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Role of Protein Flexibility in Enzymatic Catalysis: Quantum Mechanical−Molecular Mechanical Study of the Deacylation Reaction in Class A β-Lactamases

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Abstract: We present a theoretical study of a mechanism for the hydrolysis of the acyl-enzyme complex formed by a class A β-lactamase (TEM1) and an antibiotic (penicillanate), as a part of the process of antibiotic's inactivation by this type of enzymes. In the presented mechanism the carboxylate group of a particular residue (Glu166) activates a water molecule, accepting one of its protons, and afterward transfers this proton directly to the acylated serine residue (Ser70). In our study we employed a quantum mechanics (AM1)−molecular mechanics partition scheme (QM/MM) where all the atoms of the system were allowed to relax. For this purpose we used the GRACE procedure in which part of the system is used to define the Hessian matrix while the rest is relaxed at each step of the stationary structures search. By use of this computational scheme, the hydrolysis of the acyl-enzyme is described as a three-step process: The first step corresponds to the proton transfer from the hydrolytic water molecule to the carboxylate group of Glu166 and the subsequent formation of a tetrahedral adduct as a consequence of the attack of this activated water molecule to the carbonyl carbon atom of the β-lactam. In the second step, the acyl-enzyme bond is broken, obtaining a negatively charged Ser70. In the last step this residue is protonated by means of a direct proton transfer from Glu166. The large mobility of Glu166, a residue that is placed in a Ω-loop, is essential to facilitate this mechanism. The geometry of the acyl-enzyme complex shows a large distance between Glu166 and Ser70 and thus, if protein coordinates were kept frozen during the reaction path, it would be difficult to get a direct proton transfer between these two residues. This computational study shows how a flexible treatment suggests the feasibility of a mechanism that could have been discounted on the basis of crystallographic positions.

1. Introduction

Theoretical studies of enzyme-catalyzed reactions have recently received much attention, because they provide detailed information at the microscopic level. Gas-phase studies can be useful to get a first insight into the reaction mechanism, but it is quite clear nowadays that environmental effects must be included in some way. One simple way is just to consider some key residues in the calculation with some atoms fixed at their crystallographic positions. The development of several hybrid methodologies,[1] where only part of the system is described by quantum mechanics (QM) while the rest is considered usually by means of charges and van der Waals parameters (MM), has allowed the inclusion of larger parts of the protein environment into the study of the reaction mechanism.[2] It has been usual in these studies to retain a great part of the enzyme at fixed positions obtained from crystallographic data or partial geometry relaxation. However, this could be not always a good selection as far as proteins can be quite flexible systems and thus movements of their constituents could play an important role in catalysis. Searching for stationary structures considering relaxation of the full system (substrate plus enzyme) could be decisive in order to obtain realistic descriptions of some enzymatic reaction mechanisms.[3]

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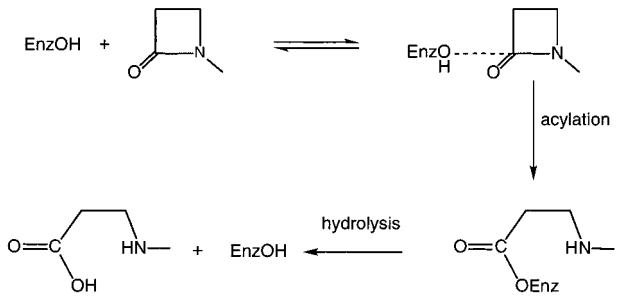
Scheme 1



A good example for testing the importance of protein flexibility in the study of the reaction mechanism is that of class A β-lactamases, a family of β-lactamases in which at least one of the key residues is placed in a highly mobile part of the protein.[4] β-Lactamases are produced by pathogenic bacteria to resist the attack of β-lactam antibiotics such as penicillins and cephalosporins, by catalyzing the hydrolysis of the amide group of the β-lactam ring.[5,6] These antibiotics kill the bacteria through an irreversible inhibition of a number of enzymes essential to the synthesis of the cell walls,[7] enzymes that are generically termed as penicillin binding proteins (PBPs). In recent years, the therapeutic effectiveness of this kind of antibiotics has been decreased by the appearance of strains of bacteria that are resistant to β-lactams. This resistance demands a continuous strategy of modification of known antibiotics, synthesis of new β-lactams, and development of inhibitors of β-lactamases to be coadministered with normal β-lactam antibiotics. Therefore, understanding of the catalytic mechanism of β-lactamases is crucial for the design of new antibiotics.

While antibiotics react with PBP proteins, forming a stable acyl-enzyme intermediate, β-lactamases destroy the antibiotic's efficacy by cleaving and hydrolyzing the sensitive four-membered ring (see Scheme 1) in a two-step (acylation and hydrolysis) mechanism to produce inactive acids. Whether a β-lactam antibiotic acylates, and thereby inhibits, an enzyme or is finally hydrolyzed and therefore inactivated depends on the relative values of the rate constants associated with these two processes. Thus, in the case of β-lactamases the hydrolysis of the acyl-enzyme intermediate is faster than for PBPs.

