A quantum mechanics/molecular mechanics study of the acylation reaction of TEM1 β-lactamase and penicillanate

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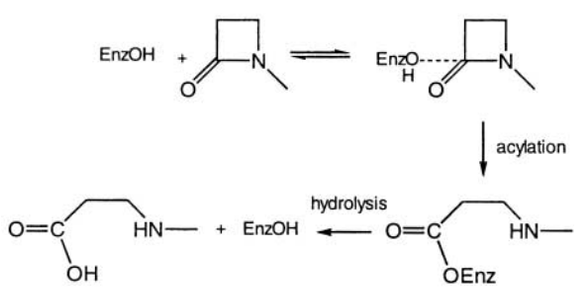
The acylation step in β-lactamase catalyzed hydrolysis of β-lactams has been explored by means of a quantum mechanics/molecular mechanics approach (AM1/CHARMM). The TEM1 enzyme, a class A β-lactamase, and the penicillanate constitute the system employed in our study. The entire molecular system is divided into a quantum and a classical region: the quantum part is composed by the substrate, the serine Ser70 and the essential moieties of key active site residues, Lys73, Ser130 and Glu166, as well as a water molecule present in the active site region, while the classical part is formed by the remaining residues and structural waters of the enzyme. In particular, the sequence of steps proposed by Strynadka *et al.* (*Nature*, 1992, **359**, 700) for the acylation reaction is analyzed. Minimal and transition structures for the mechanism are reported and an energy activation of 18.29 kcal mol^{-1} has been calculated for the rate-limiting step, the formation of an initial tetrahedral adduct. From this structure, two different mechanistic routes have been found to achieve the acyl–enzyme intermediate. In the first of them a simultaneous β-lactam ring opening and proton transfer from Ser130 to the β-lactam nitrogen atom occurs, presenting an energy barrier of 12.91 kcal mol^{-1} with respect to the tetrahedral intermediate. In the second route, these processes take place in a sequential way. From an energetic point of view, the sequential mechanism is favored, requiring the ring opening step (7.66 kcal mol^{-1}) and the subsequent nitrogen protonation (2.76 kcal mol^{-1}). Some reflections arising from the preference of sequential processes in this system are exposed.

Introduction

The most common mechanism of resistance to β-lactam antibiotics is the ability of bacteria to produce β-lactamases. The presence of β-lactamases in the bacteria was first discovered in 1940,[1] before the introduction of penicillin into clinical practice. These enzymes catalyze the hydrolysis of the sensitive β-lactam moiety of these kinds of antibiotics. In this way the compound is inactivated before it can reach its target enzymes, transpeptidases and carboxypeptidases (collectively so-called penicillin binding proteins, PBPs) involved in the synthesis of the bacterial cell wall. It is well known that the inactivation of the PBPs is due to the irreversible acylation of a serine residue.[2,3] The more usual β-lactamases, classes A and C, are active site serine enzymes (Ser70 in the class A enzymes: sequence numbering of Ambler *et al.*[4]) and their catalytic pathway also involves the formation of an acyl–enzyme intermediate. However, as opposed to the PBPs, the β-lactamases undergo deacylation in an easy manner, regenerating the enzyme and releasing the inactive drug, see Scheme 1.[5]



Scheme 1

The β-lactam ring opening, which takes place during the acylation step, renders the antibiotic completely inactive, while the enzyme can recover its activity by hydrolyzing the acyl bond. This process has been the subject of numerous studies. Experimental[6–9] and theoretical[10–14] works have been devoted to non-enzymatic β-lactam ring opening. The particular reaction mechanism for such a process depends on the nucleophilic agent and several possibilities have been theoretically explored: hydroxy anion,[10,13] water,[13,14] methanol[14] and ammonia.[11] On the other hand, the study of the enzymatic acylation reaction has been tackled in different disciplines. Structural data are available for several of the β-lactamase proteins.[15–30] For the class A β-lactamases a considerable amino acid sequence homology has been found, in particular at the active site region, which contains a set of conserved residues presumably crucial for catalysis, Ser70, Lys73, Lys234, Ser130, Glu166. A fundamental question is—what role do these residues perform at a microscopic level. The acylation of Ser70, as well as the global mechanism shown in Scheme 1, seems to be a well-established fact. However, the specific path followed by the proton (or protons) during the acylation step is still unclear and different possibilities have been proposed. Some reviews are available summarizing the different proposed mechanisms for the acylation.[31,32] Two important differences can be found among them with respect to the proton transfer paths: in the base accepting the Ser70 proton, and in the residue acting as proton donor with the β-lactam nitrogen atom. With respect to the former, different possibilities have been proposed. In one of them, the proton of Ser70 is transferred to the carboxylate of Glu166.[33,34] The too-long distance between the Glu166 carboxylate and the Ser70 hydroxy is the most unfavorable feature of this mechanism. However, a conserved water molecule located between both residues could act as a proton relay for this transfer.[26,35,36] Moreover, a molecular dynamics study[36] of PC1, a class A β-lactamase, has shown that Glu166 is highly mobile, which could favor the approach to Ser70. In a second possibility, the Lys73 residue would act as a general base.[37–39] This implies a deprotonated side chain amino group of Lys73. Different studies have been devoted to discerning the proton-

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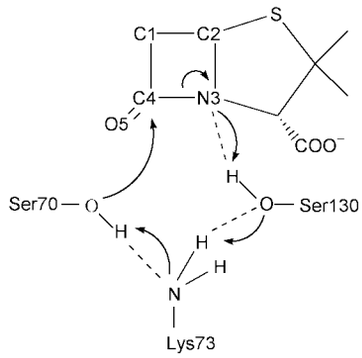


Fig. 1 Schematic representation of the electron and proton transfer processes that take place in the acylation mechanism proposed by Strynadka *et al.* The figure is also used for introducing the numeration of some substrate atoms used along the paper.

