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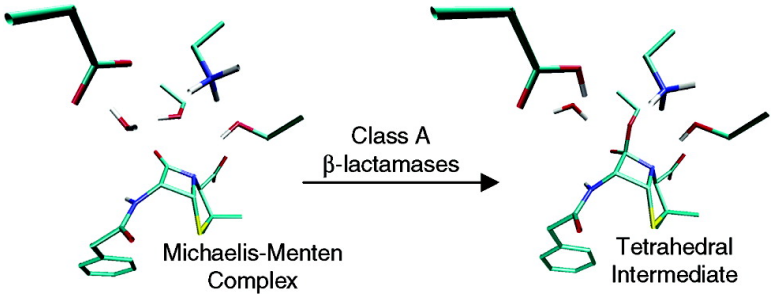


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# Identification of Glu166 as the General Base in the Acylation Reaction of Class A $\beta$-Lactamases through QM/MM Modeling

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Class A $\beta$-lactamases represent the most widespread form of resistance to $\beta$-lactam antibiotics. These enzymes, which evolved in bacteria from DD-transpeptidases (which are the natural targets of these compounds), hydrolyze the $\beta$-lactam ring and release the cleaved antibiotics. As such, $\beta$-lactamases pose a threat to human health and form a target for drug design. Improved mechanistic understanding of these enzymes should assist in the development of inhibitors and new antibiotics. The $\beta$-lactamase mechanism consists of two steps: acylation (covalent attachment of the $\beta$-lactam to an active site serine, Ser70[1]), followed by deacylation. Although the proposed mechanism of deacylation[2] is widely accepted, the mechanism of the acylation step is uncertain, with a number of mechanistic proposals having been made.[3−5] The rate-limiting step is the initial formation of a tetrahedral intermediate by nucleophilic attack of Ser70 on the $\beta$-lactam carbonyl group.[4−6a] Two possible candidates for the catalytic general base are the structurally conserved residues Glu166 and Lys73. Mutation of either residue results in greatly reduced activity.[7a−d] However, to act as the base, Lys73 would have to exist in the neutral form. This possibility, and the subsequent deacylation, has been modeled,[6a,b] but from experimental data[8] and theoretical investigations,[4,9] it seems unlikely that Lys73 is neutral. An alternative proposal involves Glu166 as the base[7b,10] in both acylation and deacylation acting via a structurally conserved water molecule.

The first step in the acylation mechanism in the enzyme, the formation of the tetrahedral intermediate, has been modeled here using a well-tested combined quantum mechanics/molecular mechanics (QM/MM) approach.[11a,b] The calculated potential energy surface (PES) shows that Glu166 acts as the general base (as in the deacylation step), deprotonating Ser70 via a water molecule. Deprotonation is found to be concerted with the nucleophilic attack on the lactam ring. The modeled mechanism is both energetically and structurally reasonable and is consistent with recent experimental structural investigations.[12a,b]

For this purpose, we prepared the crystal structure of the deacylation-defective benzylpenicillin acylated E166N-mutant TEM1 $\beta$-lactamase from *Escherichia coli* (PDB entry code 1FQG[13]) for the treatment with the QM/MM-module[14a] of the CHARMM software package 27b2[14b] (similar to the procedure described in ref 11b). Residue 166 was changed back to glutamate. Four important side chains (Ser70, Lys73, Ser130, and Glu166), the catalytic water (Wat290), and the substrate benzylpenicillin (70 atoms in total) were treated quantum mechanically on the basis of the semiempirical AM1 Hamiltonian, which has been shown to perform well on this system.[6a−c] Four "link atoms" were introduced to saturate the shells of QM-atoms covalently bonded to MM-atoms.[14a] All other atoms (3249 in total) were treated at the MM level with the CHARMM22 force field.[14c] QM/MM calculations were used to explore a number of mechanistic possibilities, by modeling the associated PESs. However, of the various mechanistic possibilities tested, only one was found to be both energetically and structurally reasonable, and in accord with experimental data[7a,b,9,12a,b] and recent molecular dynamics results.[15] In this model, formation of the tetrahedral intermediate (Figure 1) involves nucleophilic attack of Ser70 on the $\beta$-lactam ring (**Z**) and activation of Ser70 through abstraction of a proton by the general base Glu166, via a conserved water molecule Wat290 (**X**,**Y**).