The classification of β-lactamases is based on comparison of the amino acid sequences. This separates the serine enzymes (in which its hydroxyl group is acylated by β-lactam substrate) into three classes A, C, and D, while the zinc metalloenzymes (where it is the zinc ion that hydrolyzes the antibiotic) are grouped together into class B. The most common β-lactamases are class A and, therefore, they constitute the group most intensively studied by means of X-ray crystallography,[8−10] kinetics,[11,12] mutagenesis experiments,[13−15] and molecular simulations.[4,16−22] From these studies, it has been found that the following residues play an important catalytic role in the mechanism of all the class A β-lactamases: Ser70, Lys73, Lys234, Glu166, and Ser130 (sequence numbering of Ambler et al.[23]). The acylation of Ser70 seems to be a well-established fact. However, the specific path followed by the proton (or protons) during the acylation step is still unclear. At least two important differences can be found among the proposed mechanisms for acylation.[24,25] The first one is the residue acting as the general base that accepts the proton coming from Ser70, and the second one is the residue acting as proton donor to the β-lactam nitrogen atom. With respect to the first question, there are at least two different candidates, Glu166 and Lys73. An acylation step assisted by Glu166 seems disfavored by the structural finding of a too-long distance between Glu166 and the hydroxyl oxygen of Ser70. However, a conserved water molecule could act as proton relay for the proton transfer between these two units.[4,16,26] Moreover, molecular dynamics simulations of PC1, a class A β-lactamase, has shown that the Ω-loop, a common feature of this kind of β-lactamases in which Glu166 is found, presents a high degree of mobility, which could favor the approach to Ser70.[4] With respect to Lys73, its role as general base would imply a deprotonated state for this residue. A general consensus has not been reached about this question. Some theoretical[27,28] and experimental data[26] support an initially protonated ϵ-amino group for this residue, and in particular, studies on the mutation of Lys73 and on the pH dependence of the catalytic constants suggest a protonated state for this residue.[19] Other studies found a pK_a shift as the substrate binds into the active site, suggesting a deprotonated amino group.[29] A combined quantum mechanics/molecular mechanics (QM/MM) optimization of an acyl-enzyme intermediate with both protonation states showed a better agreement with the X-ray structure in the case of a deprotonated Lys73.[19] Moreover, these calculations showed the possibility of a close contact between a protonated Lys73 and Glu166, which would facilitate a proton transfer from the Lys73 amino group to the carboxylate group of Glu166. As said before, the second controversial question is the protonation of the β-lactam nitrogen atom. This protonation can be reached after a direct proton transfer from Ser70 or, alternatively, by means of several proton-transfer events involv-

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ing several residues of the active site. Theoretical calculations seems to discard the direct concerted mechanism[19] while several paths are possible for stepwise proton transfer.[17,20] In a mechanism proposed by Strynadka et al.,[10] protonation would imply a sequence of proton transfers involving Ser70, Lys73, and Ser130. The feasibility of this mechanism has been demonstrated computationally by us,[20] showing energy barriers of modest size. Another recent proposal would imply a proton transfer from Ser70 to the carboxylate moiety of the antibiotic molecule assisted by the hydroxyl group of Ser130. This mechanism, which has also been theoretically explored, does not need Glu166 or Lys73 as general base for the deprotonation of the nucleophilic Ser70.[30]

How the serine-bound intermediate is deacylated is also unclear, but structural, kinetics, site-directed mutagenesis, and modeling studies have indicated that the conserved residue Glu166 is involved.[31−33] Strynadka et al.[10] proposed that the deacylation process could be accomplished by the nucleophilic attack of a water molecule assisted by a general base, Glu166, on the ester carbonyl carbon of the acyl-enzyme intermediate. This proposal lost support because of the distance between Glu166 and Ser70 residues in the crystal structure of the acyl-enzyme.[34] However, the structure of the active site in crystalline class A β-lactamases enzymes accommodates a water molecule closely associated with the carboxylate group of Glu166($O\epsilon_2$), the hydroxyl group of Ser70, and the amide group of Asn170. It has been suggested[9,35] that this water molecule is the one involved in hydrolysis (the hydrolytic water molecule). In a theoretical study using a reduced model of the active site, with some atoms fixed at their crystallographic positions, Hata et al.[36] found that an initially protonated Lys73 transfers the proton, via Ser130, to the carboxylate group of the β-lactam molecule. From this structure the hydrolytic water molecule, activated by a proton transfer to Glu166, approaches the carbonyl carbon of the antibiotic, forming a tetrahedral intermediate. From analysis of the resulting structure, a direct proton transfer from Glu166 $O\epsilon$ to Ser70 $O\gamma$, to give the inactivated antibiotic plus the free enzyme, was excluded on the basis of the large distance between these two atoms (4.1 Å).

In this paper we continue our exploration of the Strynadka mechanism for the hydrolysis of β-lactam antibiotics.[10] We have previously studied the acylation reaction by means of a QM/MM approach to the TEM1 enzyme and penicillanate (3α-carboxypenam) system.[20] Here we present the study of the deacylation or hydrolysis reaction by using the same QM/MM hybrid method, in which the full system is free to relax. We will show that, if geometry relaxation is allowed all along the reaction path, Glu166 can play a role not only in activating the hydrolytic water molecule but also in the proton transfer to Ser70 $O\gamma$. As we will show below, the reaction energy barriers obtained for this mechanism are of modest size, and thus it cannot be discarded only on the basis of the geometry analysis of some particular structures of the whole reaction path. Active-site residues implicated in catalysis are capable of displacements from their positions by several angstroms.