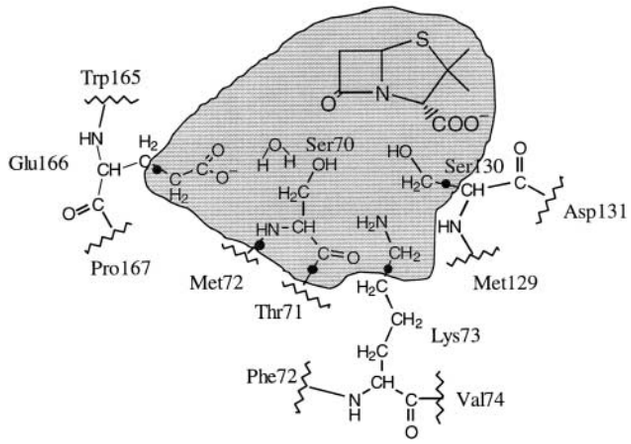


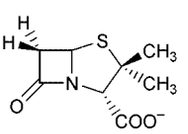
Fig. 2 Schematic representation of the active site: the shaded region corresponds to the QM atoms. The five link atoms are indicated as "●".

ation state of Lys73, but a general agreement has not been reached. Some data support an initially protonated ε-amino group for this residue,[35,40,41] and concretely, continuum electrostatic calculations for several β-lactamases in the absence and presence of different types of β-lactam antibiotics have rendered the pK_a for the Lys73 above 10.[40,41] Other studies found a pK_a shift from 8 to 14 as the substrate binds, suggesting an initially deprotonated amino group.[39] On the other hand, we have previously observed[42] that an initially protonated Lys73 establishes a close contact with the Glu166 carboxylate and could easily transfer a proton to this residue. In this way an initially protonated Lys73 would not rule out a mechanism where this residue acts as the general base.

The second controversial question refers to the protonation of the β-lactam nitrogen. Acylation implies not only the formation of an acyl bond with the carbonyl carbon, but also β-lactam ring opening and nitrogen protonation. This nitrogen protonation can be reached after a direct proton transfer from Ser70 or, alternatively, by means of several proton transfer events involving different residues of the enzyme.

In this work we carry out a computational study of a mechanism recently proposed by Strynadka *et al.*[37] for the acylation of β-lactam antibiotics in β-lactamases. This mechanism would explain both the deprotonation of Ser70 and the nitrogen protonation by means of a sequence of proton transfers involving Ser70, Lys73 and Ser130. In Fig. 1 a schematic representation of these processes is given. In this mechanism the Lys73 residue acts as the general base and accepts the proton from Ser70. Then, a proton is transferred from Ser130 to the β-lactam nitrogen and from Lys73 to Ser130. Wladkowski *et al.*[43] have explored this mechanism using an *ab initio* quantum mechanical approach. Their model only incorporates a simple β-lactam substrate and essential fragments of some key residues needed to analyze the mechanism. Energy barriers of modest size were obtained despite the limitations of the model, but several aspects of the role of some residues in the mechanism remained unsolved. This encouraged us to extend the analysis to include the whole protein and a more realistic substrate, exploiting the capabilities of the new generation of hybrid quantum/classical methods for treating large chemical systems.

These quantum/classical methods treat a reduced part of the system at a quantum mechanical level while the rest is described using molecular mechanics. The location of the stationary points, minima and transition structures that characterize a reaction mechanism must be carried out taking into account the coupling between the quantum and classical subsystems. Nowadays, there are some procedures capable of locating both transition structures and minima in this way, such as the GRACE procedure[44,45] in combination with the CHARMM program.[46] We have recently applied the CHARMM/GRACE capabilities to study the β-lactamase acylation by considering a concerted mechanism.[42] In that mechanism the proton of Ser70 is directly transferred to the β-lactam nitrogen as acylation occurs. Although the concerted mechanism was shown not to be a plausible enzymatic mechanism on the basis of its high energy barrier and some geometrical considerations, the methodology proved to be especially suitable for these kinds of mechanistic studies. The goal of this paper is to take advantage of this methodology to explore a more elaborate and feasible mechanism for the acylation, such as that proposed by Strynadka *et al.* For this purpose, we selected the clinically relevant TEM-1 β-lactamase, a prototypical class A enzyme, and the penicillanate **1**, a substrate easily hydrolyzed by this enzyme, as an adequate system for our study.



