Theoretical Evidence for the Nonoccurrence of Tetrahedral Intermediates in the Deacylation Pathway of the Oxacillinase-24/Avibactam Complex

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ABSTRACT: Oxacillinas (OXAs) β-lactamases are of special interest because of their capacity to hydrolyze antibacterial drugs such as cephalosporins and carbapenems, which are frequently used as the last option for the treatment of multidrug-resistant bacteria. Although the comprehension of the involved mechanisms at the atomic level is crucial for the rational design of new inhibitors and antibiotics, currently there is no study on the acylation/deacylation mechanisms of the OXA-24/avibactam complex from first principles; therefore, mechanistic details such as activation barriers, characterization of intermediates, and transition states are still uncertain. In this article, we address the deacylation of the OXA-24/avibactam complex by molecular dynamics simulations and hybrid quantum mechanics/molecular mechanics computations. The study supplies mechanistic details not available so far, namely, topology of the potential energy surfaces, characterization of transition states and intermediates, and calculation of the respective activation barriers. The results show that the deacylation occurs via a mechanism of two stages; the first one involves the formation of a dianionic intermediate with a computed activation barrier of 24 kcal/mol. The second stage corresponds to the cleavage of the O581−C7 bond promoted by the protonation of the O581 atom by the carboxylated Lys84 and the concomitant formation of the C7−N6 bond, allowing the liberation of avibactam and recovery of the enzyme. The calculated activation barrier for the second stage is 13 kcal/mol. The structure of the intermediate, formed in the first stage, does not fulfill the characteristics of a tetrahedral intermediate. These results suggest that the recylization of avibactam from the OXA-24/avibactam complex may occur without the emergence of tetrahedral intermediates, unlike that observed in the class A CTX-M-15.

INTRODUCTION

Gram-negative bacteria have built-in capabilities to find new manners to be resistant and can pass along genetic materials that permit other bacteria to become drug resistant as well. This resistance is facilitated by the production of enzymes, known as β-lactamases, which make the antibiotic inactive by hydrolyzing the amide bond of the β-lactam ring. According to the literature, β-lactamases share a common characteristic in their catalytic mechanism consisting of the emergence of one or two tetrahedral intermediates along the reaction pathway.1−3

β-Lactamases are cataloged in serine β-lactamases, SBLs, in which a serine residue plays the role of a nucleophile to attack the carbonyl carbon of the β-lactam ring; and in the zinc metallo-enzymes, MBLs, in which a zinc ion catalyzes the hydrolysis of a wide variety of β-lactam antibiotics. The SBLs are classified according to their amino acid sequence in three groups, class A, class C, and class D, whereas the MBLs are categorized together into the class B. Because of their preference for isoxazolyl-type penicillins, like oxacillin, the class D enzymes are known as oxacillinas, or simply OXAs. Nowadays, more than 250 class D β-lactamases have been identified and are classified into three subfamilies according to the following: (a) narrow-spectrum enzymes exhibit a preference for penicillins; (b) extended-spectrum β-lactamases are those having the capability to hydrolyze advanced-generation cephalosporins, besides penicillins; and (c) carbapenem-hydrolizing class D β-lactamases such as OXA-23, OXA-24, and OXA-48.4−7 Unlike class A and class C β-lactamases, the structure of class D β-lactamases lacks an omega loop Glu166, and in its place involves a fully carboxylated lysine under biological conditions. Presumably because of the hydrophobic character of the active site that decreases the pKα of Lys84, permitting in this way the deprotonation that is required for the attack of CO₂. Thus, at physiological conditions, it should be completely carboxylated.
although the crystallization pH may influence the grade of carboxylation.3,8

One effective stratagem to fight the resistance is to supply an antibacterial drug along with a β-lactamase inhibitor. Thus, in the late seventies and early eighties of the last century, three β-lactam-inhibitors were incorporated into clinical use: tazobactam, sulbactam, and clavulanic acid, all of them comprise a β-lactam core. These inhibitors are known as suicide inactivators through the formation of a stable acyl-enzyme intermediate with the catalytic serine, producing near permanently inactivated species.9 On the other hand, avibactam,10 a diazobicyclo heterocyclic inhibitor that reversibly acylates serine beta lactamases, shows exceptional inhibitory activity against both class A and class C enzymes and variable levels of inhibition against class D enzymes. Although the comprehension of the involved mechanisms at the atomic level is fundamental for the rational design of new inhibitors and antibiotics, currently there is no study on the deacylation mechanism of the OXA-24/avibactam complex from first principles, therefore the mechanistic details are a matter of controversy.

