

QM/MM Modeling of Class A β -Lactamases Reveals Distinct Acylation Pathways for Ampicillin and Cefalexin

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16 **Abstract**

17 Efficient mechanism-based design of antibiotics that are not susceptible to β -lactamases is hindered by
18 the lack of comprehensive knowledge on the energetic landscapes for the hydrolysis of various β -lactams.
19 Herein, we adopted efficient quantum mechanics/molecular mechanics simulations to explore the
20 acylation reaction catalyzed by CTX-M-44 (Toho-1) β -lactamase. We show that the catalytic pathways
21 for β -lactam hydrolysis are correlated to substrate scaffolds: using Glu166 as the only general base for
22 acylation is viable for ampicillin but prohibitive for cefalexin. The present computational workflow
23 provides quantitative insights to facilitate the optimization of future β -lactam antibiotics.

24

25 Antibiotic resistance undermines the effective treatment of bacterial infections. The application of β -
26 lactam drugs has elevated many bacterial strains to inactivate common β -lactam based antibiotics families.
27 One major source of β -lactam resistance stems from β -lactamases, bacterially-produced enzymes that
28 effectively hydrolyze β -lactam drugs.¹⁻³ β -lactamases are generally classified into four groups: classes A,
29 C, D are serine-based, and class B are zinc-based. Class A serine β -lactamases (AS β Ls) represent a severe
30 threat due to their prevalence in infectious strains and affinity to a wide range of β -lactams.^{4,5} The
31 inactivation of β -lactams by AS β Ls has been extensively explored by pioneering computational and
32 experimental studies. Conserved in most AS β Ls, a widely-accepted catalytic mechanism has been
33 proposed that β -lactamase-promoted hydrolysis is a serine-mediated acylation-deacylation process.⁶⁻¹⁹
34 The acylation pathways have shown flexibility as this process could be mediated by either Lys73 or
35 Glu166 acting as the general base (Fig 1a).⁶⁻⁸ While the acylation process is believed to be conserved in
36 all AS β Ls, their catalytic efficiency (k_{cat}/K_M) against different β -lactam substrates has been shown to be
37 diverse.^{2,3,9} Among hundreds of β -lactam-based antibiotics being developed, the most successful efforts
38 involve engineering the β -lactam cyclic scaffold.²⁰ In this regard, understanding the underlying interaction
39 landscapes resulting from modifications on substrate structures can be informative for future optimization
40 and design of novel antibiotic series.

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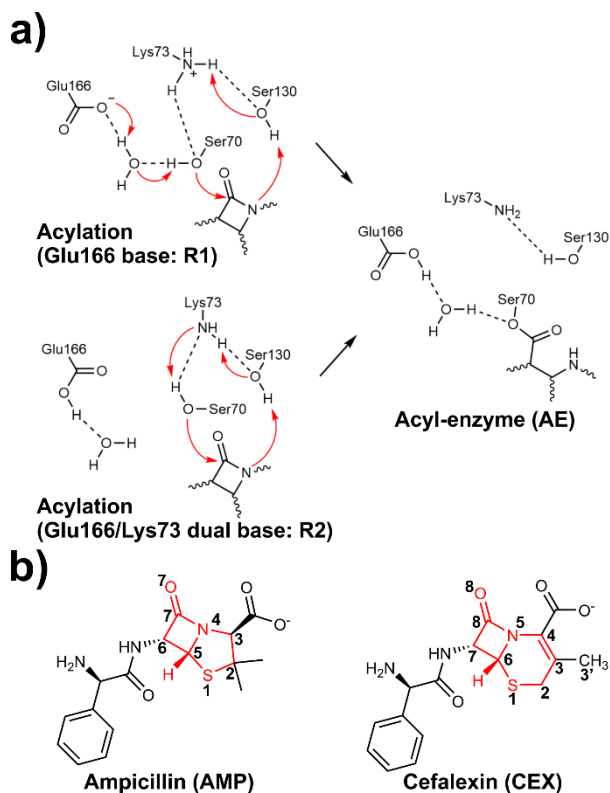


Figure 1. Mechanisms of acylation in ASBLs and structures of the model substrates. (a) The general mechanism of β -lactam acylation mediated by ASBL; (b) Structures of ampicillin (AMP) and cefalexin (CEX).

