximum along the PES and to obtain the thermochemical analysis, frequency calculations were accomplished at the same level of theory used for optimizations. With the aim of assessing whether the localized transition states (TSs) correctly connect to the corresponding minima along the imaginary mode of vibration, the intrinsic reaction coordinate (IRC)[38] method was used. For TSs the vibrational mode associated with the imaginary frequency was checked. Natural bond order (NBO) analysis was performed on every stationary point of the PESs.[39]

To restore the active enzyme, water exchange with both the bicarbonate and ureate products was also investigated at the QM level. For this purpose, relaxed PES scans (with geometry optimization at each point), using 10 steps with a step size value of 0.1 Å on the Zn−O_w distance, were performed. Results are reported in Supporting Information.

For both QM and QM/MM calculations the structure of the enzyme was retrieved from the crystallographic structure.[40] The cluster for QM study was built considering the amino acid residues directly and indirectly involved in the catalytic mechanism. More specifically, it includes the Zn^{2+} cation with its four primary ligands of a water molecule and three histidine residues (His94, His96, and His119) and the hydrophilic binding cavity formed by Glu106, Thr199, and Thr200 residues belonging to the outer coordination sphere. Their presence in the catalytic pocket is strictly related to the management of the substrate in a way that is correct and suitable for establishing favorable interactions with the environment surrounding the active site. Another water molecule (so-called deep water) was incorporated into the cluster. Following a consolidating procedure,[20−24,32,35] in the QM cluster, all the amino acid residues were truncated to reduce the size of the model, and an atom for each residue was fixed during the geometry optimizations (circled in Scheme 1).[20−24,32]

To further verify the goodness of this approximation, we also optimized the first intermediate (INT1) of the carbodiimmide reaction by using the entire amino acids. Comparison between the two structures shows good agreement in the main structural parameters (see Supporting Information, Figure S1).

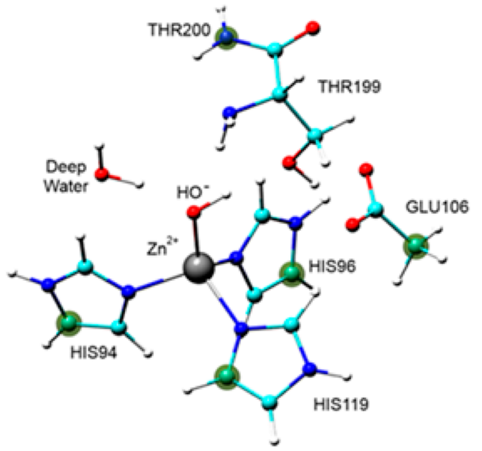
In the QM/MM computations, starting from the crystallographic structure,[40] the QM region includes the first and second coordination shells around the zinc cation (His94, His96, His119, Glu106, Thr199, and Thr200) and a deep water molecule. The remaining portion of the enzyme, along with 219 water molecules, is described at the MM level.

■ RESULTS AND DISCUSSION

The comparison of the PESs of the reaction mechanism for two considered substrates by means of the combined use of QM and QM/MM approaches can allow the rationalization of the “promiscuous” activity of this enzyme and could evidence some reasons for which the enzymatic turnover is reduced in the case of carbodiimide hydration. The carbodiimide tautomer was chosen as initial substrate since previous studies in the gas phase have demonstrated it to be the more stable one.[19]

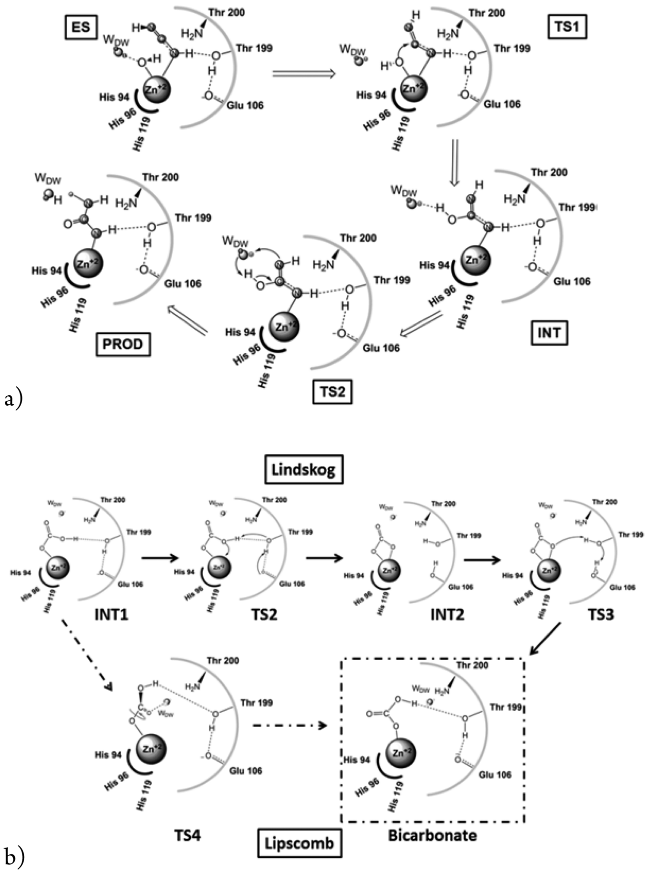
The reaction mechanism derived by the available experimental data[2,41−44] and by previous theoretical[14−16,45−48] studies on the native substrate was followed in the carbodiimide substrate (Scheme 2). In this mechanism, the carbodiimide

