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Review

Carbonic Anhydrases: Versatile and Useful Biocatalysts in Chemistry and Biochemistry

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Abstract: Metalloenzymes such as the carbonic anhydrases (CAs, EC 4.2.1.1) possess highly specialized active sites that promote fast reaction rates and high substrate selectivity for the physiologic reaction that they catalyze, hydration of CO_2 to bicarbonate and a proton. Among the eight genetic CA macrofamilies, α-CAs possess rather spacious active sites and show catalytic promiscuity, being esterases with many types of esters, but also acting on diverse small molecules such as cyanamide, carbonyl sulfide (COS), CS_2, etc. Although artificial CAs have been developed with the intent to efficiently catalyse non-biologically related chemical transformations with high control of stereoselectivity, the activities of these enzymes were much lower when compared to natural CAs. Here, we report an overview on the catalytic activities of α-CAs as well as of enzymes which were mutated or artificially designed by incorporation of transition metal ions. In particular, the distinct catalytic mechanisms of the reductase, oxidase and metatheses-ase such as de novo designed CAs are discussed.

Keywords: carbonic anhydrase; metalloenzyme; reductase; artificial enzyme; oxidase

1. Introduction

Enzymes play a pivotal role in the life of every living organism by efficiently catalyzing a large number of biological reactions and thus assuring that metabolic needs are met. In some cases, the rate of biotransformation is increased up to 10^{17}-fold [1]. Often the elevated catalytic turnover of enzymes requires minimal energy to happen and in comparison to the uncatalyzed chemical transformation, no influences on the equilibrium constants are encountered, whereas a residual amount of side products is formed, and the stereospecific information within starting materials and/or products are highly conserved. These features have attracted researchers in all chemistry fields due to the possibility of developing enzymes capable of catalyzing transformations difficult or impossible to perform using classical synthetic organic tools. The appealing properties of the enzymes prompt researchers to both look for new applications of the naturally occurring ones and to re-engineer them in order to generate new bio-inspired catalysts, possibly endowed with novel and features not previously reported [2–5]. Among all known proteins, Nature offers a well-studied superfamily of metalloenzymes, the carbonic anhydrases (CAs, EC 4.2.1.1), which have genetically evolved independently into at least eight families (i.e., the α-, β-, γ-, δ-, ζ- η-, θ- and ι-CAs) according to a convergent evolution process [6–13]. The α-CAs are present in vertebrates, protozoa, algae, cytoplasm of green plants and in many Gram negative bacteria [6,7,14]; the β-CAs are found in both Gram negative and positive bacteria, algae and chloroplasts of mono- as well as di-cotyledons, and also in many fungi and some *Archaea* [6–10,15]. The γ-CAs are found in *Archaea*, cyanobacteria and most types of bacteria [8,11,16],

the δ-, ζ- and θ-CAs seem to be present only in marine diatoms [9,10,13], whereas the η-Cas are present in protozoa [12]. Finally, ι-CAs were discovered in marine phytoplankton but are also present in bacteria [17]. The present review outlines the different catalytic mechanisms of natural and engineered CAs and their application within different chemistry fields.

2. Catalytic Mechanism of Carbonic Anhydrases

CAs were first discovered in 1933, when the erythrocytes were observed to contain stoichiometric amounts of zinc and an abundant protein (later denominated carbonic anhydrase), which was also proven essential for the enzymatic activity of CO_2 hydration [18]. CAs are ubiquitously expressed within all life kingdoms and, as mentioned above, are encoded by eight distinct gene families. All of them differ for their catalytic activities, subcellular localizations, and tissue distributions. All the catalytically active CAs reversibly hydrate carbon dioxide to the bicarbonate ion and a proton. Although this reaction may also occur spontaneously, at physiological pH values it is too slow to meet the metabolic needs of most organisms/cells. The α-CAs catalytic mechanism occurs in two main steps according to Figure 1 [19].