CTX-M is a representative ASBL group and has been identified as an immediate menace to commonly prescribed β -lactam antibiotics.⁴ The CTX-M enzyme class is characterized by its enhanced catalytic efficiency (k_{cat}/K_M) against cephalosporin antibiotic families.⁵ The hydrolysis of most cephalosporins deviates from that of other β -lactams by bearing a leaving group at its C3' position. Expelling the C3' leaving group would trigger a series of rearrangements, allowing its dihydrothiazine nitrogen to stay as an unprotonated imine after the acylation. However, an exception is cefalexin (CEX) which adopts a C3' methyl as a poor leaving group (Fig. 1b); The protonation of the CEX cephem amine is thus inevitable. CEX also poses enhanced resistance against CTX-M hydrolysis compared to other early generations of

55 penicillin or cephalosporins. In particular, Nitnai *et al.*⁹ showed that the catalytic efficiency (k_{cat}/K_M) of
56 CEX hydrolysis mediated by Toho-1 (also known as CTX-M-44) is $0.119 \mu\text{M}^{-1} \text{s}^{-1}$, which is 17-fold lower
57 than that of ampicillin (AMP, $2.11 \mu\text{M}^{-1} \text{s}^{-1}$). Whereas AMP and CEX structurally differ only in their
58 signature penam/cephem bicyclic rings (Fig. 1b), the cephem scaffold of CEX evidently showed higher
59 hydrolysis resistance even to the CTX-M enzyme class.

60 Pioneering computational efforts applying hybrid Quantum Mechanical/Molecular Mechanical
61 (QM/MM) techniques have provided fruitful insights into antibiotic resistance driven by AS β LS.⁶⁻⁸
62 Compared to other methods, the QM/MM Chain-of-States (CoS) approaches²¹⁻²⁴ are inherently
63 advantageous for computational efficiency and accuracy. As the CoS methods optimize the transition path
64 in the original conformational space, exhaustive exploration in the reaction-coordinates or collective-
65 variables reduced space can be avoided. Moreover, we demonstrated in a recent study¹¹ that the constraint-
66 based Replica Path Method^{21,22} optimized minimum energy pathways (MEPs) could provide barrier
67 heights that are compatible to experimentally determined k_{cat} for AS β L-catalyzed hydrolysis. In this study,
68 the acylation pathways of AMP and CEX hydrolysis in Toho-1 was investigated using QM/MM CoS
69 calculations.

70 The high-resolution crystal structures of Toho-1/benzylpenicillin (PDB entry: 5KMW, $1.10 \text{ \AA}$)¹⁰ and
71 Toho-1/cephalothin (PDB entry: 2ZQ9, $1.07 \text{ \AA}$)⁹ acyl-enzyme complexes were used as template systems
72 to create structures for Toho-1/AMP and Toho-1/CEX complexes. The topology files of AMP and CEX
73 were derived from CHARMM General Force Field (CGenFF)²⁵⁻²⁷. The ligand topologies in the template
74 systems were then substituted to create initial structures for Toho-1/AMP and Toho-1/CEX complexes.
75 As Lys73 and Glu166 are both potential general bases during the acylation step, systems with alternative
76 protonation states on Lys73 and Glu166 were prepared to account for acylation pathways via different
77 general base residues: first with protonated Lys73 and deprotonated Glu166 (noted as R1), and the other

78 with deprotonated Lys73 and protonated Glu166 (noted as R2). The protonation states of other titratable
79 residues are assigned referring to additional pKa calculations (Table S1) and neutron diffraction data of
80 the *apo*-state Toho-1¹². A total of 4 enzyme-ligand models were created, protonated, optimized, and
81 equilibrated using a semi-empirical QM/MM scheme with the third-order Density Functional Tight
82 Binding theory with the 3OB parameter set (DFTB3/3OB)^{28,29} as the QM potential and CHARMM36
83 force field (C36)³⁰ as the MM counterpart (see Supporting Information, SI, Fig. S1, Fig. S2 for details).
84 The interatomic distances between the key reacting heavy atoms during a 100 ps molecular dynamic
85 simulation using the DFTB3/3OB/C36 potential are shown in Table 1; it is noted that the distribution of
86 key reacting distances does not significantly differ between the 2 systems. The initial structures of the
87 pathway calculations were selected as the snapshots that have the minimal inter-heavy-atom distances
88 between the reacting functional groups of the four residues (Ser70, Lys73, Ser130, and Glu166), the
89 catalytic water and the β -lactam.