Scheme 1. Catalytic Site Model Used in the QM calculations[a]



[a]The circled atoms were frozen during the optimization procedure.

Scheme 2[a]



[a](a) Proposed reaction mechanism for addition of water to the carbodiimide; (b) Lipscomb and Lindskog mechanisms followed in the case of CO_2 substrate.

along with the hydroxyl group (OH^- arising from the water ligand) is coordinated to the zinc ion. In such a way, the nucleophile species (OH^-) is ready to perform the nucleophilic attack on the carbodiimide substrate, generating a pentacoordinated adduct. The formed intermediate suffers a proton shift at the expense of a water molecule that gives rise to the ureate anion as final product. For both substrates the activated water (Zn−OH) was used as nucleophile agent responsible for the attack on the substrate and not the neutral water as suggested by Thoms.[44] In fact, recent calculations[16] based on the use of zinc-bound water rather than zinc-bound hydroxide did not support Thoms' hypothesis, because the present hydrogen bond network is not sufficient to enhance either the electrophilic character of the carbon on CO_2 or the nucleophilic character of the metal-bound water. In the case of CO_2 hydration, both the Lipscomb mechanism, favoring a proton shift between the two oxygens,[45] and the Lindskog one,[46] favoring a rotation step, were taken into account (see Scheme 2b). In Figure 1 are shown the PESs obtained for both studied substrates at QM (solid line) and QM/MM (dotted line) theoretical levels.

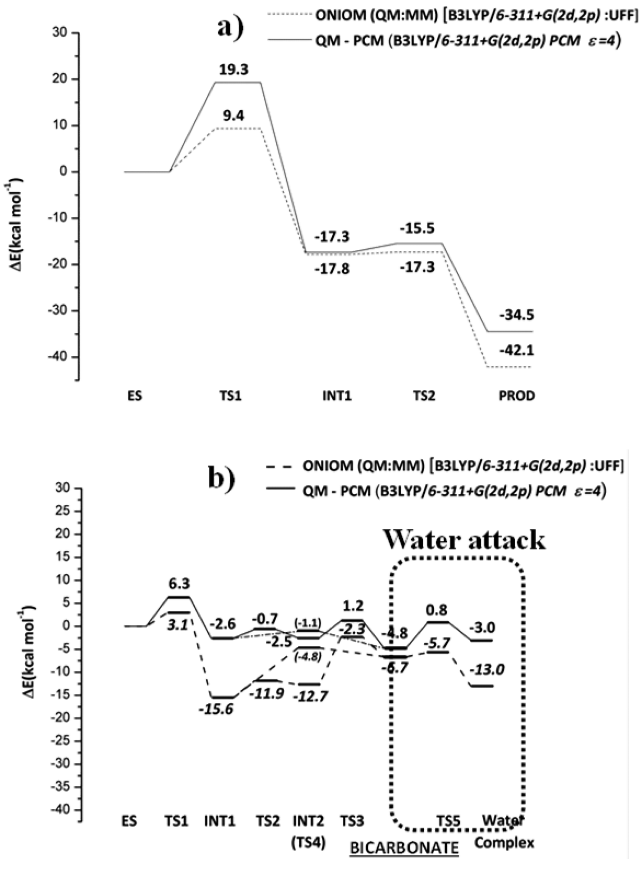


Figure 1. PESs for the hydrolysis of carbodiimide (a) and carbon dioxide (b) by hCAII at QM and QM/MM levels.

