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Probing the Binding Site in the Antibiotic Resistance Enzyme, AmpC β-lactamase

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Abstract

β-lactam drugs, such as penicillins and cephalosporins, are widely used to treat bacterial infections, but resistance to these drugs is increasingly becoming a problem. One of the main causes of resistance to these β-lactam drugs is the bacterial production of β-lactamase enzymes, such as AmpC. These enzymes are capable of breaking down the drug within their active sites, rendering the antibiotic unable to harm the bacteria. The exact roles that the active site amino acid residues play in the recognition and breakdown of the drug are not fully understood. Here, we investigate the role of the active site residue asparagine-152 (Asn152) in AmpC by mutating it to a glycine, serine, or threonine residue and examining the effect that these mutations have on kinetic and structural properties. Uncovering the specific role of Asn152 in the function of AmpC will be useful in the development of inhibitors to these enzymes in order to combat bacterial resistance.

Introduction

β-lactam drugs, named for their β-lactam ring, have been used to treat bacterial infections for decades; however, bacteria are continually becoming resistant to these drugs, creating a growing problem in today's treatment of bacterial infections. Resistance requires the need for stronger, more potent antibiotics and these may not even be able to combat the infection. One of the primary mechanisms of resistance to these β-lactam drugs is the production of β-lactamases by the bacteria. These enzymes are able to hydrolyze the β-lactam ring, turning the drug into a molecule that is harmless to the bacteria[1].

One important β-lactamase that is a problem in many pathogens is AmpC[2]. This class C β-lactamase is able to hydrolyze extended-spectrum antibiotics, which are usually active against a wide range of bacteria[3]. The proposed mechanism of action of AmpC is shown in figure 1 and involves an acyl-intermediate where the substrate is covalently bound to the enzyme[4].
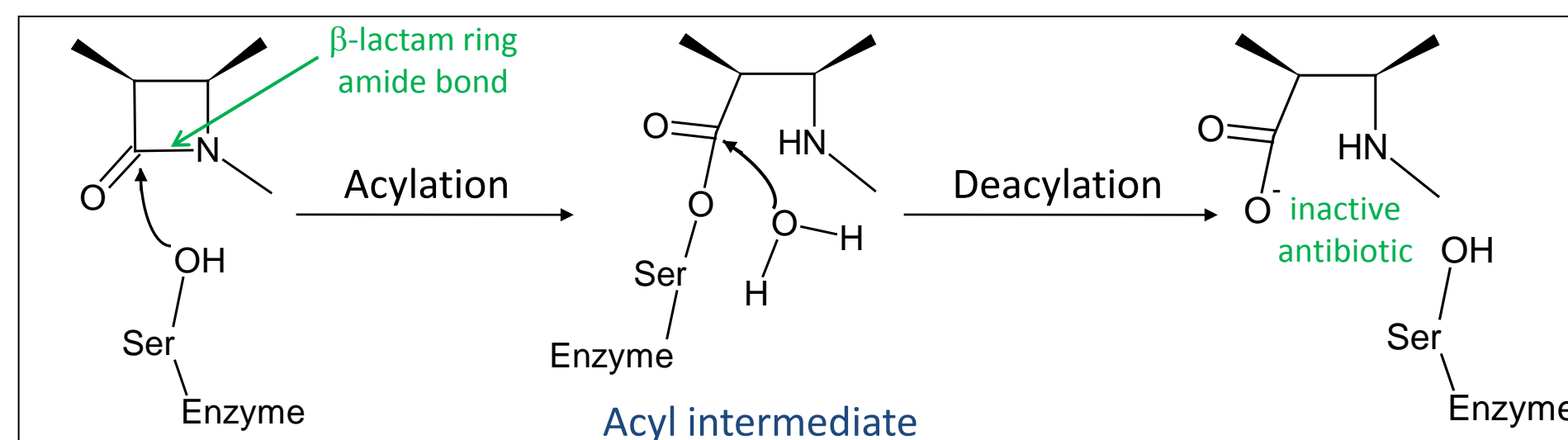

Figure 1: Proposed mechanism of β-lactam hydrolysis for AmpC

Although the general mechanism is understood, it is unknown how specific amino acid residues of the enzyme active site contribute to the catalysis, or how they are involved in recognition of the substrate. Elucidating the roles of each of the key residues would not only provide a clearer picture of the mechanism of hydrolysis of the drugs, but could also provide details of substrate recognition and binding for the development of new drugs that might combat antibiotic resistance.

One of the key residues in the active site is asparagine-152. The Asn152 residue is highly conserved in class C β-lactamases and takes part in the active site hydrogen-bonding network[5,6]. This residue appears to play a role in substrate selectivity, as a study done on P99 cephalosporinase, another class C β-lactamase, revealed that certain mutations at this site led to a switch in substrate selectivity, causing some substrates to be hydrolyzed more quickly than the wild type enzyme but others to be hydrolyzed more slowly[6]. Understanding the exact role of this residue could help determine the reason for the substrate specificity of AmpC. This information could be used to develop inhibitors that bind very well to the active site but are not hydrolyzed (released), thereby inactivating the β-lactamase and helping to kill the infectious bacteria.

