An Efficient Computational Assay for β -Lactam Antibiotic Breakdown by Class A β -Lactamases

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Breakdown of β -lactam antibiotics by β -lactamases is one of the most common resistance mechanisms against these drugs. Here, we present a computationally efficient combined quantum mechanics/molecular mechanics simulation protocol for hydrolysis of the β -lactamase acylenzyme with meropenem, a carbapenem antibiotic. Starting from the previously published computational assay, we have successfully reduced both the conformational space and the sampling time, which leads to a more than 99% reduction on computer resources needed for this assay (less than 24 CPU hours per reaction). This simplified in silico protocol distinguishes accurately between class A β -lactamases that are able or unable to break down carbapenems. We further demonstrate that these protocols can be used to elucidate mechanistic details of β -lactam breakdown. The new efficient computational assay may be used to predict carbapenem hydrolytic activity of new β -lactamase variants as they arise, and in identifying routes to development of new, more resilient β -lactam antibiotics or new generations of mechanism-based β -lactamase inhibitors.

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An efficient computational assay for β -lactam antibiotic breakdown by class A β -lactamases

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ABSTRACT: Breakdown of β -lactam antibiotics by β -lactamases is one of the most common resistance mechanisms against these drugs. Here, we present a computationally efficient combined quantum mechanics/molecular mechanics simulation protocol for hydrolysis of the β -lactamase acyl-enzyme with meropenem, a carbapenem antibiotic. Starting from the previously published computational assay, we have successfully reduced both the conformational space and the sampling time, which leads to a more than 99% reduction on computer resources needed for this assay (less than 24 CPU hours per reaction). This simplified *in silico* protocol distinguishes accurately between class A β -lactamases that are able or unable to break down carbapenems. We further demonstrate that these protocols can be used to elucidate mechanistic details of β -lactam breakdown. The new efficient computational assay may be used to predict carbapenem hydrolytic activity of new β -lactamase variants as they arise, and in identifying routes to development of new, more resilient β -lactam antibiotics or new generations of mechanism-based β -lactamase inhibitors.

Antibiotic resistance is one of the most concerning phenomena of the 21st century.^{1,2} To some extent, this resistance occurs naturally, but in recent decades its spread has been accelerated by the excessive use of antibiotics.³ β -lactam drugs are one of the largest groups of commercially available antibiotics and they remain the most prescribed ones,⁴ but they also suffer from increasing clinical resistance.⁵ The key source for this resistance, especially in Gram-negative bacteria, are the β -lactamase enzymes.⁶⁻⁸

β -lactamases can be divided into four different classes based on their primary amino acid sequence homology (Ambler classification): classes A, C, and D are serine β -lactamases with an active site serine residue, whilst class B are metallo- β -lactamases with active site zinc ion(s).⁹ Of the four classes, class A β -lactamases form the largest group with many clinically significant enzymes. These enzymes are frequently encoded on mobile plasmids, which makes the transfer of resistance genes between pathogens easy and the enzymes widespread globally. Additionally, they readily expand their hydrolysis profiles via point mutations.¹⁰ This means that class A β -lactamases can inactivate a broad range of β -lactam substrates: in addition to hydrolyzing penicillins and cephalosporins, some family members can also mediate resistance against carbapenems.¹¹⁻¹³ Carbapenems are mainly used as 'last resort' antibiotics or for difficult infections,¹⁴ hence the Centers for Disease Control and Prevention (CDC) have categorized the clinical importance of

carbapenemase-producing Enterobacteriaceae (the group of Gram-negative bacteria that includes *Escherichia coli* and *Klebsiella pneumoniae*) as "an immediate public health threat that requires urgent and aggressive action".¹⁵

Scheme 1. First step of deacylation in class A β -lactamases. Glu166 acts as a proton acceptor allowing the deacylating water to perform nucleophilic attack on the acyl-enzyme (1), which results in tetrahedral intermediate formation (2).

β -lactamases inactivate β -lactam antibiotics by hydrolyzing the β -lactam amide (Scheme 1). In serine β -lactamases, this consists of two consequent reactions: acylation, resulting in formation of a covalently-bound acyl-enzyme, and deacylation.¹³ In acylation, a nucleophilic attack by the active site serine upon the β -lactam carbonyl carbon occurs to form the acyl-enzyme, via a tetrahedral intermediate.¹⁶ Deacylation is analogous to acylation including a tetrahedral intermediate (TI), but the nucleophile is an active site water molecule (the deacylating water, DW). In class A enzymes, both nucleophiles are activated via proton abstraction by an active site glutamate residue (Glu166 in the class A β -lactamase numbering scheme).^{17, 18} β -lactamases are typically inhibited when the acyl-enzyme intermediate is long-lived due to slow deacylation rates.¹⁹ For many β -lactam drugs, including carbapenems, TI formation in deacylation of the acyl-enzyme is most likely the rate-limiting step.¹⁴ Therefore, to determine the carbapenemase activity of class A β -lactamases, only this reaction needs to be modelled.