Enzyme−Substrate Complexes. In both QM and QM/MM structures of the Michaelis−Menten complex (ES), the carbodiimide substrate is coordinated to the zinc ion by nitrogen atom N1 (the Zn−N1 distance = 2.759 Å), giving a pentacoordinated complex (Figure 2). In the case of natural substrate (CO_2) the ES structure is different, since the CO_2 lies at a Zn−O1 distance of 3.63 Å. This finding agrees with the cryocrystallographic study[13] where the carbodiimide, in spite of owing isoelectronic and isostructural with the native substrate, binds with the active site in a different manner from CO_2. In addition, in the case of carbodiimide, the ES is stabilized by a network of hydrogen bonds with the surrounding groups (Thr199, Thr200, and the water molecule, Figure 2).

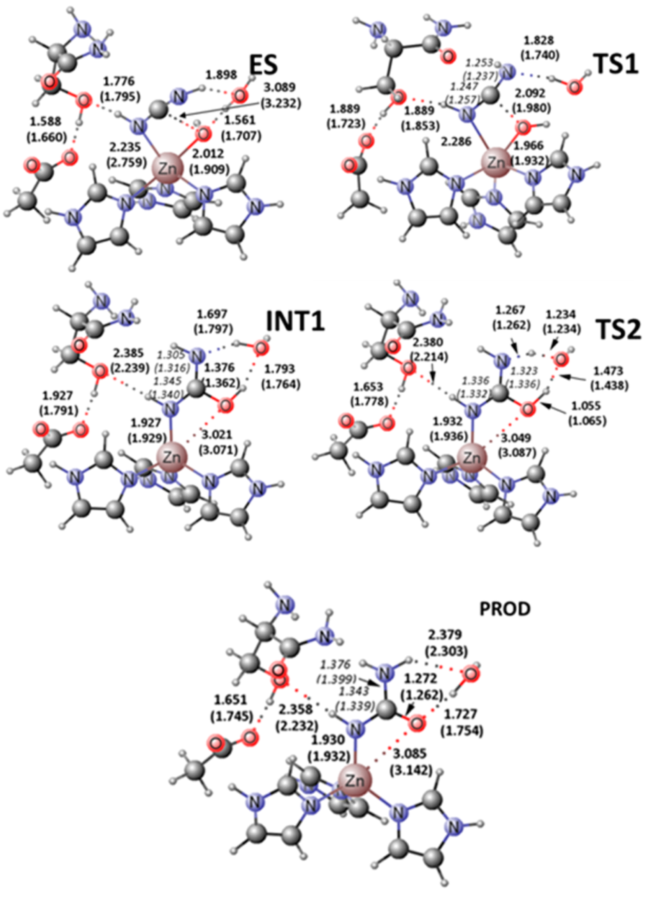


Figure 2. Optimized structures of the stationary points along the PES of carbodiimide. All bond lengths are given in Å. Values refer to the QM and the QM/MM approach, in parentheses.

Nucleophilic Attack. The subsequent stationary point on the reaction path (Figures 1 and 2) is the transition state (TS1) for the nucleophilic attack on the carbon of two substrates. The distance of the nucleophilic OH group to the substrate carbon atom is 1.980 Å for carbodiimide, indicating that the C−O bond is starting to occur. The corresponding distance in CO_2 is 1.750 Å. The occurred activation of the linear geometries characterizing the two considered substrates is also indicated by the valence angle value that is 152.2° in carbodiimide and 146.5° in CO_2, at QM/MM level. The imaginary frequency of 161.5*i* and 321.1*i* obtained in CO_2 and carbodiimide, respectively, is clearly associated with the stretching of the C−OH moiety describing the nucleophile attack by OH group on the electrophile atom of the two considered substrates ($O{=}\underline{C}{=}O$ and $HN{=}\underline{C}{=}NH$).

In the TS1 of carbodiimide, the Zn−N1 (nitrogen atom of the substrate) is 3.825 Å, while the corresponding value in ES is 2.286 Å. This is because the carbodiimide is rotated (about 60°) with respect to the position assumed in the ES. The hydrogen bond network present in the ES is retained, although with longer distances, and contributes to stabilize the TS1. Furthermore, in this species is observed an elongation of the two C−N bonds (C−N1 1.257 Å and C−N2 1.237 Å) (Figure

2). The same behavior is observed in the corresponding C−O bonds in CO_2 activation.