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2. Choi, S., Lee, J. E., Park, S. J., Choi, S., Lee, S., Jeong, J., & Kim, M. (2008). *Antimicrobial Agents and Chemotherapy, 52*(3), 995-1000.
3. Thomson, K. S. (2010). *Journal of Clinical Microbiology, 48*(4), 1019-1025.
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5. Goldberg, S. D., Iannuccilli, W., Nguyen, T., Ju, J., & Cornish, V. W. (2003). *Protein Science, 12*, 1633-1645.
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In this study, we mutated the Asn152 residue to either a glycine, serine, or threonine, shown in figure 2, and examined the effects the mutations have on the activity of AmpC from *E. coli*.
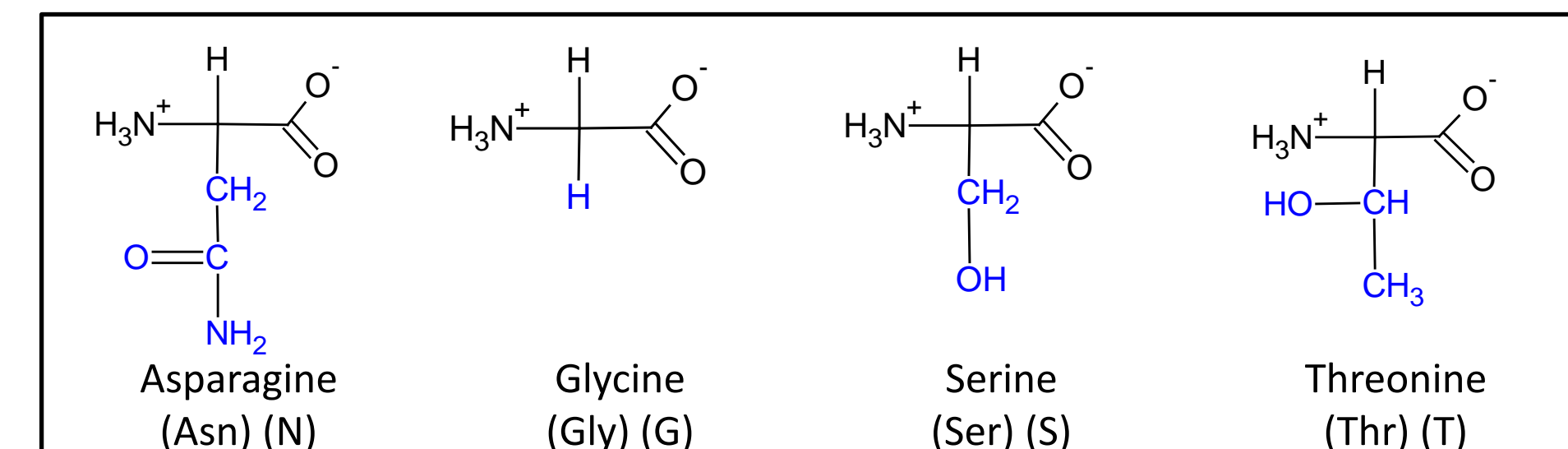