In this article, we address the deacylation of the OXA-24/Avibactam complex by means of molecular dynamics (MD) simulations along with hybrid quantum mechanics/molecular mechanics calculations. The results suggest that the deacylation of the OXA-24/avibactam complex occurs via a two stage-mechanism. The first one, comprises the abstraction of the proton from the atom N6 by Lys218, through Ser128, entailing the formation of a dianionic intermediate in the first stage; whereas the second stage involves the nucleophilic attack of the N6 atom on the C7 carbonyl atom, causing the break of the Oδ1−C7 bond and the release of avibactam and the recovery of the enzyme. On the other hand, the results also show that the deacylation of the OXA-24/avibactam complex occurs without the emergence of any tetrahedral intermediate.

RESULTS AND DISCUSSION

The results of the 100 ns MD simulations are shown in Figure 1. The obtained plots of root-mean-square deviation (rmsd) for the active site and the root-mean-square fluctuation (RMSF) show a very stable behavior along the simulation. The interactions among the avibactam and the active-site residues are described as follows. The most important observed interaction is between Arg261 and the sulfate group of avibactam, Figure 2a, whose calculated MD interactions energy is about $-75$ (kcal/mol) on average. This interaction keeps the aminosulfate moiety of avibactam firmly inside the active site, and in this way hinders its rotational freedom. The other important residue is Ser128, which interacts with both avibactam and Lys218 because of the different possible orientations of the hydroxyl group, Figure 2b. Thus, the angle Ser128(O−H−N)Lys218 may take two possible orientations, that with values below 130° and that with values greater than this value. In the first case, the hydroxyl group is oriented toward the sulfate group, whereas in the second case, the hydroxyl proton points to the nitrogen atom of Lys218, forming an adequate angle for the proton transfer. In addition, Ser128 interacts through the hydroxyl oxygen with the proton of the aminosulfate nitrogen N6 by means of hydrogen bonding.

As it was mentioned above, the proton of the aminosulfate nitrogen N6 may interact with the hydroxyl oxygen of Ser128. During the dynamics, the angle of the N6−H bond fluctuates among different possible directions as shown in Figure 3. For
values less than 100°, the proton points to outside of the active site; whereas for values greater than 100°, the proton points to inside the active site. In the first case, the orientation does not lead to any reaction, whereas in the second case, the orientation allows the hydrogen bonding interaction with the hydroxyl oxygen of Ser128, favoring in this way the deprotonation of the N6 atom via the proton shuttle toward Lys218 via Ser128.

In the present study, a cluster analysis was carried out in terms of the dihedral angles $\alpha$, $\beta$, $\gamma$, defined as follows: $\alpha = C_8\text{--}C_5\text{--}N_6\text{--}H_A$, $\beta = C_4\text{--}C_5\text{--}N_6\text{--}H_A$, and $\gamma = C_5\text{--}N_6\text{--}O\text{--}S$; the first two dihedrals account for the orientation of the proton attached to the N6 atom, whereas the last one accounts for the orientation of the sulfate moiety. In cluster analysis, data is ordered into descriptive groups based upon comparable features. The elements of each group have more in common among them than with those of other groups. The component most typical of each group is known as the centroid of the cluster.

The results of the cluster analysis are shown in Figure 4; the red dot shows the centroid of the cluster. This centroid corresponds to the structure at 76 ns of the simulation. A reduced representation of the centroid is depicted in Figure 5. On account of the positions of Ser128, Ser81, Lys218, Lys84, and Arg261, along with the protonation state of Lys218, according to Propka3; we postulate that the deacylation mechanism may be described by two potential energy surfaces, Scheme 1.