90

91 Table 1. The mean interatomic distances between key reacting heavy atoms in the DFTB3/3OB/C36
92 dynamics. Parenthesis denote the standard deviation (unit: Å).

Atom pairs	Toho/AMP:R1	Toho/CEX:R1	Toho/AMP:R2	Toho/CEX:R2
Ser70 O γ – AMP C7 or CEX C8	2.43 (0.17)	2.58 (0.18)	2.44 (0.17)	2.57 (0.18)
Lys73 N ζ – Ser130 O γ	2.85 (0.15)	2.95 (0.32)	3.07 (0.25)	3.15 (0.32)
Ser130 O γ – AMP N4 or CEX N5	3.60 (0.23)	3.86 (0.26)	3.67 (0.31)	3.63 (0.31)
Ser70 O γ – Water _{cat} O	2.65 (0.10)	2.65 (0.09)	–	–
Glu166 O ϵ 2 – Watercat O	3.06 (0.23)	2.77 (0.17)	–	–
Ser70 O γ – Lys73 N ζ	–	–	2.88 (0.13)	2.93 (0.17)

93

94 A total of 5 structures (noted as Toho/AMP: R1, R2, and Toho/CEX: R1, R1a, R2) were chosen from
95 the production trajectories. These 5 frames were then subjected to calculations at Density Functional
96 Theory (DFT) level. The DFT QM region covers important active site fragments: β -lactams, the catalytic
97 water, the surrounding residues (Ser70, Lys73, Ser130, Glu166, Asn170, Lys234, Thr235, Ser237),
98 together with a surrounding solvent molecule for the reaction pathway calculations. The hybrid density
99 functional B3LYP³¹ was used in conjunction with Pople's 6-31G double ζ basis set³² for the QM atoms
100 (B3LYP/6-31G/C36). The experimentally known stable states (reactant and acyl-enzyme) were first
101 subjected to geometry optimizations at the DFT/MM level. The optimized states were then connected by
102 a series of replicated conformations (replicas) that linearly intercepted the Cartesian space. The Replica
103 Path Method with holonomic constraints²¹ implemented in CHARMM³³ was applied for all pathway
104 optimizations through its interface³⁴ to Q-Chem³⁵. In order to comprehensively explore the stable
105 intermediates along the reaction, the replicas on the initial MEPs were independently minimized to the
106 nearest local minimum states. The final MEPs were then obtained by re-optimizing the chain-of-replicas
107 that connects the local minimums identified from the initial pathways. The energetic profiles on the
108 B3LYP/6-31G/C36 optimized MEPs were further refined with the augmented 6-31++G** basis set³⁶.
109 Lonsdale *et al.*^{37,38} proposed that the contribution from the dispersion effect is critical to accurately account
110 for enzymatic reaction profiles, therefore the D3 dispersion correction of Grimme³⁹ was also applied in
111 the single point energy calculations (B3LYP-D3/6-31++G**/C36). The locations of the transition states
112 are approximated by the replica with the highest energy on the optimized minimal energy path. This dual-
113 level DFT/MM workflow has been previously validated for closely resembling the catalytic barriers in
114 similar AS β L systems.¹¹ The ChElPG scheme⁴⁰ was employed for the charge population analysis along
115 the chain-of-states.

116 Pioneering theoretical studies proposed that the acylation of β -lactams could be mediated by either
117 Glu166 along or concertedly with Lys73. Hermann *et al.*⁶ first reported that the acylation reaction could
118 be mediated using Glu166 as the basic proton host in AS β L hydrolysis. In a similar AS β L/penam system,
119 Meroueh *et al.*⁸ further proposed that Lys73 is a viable alternative for the general base that accepts the
120 Ser70 hydroxyl proton. Augmented by extensive Machine-Learning regression analysis, our previous
121 work¹¹ on TEM-1 acylation pathways bridged the discrepancies between the energetics reported from the
122 above pioneer studies. In the present study, both pathways for acylation were investigated for AMP and
123 CEX.