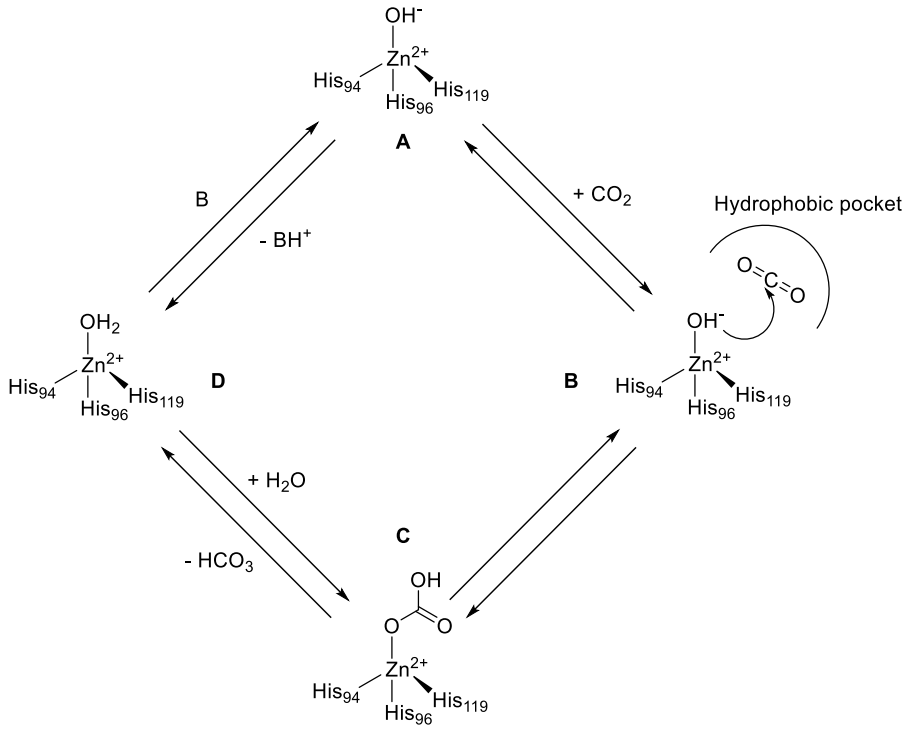

Figure 1. Catalytic mechanism of reversible hydration of CO_2 to HCO_3^- and a proton in the presence of α-CAs.

The first step is the nucleophilic attack of the Zn^{2+}-bound hydroxide ion to a CO_2 with the consequent formation of the enzyme-HCO_3 adduct (**B** to **C**), which is thereafter displaced from the active site by a water molecule (**C** to **D**). The last step (**D** to **A**), which is the kinetically rate limiting one, regenerates the catalytically active Zn^{2+}-bound hydroxide ion through a proton transfer reaction from the Zn^{2+}-bound water molecule to an exogenous proton acceptor or to an active site residue (Figure 1). CO_2 is not only an essential molecule in physiological processes, but also one of the principal products in combustion reactions, being produced in a large quantity by anthropogenic emissions, becoming thus one of the most significant and long-lived greenhouse gases in the earth's atmosphere. As a result, CO_2 capture and sequestration is becoming a critical point and has started to be highly investigated in the last decade. Recently, CAs have been reported to have the potential to accelerate CO_2 capture from large combustion emitters [20]. However, the poor stability and activity of mammalian CAs

under the harsh conditions of these processes such as temperatures ranging from 50 °C to over 125 °C, the high concentrations of organic amines, trace contaminants such as heavy metals, sulfur and nitrogen oxides, have drastically limited their use [21]. Approaches to overcome these limitations have included sourcing CAs from thermophilic organisms, or the use of protein engineering techniques to create thermo-tolerant enzymes [22–25]. However, CAs are not only highly effective catalysts for the interconversion between carbon dioxide and bicarbonates, but show catalytic versatility, participating in several other hydrolytic processes, which are reported below in this review. To date, only α-CAs and a few β-CAs were reported to have other catalytic functions than the CO_2 hydration one.

3. Hydrolysis Reactions Catalyzed by CAs

In addition to the physiologically relevant reaction, α-CAs were reported to catalyze other reactions involving carbonyl systems such as the hydrolysis of esters [26,27]. Catalysis takes place in a 15-Å wide by 15-Å deep hydrophobic pocket, close to the zinc ion, that can accommodate ligands much larger than CO_2. This esterase activity probably stems from the mechanistic similarity between hydration of CO_2 and hydrolysis of an ester, i.e., nucleophilic attack by a zinc-coordinated OH^- ion to the CO and the stabilization of the resulting oxyanionic intermediate, as shown in Figure 2.