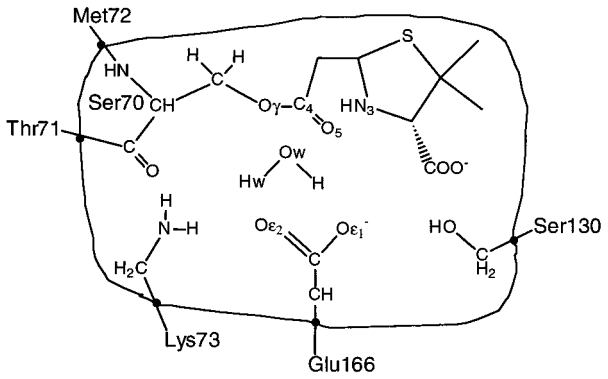
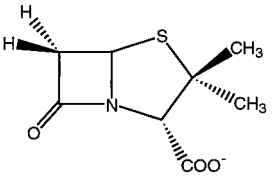


Figure 1. Schematic representation of the active site. The five link atoms are indicated as ●.

2. Computational Details

The initial geometry was obtained from the 1.95 Å resolution crystallographic structure of an *Escherichia coli* acyl-enzyme intermediate registered in the Protein Data Bank (ID code 1TEM). The complex is formed by TEM-1 β-lactamase enzyme and 6α-(hydroxymethyl)-penicillanate, a novel inhibitor for this enzyme. The hydroxymethyl moiety was manually removed to obtain the penicillanate **1**, a known substrate of TEM-1.



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The hybrid QM/MM treatment was carried out by means of the CHARMM25b2 program,[37] using the semiempirical AM1 Hamiltonian[38] with the CHARMM25b2 protein parameter set.[39,40] The entire molecular system, containing 4827 atoms, was divided in two different subsystems: the QM and MM regions. The QM region is formed by the penicillanate and the essential moieties of key active-site residues Ser70, Lys73, Ser130, and Glu166, as well as a structural water molecule located between the Glu166 and Ser70 residues (the hydrolytic water molecule). The MM region is composed by the rest of the protein and water molecules present in the crystallographic structure. Five hydrogen link atoms[41] were added to satisfy the valence of the QM fragments. Figure 1 shows the QM/MM division of the active site and the numbering used for some relevant atoms of the substrate.

In our calculations not only the QM atoms but also all the MM atoms were free to relax during the studied process. QM/MM energy optimizations were performed to obtain the potential energy profile. The minima presented a root-mean-square (rms) residual gradient of less than 0.001 kcal mol^{-1} $Å^{-1}$. A grid scan was used as a first step of the saddle-point search procedure. This exploration was carried out by

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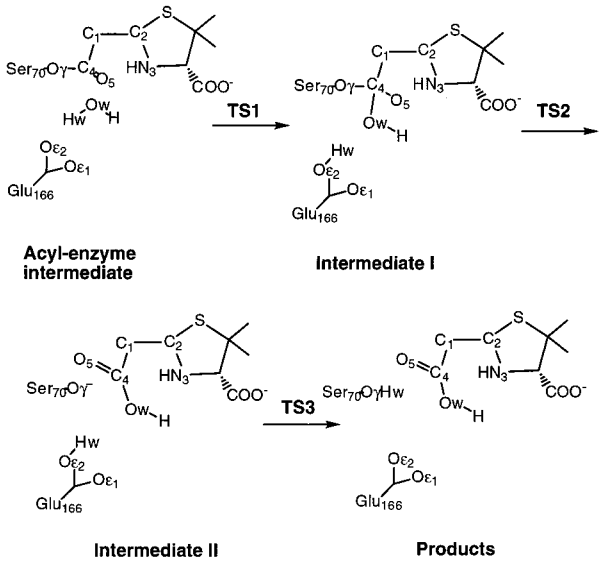
Table 1. Selected Geometrical Parameters[a] of the Stationary Structures

	acyl-enzyme	TS1	intermediate I	TS2	intermediate II	TS3	products
C_4–Oγ(Ser70)	1.371	1.430	1.460	2.116	2.646	2.631	2.543
C_4–N_3	2.563	2.829	2.873	2.914	2.911	2.961	2.944
C_4–O_5	1.236	1.283	1.302	1.248	1.240	1.241	1.241
C_4–Ow	2.491	1.537	1.437	1.367	1.348	1.344	1.343
Ow−Hw	0.966	1.113	2.094	2.182	2.556	2.829	2.614
Hw−Oϵ(Glu166)	2.025	1.389	0.984	0.987	0.985	1.090	2.112
Hw−Oγ(Ser70)	2.242	2.287	2.888	2.371	2.143	1.414	0.976
Oϵ2(Glu166)−C_4	4.779	3.592	4.043	4.128	4.610	4.582	4.449
Oϵ2(Glu166)−Oγ(Ser70)	4.188	3.191	3.575	3.152	2.985	2.486	3.032
Oγ(Ser70)−Nζ(Lys73)	3.318	3.210	3.345	3.024	3.887	3.174	3.181
Oϵ2(Glu166)−Nζ(Lys73)	4.010	3.923	3.849	3.349	3.257	3.244	3.659
C_4–C_1–C_2–N_3	−15.82	−31.41	−36.28	−39.25	−54.01	−58.57	−57.40

[a] Distances are given in angstroms; angles are given in degrees.