Previously, a quantum mechanics/molecular mechanics (QM/MM) protocol for modelling TI formation in the deacylation step for class A enzymes with meropenem was shown to correctly distinguish between carbapenemases and non-carbapenemases.¹⁹ Using the proposed protocol, carbapenem-inhibited enzymes showed deacylation barriers of 17.0-18.9 kcal/mol, whilst for carbapenem-hydrolyzing enzymes the values were 7.5-10.5 kcal/mol. A similar computational protocol has been used to study class A β -lactamase inhibition by clavulanate, which identified the covalent clavulanate complex

responsible for irreversible β -lactamase inhibition.²⁰ Despite the promising results of these protocols, the carbapenem assay requires significant computational resources due to extensive sampling of the free energy surface. Hence, it cannot be used for more rapid, computationally efficient, screening of a variety of different enzyme-antibiotic combinations.

Starting from our previous work, here we present and validate a more rapid computational assay that can discriminate between carbapenemase activities using only limited computational resources. The resources are minimized first by limiting the area on the free energy surfaces (FES) to be sampled, and then by reducing the sampling time. We demonstrate that this reduced protocol, requiring less than 1 % of the computational resources of the original assay, can still correctly distinguish between carbapenemases and carbapenem-inhibited class A β -lactamases. It therefore provides an efficient computational diagnostic towards *in silico* screening of β -lactamase activity.

We investigate eight class A β -lactamases, including the widely distributed *K. pneumoniae* carbapenemase (KPC), and the TEM, SHV and CTX-M enzymes (Table 1), selected for their clinical relevance and their ability (or inability) to hydrolyze carbapenems. Acylenzyme systems were set up, heated and equilibrated as described previously.¹⁹ Briefly, structures were solvated in water (TIP4P-Ew) and then minimized for 1000 steps, followed by heating from 50 K to 300 K in 50 ps using Langevin dynamics. 50 ps (unrestrained) QM/MM molecular dynamics was performed for equilibration, with followed by 400 ps of unrestrained QM/MM MD to generate starting structures for umbrella sampling (US) calculations. All further calculations were done employing QM/MM US simulations along two reaction coordinates: one for describing the proton transfer between DW and Glu166, and one for describing the nucleophilic attack of DW on the acylenzyme carbonyl. The semi-empirical SCC-DFTB method was used for the QM region,²¹ with the ff14SB and GAFF force-fields to describe the MM region of the protein and ligand, respectively. All calculations were done with the Amber16 program package.²² The weighted-histogram analysis method (WHAM)²³ was used to analyze US results and to obtain calculated barriers for each reaction ($\Delta^\ddagger G_{\text{calc}}$). Three independent US simulations were run for each acylenzyme (using different starting structures) to test differences in convergence of $\Delta^\ddagger G_{\text{calc}}$ (details in ESI; meropenem parameters at DOI 10.6084/m9.figshare.8158097).

Several modifications to the protocol were evaluated in order to improve computational efficiency. First, the amount of sampling was reduced by sampling only at those US windows corresponding to the approximate minimum free energy path (MFEP) on the FES. Based on our earlier work,¹⁹ the calculated MFEPs on full FESs across all eight studied β -lactamases are similar, which implies that only a partial FES needs to be calculated to compare the deacylation rates between studied enzymes. The “standard” MFEP used for partial sampling along the FES is presented in Figure 1 (and in the ESI). Sampling only in windows along this MFEP reduces the amount of US calculations from the original 374 to 28 per (partial) surface. The more limited sampling along the surface does not change the resulting $\Delta^\ddagger G_{\text{calc}}$ values significantly, with the largest calculated change between full and partial surface calculations being 2.5 kcal/mol (Table 1). Despite some changes in $\Delta^\ddagger G_{\text{calc}}$ values for all enzymes, the correct division into two groups is maintained. Carbapenemases (KPC, SFC, SME and NMC) have $\Delta^\ddagger G_{\text{calc}}$ values

between 7.8-10.4 kcal/mol, and carbapenem-inhibited enzymes (SHV, TEM, BlaC and CTX-M) between 15.5-16.8 kcal/mol. This suggests that the standard MFEP describes deacylation sufficiently well for differentiating between different carbapenem-hydrolyzing abilities.