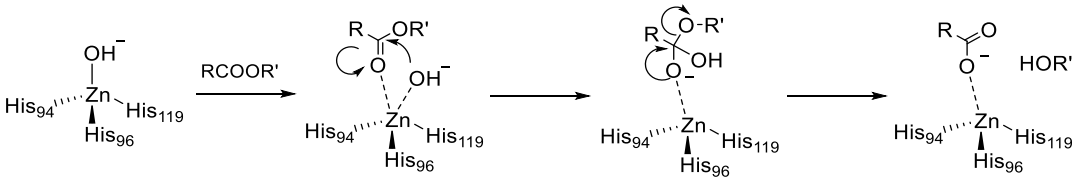

Figure 2. Esterase Activity of carbonic anhydrases (CAs).

In several studies, the increase in the esterase activity through different mutations in the CA active site was investigated, which led to a 40-fold improvement in the activity towards several carboxylic acid esters [28,29]. These mutations enhanced the activity principally through steric adaptation of the engineered CA active site, which enhanced the ability to accommodate bulkier ligands. In addition, in the last 10 years this activity was used to generate novel and highly selective CA inhibitors targeting the tumor associate isoforms (CA IX and XII). Indeed, the coumarins and their isosters were shown to act as prodrug inhibitors. Such compounds undergo the hydrolysis of the lactone ring due to the esterase activity of these enzymes [30–33]. Recently, Supuran's group also showed CAs to possess thioesterase activity [34], whereas in the last year, CAs were found to hydrolyze selenoesters, acting in the same manner with regard to coumarin scaffolds, and generate CA inhibitors of the selenol type [35].

The hydration of other carbonyl systems was not limited to esters, but was extended to aldehyde moieties [36,37]. The reaction follows pseudo-first-order kinetics and is enhanced when the enzyme is in imidazole buffers, which probably act as CA activators [38].

The availability within the CAs catalytic site of a strong nucleophile, the Zn(II)-bound hydroxide ion, gives the opportunity to hydrolyze other molecules such as cyanamide. This is a linear molecule isoelectronic with CO_2, and the CA-catalyzed hydration leads to the formation of urea, bound as a ureate anion to the Zn(II) from the enzyme active site. The conversion of cyanamide to urea is a very slow reaction and it proceeded until a stoichiometric amount of enzyme-urea adduct was formed [39,40].

In recent years, the hydrolysis reaction of CAs has been employed to generate hydrogen sulfide (H_2S). A small gaseous biomolecules such as H_2S has attracted significant attention due to its important physiological role as a signaling molecule. Both endogenous H_2S synthesis and exogenous H_2S administration exhibited promising protections in different stages of diverse diseases [41]. In this scenario, researchers modulated biological levels of H_2S developing novel class of H_2S-donor molecules by engineering the release of carbonyl sulfide (COS) from different scaffolds (Figure 3), as an intermediate which can be quickly converted to H_2S by CAs [42,43].

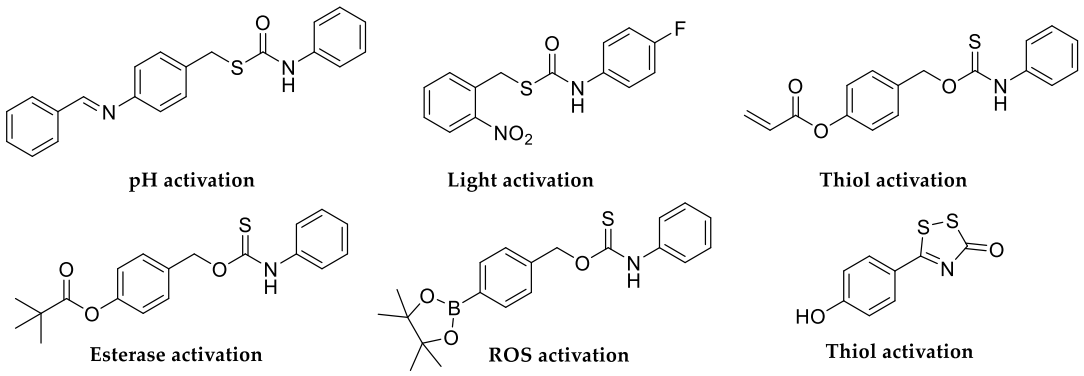

Figure 3. Selected small molecule incorporating H_2S donors and their chemical motifs.