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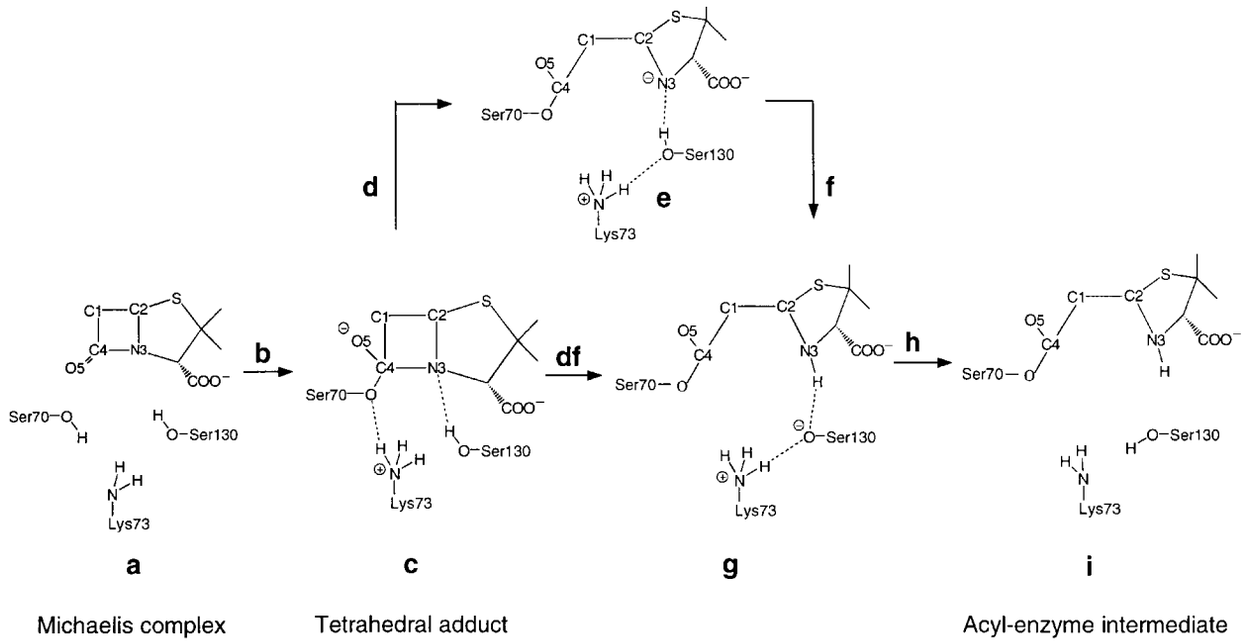
Methods

Initial coordinates for the system have been obtained from the crystallographic structure of an acyl–enzyme intermediate recently reported.[47] It is known that the intermediate is formed by TEM-1 β-lactamase enzyme and the 6α-(hydroxymethyl) penicillanate, a novel inhibitor for this enzyme. The structure is available in the Protein Data Bank (ID code, 1TEM). The hydroxymethyl moiety was manually removed to obtain the penicillanate **1**. A deprotonated ε-amino group for Lys73 was considered. This assumption is a requirement for studying the mechanism proposed by Strynadka *et al.* where the Lys73 acts as the general base which deprotonates Ser70.

The hybrid quantum mechanics/molecular mechanics (QM/MM) treatment was performed by means of the CHARMM 25b2 program,[46] using the semiempirical AM1 hamiltonian[48] with the CHARMM25b2 protein parameter set.[49,50] The AM1 ability to describe carbon–sulfur bonds has been tested in a previous work.[42] The entire molecular system was divided into QM and MM regions. In the Fig. 2 we show the quantum region. It is composed of the substrate, the serine Ser70, and the essential moieties of key active site residues, Lys73, Ser130, Glu166. A structural water molecule located between the Glu166 and Ser70 residues is also included in the QM region. The rest of the protein and water molecules present in the crystallographic structure were treated by the classical force field. The whole TEM-1 protein was considered in the calculations and the positions of all the atoms of the system were allowed to relax. Five link atoms[51] were inserted where the QM/MM boundary intersected covalent bonds. Their positions are shown in Fig. 2. The QM region contained a total of 64 atoms,



Scheme 2 Schematic representation of the minimum energy structures appearing along the reaction path.

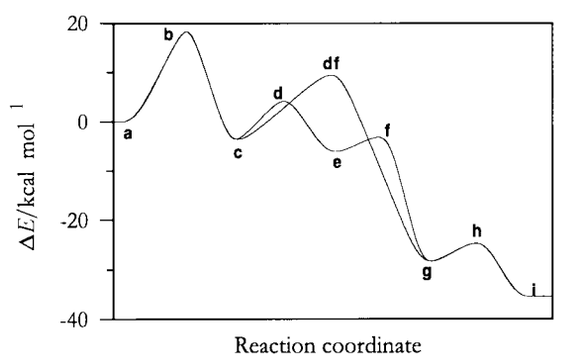


Fig. 3 Energy profile of the acylation process.

