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Theoretical studies of the hydrolysis of antibiotics catalyzed by a metallo-β-lactamase

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ABSTRACT

In this paper, hybrid QM/MM molecular dynamics (MD) simulations have been performed to explore the mechanisms of hydrolysis of two antibiotics, Imipenen (IMI), an antibiotic belonging to the subgroup of carbapenems, and the Cefotaxime (CEF), a third-generation cephalosporin antibiotic, in the active site of a mono-nuclear β-lactamase, CphA from *Aeromonas hydrophila*. Significant different transition state structures are obtained for the hydrolysis of both antibiotics: while the TS of the CEF is a ionic species with negative charge on nitrogen, the IMI TS presents a tetrahedral-like character with negative charge on oxygen atom of the carbonyl group of the lactam ring. Thus, dramatic conformational changes can take place in the cavity of CphA to accommodate different substrates, which would be the origin of its substrate promiscuity. Since CphA shows only activity against carbapenem antibiotic, this study sheds some light into the origin of the selectivity of the different MbL and, as a consequence, into the discovery of specific and potent MβL inhibitors against a broad spectrum of bacterial pathogens. We have finally probed that a re-parametrization of semiempirical methods should be done to properly describe the behavior the metal cation in active site, Zn^{2+}, when used in QM/MM calculations.

Introduction

β-lactam antibiotics are the most effective chemotherapeutic agents for the treatment of bacterial infections, accounting for more than half of the world's antibiotic market [1,2]. The introduction of β-lactam antibiotics into clinical medicine has had a profound impact on our civilization [3]. The mechanism of the antibacterial activity of β-lactames involves the inhibition of the biosynthesis of the bacterial cell wall peptidoglycan. Nevertheless, despite much progress in antibiotics design has been done during the past decades, the increasing use of these compounds has induced the development of different resistance mechanisms in pathogenic microorganisms [1]. One strategy developed by bacteria to resist the action of antibiotics is the expression of β-lactamases [4] that hydrolyze the four-membered ring of β-lactam antibiotics. It is accepted that hydrolysis involves nucleophilic attack on the carbonyl group of the β-lactam ring and protonation of N atom with concomitant scission of the carbon–nitrogen bond. Nevertheless, the timing of carbon–nitrogen scission bond and the protonation of the N, which could even take place concertedly (see Scheme 1), is an open question of debate. A detailed knowledge of the hydrolysis of the four-membered ring of β-lactam antibiotics reaction mechanism is required in order to know the possible ways of inhibiting bacteria activity. Nevertheless, this is not an easy task due to the plethora of different β-lactamases identified up to now. Today, more than 500 β-lactamases are known, classified into four groups [5], A–D, according to their amino acid sequence [6]. Groups A, C and D, also called serine-β-lactamases (SβLs)[1], utilize an active site serine as a nucleophile [1], while B group, or metallo-β-lactamases (MβLs), required 1 or 2 Zn(II) ions to perform the hydrolysis.

The MβLs family was defined in 1997 as a new superfamily of metallohydrolases [7]. There has been a growing concern on this zinc-dependent β-lactamases since, despite catalyzing the same reaction, it seems that SβLs and MβLs do not share any structural nor mechanistic similarity [8] and, in fact, the latter are unaffected by all clinically useful inhibitors of the serine-active enzymes [9]. In fact, no MβL inhibitors are available for clinical use [10]. Another feature of MβLs is their capability of hydrolyzing different

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[1] *Abbreviations used*: MD, molecular dynamics; IMI, Imipenen; CEF, Cefotaxime; SβLs, serine-β-lactamases; MβLs, metallo-β-lactamases; PBC, periodic boundary conditions; ABNR, Adopted Basis Newton Raphson; RC, reaction coordinates; WHAM, weighted histogram analysis method; HL, high-level, LL, low-level; DFT, density functional theory; MC, Michaelis complex.

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antibiotics which means a remarkable substrate promiscuity [11]. Further studies are required to elucidate whether they also show catalytic promiscuity.

Three subgroups of MβL have been further identified depending on sequence structure and activity similarities. B1 and B3 subclasses posses a binuclear active site, which requires one or two Zn(II) ions for full activity and are able to hydrolyze carbapenems, pelicinillins and cephalosporins [10]. B2 subclass, unlike those from subclasses B1 and B3, are fully active with one zinc ion bound and possess a narrow spectrum of activity, hydrolyzing carbapenem substrates almost exclusively [12]. Initially, a reduced number of structures of B2MβL, CphA from *Aeromonas hydrophila* [13], ImiS from *Aeromonas* veronii bv. Sobria [14] and Sfh-I from *Serratia* fonticola [12], have been crystalized, being the CphA the most studied one. In particular, three different structures, two of them in the apo form and the last one corresponding to the N220G mutant in complex with a biapenem (Bia) derivative, were obtained. Nevertheless, there are some concerns related with these structures. As commented by Garau et al., the electron density could not be interpreted as either biapenem or a hydrolyzed biapenem molecule, although it was clear the presence of two fused rings near the zinc ion and both, C2 and C3 carbon atoms of the intermediate exhibiting sp3 hybridization [13]. Then, it appears that the molecule has lost the double bond established between these two atoms. Consequently, it is difficult to associate this complex to an intermediate or a product of the antibiotic hydrolysis, as suggested by experimental studies of Sharma et al. for the reaction catalyzed by ImiS [14]. The CphA-Bia complex structure has shown how the zinc metal accommodates in the Zn2 site, with a trigonal bipyramidal coordination formed by Asp120, Cys221, His263, the carboxylate oxygen and the N4 atoms of Bia. Based on these X-ray structures, Garau et al. suggested a mechanism involving a non-metal-binding water nucleophile, activated by His118 (depicted as ":B" in Scheme 1), that would attack the carbonyl carbon of the substrate, leading to cleavage of the C7–N4 bond of the lactam ring (mechanism "2" in Scheme 1). This proposal has been supported by QM/MM theoretical calculations of Xu et al. [15,16] although suggesting that Asp120 would be the base activating the water molecule, instead of His118. In a more recent paper, Wu et al. [17] proposed a complete reaction mechanism for the hydrolysis of biapenem antibiotic catalyzed by CphA, arguing that the CphA-Bia complex determined by Garau et al. would belong to a minor pathway, in contrast to the original suggestion. In this regard, simulations performed by Gatti [18] suggest that the bicyclic derivative of Garau et al. would not be formed inside the enzyme active site. Hydrolyzed biapenem might be released first, cyclization would occur in solution and then the bicyclic compound would bind back to the active site.