Although there was no single universal "ideal donor," certain donor classes provided distinct advantages and useful properties. First, the donors should be stable until the activating group is cleaved or modified. Subsequently, they should have readily accessible control compounds that can be used to clearly delineate observed biological activities and outcomes associated with H_2S from those of donor by-products. Similarly, donors that respond to specific stimuli enable experiments in which H_2S delivery can be controlled or triggered by specific activators such as light, various pH ranges, other enzymes, biological thiols, and hydrogen peroxide (H_2O_2) [44–47]. COS shares an interconnection with H_2S generation through the action of CAs, which convert it to H_2S. Growing evidence shows that COS may play roles in sulfide transport in several disease pathologies. Indeed, the consumption of COS by many organisms was found to be CA-dependent. Furthermore, CA played an important role in explaining the toxicity of COS. During COS consumption, H_2S is formed, which was recognized as being the actual toxin responsible for the noxious effect of some heterocumulene derivatives [48]. The mechanism by which CAs catalyze the irreversible hydration of COS, even though it is not their natural substrate, has been investigated by computational model by Anders et al. (Figure 4) [48].

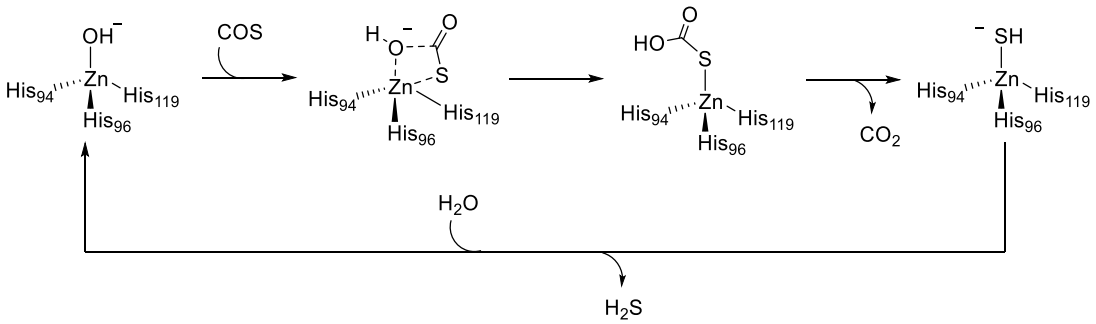

Figure 4. Catalytic hydration mechanism of carbonyl sulfide (COS).

The reaction follows the same principle as the CO_2 hydration reaction. COS is attacked by the zinc-bound hydroxide, which occurs exclusively at the C=S bond. The result is a four-center transition structure, whereby a zinc bound thiocarbonate is formed (Figure 4). CO_2 is formed by a water-assisted proton transfer, then expelled, and the zinc hydrosulfide complex is obtained. This species is stabilized by a strong Zn-S bond. Finally, the mode of reactivation of the CA catalytic domain required a water molecule [48].

4. CA as a New Reductase Enzyme

A common and useful reaction in organic chemistry is the direct hydrogenation of reagents using hydrogen gas. Nature, on the other hand, does not utilize this technique, and reduces organic molecules during metabolic pathways involving cofactors such as NADPH or $FADH_2$, which provide evolutionary advantages over the direct use of hydrogen [49]. This likely makes the enzymes more

efficient than they would be if H_2 was used. In 2009, Kazlauskas and coworkers replaced the zinc ion in the CA active site with a rhodium ion, introducing new catalytic activity to the enzyme. [50] This choice was made on the basis of different considerations: first, ionic radii, similar in both zinc and rhodium conserve the native structure of the active site [51]. Second, histidine residues in the active site may also act as good ligands for rhodium [52]. Finally, CA II lacks cysteine residues within its active site and consequently may have less anchoring points for additional rhodium ions. The generated Rh-CA has been studied for its reductase ability. In the first work, the authors reported stereoselective hydrogenation of *cis*-stilbene over *trans*-stilbene. Human Rh-CA II with two mutated histidines, in order to minimize the side anchored points for rhodium ions, was the best engineered enzyme with the new reductase function. An interesting point was the lower amount of isomerization of *cis*-stilbene to *trans*-stilbene during the hydrogenation reaction, as outlined in Figure 5(**1**) [53].