Scheme 2



optimizing all the geometrical variables except the distinguished coordinates of the reaction path. Once the potential energy surface was obtained, the approximate stationary points (minima and transition structures) were refined by use of the developed GRACE software.[3] This procedure allows for an efficient exploration of potential energy surfaces with very high dimensionality. With this purpose, a core was defined for which the Hessian matrix was calculated. This Hessian matrix was used to look for stationary structures, while the rest of the system was minimized at each step of the stationary structure search. In this case, a Newton−Raphson method was employed, utilizing a Hessian matrix of order 64 × 3, describing the curvature of the QM/MM energy hypersurface for a subset of the system, the QM atoms, together with a diagonal Hessian plus updates for the rest of the system. The rms residual gradient on the 64 atoms in the subset was less than 0.001 kcal mol^{-1} Å^{-1} in the optimized structure, while on the remaining atoms it was less than 0.005 kcal mol^{-1} Å^{-1}. Finally, the intrinsic reaction coordinate path (IRC)[42] was traced down from each refined transition structure by use of the GRACE program, to demonstrate conclusively that the reported structures were indeed the correct ones.

The results previously obtained by the same methodology for the acylation reaction[20] were in quite good agreement with ab initio calculations on reduced-protein models.[17] For example, the energy barrier for the rate-determining step during the deacylation process were 18.3 and 23.6 kcal mol^{-1} at the AM1 and MP2//HF/6-31+G(d) levels, respectively. Calculations were carried out on Cray-Silicon Graphics Origin 2000 at the Computation Centers of the Universitat of Valencia and Universitat Jaume I of Castellón.

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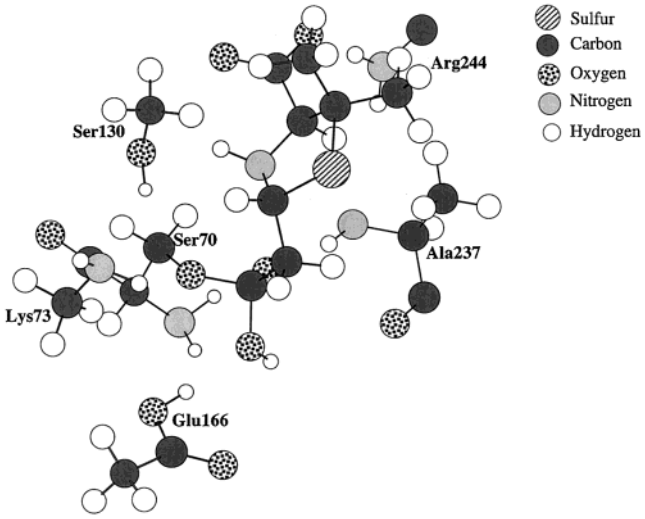

Figure 2. Structure of intermediate I obtained after QM/MM minimization.

3. Results and Discussion

Stationary Structures. The initial structure to study the deacylation reaction is the acyl-enzyme intermediate obtained from acylation of Ser70 by penicillanate, which was previously reported in ref 20. After exploration of the potential energy surface we found a deacylation reaction mechanism taking place in three steps. A representation of the reaction mechanism is shown in Scheme 2. The detailed structure of one of the stationary structures found (the first reaction intermediate) is given in Figure 2. Table 1 reports some relevant geometrical parameters of the stationary structures. Deacylation is initiated by the nucleophilic attack of a water molecule (the hydrolytic water molecule) on the ester carbonyl carbon of the acyl-enzyme intermediate through a unique transition structure, obtaining a tetrahedral intermediate. In this step the Glu166 carboxylate acts as a general base accepting the proton from the water molecule. The second step consists of the bond-breaking between the Ser70 Oγ of the enzyme and the β-lactam carbonyl carbon atom. The last step is the hydrogen transfer from the Glu166 carboxylate to the Ser70 Oγ to regenerate the protonation state of the enzyme, leading to a deprotonated Glu166. Relative and total energies of corresponding QM/MM optimized structures are given in Table 2. A qualitative representation of the energy profile is given in Figure 3.

In the acyl-enzyme intermediate, the β-lactam ring is already opened and bonded to the enzyme through Ser70 Oγ. The water molecule involved in the deacylation reaction forms a hydrogen

Table 2. Total and Relative Energies of the Stationary Structures Located on the QM/MM Potential Energy Surface

	total energy	relative energy	relative energy	relative energy
acyl-enzyme	−12271.73	0.00		
TS1	−12260.17	11.56		
intermediate I	−12280.07	−8.34	0.00	
TS2	−12263.73	8.00	16.34	
intermediate II	−12265.39	6.34	14.68	0.00
TS3	−12258.56	13.17	21.51	6.83
products	−12280.56	−8.83	−0.49	−15.17

[a] Energies are given in kilocalories per mole.

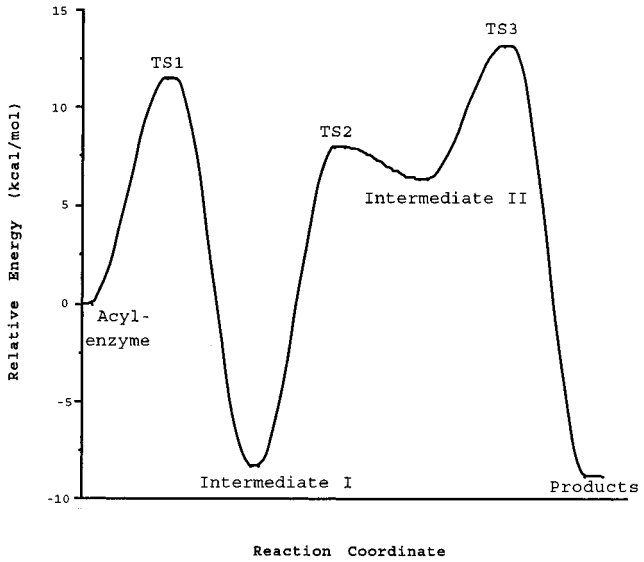

Figure 3. Energy profile for the deacylation process.