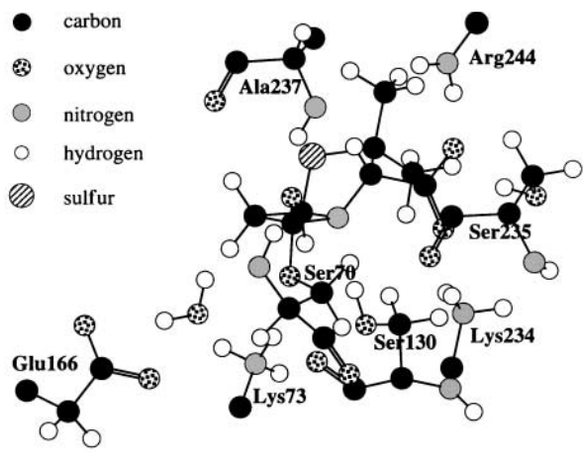


Fig. 4 Structure of the tetrahedral adduct **c** obtained after QM/MM minimization. For clarity, only some key residues are shown.

including the quantum link atoms, and the MM region a total of 4763 atoms.

QM/MM energy minimizations were performed to obtain the potential energy profile. The minima presented a rms residual gradient of less than 0.001 kcal mol^{-1} Å^{-1}. Searching for transition structures was carried out starting from a guess structure obtained from an initial exploration of the potential energy surface. This exploration was carried out by scanning on distinguished coordinates of the reaction path. During these scans all the geometrical variables, except the distinguished ones, were optimized. The obtained guess structure was further refined by using the GRACE program.[44,45] A Newton–Raphson method was employed using a Hessian matrix that describes the curvature of the QM/MM energy hypersurface for a sub-set of the system, the QM atoms, together with a diagonal Hessian plus updates for the rest of the system. In the optimized structure the rms residual gradient on the 64 atoms of the sub-set was less than 0.001 kcal mol^{-1} Å^{-1}, while on the remaining atoms it was less than 0.005 kcal mol^{-1} Å^{-1}. Finally, the intrinsic reaction coordinate[52] (IRC) path was traced from the refined transition structure in each direction using the GRACE capabilities. The calculations were carried out on a Cray-Silicon Graphics Origin 2000 at the Computer Centers of University of Valencia and University Jaume I of Castellon.

Results and discussion

In the following sections we will describe separately the different steps found for the acylation reaction. For clarity, it is convenient to introduce here the global features of the mechanistic routes. In Scheme 2 the reaction path is presented in a schematic way and in Fig. 3 the energy profile that interconnects the different structures is shown. Starting from the Michaelis complex, structure **a**, the first process that takes place is the formation of a tetrahedral adduct **c**, where the acyl–enzyme bond is already formed, with the β-lactam ring remaining closed. The formation of **c** is achieved through a transition structure **b**, where the hydroxy proton of Ser70 is transferred from the serine to Lys73 as the serine becomes acylated. In the energy profile we observe the rate-limiting nature of this step. A representation of structure **c** is given in Fig. 4. This figure is also useful for keeping in mind throughout the paper an image of the relative disposition of some key residues. From the tetrahedral adduct we have located two possible pathways to achieve the acyl–enzyme intermediate. In one of them the β-lactam ring opening and the β-lactam nitrogen protonation takes place in a simultaneous way through a single transition structure, **df**. In the second one these processes are sequential: first, the ring opening takes place through the transition structure **d** leading to a minimum **e**. Then a hydrogen is transferred from Ser130 Oγ to the β-lactam nitrogen atom. Both mechanisms, sequential and concerted, lead to the same minimum, **g**, where the ring is opened and the nitrogen atom protonated. The **g** structure is not the final acyl–enzyme intermediate because the Ser130 Oγ is deprotonated. The protonation of this atom takes place by

Table 1 Relative energy of the stationary structures (kcal mol^{-1}), Mulliken charges on some atoms (au), β-lactam ring dihedral angle (degrees) and selected interatomic distances (Å) obtained by means of the QM/MM calculations

	a	**b**	**c**	**d**	**e**	**f**	**df**	**g**	**h**	**i**
ΔE/kcal mol^{-1}	0.00	18.29	−3.56	4.10	−6.05	−3.29	9.38	−28.29	−24.74	−35.47
Mulliken charges/au										
N3	−0.26	−0.27	−0.34	−0.53	−0.68	−0.60	−0.31	−0.33	−0.32	−0.32
O5	−0.37	−0.37	−0.70	−0.58	−0.44	−0.44	−0.63	−0.43	−0.44	−0.45
Ser70 Oγ	−0.35	−0.67	−0.38	−0.34	−0.31	−0.30	−0.34	−0.27	−0.26	−0.23
Lys73 Nζ	−0.42	−0.21	−0.10	−0.10	−0.10	−0.10	−0.10	−0.11	−0.21	−0.41
Ser130 Oγ	−0.37	−0.37	−0.41	−0.44	−0.45	−0.61	−0.67	−0.81	−0.70	−0.40
Geometrical parameters										
C4–N3/Å	1.45	1.46	1.57	1.96	2.58	2.56	1.72	2.58	2.57	2.57
C4–Ser70 Oγ/Å	2.45	2.35	1.46	1.41	1.39	1.38	1.43	1.38	1.37	1.37
Ser70 Oγ–Lys73 Nζ/Å	3.20	2.58	2.98	2.96	2.97	2.91	2.89	2.98	3.04	3.29
Lys73 Nζ–Ser130 Oγ/Å	3.26	3.36	3.07	3.05	3.08	3.17	3.05	2.89	2.60	3.04
Ser130 Oγ–N3/Å	3.22	3.18	2.94	2.81	2.82	2.55	2.55	2.96	3.02	2.99
Ring torsion angle/°	3.45	3.85	−1.72	−4.62	−16.04	−15.15	−3.72	−21.51	−20.71	−18.27

means of a proton transfer from Lys73 to Ser130, leading to the acyl–enzyme intermediate, **i**. In Table 1 the relative energy values for the different structures, the Mulliken charges on some atoms and selected interatomic distances useful for the following discussion are gathered.