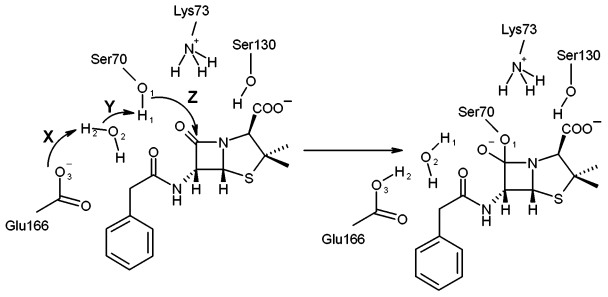


***Figure 1.*** Acylation mechanism of class A $\beta$-lactamases. Step 1: formation of the tetrahedral intermediate.

A PES was calculated as a function of two reaction coordinates $R_{\mathbf{Y}}$ and $R_{\mathbf{Z}}$. The first coordinate was defined as the difference between two interatomic distances (one for the bond to be cleaved, the other for the bond to be formed) ($R_{\mathbf{Y}} = d[O_1;H_1] - d[O_2;H_1]$). The second was defined as the distance between the $\beta$-lactam carbonyl carbon and the nucleophilic oxygen of Ser70 ($R_{\mathbf{Z}} = d[C_1;O_1]$). $R_{\mathbf{Y}}$ and $R_{\mathbf{Z}}$ were harmonically restrained and varied in steps of 0.2 and 0.1 Å, respectively. Energy minimizations of the structures were performed at each grid point to a gradient tolerance of 0.01 kcal mol$^{-1}$ applying the ABNR method (cutoff 13 Å, dielectric constant 1.0). The intermediates (energy minima) were determined more precisely by performing additional geometry optimizations with neither of the reaction coordinates restrained.

The shape of the resulting surface shows two minima representing the Michaelis−Menten complex (1) and a hydroxonium-ion structure (2). The other two corners of the surface are high-energy areas representing unstable structures: negatively charged Ser70 and protonated ester geometries. The approximate transition state (TS) can be identified with a barrier of 26 kcal mol$^{-1}$.

From the surface, it can be seen that the acylation follows a concerted reaction mechanism, where activation of Ser70 and nucleophilic attack happen simultaneously.[16] In the transition state, the distance between the oxygen and the carbonyl carbon is only 1.65 Å, and the proton is nearly equidistant (1.2 Å) between the oxygens of Ser70 and the catalytic water. The reacting $\beta$-lactam carbon changes from sp$^2$ to sp$^3$ hybridization. The carbonyl oxygen points into the so-called "oxyanion hole" where its charge is stabilized by two backbone nitrogen hydrogens (Ser70, Ala237).[3]