The first one, PES-1, should account for the abstraction of the proton, from the N6 atom, by Lys218 through Ser128 to form an intermediate, which in turn should lead to the liberation of the enzyme and recyelization of avibactam by the break of the Ser81--C bond, PES-2.

For the exploration of PES-1, the following reaction coordinates were used, $R_1$ defined as the distance between the hydroxyl proton of Ser128 and the nitrogen atom of Lys218 and the second reaction coordinate $R_2$ is defined by the distance between N6 and C7 atoms. While for the PES-2, the reaction coordinates are defined as follows: the distance between atoms N6 and C7 defines the reaction coordinate, $R_3$; and the distance between the proton of the N-carboxylated lysine and the oxygen of Ser81 defines the reaction coordinates, $R_4$.

The so-obtained PES-1 is shown in Figure 6a. The representative structure, taken from the cluster analysis, lies in the upper right corner of the surface. From this point, the topology of PES-1 suggests a reaction pathway in which both coordinates vary in a nearly symmetrical way until the complex $R$ is formed at coordinates $R_1 \approx 2.1$ Å and $R_2 \approx 2.8$ Å. From this point the reaction pathway shows the inversion of the pyramidal structure of the N6 atom to position its electron-lone pair pointing to the C7 carbonyl carbon, along with the rotation of the amino group of Lys218 to dispose the electron
pair pointing to the hydroxyl proton of Ser128. As result of the above steps, the Michaelis Complex, MC-1 is formed at coordinates $R_1 \approx 2.1 \text{ Å}$ and $R_2 \approx 2.0 \text{ Å}$.

From the MC-1, the reaction follows with the deprotonation of the N$_6$ atom by Lys218, through Ser128, to reach a dianionic intermediate, INT, via the formation of the transition state, TS-1. The reaction path shows an activation energy of 24.0 kcal/mol corresponding to the saddle point located at $R_1 \approx 1.5 \text{ Å}$, and $R_2 \approx 2.1 \text{ Å}$. The structure of the respective transition state is shown in Figure 6b. It is possible to observe an optimum alignment among N$_6$−H, Ser128, and Lys218, allowing in this way the proton transfer from the N$_6$ atom to Lys218 through Ser128. On the other hand, the distances and bond lengths of the participating species are evidence that the proton transfer is in course.

To characterize TS-1, and in order to save computational time, we considered a clusterized model including only the species of the QM region, namely, avibactam, Ser81, Lys84, Ser128, Lys218, and Arg261. In addition, Ser128 was replaced by methanol, the Lys218 was replaced by methylamine, and Arg261 was replaced by N-methylguanidinium. The use of clusterized models including the residues involved directly in the catalytic task has been reported to be adequate to describe electronic structures and related energetic profiles.11

The transition state was characterized by frequency calculations, at the M06-2X/6-31+G* level of theory. The results show one imaginary frequency ($\nu = 1541.0 \text{ cm}^{-1}$, intensity = 4110), in addition to some spurious frequencies because of the absence of the proteic environment in the clusterized model. The observed imaginary frequency corresponds to the (Ser128)−O−H and N$_6$−H$_A$ bond stretching, accounting for the protonation of Lys218 and the abstraction of the N$_6$ proton by Ser128. An animation of the associated imaginary frequency is shown in Movie S1 in the Supporting Information. The results of the natural bond orbital, NBO, calculations, for the same model, are summarized in Table 1.

It is observed that the NBO charges of key atoms (N$_6$, C$_7$, O$_{3218}$ N$_{218}$) vary according to the progress of the reaction; thus, the atomic partial charges on the key N$_6$ and C$_7$ atoms take extreme values in the intermediate INT, as expected for

| atom | MC-1 | TS-1 | INT |
|------|------|------|-----|
| C$_7$ | 0.96 | 0.98 | 1.00 |
| N$_6$ | -0.36 | -0.045 | -0.61 |
| O$_{3218}$ | -0.84 | -0.62 | -0.62 |
| N$_{218}$ | -0.96 | -0.91 | -0.87 |
| O$_A$ | -0.73 | -0.72 | -0.78 |