Michaelis complex

In the Michaelis complex, structure **a**, the β-lactam carbonyl oxygen interacts with the enzyme by means of two hydrogen bonds with the amine backbone of Ala237 and Ser70, which constitute the so-called oxy-anion hole by analogy to this feature of the serine proteases.[53,54] These atoms, Ala237 N and Ser70 N, are placed at 2.82 and 3.14 Å from the β-lactam carbonyl oxygen atom. The β-lactam carboxylate group is hydrogen bonded to Arg244 Nη$_1$, Ser235 Oγ and Lys234 Nζ. The distances from these atoms to the closest carboxylate oxygen are 2.72, 2.84 and 2.75 Å respectively. No H-bond between carboxylate and Ser130 is found. Ser130 Oγ is placed at 3.22 Å from the nitrogen atom and at 3.26 Å from Lys73 Nζ. Though close to both atoms, Ser130 hydroxy does not establish a strong H-bond interaction either with Lys73 Nζ or with the β-lactam nitrogen and a little variation in the charge distribution could favor the interaction with either of these two atoms. Effectively, as we will discuss below, the formation of the tetrahedral adduct, and even more the ring opening, leads to a strong H-bond interaction between the β-lactam nitrogen and Ser130.

The Ser70 Oγ atom is found at 2.45 Å from the β-lactam carbonyl carbon atom. The hydroxy hydrogen is not pointing directly to Lys73 Nζ but to the Glu166 carboxylate. The distance between the hydroxy hydrogen of Ser70 and the closest carboxylate oxygen of Glu166 is quite large, 3.24 Å, though there is a water molecule (belonging to the quantum core) between both residues. A water hydrogen from this molecule is placed at 2.03 Å from the closest carboxylate oxygen of Glu166 and the water oxygen atom is at 2.05 Å from the hydroxy hydrogen of Ser70. In fact, this structure seems suitable for a proton transfer between Glu166 and Ser70 through the structural water molecule, as in some of the proposed mechanisms.[26,35,36] However, an IRC calculation shows that this minimum connects with the transition structure that corresponds to the proton transfer from Ser70 to Lys73.

These results seem to indicate that this Michaelis complex could be expected to be the same for both mechanistic routes. In addition, in a previous work,[42] when we employed a reduced quantum core, the hydroxy hydrogen atom of Ser70 was directly pointing toward the β-lactam nitrogen atom, which was coherent with the concerted mechanism studied on that occasion, based on a direct proton transfer from Ser70 to the β-lactam nitrogen atom. From our experience several possibilities exist for the orientation of a given hydrogen atom if several proton acceptors atoms are close to its position.

Formation of a tetrahedral adduct

In the tetrahedral adduct **c** the acyl–enzyme bond is already formed while the β-lactam ring remains closed. The energy barrier involved in the **a** to **c** process, 18.29 kcal mol^{-1}, is the highest along the studied acylation mechanism. Though we have not yet commented on the steps that follow the formation of the tetrahedral adduct, *i.e.*, ring opening and proton transfer to the β-lactam nitrogen, they are less expensive from an energetic point of view. Experimental data on the non-enzymatic alkaline hydrolysis of β-lactam compounds point to the formation of the tetrahedral intermediate as the rate-limiting step.[6,55] Wladkowski *et al.*[43] obtain an activation energy of 23.6 kcal mol^{-1} for the equivalent reaction step in their reduced model of protein at the MP2/6-31+G(d)//RHF/6-31+G(d) level of theory.

In the transition structure **b** the hydroxy proton is being transferred from Ser70 to Lys73 and simultaneously the serine is being acylated. The proton transfer is more advanced than the acyl–bond formation. The distance between Lys73 Nζ and Ser70 Oγ is shortened from 3.20 in **a** to 2.58 Å in **b**. The Ser70 Oγ approaches closer to the β-lactam carbonyl carbon by only 0.1 Å with respect to its distance in the Michaelis complex, 2.45 Å. This leads to the development of a large negative charge on the Ser70 Oγ atom in the transition structure **b**, −0.67 au. When the acyl bond is formed, structure **c**, the charge on Ser70 Oγ diminishes to −0.38 au. We shall return to the discussion of charge distribution later.