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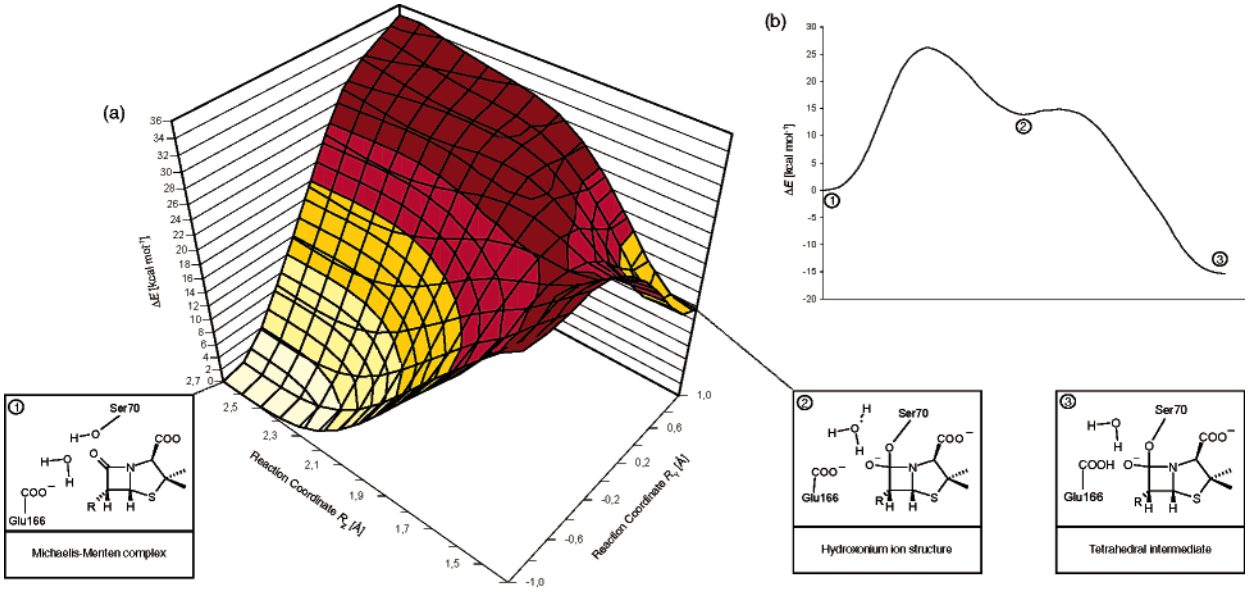


***Figure 2.*** (a) QM/MM potential energy surface of the first step of acylation. (b) Overall reaction energy profile of the formation of the tetrahedral intermediate.

Geometry (2) has a hydroxonium ion close to the general base Glu166. In further investigations, the reaction pathway for the proton transfer to Glu166 was calculated by restraining and varying reaction coordinate *X*, while *Y* and *Z* were not restrained. The resulting profile shows (as expected) a very small barrier of less than 1 kcal mol$^{-1}$ (Figure 2b). This suggests that this $H^+$ transfer happens directly after nucleophilic attack and that the structure containing $H_3O^+$ should not be considered an intermediate with observable lifetime. The resulting tetrahedral intermediate (3) with Glu166 protonated is calculated to be more stable than the Michaelis−Menten complex. This finding is in contrast to the results of a recent QM-only study[4] of a reduced active site model, demonstrating the importance of specific protein interactions in stabilizing the tetrahedral intermediate. The protein environment is included in the present QM/MM study, which accounts for the stabilizing influence of all surrounding protein residues through QM/MM interactions (especially key residues such as Asn132, Arg243, and Ala237, which were not included in the previous QM-study).

We present here the first QM/MM-study of the acylation step in class A $\beta$-lactamase with Glu166 as the active site base. The results from a large, realistic model, including all mechanistically important residues, provide new insights into how the active site residues work together. Several calculations were carried out under different conditions and were repeated in both reaction directions. We varied the restraints and applied alternative reaction coordinates for this mechanism, without observing any qualitative differences. Although we find no chemical role for Lys73 in this reaction step, the results do support its importance for catalysis, in agreement with experimental mutation studies.[7b,d] The function of Lys73 in the first step of acylation is electrostatic stabilization of the TS. On the basis of the present model, this TS stabilization is estimated to be catalytically significant (4 kcal mol$^{-1}$, by single point calculations on K73A mutant structures of the reactants and TS). Furthermore, Lys73 is in a good position to act as a proton shuttle, very important in subsequent reaction steps when the leaving $\beta$-lactam nitrogen is protonated by Ser130. The reduced activity of mutants lacking Lys73 can be attributed to loss of TS stabilization in acylation as well as likely impaired proton transfer in later reaction steps.[7b,d]

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