| bond | MC-1 | TS-1 | INT |
|------|------|------|-----|
| N$_6$−C$_7$ | 0.21 | 0.18 | 0.08 |
| C$_7$−O$_A$ | 1.58 | 1.60 | 1.64 |
| N$_6$−H$_A$ | 0.75 | 0.56 | 0.19 |
| H$_A$−O$_{3218}$ | 0.02 | 0.18 | 0.50 |
| O$_{3218}$−H$_{3218}$ | 0.63 | 0.29 | 0.06 |
| H$_{3218}$−N$_{218}$ | 0.05 | 0.41 | 0.59 |
the formation of the dianionic intermediate. On the other hand, the charges on the N_{K218} and the O_{\alpha} atoms follow the same tendency, making evident the following stage of the mechanism. Meanwhile, the Wiberg bond indexes vary accordingly; that is, the decrease in bond indexes of the bonds subject to cleavage, and the strength of those bonds being formed. It is especially remarkable that the index of the C_{7}−O_{A} bond remains practically unchanged during the reaction, reaching the value of 1.64 in the intermediate, 1.60 in the transition state TS-1, and 1.58 in the Michaelis complex, MC-1. Correspondingly, the bond length of the C_{7}−O_{A} keeps constant at 1.25 Å, and the NBO charge on the carbonyl oxygen changes slightly from −0.73 to −0.68 when going from the Michaelis complex, MC-1 to the intermediate, INT.

Once the transition state TS-1 is formed, the reaction continues with the formation of the intermediate INT, at coordinates R_{1} ≈ 1.0 Å and R_{2} ≈ 2.5 Å, Figure 6c. This intermediate is characterized with real frequencies, with the exception of some spurious frequencies arising because of the clustered model. The bond angles of the C_{7} carbonyl atom are 128.7, 113.8, and 115.5°, whereas the C_{7}−O_{A} bond length is 1.25 Å. The bond orders obtained by NBO calculations are: C_{7}−O_{A}: 1.64, C_{7}−N_{6}: 0.08. All of the above values do not meet the expected values corresponding to sp^{3} hybridization, but to an sp^{2} carbon. In consequence, it is possible to conclude that the hybridization of the C_{7} carbonyl carbon does not change along the reaction path and keeps as sp^{2} all of the way.

On the other hand, if a tetrahedral intermediate were formed in this stage, it would be observed at coordinates R_{1} ≈ 1.1 Å and R_{2} ≈ 1.5 Å, on the PES-1. However, no local minimum is observed at this point; moreover, the energy at these coordinates is 32.0 kcal/mol higher than the dianionic intermediate. These results suggest the nonoccurrence of a tetrahedral intermediate in the deacylation of the OXA-24/avibactam complex. Moreover, the ambient around the carbonyl carbon in class D enzymes is more hydrophobic than those found in class A and class C \( \beta \)-lactamases. In OXA-24, the more hydrophobic nature of the binding pocket results in fewer polar interactions with avibactam when compared to other \( \beta \)-lactamases. A schematic representation of the interactions between avibactam and the protein for the 100 ns MD simulation, are shown in Figure 7.

The hydrophobic barrier formed by Tyr112, Trp115, Trp221, Trp167, Ala126, Ala80, and Leu127 is clearly observed; accordingly, there is no water molecules interacting with the O_{\alpha} atom of the carbonyl group of avibactam. This finding is in agreement with that reported in the literature for OXAS. Under these conditions, the formation of a potential oxyanion, such as that required for the formation of tetrahedral intermediates, would be highly hindered.

The intermediate INT so formed is located in the upper right corner of PES-2, Figure 8a. From here, the reaction continues fundamentally with a conformational rearrangement, that is, the approaching of the carboxylic proton of Lys84 toward the oxygen O_{Ser81} to reach the prereactive complex MC-2, located at coordinates of about (R_{1}~2.0 and R_{2}~1.8) in the PES-2.