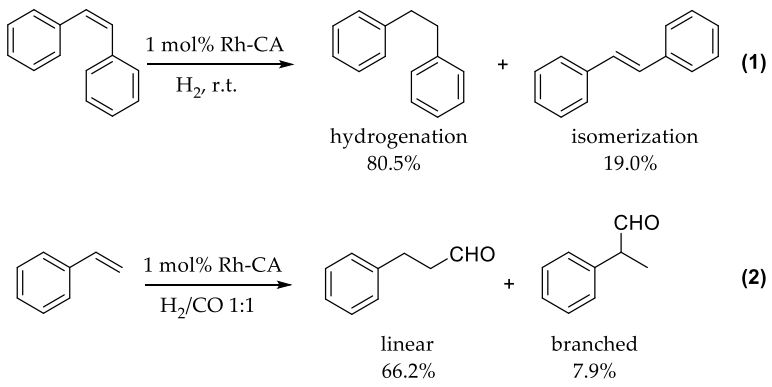

Figure 5. Hydrogenation and isomerization of cis-stilbene (**1**) and hydroformylation of styrene (**2**).

Subsequently, the same authors reported the Rh-CA catalyzed hydroformylation of an unfunctionalized olefin with a regioselectivity of approximately 8.4 for a linear versus branched aldehyde product, as shown in Figure 5(**2**) [53]. Moreover, in this case the rhodium outside the active site generated most of the side products (branched aldehyde), whereas the rhodium within the active site produced mostly linear product. Consistent experiments with different variants of Rh-CA were conducted, which confirmed this hypothesis. Due to the novelty of these results and the lack of mechanistic information, Piazzetta et al., explored by computational methods the reaction mechanisms beside the reductase catalytic process of CO_2 [54,55]. CO_2 is thermodynamically stable, and its transformation into formic acid can be considered as an innovative means for hydrogen storage [56]. The reduction of CO_2 to HCOOH can be done by a hydrogenation reaction by using efficient and specific catalyst such as Rh-CA. This process involved the roles of H_2 and H_2O. The carbon dioxide reduction process started with the activation of the enzyme through the substitution of a coordinated water molecule by the insertion of H_2 (**1A**) as outlined in Figure 6.

The subsequent insertion of a CO_2 molecule allowed the formation of the rhodium complex **1B** followed by the formation of the penta-coordinated complex **1C**. A concerted transition state, δ-bond metathesis, and rotation of the formate anion were triggered by a hydrogen molecule, which restored the octahedral geometry **1E**. The **1E** species, with formic acid coordinated to the Rh, was then released with concomitant insertion of a CO_2 molecule, which restored the Rh octahedral geometry. This was the rate-determining step of the process and governed the regeneration of the catalyst [55].

A second type of CA with reductase function is Cu-CA [57]. This enzyme showed nitrite reductase reducing nitrite (NO_2^-) to nitric oxide (NO), and thus, may play a role in vasodilation and regulation of blood pressure [58–60]. Interestingly, this enzyme has reductase function only if two sites are occupied by copper, one within the active site and, and the second in the T-2 site, which is situated at the entrance of the cavity. The mechanism is poorly understood at the moment and the oxidation state of the two copper ions (if Cu(I) or Cu(II), or mixed) has also not been elucidated [57].