From the PES in Figure 1, the activation barrier (TS1) with respect to the energy of the reactants (9.4(3.1) kcal/mol at the QM/MM level and 19.3(6.3) kcal/mol at the QM level for carbodiimide (CO_2)) represents the rate-determining step (RDS) of the reaction at both levels of theory used and for both investigated substrates. The calculated barrier value for processing the carbodiimide (9.4 kcal/mol) shows that hCAII is able to hydrolyze the substrate with acceptable kinetics for an enzyme-catalyzed reaction.

Referring to CO_2, the corresponding barrier proposed by Bottoni et al.[14] in his QM cluster calculations was 0.6 kcal/mol, which became 7.6 kcal/mol in a most recent paper,[16] but in both works the TS is characterized by a longer distance (2.191 and 2.030 Å) between the oxygen hydroxyl group and carbon atom with respect to our TS geometries (1.850 and 1.750 Å at QM and QM/MM levels, respectively). For CO_2 substrate a comparison with the experimental value is possible since it is possible to extract from the measured available kinetic data (turnover number, 10^6 s^{-1}) the corresponding activation barrier (4.02 kcal/mol). If we consider the corresponding barrier computed at QM and QM/MM levels (6.3 and 3.1 kcal/mol, respectively), we can conclude that the QM/MM value is closer to the experimental data. In any case the obtained value for QM barrier gives deviations of about 2 kcal/mol, which falls in the range of error previously established for QM cluster calculations.[49]

We note that the barrier obtained at QM level is 9.9 kcal/mol higher than the corresponding QM/MM one. If we consider that in the case of natural substrate the QM barrier (TS1) is overestimated and the QM/MM one is underestimated with respect to experimental counterpart, the difference between the energy barriers computed employing the two computational protocols should decrease. Furthermore, we underline that this difference was found also in other enzymes,[28] and in our case it is essentially due to the more extended H-bond network present in QM/MM that contributes to stabilize the TS1. On the basis of our two results it seems that QM/MM gives better results, but this conclusion cannot be generalized without additional evidence that is consistent with these findings.

Lipscomb and Lindskog Mechanisms. The next stationary point suggested by the intrinsic reaction coordinate procedure is INT1, where the C−O bond is completely formed. NBO analysis shows that this bond is covalent in nature with the carbon and oxygen atoms overlapping their s(25%) p(75%) and s(32%) p(68%) orbitals for both substrates. The same analysis for carbodiimide reveals as charge values on the nucleophilic species (OH^-) suffer a drop in going from the starting complex (−1.310 |e|) to the INT1 (−0.808 |e|) as generally occurs in a nucleophilic attack. The energy stabilization of INT1 related to the Michaelis complex is 17.8 kcal/mol (Figure 1). After the INT1 species, the catalytic reaction of hCAII or the Lipscomb or Lindskog mechanism (see Scheme 2b) can follow, but in the case of HN═C═NH the Lindskog mechanism must be ruled out to make possible the ureate formation. Consequently in carbodiimide after INT1 formation the catalytic process evolves toward the ureate (PROD) throughout the Lipscomb-like TS (TS2, see Scheme 2a). In this step the assistance of a deep water molecule is indispensable for acting as proton acceptor and proton donor contemporaneously (see TS2 Figure 2). The proton shift concerning the NH group is characterized by the distances of 1.262 Å (N···H) and 1.234 Å (H···O), while the other one presents a pronounced shortening of the O(water)−H (1.438 Å compared to that present in INT1 (1.764 Å). The analysis of the calculated imaginary frequency (1097.2*i* cm^{-1}) properly depicts the reorganization just described. The TS2 energy (−17.3 kcal/mol) lies below the energy of reactants, which underlines that the interconversion between the two ureate tautomer forms, present in the INT1 and PROD species, can occur without a great energetic effort. The computed PESs at the QM and QM/MM levels clearly indicate the reaction is highly exothermic (−42.1 kcal/mol at the QM/MM level and −34.5 kcal/mol at the QM one), meaning that the ureate product is sensibly stabilized in the active site. Referring to the geometrical parameters, the ureate in PROD shows coordination to the Zn^{2+} by deprotonated nitrogen (N1) (1.932 Å) and by carbonyl oxygen (3.142 Å). The NBO charge values of the nitrogen (N1) show its negative increase on every point of the PES. The N1 of ureate remains engaged in hydrogen bonds with the side chain of Thr199, while the N2 with the side chain of Thr200, water, and the oxygen atom of ureate is mainly implicated in the hydrogen bond with the water molecule. This arrangement agrees well with that observed in the experimental counterpart.[11,13]