bond with Glu166 Oε (2.025 Å) and interacts also with the carbonyl carbon atom of the β-lactam (the C_4−water oxygen distance being 2.491 Å). In fact, this structure is correctly oriented for a nucleophilic attack of this water molecule to the carbonyl carbon atom and a proton transfer from the water molecule to Glu166. The distance between Glu166 Oε and the C_4 atom of the β-lactam is quite large, 4.779 Å, and the water molecule is placed in this hole. Obviously, the Ser70Oγ-Glu166Oε distance is also quite large (4.188 Å), in good agreement with the crystallographic structure.[34] This acyl-enzyme intermediate is 35.47 kcal mol^{-1} more stable than the Michaelis complex formed between the TEM-1 and the penicillanate, according to a previous study with the same computational model.[20]

TS1 is the transition structure that connects the acyl-enzyme and the tetrahedral adduct, called intermediate I. The distances from the oxygen of the hydrolytic water to C_4 and to Glu166 Oε are 1.537 and 2.502 Å, respectively. In this structure we can note the partial loss of double-bond character of the bond between the carbonyl carbon and O5 due to the approximation of the water molecule. One of the hydrogen atoms of this water is being transferred to the carboxylate group of Glu166 and the distance from this hydrogen to the acceptor oxygen atom is only 1.389 Å. Moreover, the distances from Glu166 Oε to C_4 and to Ser70 Oγ are shortened from 4.779 and 4.188 in the acyl-enzyme to 3.592 and 3.191 Å, respectively. This indicates that the Glu166 residue is closer to the substrate in the transition structure than in the acyl-enzyme. The energy barrier for this step is 11.56 kcal mol^{-1}.

Intermediate I is the tetrahedral structure for the deacylation process. This intermediate is 8.34 kcal mol^{-1} more stable that the acyl-enzyme complex. The carbonyl carbon of the β-lactam is bonded to the oxygen atom of the water molecule (1.437 Å), while the acyl bond (Ser70Oγ-C_4) is slightly lengthened (up to 1.460 Å). Therefore, the hybridization of the C_4 carbon atom changes from sp^2 in the acyl-enzyme intermediate to sp^3 in intermediate I. The C_4–O_5 distance has been also slightly increased, from 1.236 Å in the acyl-enzyme intermediate to 1.302 Å in the tetrahedral intermediate, as a consequence of this change in the electronic distribution around the carbon atom. Once the transfer of the proton from the water molecule to Glu166 has been completed, this residue moves away from the active site—both Glu166Oε-C_4 and Ser70Oγ-C_4 distances are lengthened—but a hydrogen bond is maintained between its acidic hydrogen and the oxygen of the water molecule (2.094 Å).

The normal-mode vibrational analysis of the unique imaginary frequency in the structure TS2 shows that this is associated with the distance between Ser70 Oγ and the carbonyl carbon atom of the β-lactam. This distance increases from 1.460 Å in intermediate I to 2.116 Å in TS2. This bond-breaking is assisted by a new hydrogen bond formed between Ser70 Oγ and the acidic hydrogen of the protonated Glu166, the distance being reduced from 2.888 to 2.371 Å when passing from intermediate I to TS2. That is, the Glu166 approaches again to the enzyme active site. Simultaneously with this bond-breaking, the two other bonds of C4 with oxygen atoms (O_5 and O_w) are shortened. The energy barrier for this second step, 16.34 kcal mol^{-1}, is the highest for a single step along the studied mechanism.

In the structure here called intermediate II there is no bond between the enzyme and the β-lactam antibiotic, the penicillanate remaining in its inactive acid form. The Glu166 is located far away from the substrate, the distance between Glu166 Oε and the C_4 of the β-lactam is 4.610 Å, and a hydrogen bond is formed with Ser70 Oγ (2.143 Å). Thus, by optimization of the full system we have arrived at a structure where the initial protonation state of the enzyme can be recovered from a simple and direct proton transfer from Glu166 to Ser70. It should be taken into account that this structure is only 1.64 kcal mol^{-1} more stable than the TS2. Furthermore, this energy difference is reduced to 1.35 kcal mol^{-1} when zero-point energies are included and to 0.68 kcal mol^{-1} if thermal contributions to the enthalpy (calculated at 298 K) are considered. Thus we cannot discard a possible two-step mechanism in which a unique transition structure would directly lead from intermediate I to products with adequate protonation states for Glu166 and Ser70. The TS3 structure interconnects intermediate II and products, the reaction coordinate being dominated by proton transfer from Glu166 Oε to Ser70 Oγ, recovering in this way the enzyme in its initial state and leaving the antibiotic in its inactive (opened) form. The distances between the hydrogen atom and the donor and acceptor oxygen atoms in this transition structure are 1.090 and 1.414 Å, respectively. The energy barrier for this step is only 6.83 kcal mol^{-1}. However, TS3 is the highest point along our energy profile and thus determines the rate of the global process. The energy needed to reach this structure is 21.51 kcal mol^{-1} from intermediate I but only 13.17 kcal mol^{-1} from the acyl-enzyme. These classical potential energies are reduced up to 17.50 and 10.65 kcal mol^{-1}, respectively, if zero-point