124 The optimized reactant structures of Toho/AMP differ from Toho/CEX by the hydrogen bonding
125 networks between the penam/cephem carboxylate and the residues Thr235, Ser237 (Fig 2, Fig. S3).
126 Practically, the Ser237 hydroxyl is generally outside of the H-bonding region of the AMP carboxylic
127 group. The reactant configuration is therefore stabilized by a water molecule serving as the H-bond bridge
128 between the Ser237 hydroxyl and the AMP carboxylate (Toho/AMP:R1, Fig. 2a). Meanwhile, the CEX
129 adopts a more flexible binding pattern: the hydroxyl group from Ser237 could either form direct hydrogen
130 interacting to the substrate carboxyl group (Toho/CEX:R1, Fig. 2b) or to a solvent water molecule
131 (Toho/CEX:R1a, Fig. 2c). The superimposed conformations of the reactant states show that the QM
132 residues, the substrates and the catalytic water share a similar orientation (Fig. S4), indicating that the
133 optimized reactant structures are in the equivalent stationary potential energy state. As for the product
134 acyl-enzyme states, Vandavasi *et al.*¹² observed two Lys73 conformers in the perdeuterated acyl-enzyme
135 complex of Toho(Glu166Ala)/cefotaxime (PDB entry: 5A93, 2.20 Å). In our study, the conformations of
136 all AE1 states agree with the B conformer that carries a deprotonated Lys73 amine with its sidechain
137 resting in an extended configuration (Fig. S5). Notably, we observed an alternative Lys73 deprotonated
138 acyl-enzyme local minimum state (AE2) on all acylation pathways. The AE2 states slightly differ from

the AE1 states by the configuration of the deprotonated Lys73 amino (Fig. S6): the AE2 Lys73 N ζ adopts an extra hydrogen interaction to Ser70 O γ , while the AE1 Lys73 does not form the H-bonds to the acyl-serine complex. While the conversion between AE1 and AE2 are found to be barrier-less on all acylation pathways, we note that the AE1 states are shown to be slightly more energetically favorable as their energies are generally 2-4 kcal mol⁻¹ lower than the AE2 states (Table S2).

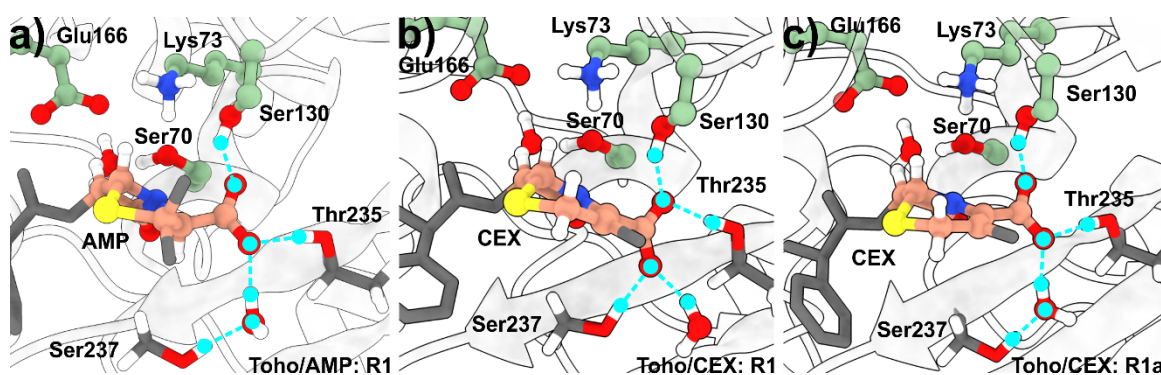


Figure 2. Conformations of R1 reactant states. The conformations of (a) Toho/AMP:R1; (b) Toho/CEX:R1; (c) Toho/CEX:R1a. The hydrogen bonding interactions are noted as blue dashed lines.

Our calculated Toho/AMP acylation pathways (Fig. 3a) closely resemble the potential energy landscapes reported by Meroueh *et al.*⁸: the energy barrier for the acylation using Glu166 as general base (14.0 kcal mol⁻¹) is moderately higher than that of Lys73/Glu166 concerted base (8.7 kcal mol⁻¹). The Toho/AMP acylation pathways agree with both acylation mechanisms, indicating that either Lys73 or Glu166 could mediate the acylation process in Toho/AMP hydrolysis. The ChEIPG charge profiles of the Toho/AMP pathways align with the intuitive understanding of the reaction mechanism. As shown in Fig. 3b and 3c, the decreasing charge population on AMP O7 between replica 20 to 27 is synergetic to the increasing charge on Ser70 O γ , suggesting the formation of tetrahedral intermediate (with a formal charge of -1 on AMP O7) during the serine addition. Furthermore, the locations of maximal charge profiles on

AMP N4 are also correlated with the replica with the highest energy along the reaction progress, showing that the protonation of AMP N4 is strongly correlated with the rate of acylation, agreeing with previous observations¹¹.