As mentioned above, the mechanism proposed for HN═C═NH differs from the CO_2 one in the number of elementary steps. In fact, in the case of CO_2, starting from INT1, the Lipscomb or Lindskog mechanism can occur with activation barriers lying below the energy of reactants (see Scheme 2b). The one-step Lindskog mechanism involves a rotation around the Zn−O1 bond with simultaneous breaking of the Zn−OH bond (TS4) giving rise to the rearranged bicarbonate fragment in the active site. The PES in this region is flat enough due to the low barriers found in both theoretical levels used (−6.7 and −4.8 kcal/mol at QM/MM and QM level, respectively, related to the ES). In the Lipscomb mechanism the rearrangement of bicarbonate takes place via a double proton transfer mechanism involving the Thr199 that acts as a proton shuttle. In particular, the TS2 describes a proton transferred from the original hydroxide oxygen to the oxygen of the OH group of the Thr199 that in turn transfers its proton to the Glu106. The energy barrier for this step is low at both levels of theory (3.7 and 1.9 kcal/mol at QM/MM and QM level, respectively, obtained with respect to the INT1 species).

The INT2-TS3-bicarbonate step describes the double proton transfer: from Glu106 to Thr199 and from Thr199 to the bicarbonate moiety. The barrier for the second proton transfer (TS3) is 10.4 and 3.7 kcal/mol at QM/MM and QM levels, respectively. On the basis of the calculated TS4 (Lindskog) and TS2/TS3 (Lipscomb) activation barriers, it is evinced that the competition between the two possible rearrangements is still feasible, contrary to what was proposed by a recent QM work on the catalytic mechanism of hCAII.[16]

Since all the calculations indicate a great stability of the ureate with respect to HCO_3^- on the active site of the enzyme, we have evaluated the BEs (enthalpy values) at the QM level. Results show that the BE for ureate is 62.9 kcal/mol, while the corresponding one for HCO_3^- is 42.6 kcal/mol. These data suggest that the release of the product is more difficult when hCAII explicates its promiscuous activity catalyzing the carbodiimide. This is not an innocent consequence because of the difficulty in restoring the enzymatic turnover.

Attack of the Water Molecule. For both native and promiscuous substrates the last step of the reaction closing the catalytic cycle and describing the displacement of the products (ureate and bicarbonate anions), respectively, by water molecule was investigated. For CO_2 the PES concerning this step is evidenced in Figure 1b. In the 4-coordinated carbonate complex at both QM (QM/MM) levels the entering water lies at 1.967 (1.949) Å from the oxygen of the bicarbonate moiety and at 3.934 (3.706) Å from the Zn ion. The water attack leads to the formation of the pentacoordinated water complex throughout the TS5 located at 0.8 (−5.7) kcal/mol relative to the reactant. This TS is characterized by a water oxygen−Zn^{2+} distance of 2.608 (2.806) Å, and the HCO_3^- is now at 1.986 (1.958) Å from the Zn^{2+}; the H-bond network involving the water and neighboring atoms both of the carbonate 1.945 (1.841) and of Thr199 1.862 (2.771) is more appropriate (Figure 3). In the obtained pentacoordinated zinc complex (water complex) both the water and the bicarbonate are firmly bonded to the metal (the two O_w−Zn and O−Zn bonds are 2.256 (2.365) and 2.043 (2.011) Å, respectively (Figure 3). This intermediate lies at only 3.0 (13.0) kcal/mol above the reactants; thus, the reaction can proceed, releasing the bicarbonate and consequently restoring the catalytic cycle. A different behavior was observed in the carbodiimide. In fact, all the attempts to localize a water−ureate final complex failed at both the QM and QM/MM levels. As previously noted this intermediate is strongly due to the presence of Zn−N covalent bond as indicated by the NBO analysis. The potential energy scan on the Zn−O_w bond at the QM level for both substrates is provided in Supporting Information, Figures S2 and S3. The scan for carbodiimide reveals that as the water approaches the Zn^{2+}, the His119 is detached by the zinc coordination sphere. Figure 3 shows that at a distance of 2.203 Å (similar to that found in the case of carbonate) the Zn−NHis119 one becomes 2.512 Å. In the case of the pentacoordinated complex with the bicarbonate the corresponding Zn−NHis119 distance is 2.043 (2.075) Å at QM (QM/MM) levels. These findings suggest that the release of the reaction product in the carbodiimide reaction is more difficult. Considering that the barriers for the RDS for the two reactions (9.4 and 3.1 kcal/mol for carbodiimide and CO_2, respectively at the QM/MM level) are not so large to justify the different catalytic efficiency between the two substrates, we think that the QM and QM/MM results indicate that the release of the product should be considered for a more convincing explanation of the reduced turnover for the carbodiimide substrate.