Figure 1. Full free energy surface of deacylation for KPC-2 with meropenem. Red circles show the positions of umbrella sampling windows along the approximate “standard” minimum free energy path (MFEP) used for all enzymes. AC = acylenzyme, TS = approximate transition state, TI = tetrahedral intermediate.

Even when the amount of US windows is significantly reduced, the computational time for each window remains high. Hence, the possibility of utilizing shorter sampling times was first tested using the original 20 ps US results. This was done by using only the first 0.5, 1, 2, 5 or 10 ps of each 20 ps US window to calculate $\Delta^\ddagger G_{\text{calc}}$ values. (Table S1). This differs from sampling each window for a shorter time only, since the system is still allowed to equilibrate for 20 ps before changing the reaction coordinate restraints. Nonetheless, this preliminary analysis can be used to study the effects of shorter sampling. The results indicate that sampling for only a fraction of 20 ps is enough to distinguish between the two groups of β -lactamases. In most cases, the preliminary shorter sampling times yield somewhat higher barriers (<2.5 kcal/mol), which is expected due to reduced sampling of the phase space. However, this does not affect the distinction between carbapenemases and non-carbapenemases. When reducing sampling to 1 ps per window or less, more significant increases of the barrier heights (up to 20%) become common. We thus decided to use 2 ps US sampling per window. Sampling for 2 ps also ensures enough overlap between adjacent US windows.

Table 1. Experimental ($\Delta^\ddagger G_{\text{exp}}$) and calculated free energies of activation ($\Delta^\ddagger G_{\text{calc}}$) for the first step of deacylation of meropenem by eight enzymes using different protocols. The first four enzymes listed (KPC-2, SFC-1, SME-1, and NMC-A) are carbapenemases, whilst the rest (SHV-1, TEM-1, BlaC, and CTX-M-16) are not. $\Delta^\ddagger G_{\text{calc}}$ barriers for MFEP calculations are calculated from three simulations using WHAM as described in the ESI, standard deviations in the parenthesis. Computer resources required are estimated by extrapolating the time needed for all required QM/MM simulations from 2 ps calculations: 374 umbrella sampling windows for the whole surface and 20 ps per window (374 x 20) for “Full”, 28 windows x 20 ps for “MFEP (20ps)” and 28 windows x 2 ps for “MFEP (2ps)”.

β -lactamase	k_{cat} (s^{-1}) ^a	$\Delta^\ddagger G_{\text{exp}}$ (kcal/mol) ^b	$\Delta^\ddagger G_{\text{calc}}$ (kcal/mol)		
			Full ^a	MFEP (20ps)	MFEP (2ps)
KPC-2	3.6	16.8	10.5 (0.88)	8.5 (2.2)	9.1 (1.1)
SFC-1	6.5	16.6	10.9 (0.86)	10.4 (1.2)	9.9 (1.3)
SME-1	3.2	16.9	10.3 (2.8)	7.8 (0.2)	9.5 (1.4)
NMC-A	12.0	16.1	7.5 (0.43)	8.8 (0.4)	10.4 (0.7)
SHV-1	0.0013	21.6	17.0 (0.43)	16.1 (1.0)	19.5 (0.5)
TEM-1	0.0023	22.7	17.1 (0.43)	16.3 (2.2)	23.6 (1.4)
BlaC	0.0017	21.5	17.9 (0.08)	15.5 (2.2)	24.7 (1.1)
CTX-M-16	0.0042	20.8	18.9 (1.09)	16.8 (1.5)	17.2 (1.6)
Computer resource (%)			100	7.5	0.75

a) Values taken from ref. 19

b) Calculated from experimental rate constants using the Eyring equation.

Sampling for only 2 ps per window along the MFEP gives similar overall results to sampling for 20 ps (Table 1). With the shorter protocol, carbapenemases have $\Delta^\ddagger G_{\text{calc}}$ values of 9.1–10.4 kcal/mol, whilst carbapenem-inhibited enzymes have $\Delta^\ddagger G_{\text{calc}}$ values of 17.2–24.7 kcal/mol. Shorter US gives higher deacylation $\Delta^\ddagger G_{\text{calc}}$ values for all enzymes, with most significant increases seen for carbapenem-inhibited enzymes (0.4–9.2 kcal/mol) when compared to calculations using 20 ps sampling along the MFEP. However, the increased deacylation barriers do not change the division of the enzymes into the two distinct groups. Furthermore, the shortened protocol enables all running calculations on modest computing resources (e.g. on a desktop with one CPU) in a reasonable time. For the largest system (SFC-1, containing 54.9k atoms), US takes on average 23 hours to finish on one 2.6 GHz CPU. In general, the more efficient assay requires less than 1 % of the computer resources needed for the original assay (Table 1).