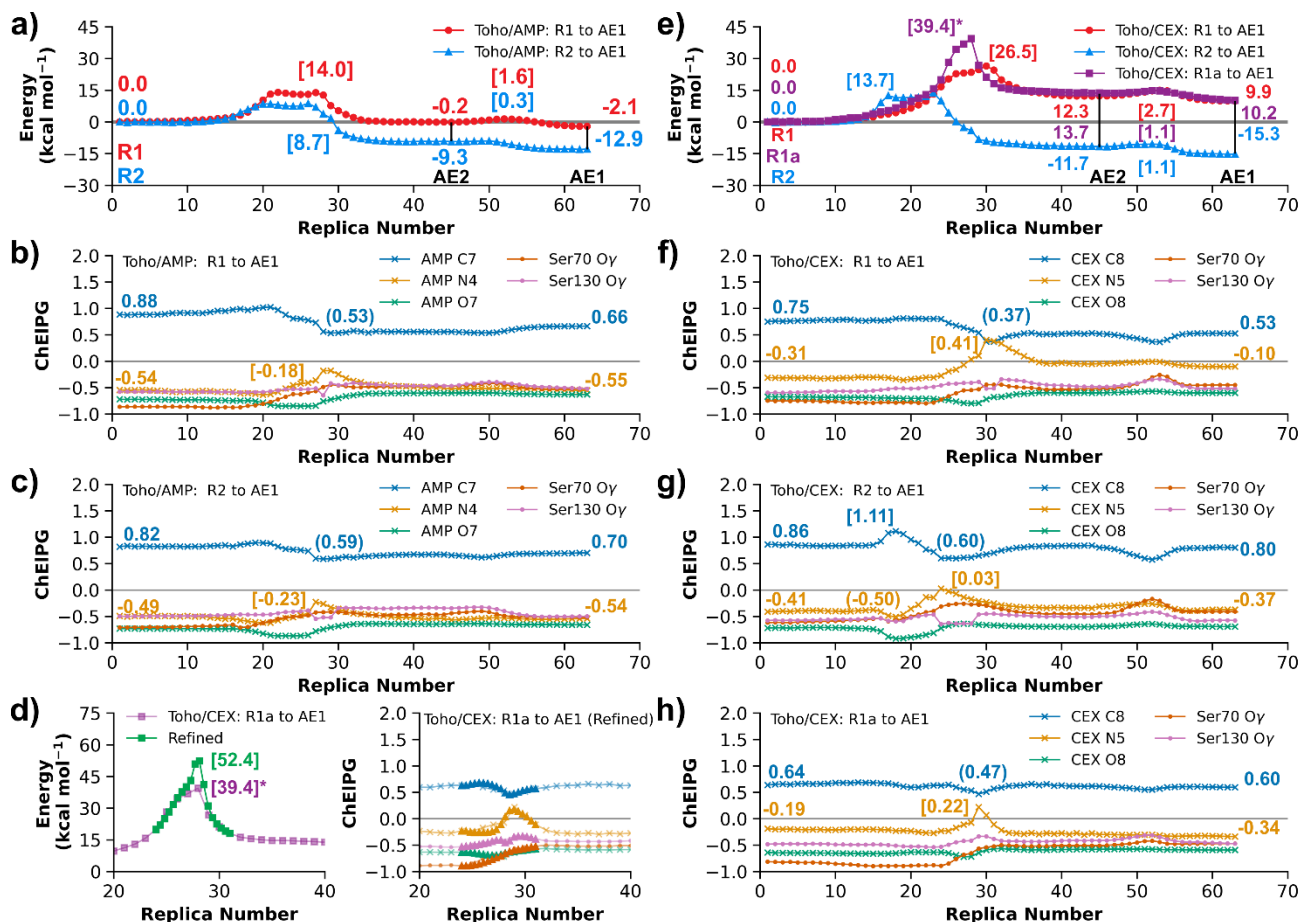


Figure 3. Energy profiles and the ChElPG charges of key atoms along the acylation pathways in Toho-1 hydrolysis. (a) The acylation profiles of Toho/AMP; The ChElPG charges along (b) the Toho/AMP: R1 to AE1 pathway, and (c) the Toho/AMP: R2 to AE1 pathway; (d) The energy profile and the ChElPG charge profiles of the refined Toho/CEX: R1a to AE1 pathways, which is calculated from inserting 18 replicas between replica 24 and 31 (see SI); (e) The acylation profiles of Toho/CEX; The ChElPG charges along (f) the Toho/CEX: R1 to AE1 pathway, (g) the Toho/CEX: R2 to AE1 pathway, and (h) the Toho/CEX: R1a to AE1 pathway. The vertical black solid lines in (a) and (d) indicate the

location of AE1 and AE2. Numbers in parentheses and brackets denote the local minimum and maximum values of important states along the reaction path. Note that only ChElPG charge values of β -lactam carbonyl carbon (blue) and nitrogen (orange) are shown in (b), (c), (f), (g), (h). See also Table S3-S7 for detailed replica-wise energy components and ChElPG charges on key atoms.

However, Toho/CEX acylation demonstrates a different catalytic mechanism, as shown in Fig. 3e. The acylation barrier using Glu166 as the general base is prohibitively high (26.5 kcal mol⁻¹). In particular, the corresponding barrier further increases to 52.4 kcal mol⁻¹ when cefalexin substrate adopts a similar binding pattern as ampicillin (Toho/CEX:R1a to AE1, Fig. 3d). These leave Lys73 as the inevitable candidate to mediate deprotonation of the Ser70 hydroxyl during CEX acylation, which confers an energetic barrier of 13.7 kcal mol⁻¹ (Toho/CEX:R2 to AE1). Further mechanistic insights can be derived from the ChElPG charge profiles. On the Glu166-mediated Toho/CEX acylation pathways (Fig. 3e, 3g, 3h), a stable tetrahedral intermediate indicated by the temporarily decreased charge on β -lactam carbonyl oxygen (as in the corresponding Toho/AMP pathways) is less synergetic to the formation of the tetrahedral intermediate. Moreover, the charge on the