In the tetrahedral adduct **c** the CN distance, 1.57 Å, is slightly lengthened with respect to that of the Michaelis complex, 1.45 Å, as a consequence of the change from sp^2 to sp^3 hybridation of the C4 atom on going from **a** to **c**. The CO acyl bond is completely formed, 1.46 Å. The ring remains closed and keeps its planarity, as manifested by a C4–C1–C2–N3 torsion angle of −1.72°. In **a** and **b**, the β-lactam nitrogen atom is found slightly outside the plane formed by the C1, C2 and C4 atoms, on the same side as the Ser70 residue (α face), but when the ring opening takes place, the nitrogen atom moves to the opposite side of the plane, with respect to the acylated Ser70. Thus, the tetrahedral adduct **c** found here is an intermediate situation between the positive values for the torsion angle in **a** and **b** and the negative values in all the following structures.

As a consequence of the proton transfer from Ser70, the Lys73 Nζ atom is protonated in structure **c**. This atom is now able to form strong H-bond interactions with the closest

residues, Ser70, Ser130 and Glu166. Though the distance to Ser70 Oγ, 2.98 Å, is similar to that found in the reactant complex, 3.20 Å, the hydrogen atom is now placed between the proton donor and proton acceptor atoms, indicating the existence of a strong linear hydrogen bond. The Lys73 Nζ is closer to both Ser130 O, 2.84 Å, and Ser130 Oγ, 3.07 Å, though only the Ser130 O forms a H-bond with the lysine. The protonated Lys73 is also approaching the closer carboxylate oxygen of Glu166, located at 2.83 Å from the protonated nitrogen.

On the other hand, in the tetrahedral adduct **c** a hydrogen bond appears between the β-lactam nitrogen and the Ser130 Oγ placed at 2.94 Å. In **a** and **b** structures this residue is also close to the β-lactam nitrogen, 3.22 and 3.18 Å respectively, but only in structure **c** is a H-bond clearly established. The enhancement of the nitrogen's ability to form hydrogen bonds in a tetrahedral intermediate was expected on the basis of QM/MM molecular simulations of 2-azetidinone and its hydroxylated complex in aqueous solutions.[56] In that study the hydroxylated form was used as a model structure for the tetrahedral adduct **c**. For the neutral structure, the radial distribution functions, RDFs, showed the absence of a well-defined solvation shell around the nitrogen atom. The same result was obtained by Gao *et al.*[57] in a QM/MM simulation of formamide. However, the RDFs for the hydroxylated structure showed a clear peak, confirming the ability of the nitrogen atom to form hydrogen bonds with the environment in that case.

The β-lactam carboxylate keeps the interactions with Arg244, Ser235 and Lys234 which were present in the Michaelis complex, the interaction with Lys234 being reinforced. The oxy-anion hole components, Ala237 N and Ser70 N, also reinforce their interaction with the β-lactam. Their distances to the O5 atom are shortened to 2.73 and 2.93 Å respectively. The charge distribution on some selected atoms can explain this behavior. The large negative charge present on Ser70 Oγ in the transition structure **b** is transferred towards the O5 atom in the tetrahedral adduct. In **b**, the O5 atom and Ser70 Oγ have Mulliken charges of −0.37 and −0.67 au respectively, whereas in **c** these values are practically exchanged, −0.70 and −0.38 au. This fact indicates that the oxy-anion hole components develop their stabilizing effect fundamentally on the tetrahedral intermediate and not on the transition structure. This result is in agreement with the modest effect on the energy barrier found by Wladkowski *et al.*[43] when the oxy-anion hole components are included in their computational model. They suggest that the primary role of these active site components is not the stabilization of proton transfer transition states but the binding of the substrate and its correct orientation in the active site.

The charge on Lys73 Nζ decreases, in absolute value, from −0.42 au in the Michaelis complex up to −0.21 and −0.10 au in **b** and **c** respectively, reflecting the protonation of this atom on going from **a** to **c**. The charge on the β-lactam nitrogen also increases from −0.26 au in **a** to −0.34 au in **c**. This is related to the increased ability of the β-lactam nitrogen to form hydrogen bonds in the tetrahedral adduct structure as opposed to its behavior in the Michaelis complex.

Ring opening and proton transfer to the β-lactam nitrogen

Going from the previously described tetrahedral adduct to the acyl–enzyme intermediate requires the β-lactam ring opening and the protonation of the β-lactam nitrogen atom. We have found two possible pathways, concerted or sequential, to achieve the acyl–enzyme intermediate as shown in Scheme 2. Both mechanisms lead to the same minimum, **g**, where the ring is opened and the nitrogen protonated. The structure **g** is not the final acyl–enzyme intermediate because the Ser130 Oγ is deprotonated. The protonation of this atom, the last step of the analyzed mechanism, will be discussed in the following subsection.