The efficient assay we have developed can be used to study specific mechanistic details of carbapenem hydrolysis. Glu166, the general base in deacylation, has two chemically inequivalent carboxylate oxygens: one that forms a hydrogen bond with Asn170 (OE1), and one that interacts with Lys73 (OE2) (Figure 2). The DW hydrogen bonds with OE2 in all MD simulations, and the US calculations have been performed using this oxygen as the proton acceptor. However, proton transfer to OE1 might also be relevant for deacylation. We thus used our new, efficient protocol to compare the two possible proton acceptors. When forcing the proton transfer to OE1, the $\Delta^\ddagger G_{\text{calc}}$ values increase by >3 kcal/mol for the carbapenemases (Table S2). Significant increases also incur for the carbapenem-inhibited enzymes (especially if the barrier with proton transfer to OE2 was not very high already). This consistent increase implies that the most probable proton transfer pathway in carbapenem hydrolysis is via Glu166:OE2 that interacts with Lys73, thus indicating an important role for this residue.

Figure 2. Active site of KPC-2 highlighting the hydrogen bonds between Glu-166 OE1 and OE2 and relevant residues. Meropenem in magenta.

Preference for a certain carboxylate oxygen as the general base has been observed also in other enzymes.²⁴ For the β -lactamases, DW is primarily hydrogen bonded with OE2 in the acylenzyme MD simulations and this interaction is thus present in all starting structures for US calculations. The preference for OE2 acting as the base can be explained by inspecting the additional hydrogen bonds formed by the Lys73 and Asn170 side chains. Lys73 interacts with Glu166:OE2 as well as Asn132, Ser70, meropenem and the Ser130 backbone carbonyl oxygen, whilst Asn170 interacts only with Glu166:OE1 and the backbone carbonyl of Leu167. Upon a proton transfer, other residues can balance the weakening interaction of Lys73 with Glu166:OE2, whereas Asn170 has fewer other interactions for such stabilization.

In conclusion, the carbapenemase activity of eight class A β -lactamases was assayed *in silico* using QM/MM reaction simulations with an optimized, efficient computational protocol. Efficiency is obtained by using the semi-empirical SCC-DFTB method and by limiting both conformational space and time sampled. Distinction between four enzymes known to efficiently hydrolyze carbapenems, and four enzymes that do not, can be made within a day with very modest computer resources (e.g. one CPU per enzyme). Additionally, this efficient assay can be used to inspect mechanistic aspects of carbapenem inactivation; exemplified here by comparing the $\Delta^\ddagger G_{\text{calc}}$ values of the first deacylation step for two possible proton transfer pathways. The short computational assay time with moderate computer resources now makes this assay attractive for more rapid *in silico* activity screening of different class A β -lactamase – antibiotic combinations. This will assist assessment and understanding of resistance to β -lactam drugs as conferred by β -lactamases, e.g. the effect of acquired point mutations on drug hydrolysis. As access to genome sequences of pathogen isolates becomes more routine, such information could be used in guiding prescription decisions. Furthermore, elucidation of mechanistic details of acyl-enzyme hydrolysis, as identified in simulations, may guide the development of new β -lactams or β -lactamase inhibitors designed to evade the activity of broad-spectrum and carbapenem-hydrolyzing β -lactamases.

ASSOCIATED CONTENT

Supporting Information. Simulation details, WHAM analysis and energies for the alternative proton transfer reaction are presented in the Supporting Information.

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Author Contributions

MWK & AJM designed the study; VHAH, KH, EIC and MALL performed and analyzed all simulations, with help from MWK. VHAH, KH, JS, AJM and MWK contributed to writing the manuscript.

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SUPPLEMENTARY INFORMATION

An efficient computational assay for β -lactam antibiotic breakdown by Class A β -lactamases

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Computational methods

The systems were set-up, minimized, heated and equilibrated as described in the ESI of ref. 1.

QM/MM free energy calculation

All QM/MM umbrella sampling (US) calculations were done using two reaction coordinates: one for describing the nucleophilic attack (NA, $d[\text{DW:O} - \text{ACA:C}]$), and one for describing the proton transfer (PT, $d[\text{Glu:O-DW:H}] - d[\text{DW:O-DW:H}]$).¹ Reaction coordinate values for the standard MFEP (in Å):

PT	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.7	0.6	0.5	0.4	0.3	0.2
NA	3.5	3.4	3.3	3.2	3.1	3.0	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.2	2.1

PT, continued	0.2	0.1	0.0	-0.1	-0.2	-0.2	-0.3	-0.4	-0.5	-0.6	-0.7	-0.8	-0.8
NA, continued	2.1	2.1	2.0	1.9	1.8	1.7	1.7	1.7	1.6	1.6	1.5	1.5	1.4

where PT and NA describe the values for the proton transfer and nucleophilic attack reaction coordinates, respectively.