cephem nitrogen is largely increased to 0.41 (Fig. 3e) and 0.22 (Fig. 3g) upon the barrier replica, which evidently suggests its poor proton affinity to accept the proton transfer from Ser130. Alternatively, the dual-base mediated Toho/CEX: R2 to AE1 pathways (Fig. 3f) demonstrates a similar charge profile to the corresponding AMP acylation pathway. Interestingly, an increase of ChElPG charge on CEX C8 is seen uniquely upon the formation of tetrahedral intermediate on this pathway (Fig. 3f, replica 18). Intuitively, the lone pair on Ser70 O γ in the R2 configurations are oriented towards the ligand carbonyl carbon, potentially activating the conjugated π orbital on the β -lactam bicyclic. While the π -conjugation in AMP (N4-C7=O8) is localized to the β -lactam scissile C-N bond, it is extended along the cephem bicyclic (C3=C4-N5-C8=O8) in CEX. The temporary charge increment on

CEX C8 can therefore be interpreted as the consequence of breaking the more delocalized π -conjugation on the cephem scissile bond during the nucleophilic attack of Ser70 O γ . Accordingly, this explanation is also supported by the observation that the tetrahedral intermediates on Toho/AMP and Toho/CEX pathways do not significantly differ from each other in terms of heavy atom conformations (Fig. S7).

The computational barriers are further correlated with experimental kinetic studies (Table 2). Nitana*et al.*⁹ reported that the catalytic barrier (calculated from k_{cat}) of Toho/AMP hydrolysis is ~ 14.9 kcal mol⁻¹, slightly lower by ~ 1.7 kcal mol⁻¹ than that of CEX (~ 16.6 kcal mol⁻¹). In our calculations, both acylation barriers for Toho/AMP are sufficiently lower than the experimentally determined catalytic barrier, suggesting that the acylation mechanism previously developed for AS β LS are applicable to Toho-1/AMP as well. In contrast, the only viable reaction pathway for CEX is the Lys73/Glu166 dual base mechanism. The pathway that uses Glu166 as the only general base greatly exceeded the experimental barrier (16.6 kcal mol⁻¹) by 9.9 kcal mol⁻¹.