Based on QM/MM calculations, an alternative mechanism was proposed by Simona et al. [19,20] where the nucleophilic attack and the proton transfer to the nitrogen atom of the lactam ring would occur in a single concerted step (see concerted mechanism "1" in Scheme 1). According to this proposal, the mechanism requires the activation of a second catalytic water molecule in the active site of the enzyme. This mechanism would be in agreement with experimental studies of Sharma et al. [14] based on proton inventories showing that at least one proton transfer must be involved in the rate limiting step. Nevertheless, the proposal is based on the existence of a conformation of the Michaelis complex in which the substrate binds the zinc metal through a water molecule. This model is not confirmed by the structural studies of Crowder et al. [21] based on enzyme-product complexes, that suggest a direct contact between the zinc metal and the carboxylate of the substrate. An initial structure presenting this direct contact was used by Xu et al. [15–17] to propose a step-wise mechanism that renders an estimated free energy barrier for the nucleophilic attack of ca. 14 kcal mol^{-1}, [15] a value in very good agreement with the kinetic experiments of Garau et al. [13] Nevertheless, this comparison requires the hypothesis that such step was the rate limiting step of the enzymatic cycle, apparently in contradiction with the proton inventory experiments of Crowder et al. [21] and with QM/MM computationally exploration of the full mechanism performed by Simona et al. [19] In particular, the second step related with the proton transfer from Asp120 to nitrogen atom of substrate, would become the rate limiting step, with a total free energy barrier of ca. 24 kcal mol^{-1}.

Similar debate was open on the mechanisms of binuclear B1 and B3 beta-lactamases. Thus, Dal Peraro et al. [22] proposed a mechanism with nucleophilic attack and proton transfer taking place in a concerted manner, while the simulations of Xu et al. [23] suggest that the reaction would be essentially stepwise, with a first rate limiting nucleophilic attack leading to an intermediate where the negative charge developed in the nitrogen leaving group would be stabilized by one of the Zn metal atoms (Zn2). This stable anionic intermediate, experimentally reported by Benkovic and co-workers [24] and by Vila and co-workers [25], implies a non-negligible energy barrier for the following step. Again, the studies of Dal Peraro et al., on B1 metallo beta-lactamases assumed an initial structure with the carboxylate of the substrate interacting with the zinc ions through a water molecule. This assumption could be in contradiction with reported X-ray crystallographic structures of the enzyme complex with the hydrolysis product of an antibiotic carried out by Spencer et al. that suggests a direct substrate-metal interaction also in reactant complex [26].

Interestingly, β-lactamase catalytic activity has been also studied on B1 class with only one zinc metal in the catalytic pocket based on models with the zinc placed in position 1 [22,27,28]. The activity of B1 enzymes in their mono-nuclear form has been measured for the hydrolysis of penicillin G catalyzed by Co(II) substitude B1 metallo-β-lactamase, BcII [29]. According to this study, the metal was observed in both positions, 1 and 2. Furthermore, a biochemical and biophysical characterization of a B3 class of MbL, GOB-18, has also revealed catalytic activity for the mononuclear enzyme form with the zinc ion in position 2.

In a previous paper, we carried out a computational study to explore the hydrolysis of one antibiotic, Cefotaxime (CEF), in gas phase and in aqueous solution by means of QM/MM potentials [30]. PM3 semiempirical methods rendered results in qualitative agreement with DFT calculations with B3LYP and M06-2X hybrid functionals. The free energy profiles in solution showed a step-wise mechanism kinetically determined by the nucleophilic attack of a water molecule activated by the proton transfer to the carboxylate group of the substrate. As depicted in Scheme 1, this would correspond to the first step of any of the two possible stepwise mechanisms where ":B", in Scheme 1, would represent the carboxylate group of the substrate in this case. According to the barrier obtained from the second intermediate to products, population of the second intermediate would be in agreement with experimentally detected anionic intermediates in β-lactamases [24,25]. A concerted mechanism (see Scheme 1), with a water molecule activated by the nitrogen atom of the substrate was also obtained although with a much higher free energy barrier. Keeping in mind the hypothesis that similar molecular mechanisms take place in solution and in the active site of enzymes [31], we are in this paper exploring these two mechanisms in the active site of a mononuclear β-lactamase. In particular, we are studying the hydrolysis of Imipenen (IMI), an antibiotic belonging to the subgroup of carbapenems, and the Cefotaxime (CEF), a third-generation cephalosporin antibiotic, in the active site of CphA from *A. hydrophila* (see Scheme 2). Keeping in mind that CphA shows only activity against carbapenem antibiotic, a comparative analysis of the