Outputs from US calculations were analysed using the weighted histogram analysis (WHAM)² program using 17 bins for PT and 22 bins for NA reaction coordinates. The convergence criterium was set to 0.0000000000001. This analysis was done separately for all three starting structures, and lastly by combining all three results into one WHAM calculation for calculating the overall reaction barrier (as opposed to taking the average of three snapshots). However, especially with shorter sampling times WHAM analysis was prone to give false minima on the (partial) free energy surface, due to an error in the WHAM code used. This was resolved by locating these false minima and deleting the corresponding coordinate lines from the input files. Usually the false minima were due to only few coordinates, and in each case less than 1 % of data was removed. This problem can be overcome by utilizing a different analysis method or by fixing the WHAM code.

WHAM analysis of shorter sampling

To first inspect to possibility of reducing sampling time in each US window, the US results for 20 ps sampling along the MFEP were inspected by including only the first 10/5/2/1/0.5 ps in the WHAM analysis. For all three snapshots, the desired amount of sampling was included for each US window, which was followed by the same WHAM analysis as described earlier (where the

same US windows for three different snapshots were combined into one). Results for this analysis are presented in Table S1.

Table S1. $\Delta^\ddagger G_{\text{calc}}$ values calculated using WHAM, where results from three different calculations are combined into one WHAM analysis.

	$\Delta^\ddagger G_{\text{calc}}$ (20 ps)	$\Delta^\ddagger G_{\text{calc}}$ (10 ps)	$\Delta^\ddagger G_{\text{calc}}$ (5 ps)	$\Delta^\ddagger G_{\text{calc}}$ (2 ps)	$\Delta^\ddagger G_{\text{calc}}$ (1 ps)	$\Delta^\ddagger G_{\text{calc}}$ (0.5 ps)
KPC-2	8.5	8.9	8.9	9.8	10.2	10.5
SFC-1	10.9	11.0	10.3	10.8	11.3	12.8
SME-1	9.5	7.7	7.6	7.6	8.1	8.4
NMC-A	8.8	8.6	8.9	9.0	9.6	10.5
SHV-1	16.1	16.5	16.5	16.8	16.7	17.5
TEM-1	16.3	16.8	16.9	17.2	17.9	18.6
BlaC	15.5	17.0	17.0	17.9	17.9	18.0
CTX-M-16	16.8	17.1	17.2	17.6	17.9	17.9

Alternative proton transfer pathways

The two possible proton transfer pathways were inspected using the efficient protocol. Glu166 acting as the general base has two chemically inequivalent oxygens, which can both act as the proton acceptor. As the DW hydrogen bonds with Glu166:OE2 in MD simulations, it was used as the proton acceptor in all US calculations. However, technically the proton acceptor can also be Glu166: OE1. A comparison of activation barriers for the two different proton transfers are presented in Table S2. Using OE1 as the proton acceptor increases $\Delta^\ddagger G_{\text{calc}}$ values for all enzymes 2.0-9.7 kcal/mol: new $\Delta^\ddagger G_{\text{calc}}$ values for carbapenemases are 2.0 – 7.2 kcal/mol higher, and for non-carbapenemases 3.0-9.7 kcal/mol. This suggests that Glu166: OE2 should be used as the proton acceptor when modelling deacylation in class A β -lactamases, and that the hydrogen bonding interaction with Lys73 is important for efficient catalysis.

Table S2. $\Delta^\ddagger G_{\text{calc}}$ values for the two possible proton transfer pathways (with 2 ps sampling). All energies in kcal/mol, standard deviations in parenthesis.

	$\Delta^\ddagger G_{\text{calc}}$ (OE2)	$\Delta^\ddagger G_{\text{calc}}$ (OE1)	Difference
KPC-2	9.1 (1.1)	14.5 (2.2)	+5.4
SFC-1	9.9 (1.3)	17.1 (1.8)	+7.2
SME-1	9.5 (1.4)	15.2 (2.6)	+5.7
NMC-A	10.4 (0.7)	13.8 (2.1)	+3.4
SHV-1	19.5 (0.5)	29.2 (1.2)	+9.7
TEM-1	23.6 (1.4)	26.6 (1.6)	+3.0
BlaC	24.7 (1.1)	26.7 (2.6)	+2.0
CTX-M-16	17.2 (1.6)	21.1 (0.4)	+3.9

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