Table 3. Mulliken Charges and H-Bond Distances on Some Selected Atoms for the Stationary Structures

	acyl-enzyme	TS1	intermediate I	TS2	intermediate II	TS3	products
			Mulliken Charges (au)				
Oγ (Ser70)	−0.24	−0.34	−0.36	−0.69	−0.75	−0.68	−0.35
Oϵ1 (Glu166)	−0.61	−0.54	−0.39	−0.41	−0.41	−0.47	−0.61
Oϵ2 (Glu166)	−0.63	−0.57	−0.34	−0.34	−0.34	−0.47	−0.65
C_4	0.42	0.41	0.41	0.44	0.39	0.39	0.38
O_5	−0.46	−0.69	−0.77	−0.53	−0.46	−0.47	−0.48
Ow	−0.47	−0.35	−0.48	−0.33	−0.29	−0.28	−0.27
Hw	0.23	0.36	0.31	0.32	0.32	0.35	0.27
			H Bonds (Å)				
O_5–Ala237 HN	1.89	1.90	1.79	1.89	1.88	1.89	1.95
O_5–Ser70 HN	2.08	2.02	1.97	2.13	2.19	2.15	2.13
O_5–Ser70 HB	2.49	2.41	2.31	2.56	2.63	2.56	2.88
O_5–Met69 HB1	2.63	2.50	2.46	2.39	2.40	2.46	2.42
Ser70 Oγ-Lys73 HE1	2.73	2.52	2.54	2.44	2.33	2.61	2.72
Ser70 Oγ-Lys73 HZ2	3.14	2.87	2.98	2.35	2.30	2.49	2.88
Ser70 Oγ-Hw	3.34	2.84	2.91	2.67	2.04	2.05	2.27
Glu166 Oϵ1−Asn170 HA	1.97	1.88	2.48	2.08	2.12	1.97	1.87
Glu166 Oϵ1−Pro167HA	2.51	2.38	2.44	2.57	2.69	2.83	2.50
Glu166 Oϵ1−Hw	2.48	3.40	4.03	3.38	3.15	2.69	2.89
Glu166 Oϵ1−Hw	3.19	2.52	2.30	2.35	2.63	2.51	3.72
Glu166 Oϵ2−Hw	2.14	2.88	3.69	3.23	2.98	3.05	2.70
Glu166 Oϵ2−Phe72HE1	2.34	2.51	2.45	2.44	2.41	2.38	2.43
Glu166 Oϵ2−Lys73HE1	2.90	2.82	2.83	2.54	2.46	2.23	2.47

energies are taken into account. It is also interesting to note here that, in our previous study of the acylation process, we also obtained a multiple-step mechanism, in which the rate-determining step potential energy barrier is 18.29 kcal mol^{-1}.[20] The comparison among these energy values agrees with the fact that TEM1 is a β-lactamase, and thus the hydrolysis of the antibiotic must be competitive with the acylation.

The last optimized structure corresponds to products. In this structure the Ser70 and Glu166 residues are found in the protonation states corresponding to the enzyme in its initial form. However, it should be noted that in this structure a hydrogen bond has been established between the transferred hydrogen (now bonded to Ser70 Oγ) and Glu166 Oϵ, with a Ser70Oγ-Glu166Oϵ distance of 3.032 Å, smaller than in the Michaelis complex formed between the enzyme and the substrate.[20] The absence in the products of a water molecule in the active site, hydrogen-bonded to the Glu166 carboxylate, seems to be the reason for this difference. Thus, after releasing the product, the enzyme needs to bind a new water molecule in order to be prepared for its catalytic function. This last structure is 8.83 kcal mol^{-1} more stable than the acyl-enzyme intermediate and only 0.49 kcal mol^{-1} than the tetrahedral intermediate (or intermediate I).

Role of the MM Subsystem. In our computational scheme only the QM atoms have been explicitly considered in the definition of the Hessian matrix used to locate the stationary structures of the analyzed mechanism, while the MM atoms are relaxed at each step of the geometrical search. Thus, the MM subsystem responds to the changes that are taking place into the QM part. In particular, our computational scheme allows us to analyze the specific hydrogen bonds that appear between the QM and MM subsystems in the different stationary structures. Now these interactions, together with an analysis of the charges on some atoms, will be used as a guide to get a deeper insight into the mechanism of the deacylation reaction. The H-bonds and the Mulliken charges on some selected atoms are gathered in Table 3.

Going from the acyl-enzyme intermediate to intermediate I, the most significant effect in the evolution of the atomic charges is the increase, in absolute value, of the charge on the O_5 atom of the penicillanate from −0.46 to −0.77 au and the decrease of the charges of the Glu166 Oϵ1 and Oϵ2 atoms from −0.61 and −0.63 to −0.39 and −0.34 au, respectively. The Oϵ_1 and Oϵ_2 atoms of Glu166 interact with Asn170 and Phe72 more strongly in the acyl-enzyme intermediate than in intermediate I. The distances between the oxygen and the hydrogen atoms are 1.97 and 2.34 Å in the acyl-enzyme complex and 2.48 and 2.45 Å in intermediate I, reflecting the protonation of the carboxylate group. Evidently, the large negative charge on the O_5 atom in intermediate I is a consequence of the loss of double-bond character of its bond with the C_4 atom and the formation of the tetrahedral adduct. According with this distribution of the charges, the distances of the H-bonds formed between the O_5 oxygen atom and the enzyme (Ala237 HN, Ser70 HN, Ser70 HB, and Met69 HB) are shortened from the acyl-enzyme intermediate (1.89, 2.08, 2.49, and 2.63 Å) to intermediate I (1.79, 1.97, 2.31, and 2.46 Å). Ala237 N and Ser70 N form the so-called oxyanion hole, which plays an important role stabilizing the negative charge on the O_5 oxygen atom during the acylation process.[17,20] Our results show that the oxyanion hole assists the formation of the corresponding tetrahedral adduct not only during the acylation but also during the hydrolysis or deacylation. However, there is an important difference between the role played by this oxyanion hole during the acylation and the hydrolysis reactions. In the former, the oxyanion hole develops its stabilizing effect fundamentally on the tetrahedral intermediate and not on the transition structure leading to it.[20] Here, during the deacylation reaction, we have found that the charge development on the O_5 atom is already more advanced in the transition structure, −0.69 au, as a consequence of the proximity of the hydroxyl group to C_4 and the initiation of proton transfer to the Glu166 carboxylate. Thus, the oxyanion hole must also play an important stabilizing effect on the transition structure and consequently on the energy barrier for this step. Furthermore, although the negative charge on the O_5 atom is quite important all along the reaction, the larger negative values are found for these two structures, TS1 and intermediate I.