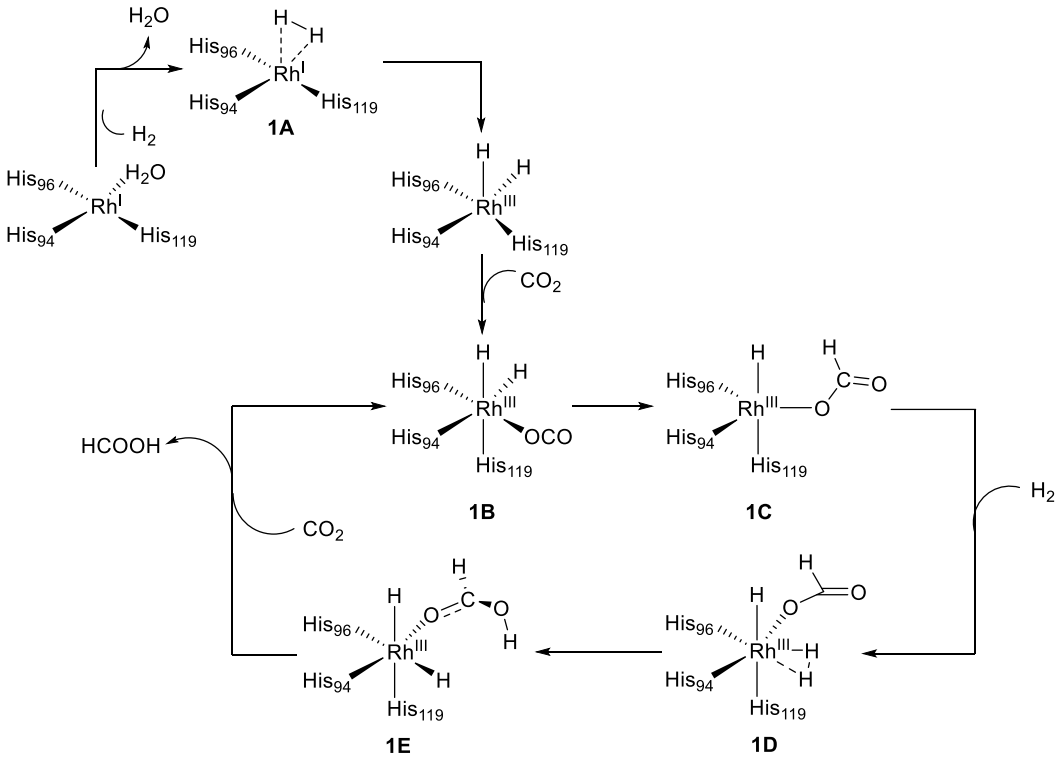

Figure 6. Proposed mechanism for the reduction of CO_2 with Rh-CA.

Alternatively, CA reductase activity developed by Ward and co-workers was obtained as a result of incorporation of an organometallic moiety within a native CA protein. The adduct was obtained by anchoring different *para*-substituted arylsulfonamides to produce an incorporate active iridium complex (Figure 7) and showed transfer hydrogenase activity [61].

Iridium complexes were chosen because they have been proven to exhibit superior reduction of imines in water than congeners with rhodium or ruthenium [62]. In addition, protein host CA II was mutated in different critical residues in order to determine the best conformation for obtaining more space within the substrate binding pocket. The complexes showed only moderate activity upon incorporation in hCA II. However, complex **2D** displayed significantly improved catalytic performance both in terms of activity and selectivity at 4 °C compared to the other complexes reported by the authors [61].

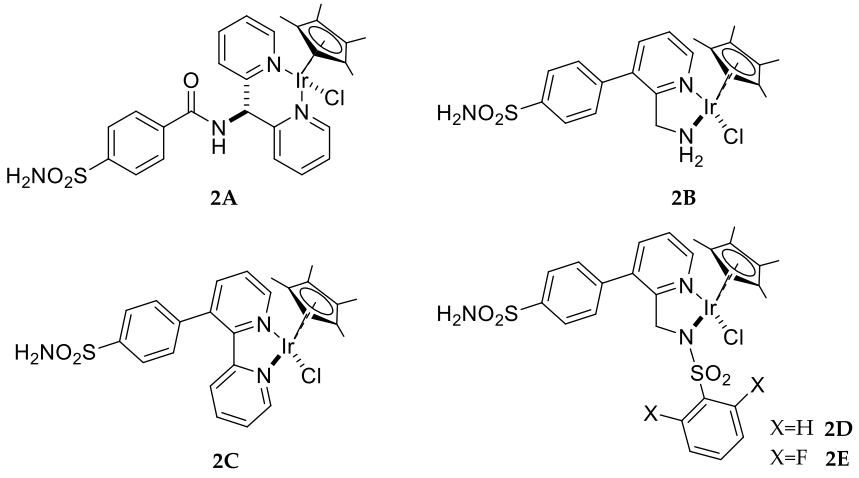

Figure 7. Pianostool complexes **2A–E**.