Table 2. The catalytic barriers of ampicillin and cefalexin hydrolysis in Toho-1.

Source ^[a]	Systems	Energy barriers (kcal mol ⁻¹)	Method ^[b]
Shimizu-Ibuka <i>et al.</i> ^{13, [c]}	Toho-1/AMP	15.5	303.15K, Exp
Nitana <i>et al.</i> ⁹	Toho-1/AMP	14.9	303.15K, Exp
This study.	Toho-1/AMP	8.7 / 14.0 ^[d]	B3LYP-D3, CoS
Nitana <i>et al.</i> ⁹	Toho-1/CEX	16.6	303.15K, Exp
This study.	Toho-1/CEX	13.7 / 26.5 ^[d]	B3LYP-D3, CoS

[a] Bold entries are computational results from this study;

[b] The experimental (Exp) catalytic barrier of Toho/AMP were derived from k_{cat} via the Eyring equations, the acylation barrier of Toho/CEX were derived from the ratio of k_{cat}/K_M to Toho/AMP;

210 [c] This study used the wild-type Toho-1 as the enzyme host while others used the Arg274Asn/Arg276Asn
211 Toho-1 mutant as the enzyme host;
212 [d] Values before “/” report the barrier of the Lys73/Glu166 concerted base acylation pathway. Values
213 after “/” report the Glu166 sole base acylation pathway.

214

215 In this study, we demonstrate that the AMP and CEX acylation energy landscapes differ from each
216 other during Toho-1 hydrolysis. Pioneering computational mechanistic studies^{6,8} suggested that acylation
217 could be mediated by either Glu166 solely or concertedly with Lys73 as the general proton acceptor(s).
218 In our calculations of both systems, the R1 pathway, which is mediated solely by Glu166 as the base,
219 confers a higher (potential) energy barrier than the R2 pathways. Using a cefotaxime bound Toho-1 system,
220 Langan *et al.*¹⁴ showed that the transition from R1 to R2 confers a free energy barrier of ~ 5 kcal mol⁻¹,
221 suggesting fast transitions between R1 and R2. This observation leads to the question of whether the R1
222 acylation pathway is mechanistically important in Toho-1 (or other AS β LS) catalysis. Herein, the R1
223 acylation pathway is shown to be energetically prohibitive for CEX (Fig. 3e, Table 2), leaving the
224 Lys73/Glu166 dual base mechanism as the main viable pathway for its acylation. In the case of AMP,
225 whereas the investigated acylation barrier via the Glu166 sole base mechanism is sufficiently lower than
226 the experimentally determined kinetics (Table 2), the viability of the R1 pathway is not evidently clear
227 from the potential barrier alone. However, unlike Toho/CEX, we note that the ChElPG charge profiles in
228 Toho/AMP acylation demonstrate a similar pattern for the R1 and R2 pathways (Fig. 3b, 3c), suggesting
229 that the R1 acylation mechanism is at least competitive to the R2 alternatives. The viability of both R1
230 and R2 pathways in Toho-1 mediated β -lactam acylation was also supported by pioneering
231 computational^{6,8} and experimental¹⁵ studies. In our assessment, the acylation mechanism developed for
232 AS β LS/benzylpenicillin, where both acylation pathways are accessible, is naturally transferable to

233 Toho/AMP catalysis. However, the acylation pathway utilizing Glu166 as the general base was shown to
234 be kinetically prohibitive for Toho/CEX as a result of the extended delocalization on N5, which is
235 introduced by the C3=C4 double bond. The viable acylation pathway for CEX is thus the Lys73/Glu166
236 dual base mechanism.

237 Our calculations with CEX acylation also shed light onto the hydrolysis of other cephalosporins. As
238 noted above, CEX mechanistically stands out in the cephalosporin family as its β -lactam nitrogen has to
239 be protonated upon the formation of the acyl-enzyme product. However, common cephalosporins such as
240 cephalothin and cefotaxime show higher catalytic efficiency (k_{cat}/K_M)^{9,16,17}, which suggests a much lower
241 acylation barrier than that of CEX. Such observations suggest that the cephem nitrogen may not be
242 protonated during the entire acylation processes of other cephalosporins. Through their crystallographic
243 study, Olmos *et al.*¹⁸ recently observed that the departure of the C3' leaving group is clearly simultaneous
244 to the serine attack during the AS β LS/cefotaxime acylation, supporting the above hypothesis. In this regard,
245 the protonation of the cephem nitrogen, which was also previously validated as the rate limiting step¹¹,
246 could be avoided, and leading to the higher acylation rates observed in other early generations of
247 cephalosporins.