The second step corresponds to the acyl-enzyme bond-breaking. In this step, the charge on the Ser70 Oγ atom is obviously increased, in absolute value, from −0.36 au in intermediate I to −0.75 au in intermediate II. This increase in the charge on Ser70 Oγ is accompanied by a reduction of the negative charge on the oxygen atom of the water molecule, from −0.48 au in intermediate I to −0.29 au in intermediate II, and a slight shortening of the two hydrogen bonds established between the Ser70 Oγ atom and Lys73, from 2.54 and 2.98 Å to 2.33 and 2.30 Å, respectively. We have seen that the O_5 atom accumulates a large negative charge in intermediate I, −0.77 au. In intermediate II a double bond is again formed between C_4 and O_5 and thus the charge on this oxygen atom is now −0.46 au. Consequently, the H-bond between the O_5 atom and Ala237 and the two H-bonds established with Ser70 (the oxyanion hole) are increased from 1.79, 1.97, and 2.31 Å in intermediate I to 1.88, 2.19, and 2.63 Å in intermediate II, respectively. The negative charge on the transition structure TS2 is distributed between Ser70 Oγ, −0.69 au, and the O_5 atom, −0.53 au, showing the advanced stage of the process from the electronic point of view.

As a consequence of the proton transfer from Glu166 Oε1 to Ser70 Oγ in the third step of the hydrolysis process, the large negative charge present on Ser70 Oγ in intermediate II is transferred toward the oxygen atoms of the Glu166 carboxylate group. In intermediate II, the Ser70 Oγ and Glu166 Oε1 and Oε2 atoms have charges of −0.75, −0.41, and −0.34 au, respectively, whereas in the products these values are practically exchanged, to −0.35, −0.61, and −0.65 au. Thus, the existing hydrogen bonds with Ser70 Oγ are increased from 2.04, 2.33, and 2.30 Å in intermediate II to 2.27, 2.72, and 2.88 Å in the products, while the hydrogen bonds formed with the carboxylate oxygen atoms of Glu166 are intensified. The distribution of this negative charge in the transition structure TS3 is −0.68 au in Ser70 Oγ and −0.47 au in each of the carboxylate oxygen atoms of Glu166. These values reflect the slightly advanced character of the proton transfer from Glu166 Oε1 to Ser70 Oγ in the transition structure.

Glu166 Mobility. As previously said, the global process of antibiotic inactivation by β-lactamases takes place in two different stages, acylation and hydrolysis or deacylation. In our study of the Strynadka mechanism we found that the Glu166 residue was kept quite far away from Ser70 and the substrate during the acylation reaction. The calculated Glu166 Oε−Ser70 Oγ distance was 4.20 and 4.19 Å in the Michaelis complex and the acyl-enzyme intermediate respectively, being even larger for intermediate structures (for example, 4.73 Å in the tetrahedral intermediate appearing during the acylation).[20] These values are in good agreement with the crystallographic data of the acyl-enzyme intermediate[34] and have been used as an argument to exclude a direct proton transfer from Glu166 to Ser70 in the last step of the hydrolysis process. However, Glu166 is placed in a highly mobile motif of the enzyme, the Ω-loop. In fact, leaving all the system free to relax, we have found important changes in the distance between Glu166 and Ser70 along the studied reaction path. So in intermediate I the Glu166 Oε−Ser70 Oγ distance has been reduced up to 3.575 Å. In intermediate II a protonated Glu166 residue forms a strong hydrogen bond with a negatively charged Ser70 Oγ, the Glu166 Oε−Ser70 Oγ distance being only 2.985 Å in this structure.

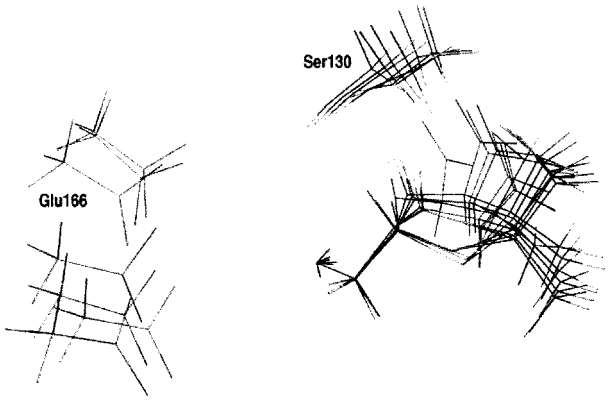

Figure 4. Superposition of the stationary structures found along the deacylation reaction, taking as reference the carbon atoms of the β-lactam ring. For clarity, only Glu166, Ser130, and the substrate are shown.