From MC-2, the reaction continues with the nucleophilic attack of the N_{6} atom on the C_{7} carbonyl carbon, along with the concomitant protonation of the O_{S81} atom by the carboxylated Lys84, reaching a saddle point at coordinates R_{3} ≈ 1.6 Å and R_{4} ≈ 1.4 Å, corresponding to the transition state, TS-2. The calculated activation barrier for this stage is about 13.0 kcal/mol. The structure corresponding to the saddle point, TS-2, is shown in Figure 8b. This transition state was characterized using a clusterized model, involving Lys218, Ser128, Lys84, Arg261, Ser81, and avibactam. The frequency calculations, at M06-2x/6-31+G* level of theory show one imaginary frequency (\( \nu = 1138.7 \) cm\(^{-1}\), intensity = 4485) corresponding to the O_{K84}−H_{K84} and O_{S81}−C_{7} bond stretching. An animation of the imaginary frequency is shown in Movie S2 of the Supporting Information.

The transition states, TS-1 and TS-2, were also characterized by NBO calculations. The obtained Wiberg bond index between key atoms are shown in Table 2. From this table, decreases in the values of the bond indexes for the bonds subject to cleavage were observed, whereas increases in the index values for the bonds being formed are observed. Thus, the value of the bond index for the C_{7}−N_{6} bond changes from 0.08 to 0.77, when going from the intermediate INT to TS-2. These values account for the nucleophilic attack of the N_{6} atom on the C_{7} carbon, entailing the recyclization of avibactam. On the other hand, the bond index for the O_{S81}−H_{S84} bond changes from 0.00 to 0.26, for INT and TS-2, respectively; on the other hand, for the C_{7}−O_{S81} bond the value goes from 0.93 to 0.66, for the intermediate and TS-2, respectively. These bond index values are indicative that two events are occurring simultaneously, namely the protonation of the O_{S81} by Lys84 and the cleavage of the C_{7}−O_{S81}.

The rate-determining step results to be the first one, corresponding to the formation of the dianionic intermediate INT. This reaction path involves the protonation of Lys218 by Ser128, and the concomitant deprotonation of the aminosulfate nitrogen N_{6} atom by Ser128, Movie S3 of the Supporting Information. The calculated activation barrier of 24.0 kcal/mol is in good agreement with the value of 25.5 kcal/mol, calculated by the Eyring equation from the experimental value of 6.3 \times 10^{-6} (s^{-1}) for the deacylation rate constant. The second stage of the mechanism corresponds to the liberation of the product, that is, the recyclization of avibactam and its release from the proteic environment. The reaction pathway of this stage is shown in Movie S4 of the Supporting Information. The calculated activation barrier for this step is 13.0 kcal/mol. Table 3 summarizes the energy of all stationary points.

![Figure 7. Avibactam-residue interactions for the 100 ns MD simulation (light green: hydrophobic, light blue: polar).](image-url)
CONCLUSIONS

Currently, it is generally accepted that all classes of β-lactamases share a common feature in their catalytic mechanism that consists of the emergence of one or more tetrahedral intermediates along the reaction pathway. The results reported herein show that the mechanism of deacylation of the OXA-24/avibactam complex involves a two-stage mechanism, in which the first stage corresponds to the formation of a dianionic intermediate with a calculated activation barrier of 24.0 kcal/mol; this high value is due to the deprotonation of the aminosulfate nitrogen. The geometric parameters, angles, and interatomic distances of the optimized structure of the intermediate INT along with the NBO results (bond orders and atomic partial charges) are consistent with a hybridization sp² for the C₇ carbonyl atom, evidencing the nonoccurrence of a tetrahedral intermediate. The second stage corresponds to the cleavage of the Ser₈₁−C₇ bond promoted by the protonation of the OS₈₁ atom through the carboxylated Lys₈₄, and the concomitant recyclization of avibactam as a consequence of the nucleophilic attack of the N₆ atom on the C₇ carbonyl carbon. The calculated activation barrier for the second stage is 13.0 (kcal/mol). In consequence, the first stage is the rate-determining step, in good agreement with that postulated by Lahiri et al.