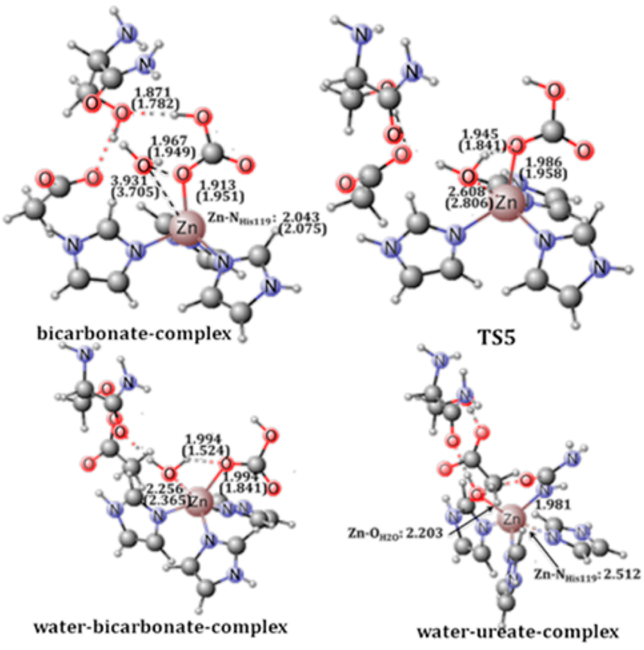


Figure 3. Optimized structures of the stationary points along the PES of CO_2 during the attack of the water molecule. Values refer to the QM and the QM/MM approach, in parentheses. Structure of the "water−ureate complex" obtained from the scan on the Zn−O_w bond at QM level. All bond lengths are given in Å.

On the other hand, as previously suggested,[50,51] enzymes reach maximum efficiency when the barrier in the RDS is lower, and when they do not reach maximum efficiency, they allow a higher stabilization of the intermediates. Our study also underlines that the stabilization of the final product should be an important step in the energetic behavior of the catalyzed reaction and more specifically in the restoring of the catalytic cycle.

■ CONCLUSIONS

QM and QM/MM methods were employed in studying, at atomistic level, the promiscuous enzymatic activity of hCAII toward the natural substrate (carbon dioxide) and the carbodiimide one. The comparative analysis of the PESs reveals interesting features that contribute to highlight the promiscuous activity of this enzyme. The following conclusions can be outlined:

All calculations show that, for both substrates, the rate-determining step is the nucleophilic attack, with a higher energy barrier in the case of the carbodiimide substrate. In particular, the QM/MM barrier is found to be 9.4 and 3.1 kcal/mol for carbodiimide and carbon dioxide, respectively. This result suggests a lower kinetic constant for carbodiimide catalysis.

Both levels of theory evidence a great thermodynamic stability of ureate product in the carbodiimide PES. The great difference in BE value (62.9 kcal/mol vs for 42.6 kcal/mol for HCO_3^-), obtained at the QM level, suggest that the release of the ureate product cannot easily occur.

Contrary to the case of bicarbonate product formation, for which we were able to ascertain substitution with a new water molecule, indispensable for the restoring of the catalytic cycle, for the case of ureate formation we fall in the determination of the pentacoordinated complex that is the crucial intermediate for the coordination of water and the release of the reaction product. This suggests that the release of the ureate should be more difficult, causing reduced turnover when hCAII carries on its promiscuous activity toward the carbodiimide substrate.

In addition, our QM and QM/MM comparison of both computed reaction PESs evidence quantitative differences in these two cases. In particular we underline that the QM/MM energy barrier for the rate-determining step is about half that of the corresponding QM value.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Cartesian coordinates of all the stationary points on the PES of carbodiimide and CO_2 hydration at QM level are reported in Tables S1 and S2, respectively. The optimized QM/MM Cartesian coordinates are available upon request. The INT1 species optimized at the QM level by taking into account different truncations of the amino acid residues are displayed in Figure S1. The results of NBO calculations performed on the bicarbonate and ureate products are shown in Figure S2. The relaxed scans on the $Zn^{2+}-O_w$ distance of the bicarbonate and ureate species are illustrated in Figures S3 and S4, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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