248 Currently, efficient mechanism-based development of new antibiotics is obstructed by the lack of
249 sufficient knowledge on the energetic landscapes of various β -lactam hydrolysis. In the present study, we
250 report that one enzyme can adopt different acylation pathways responding to different substrate structures.
251 Using AMP and CEX as the model substrates and Toho-1 as the enzyme, our QM/MM CoS pathway
252 calculations demonstrated that the acylation mechanism of Toho-1 can be substrate-dependent. The
253 acylation pathways with Glu166 acting as the only general base are shown to be viable for AMP but
254 prohibitive for CEX. We attribute the low acylation activity in CEX to the lowered proton affinity of the
255 β -lactam nitrogen induced by the extended π -conjugation from the dihydrothiazine ring. In this regard, the

256 reactivity of the scissile C–N bond could be engineered by introducing additional π -conjugations to the β -
257 lactam. Accordingly, we note that similar structural features can also be seen on other robust β -lactam
258 variants (such as carbapenems and aza- β -lactams^{19,41}). In conclusion, we report the distinct mechanistic
259 basis of the seemingly identical acylation barrier for Toho-1 mediated AMP and CEX hydrolysis. On the
260 basis of the comparative mechanistic analysis to Toho/AMP and Toho/CEX acylation profiles, it is
261 expected that the current study enlightens the flexibility of the AS β LS mediated β -lactam acylation and
262 could facilitate future optimization and development of β -lactam based antibiotic drugs.

263

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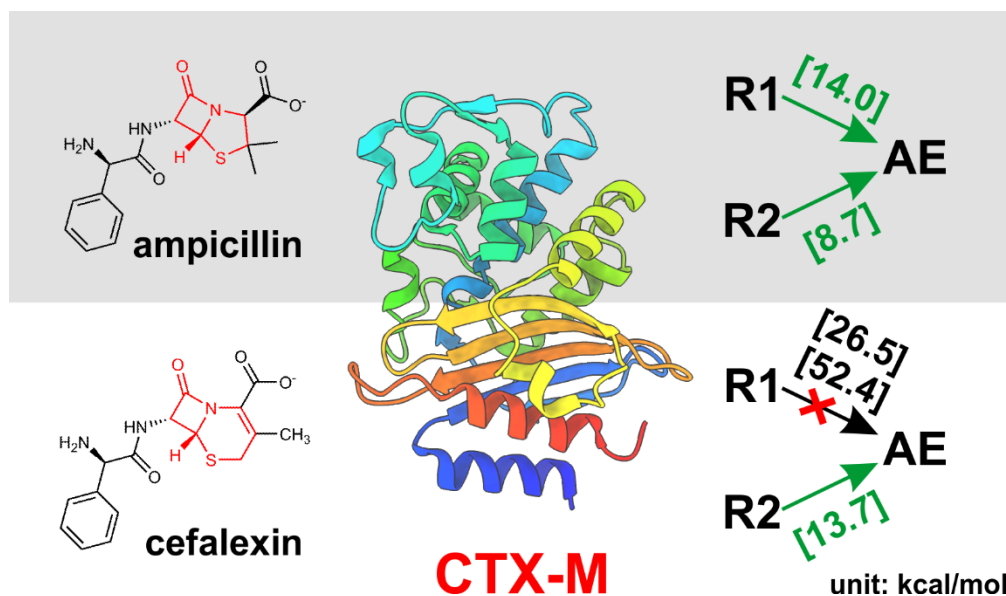
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Conflict of Interest

The authors declare no competing financial interest.

Table of Content



QM/MM chain-of-states calculations show that the acylation pathway using Glu166 as the general base is prohibited for cefalexin while it is viable for ampicillin hydrolysis, the acylation resistance observed for cefalexin attributes to decreased proton affinity induced by the delocalized π -conjugation.