During the last proton transfer, from Glu166 to Ser70, this distance becomes one of the most important components of the reaction coordinate, reaching a value of only 2.486 Å in the corresponding transition structure (TS3).

Figure 4 shows a superposition of some key atoms of the stationary structures found along the deacylation reaction, taking as reference the carbon atoms of the β-lactam ring. As can be readily seen in this figure, during deacylation Glu166 shows displacements of very large amplitude when compared, for example, to Ser130. We can rationalize these results taking into account that Glu166 is placed in the Ω-loop, a common unique feature of class A β-lactamases. This loop is believed to be quite flexible and more mobile than the bulk of the enzyme.[9,35,43] In fact, a molecular dynamics simulation of a class A β-lactamase, the PC1 from *Staphylococcus aureus*, showed that a particular movement of the Ω-loop can bring the Glu166 carboxylate group into the active site to a position maintained for the rest of the trajectory.[4] In fact, the averaged Glu166 Oε-Ser70 Oγ distance obtained from the simulation was of ca. 2.8 Å, while the initial crystallographic value was close to 4.0 Å. From our results, it seems that this conformation is essential for the reaction. Once the Glu166 residue is placed in the active site, displacements in the necessary direction can promote the reaction by stabilizing a negative charge on Ser70 Oγ and facilitating a direct proton transfer to this atom. Experimentally, it has been determined that turnover of some clinically used antibiotics is accompanied by conformational changes that have been related to the movement of the Ω-loop.[44] Our results on the deacylation mechanism agree with this experimental evidence for structural elasticity of class A β-lactamases during inactivation of antibiotics.

4. Conclusions

We have studied the hydrolysis (or deacylation) of the acyl-enzyme complex obtained from the acylation of a class A β-lactamase (TEM1) by an antibiotic model (penicillanate). The reaction path presented corresponds to the reaction mechanism proposed by Strynadka et al.[10] in which the carboxylate group of a particular residue (Glu166) activates a hydrolytic water molecule and directly transfers a proton to the acylated serine residue (Ser70).

(43) Jelsch, C.; Mourey, L.; Masson, J. M.; Samama, J. P. *Proteins: Struct., Funct., Genet.* **1993**, *16*, 364.
(44) Taibi-Tronche, P.; Massova, I.; Vakulenko, S. B.; Lerner, S. A.; Mobashery, S. *J. Am. Chem. Soc.* **1996**, *118*, 7441.

An analysis of the crystallographic coordinates of the acyl-enzyme structure[34] shows a large distance between Glu166 and Ser70 residues, and thus a direct proton transfer between these two residues seems to be excluded if geometry relaxation is not allowed. In our study we employed a QM/MM partition scheme where all the atoms of the system were allowed to relax. For this purpose we used the GRACE procedure,[3] in which part of the system is used to define the Hessian matrix while the rest is relaxed at each step of the stationary structures search. By use of this computational scheme, the hydrolysis of the acyl-enzyme is described as a three-step process. The first one corresponds to the proton transfer from the hydrolytic water molecule to the carboxylate group of Glu166 and the subsequent formation of a tetrahedral adduct (intermediate I) as a consequence of the attack of this water molecule on the carbonyl carbon atom of the β-lactam. This step is assisted by the stabilizing interactions established between the O_5 atom and the constituents of the so-called oxyanion hole. Their effect, as deduced from the Mulliken charges and H-bond distances, is important not only on the tetrahedral intermediate, intermediate I, but also in the corresponding transition structure, TS1. In the second step, the acyl-enzyme bond is broken, obtaining a negatively charged Ser70 (intermediate II), which is protonated, by means of a direct proton transfer from Glu166, in the last step. Intermediate II is only 1.6 kcal/mol more stable than the transition structure connecting intermediate I and II (TS2). Thus, we cannot discard the possibility of a two-step process where the protonation of Ser70 would take place simultaneously with the acyl-enzyme bond-breaking. Exploration of the potential energy surface at higher theoretical levels and inclusion of temperature effects would be needed to further clarify this question.

TS3 is the most energetic stationary point in our reaction path, and thus reaching this structure is the rate-determining step of the hydrolysis process. The classical potential energy needed for this is 21.51 kcal mol^{-1} from intermediate I and 13.17 kcal mol^{-1} from the acyl-enzyme complex. When zero-point energies are added, the energy differences are 17.50 and 10.65 kcal mol^{-1}, respectively. The classical potential energy barrier for the rate-determining step of the acylation process in the same system was 18.29 kcal mol^{-1}. The comparison of these energy barriers agrees with the expected behavior for a β-lactamase, where the rate of the acyl-enzyme complex hydrolysis must be competitive with the enzyme acylation.

Finally, it is important to note that this example illustrates that not only the choice of the QM and MM subsystems but also the selection of those atoms to be frozen during the reaction path can determine the result obtained in theoretical explorations of enzymatic processes. Our study indicates that the proposed mechanism, which might have been excluded a priori on the basis of the geometry of a single structure, is actually energetically feasible. In this case, purely enzymatic geometrical parameters need to be not only relaxed during the study but explicitly included into the definition of the reaction coordinate. From our analysis of the reaction mechanism presented and the aforementioned molecular dynamics study,[4] it is tempting to think of enzymatic movements as being the rate-determining step for some enzyme-catalyzed reactions.

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