The energy values of Table 1 and the energy profile of Fig. 3 show the preference for the sequential mechanism over the concerted one from an energetic point of view. In the former, the activation energy for the rate-limiting step (ring opening) is 7.66 kcal mol^{-1}, considerably smaller than the 12.94 kcal mol^{-1} found in the latter. The second step of the sequential mechanism, proton transfer from Ser130 Oγ to the β-lactam nitrogen, requires only 2.76 kcal mol^{-1}. The preference for the sequential process can be rationalized by analyzing the Mulliken charges and some of the geometrical parameters of the structures involved in the process. Going from structures **c** to **g**, the most significant effect in the evolution of the atomic charges is the considerable increase, in absolute value, of the Ser130 Oγ Mulliken charge, from −0.41 in **c** to −0.81 au in **g**. Evidently, this change is a consequence of the deprotonated state of Ser130 Oγ in **g**. This negative charge is stabilized by hydrogen bonds with the β-lactam nitrogen now protonated (Oγ–N3 distance, 2.96 Å) and with the protonated Lys73 Nζ (Oγ–Nζ distance, 2.89 Å). Though this last distance in structure **c** was only slightly greater, 3.07 Å, no hydrogen atom was pointing to Ser130 Oγ. In fact, in the tetrahedral adduct **c** the Lys73 is interacting with Ser70 Oγ, Glu166 and the carbonyl of Ser130 as described in the previous section. Going from structures **c** to **g** the interactions with Glu166 and Ser130 O are kept, but the one with Ser70 is lost and replaced by a new H-bond with Ser130 Oγ. In the sequential mechanism the change in the Lys73 Nζ H-bond interactions takes place during the first step, *i.e.*, the ring opening. Thus, when a proton is transferred from Ser130 to the β-lactam nitrogen atom during the second step, the Lys73 is already correctly oriented to provide stabilization of the excess of negative charge appearing on the Ser130 Oγ atom. However in the concerted mechanism the Lys73 is not oriented towards the Ser130 Oγ in the transition structure **df**. In **df** the charge development on the Ser130 Oγ is advanced, −0.67 au, as a consequence of the initiated proton transfer to the β-lactam nitrogen atom. The proton is at 1.24 Å from the nitrogen atom and the distance Ser130 Oγ–H is 1.32 Å. The Lys73 can not stabilize this charge because it is not yet oriented towards Ser130 Oγ. Consequently, the energy barrier of the concerted mechanism, 12.94 kcal mol^{-1}, is considerably higher than that of the rate-limiting step (ring opening) of the sequential mechanism, 7.66 kcal mol^{-1}.

Other comments can be made about the behavior of some of the substrate atoms in the β-lactam ring opening step of the sequential mechanism. In the description of the tetrahedral adduct **c** we have seen that the O5 atom accumulates a large negative charge, −0.70 au as a consequence of acyl bond formation and the loss of double bond character in the carbonyl bond. When the β-lactam ring is opened, the CN bond is broken and the carbon recovers the potential to form a carbonyl double bond with O5. Effectively, the C4–O5 distance diminishes from 1.29 in **c** to 1.23 Å in the ring-opened structure **e**. The charge on the O5 atom also diminishes in **e** to −0.44 au. In opposition, the charge of the β-lactam nitrogen increases, in absolute value, from −0.34 in **c** to −0.68 au in **e**. Thus, the existing hydrogen bond between N3 and the Ser130 hydroxy in the tetrahedral adduct is reinforced in the ring opened structure **e**. The distance from Ser130 Oγ to N3 atom diminishes from 2.94 in **c** to 2.82 Å in **e**, placing the hydroxyl group of Ser130 in a very suitable position for the subsequent proton transfer to the β-lactam nitrogen. In the transition structure **d**, the charges on the O5 and N3 atoms have intermediate values between those corresponding to **c** and **e** structures. Apart from this charge redistribution, cleavage of the CN bond and the previously discussed reorientation of Lys73 toward Ser130 Oγ, we have not appreciated other important structural changes going from **c** to **e**.

Protonation of Ser130 Oγ. Acyl–enzyme intermediate

As mentioned above, concerted and sequential reaction paths do not directly lead to the acylation product, the acyl–enzyme intermediate. Both pathways coincide in a structure, **g**, where a protonated Lys73 and a deprotonated Ser130 Oγ coexist. In the mechanism proposed by Strynadka *et al.*[37] the protonation of Ser130 Oγ is achieved by proton transfer from Lys73 Nζ. In structure **g** the Lys73 Nζ is placed at 2.89 Å from Ser130 Oγ. The favorable position of Lys73 for the proton transfer does not seem to be a specific structural feature of the **g** structure, but a more general consequence of the reorientation of the Lys73 Nζ hydrogens along the reaction path. As we have seen above, the Lys73 loses an H-bond interaction with Ser70 Oγ and establishes a new one with Ser130 Oγ along the reaction path. We have rationalized the preference of a sequential mechanism (ring opening and subsequent proton transfer to the β-lactam nitrogen) with respect to a concerted one on the basis of this reorientation of Lys73. The transition structure **h** describes the proton transfer from Lys73 to Ser130. The energy barrier is only 3.53 kcal mol^{-1}. The charge on Ser130 Oγ obviously decreases from −0.81 au in **g** to −0.40 au in the acyl–enzyme intermediate **i**. The transferred hydrogen is oriented toward the donor Lys73 Nζ and the Lys234 residue recovers the interaction with the β-lactam carboxylate, losing the hydrogen bond with Ser130 Oγ.