The above results suggest that the recyclization of avibactam from the OXA-24/avibactam complex may occur without the emergence of any tetrahedral intermediates, unlike that observed in the class A CTX-M. This finding may be explained in terms of the particularities of the active sites in class D β-lactamases. OXAs are much more hydrophobic than those of class A and C; thus, the conserved asparagine, in class A and C, is replaced by valine, isoleucine, or leucine in class D. This hydrophobic nature of class D is necessary to promote the carboxylation of Lys₈₄ to form the carbamylate. In some cases, carbamates are implicated in substrate binding or directly in catalysis, like the observed in the present study. This hydrophobic environment may explain the nonoccurrence of tetrahedral intermediates in OXAs, as their formation should involve the emergence of an oxyanion which would be highly disfavored in this hydrophobic ambient.

COMPUTATIONAL METHODS

The methodology used has been reported in previous articles, and details on the MD simulations are included in the Supporting Information. In brief, the initial structure of the OXA-24/avibactam complex determined at 2.4 Å, PDB code 4WM9, was used. All ionizable residues were set to the states corresponding to pH 7.0 using Propka, and all crystalline waters were considered. The system was solvated with a cubic box (80×96×72 Å³) of water molecules centered in the enzyme−inhibitor complex. The final system contains 46 766 atoms, of which 42 816 atoms correspond to water molecules, and the difference corresponds to the protein, avibactam, and ion atoms. The total system was divided into a quantum mechanics zone, QM, including avibactam, Ser₈₁, Lys₈₄, Ser₁₂₈, Lys₂₁₈, and Arg₂₆₁ side chains; whereas the molecular mechanics region, MM, contains the rest of the system. The total charge in the QM zone is null; and the link atom method was applied to the boundary atoms. For clarity on the labels of atoms, a reduced representation of the active site is shown in Scheme 2.

Calculations were performed at M06-2X/6-31+G(d,p) level of theory using the Q-Chem/CHARMM interface. The PESs were investigated in terms of the reaction coordinates defined above with steps of 0.1 Å.

Table 2. Calculated NBO Wiberg Bond Index for Selected Atoms of the Intermediate, INT and the Transition State, TS-2

| bond         | INT   | TS-2  |
|--------------|-------|-------|
| C₇−N₆       | 0.08  | 0.77  |
| C₇−OS₈₁     | 0.92  | 0.66  |
| H₈₄−OS₈₁    | 0.00  | 0.26  |
| O₈₄−H₈₄     | 0.69  | 0.42  |

Table 3. Energies of the Stationary Points Relative to the Michaelis Complex MC-1

|          | MC-1 | TS-1 | INT | MC-2 | TS-2 | product |
|----------|------|------|-----|------|------|---------|
| energy (kcal/mol) | 0.0  | 24.0 | 3.0 | −7.0 | 6.0  | 4.0     |

Figure 8. (a): Two-dimensional view of PES-2. (INT: intermediate, MC-2: Michaelis Complex, TS-2: transition state 2, P: products). (b): Optimized structure of the transition state TS-2, distance in Å.
The Q-Chem software was used to explore the PES by geometry optimizations of the movable part of the system using harmonic constraints (RESModulate) on the reaction coordinates described above. Energy minimizations were performed by means of relaxed scans with a gradient tolerance of 0.001 kcal/mol by the ABNR algorithm. The QM region was investigated at the M06-2X/6-31+G(d,p) level, whereas for the MM region, the CHARMM36 force field was employed. The interaction between QM and MM zones was described by the electrostatic embedding method. The search of the transition states was accomplished on a clustered model comprising avibactam and those residues in the QM zone. The level of theory used is the same mentioned above, and Jaguar was used for the respective characterization. The technique uses a simple quasi-Newton method that looks for the transition state nearest to the initial geometry. NBO analysis, to characterize the intermediate and transition states, was performed using NBO as implemented in Jaguar. The ligand–protein interactions were calculated with Desmond 5.5.

ASSOCIATED CONTENT

Supporting Information

Animation of the imaginary frequency corresponding to the transition state TS-1 formed in the first-stage of the recyclicization mechanism of avibactam (MP4)

Animation of the imaginary frequency corresponding to the transition state TS-2 formed in the second stage of the recyclicization mechanism of avibactam (MP4)

Animation of the reaction pathway corresponding to the first stage of the recyclicization mechanism of avibactam (AVI)

Animation of the reaction pathway corresponding to the second stage of the recyclicization mechanism of avibactam (AVI)

Methodology of MD simulation (PDF)

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