5. CA as a New Oxidase Enzyme

The well-characterized structure of the CA protein permitted the synthesis of several artificial isoforms with varied and novel activities. Recent studies have focused on Iridium complexes with exclusively high water-oxidizing activity [63]. The Ir-CA was prepared by binding Iridium to the apo-protein of CA and the new enzyme showed catalytic water-oxidizing activity comparable to those of other molecular catalysts [64,65]. Moreover, the oxygen-evolving catalytic activity could be displayed only when Iridium was bound to the active site of CA and this activity was sensitive to physiological conditions such as pH and temperature. Indeed, the activity was promoted by increasing the pH, which indicated that the water-oxidation activity of Ir-CA was higher under alkaline conditions [63].

Enantioselective oxidation activity was carried out by replacement of the zinc ion with manganese(II) by Soumillion et al. [66], thus discovering a Mn-CA with Epoxide Synthase activity. Several alkene substrates and variants of CAs were tested to evaluate the specificity of the epoxidation, and the best ones reached a yield of 59% and an ee of 50%. More interestingly, the replacement of Thr199 with a different residue produced amounts of styrene oxide similar to the wild type, but the enantioselectivity was severely affected. This feature showed this side chain essential for asymmetric induction [66].

6. CA as Host Protein for Metatheses-Ases Activity

Artificial metathesases activity was reported by exploiting human CA II through the creation of an adduct with benzenesulfonamide derivatives. The sulfonamide group is a well-known inhibitor of CAs. For obtaining this activity an arylsulfonamide anchored on a Hoveyda-Grubbs 2nd generation-type catalyst was synthetized (Figure 8) [67].

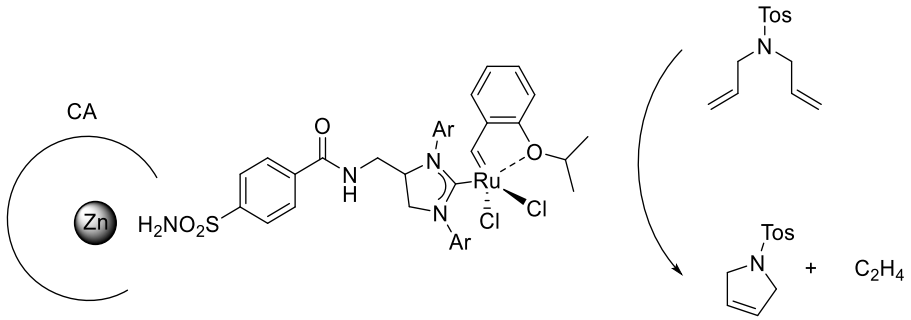

Figure 8. Artificial metalloenzyme for ring-closing metathesis.

This approach had the advantage of ensuring its localization within the CA active site so it did not require an inert atmosphere; the substrate concentration was the lowest of all reported systems to date and it was operating under physiological conditions. In addition, metathesases activity was evaluated with site-directed mutagenesis in order to modulate lipophilic, polar and coordinating amino acid residues to improve the catalytic performance [67].

7. Summary and Outlook

The CAs represents a unique example of highly investigated enzymes both for basic science studies, which allowed the understanding of intricate structure–function relationships in proteins at the atomic level, but also from the biochemistry viewpoint, with several different mutations the researchers could use for a variety of reactions not catalyzed normally by these enzymes, such as the hydratase, reductase and other catalytic propriety. All CA families use a metal hydroxide nucleophilic species of the enzyme, and possess a unique active site architecture, with half of it hydrophilic and the opposing part hydrophobic, allowing these enzymes to act as some of the most effective catalysts known in Nature. In addition, the introduction of different metal ions, mutation of key protein

residues and different molecules bound to the CA can create beneficial mutations for different activities. In this scenario, compounds containing chalcogenide atoms can be use as novel tools to catalyze different activity [68–72].

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