Some questions arise about the existence of the structure **g**. The stationary nature of this structure, with positive and a negatively charged centers placed only 2.89 Å apart, could be a consequence of the employed level of theory. It is well known in aminoacid chemistry that, using small basis sets, the glycine zwitterion is predicted to be a true minimum in the gas phase, but this minimum disappears using more flexible basis sets.[58] However, the glycine zwitterion is the absolute minimum in solid phase and aqueous solution. By means of H-bond interactions the enzyme can also stabilize two oppositely charged neighboring residues such as the protonated Lys73 and the deprotonated Ser130 residues. Thus this structure could be a true minimum on the potential energy surface also at higher levels of theory. On the other hand, disappearance of **g** in the reaction mechanism would require simultaneous proton transfers from Ser130 to β-lactam nitrogen and from Lys73 to Ser130. We have previously seen the preference of a sequential mechanism for the ring opening and proton transfer to the β-lactam nitrogen. The presence of **g** could be a consequence of similar criteria, indicating a catalytic machinery developed for avoiding complex simultaneous processes, more difficult to achieve, favoring in their place a simple sequence of elementary steps. In any case, the existence of this minimum energy structure seems to be unimportant from a kinetic point of view.

Conclusions

The capabilities of a new procedure based in a mixed quantum/classical approach have been used to tackle a very complete study of the acylation process in β-lactamase catalyzed hydrolysis of β-lactams. Minima, transition structures and two different mechanistic routes have been reported for the sequence of events proposed by Strynadka *et al.*[37] The energy barriers that have been obtained for the different steps are feasible for an enzymatic process. In fact, a moderate energy activation of 18.29 kcal mol^{-1} has been calculated for the rate-limiting step, the formation of a tetrahedral adduct.

From the obtained structures we can analyze some of the features of the enzymatic process. The role of the so-called oxy-anion hole can be better understood analyzing the charge distribution of the adduct **c** and the transition structure that leads to it, **b**. In **c** a large charge is developed on the carbonyl oxygen O5. However, this is not true of the previous transition structure, where the charge is developed fundamentally on the Ser70 Oγ. These facts seem to indicate that the fundamental role of the oxy-anion hole components, the amide backbone of Ala237 and Ser70, is not to decrease the energy barrier of the rate limiting formation of the adduct. Moreover, in a recent work on the solvation of β-lactams we have shown[56] that hydrogen bonds on the carbonyl oxygen can also have the undesirable effect of reinforcing the β-lactam CN bond, which must be broken in the subsequent steps. It has been previously claimed[43] that the role of the oxy-anion hole could be related to maintaining the substrate in the correct orientation and to help in the binding process.

On the other hand, in the tetrahedral adduct the β-lactam nitrogen has a considerably greater ability to form H-bonds than in the Michaelis complex. This fact is coincident with results of molecular dynamics simulations[56] of systems modelling the initial β-lactam and the tetrahedral intermediate. Part of the excess negative charge is located on the nitrogen atom and consequently the distance to the hydroxy hydrogen of Ser130 is shortened. This hydrogen atom can play different roles along the reaction path. Firstly, this interaction assists the cleavage of the β-lactam CN bond, favoring the location of the electron pair on the nitrogen. Secondly, the Ser130 hydroxy is located at a suitable position for the subsequent proton transfer from Ser130 to the nitrogen. Some experimental evidence on the active role of Ser130 has been observed after superimposition of the structures of the TEM-1 native enzyme and the acyl–enzyme complex with 6α-(hydroxymethyl)-penicillanate.[47] The side chain of Ser130 is displaced after complexation and acylation and the hydroxy oxygen of Ser130 is found at 3.1 Å from the nitrogen atom of the acylated substrate, in agreement with our calculated value (2.99 Å, see Table 1). Moreover, site-directed mutagenesis of Ser130 by asparagine, alanine, and glycine shows zero or reduced enzymatic activity.[59]

The tetrahedral adduct undergoes ring opening and β-lactam nitrogen protonation to achieve the acyl–enzyme intermediate, the final product of the acylation. Two mechanisms, concerted and a sequential, have been described. From an energetic point of view, the stepwise sequence of events is favored. This fact has been rationalized as a consequence of the ability of Lys73 to stabilize the large negative charge developed on Ser130 Oγ during the β-lactam nitrogen protonation. In the concerted pathway the lysine residue is not in a suitable orientation to stabilize this charge. The division of the process into several steps allows the lysine to reorient at a more favorable position before the proton transfer takes place. This feature, the preference for several elementary steps instead of a single concerted one, is also manifested in the last step of the acylation. In avoiding simultaneous proton transfers from Ser130 to the β-lactam nitrogen and from Lys73 to Ser130, a structure with close charged centers, **g**, appears in the reaction path. This structure is a consequence of a proton transfer from Ser130 to the lactam nitrogen prior to the proton transfer from Lys73 to Ser130. Some considerations have been discussed with respect to the possible relationship between these facts and the catalytic machinery developed by the enzyme. By means of hydrogen bonds the enzyme would be able to stabilize structures with neighboring oppositely charged centers. In this way, a concerted set of proton transfer events can be divided in several steps. Insofar as the positively, or negatively, charged species are better proton donors, or acceptors, than the corresponding neutral structures, these steps would have lower activation energies than the concerted process. By stabilizing certain structures, such as **e** or **g**, the enzyme can favor reaction mechanisms based on an elementary sequence of steps, which would be easier to achieve than more complex simultaneous processes.

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