Figure 2: Selected amino acids

We measured the ability of the N152 AmpC mutants to break down three different β-lactam drugs: cefotaxime, cefoxitin, and oxacillin (figure 3).
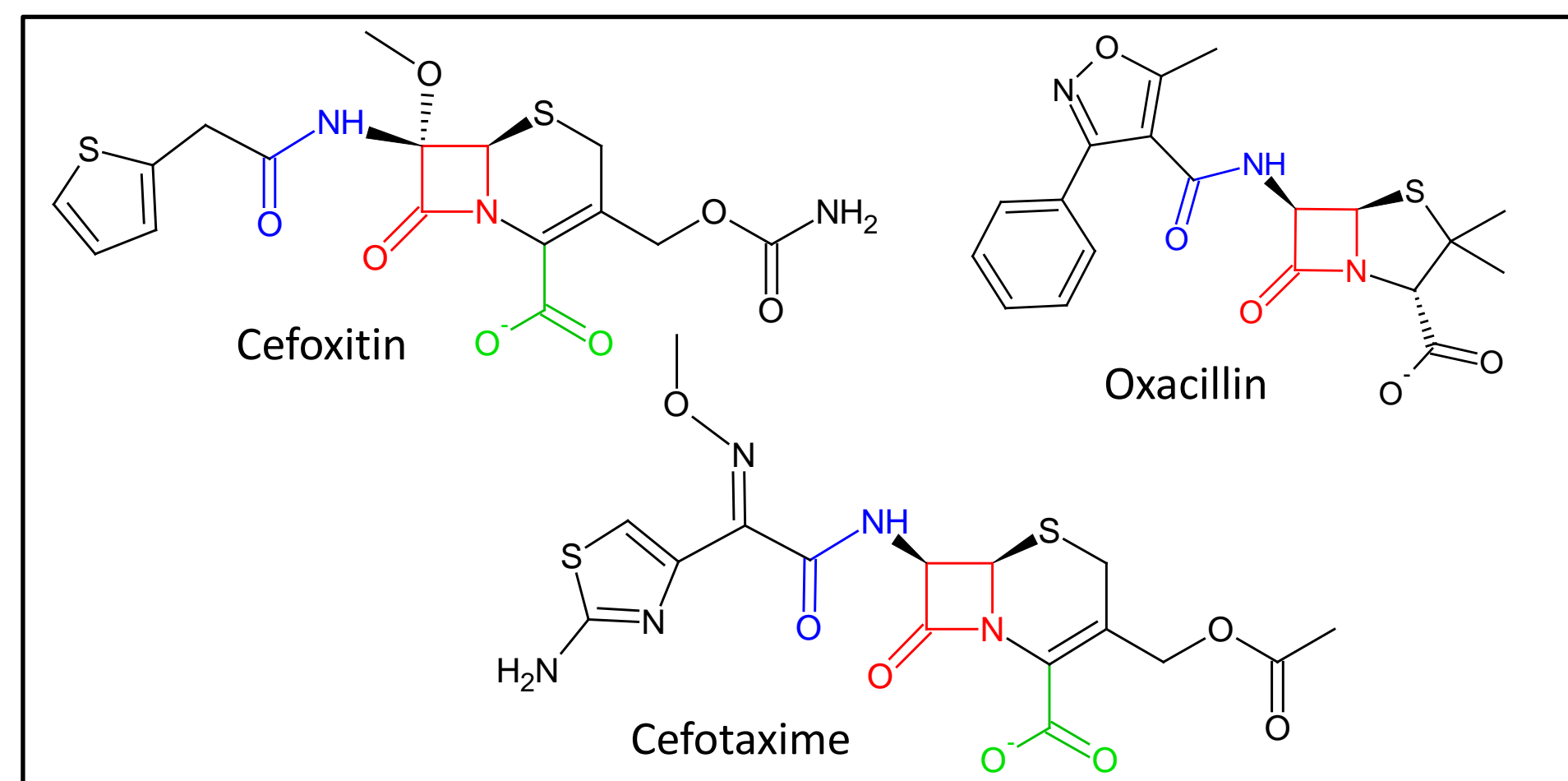

Figure 3: β-lactam drugs used in this study, with the β-lactam ring in red, C_4 carboxylate in green, and the amide of the R_1 sidechain in blue.

In order to compare the effectiveness of each of the mutants, we determined three Michaelis-Menten kinetic constants:

- K_m – "pseudo"-affinity constant – how well the enzyme binds to the substrate, a low value indicates tight binding

- k_{cat} – the "turnover number" – the total number of products that are formed by each enzyme active site per unit of time

- k_{cat}/K_m – overall efficiency – taking binding and rate of turnover into account

Results and Discussion

The Michaelis-Menten kinetic constants were determined using UV-Vis spectroscopy to follow the hydrolysis of the β-lactam amide bond. The values for the wild type AmpC enzyme and the N152G/S/T mutants with 3 different β-lactam drugs are shown below.

Cefotaxime

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1}s^{-1})
WT	1.32‡	0.012	0.009
N152G	455	1.09	0.002
N152S	47	0.56	0.012
N152T	800	1.85	0.002

Cefoxitin

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1}s^{-1})
WT	0.99‡	0.11	0.11
N152G	56	0.050	0.00090
N152S	1.16‡	0.0083	0.0071
N152T	1.12‡	0.0042	0.0038

Oxacillin

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1}s^{-1})
WT	0.053‡	ND*	ND*
N152G	0.27‡	0.41	1.52
N152S	0.068‡	0.21	3.17
N152T	1.2‡	1.38	1.15

‡Estimated as K_i *Not detected

Table 1: Michaelis-Menten kinetic constants for AmpC

Summarizing the kinetics:
- **Cefotaxime** – the mutants are faster than the wild type enzyme, but do not bind as well to the drug
- **Cefoxitin** – the N152S and N152T mutants bind the substrate as well as the wild type enzyme but are slower to break it down.
- **Oxacillin** – the wild type enzyme does not have measureable activity against this drug, but all three mutants show significant activity. The mutants do not all bind oxacillin as well as wild type, but they bind oxacillin significantly better than cefoxitin or cefotaxime, producing high k_{cat}/K_m values.

The mutations created a substrate specificity switch—allowing the enzyme to now break down oxacillin, at the cost of an activity reduction against cefoxitin, as well as affinity for cefoxitin and cefotaxime.

Focusing on the N152G mutant:
- It binds cefotaxime and cefoxitin worse than the wild type enzyme.
- It is slower to break down cefoxitin than wild type.
- It is significantly faster than the wild type enzyme when breaking down cefotaxime.

To examine the cause of this difference, the structures of AmpC N152G acylated to cefoxitin or cefotaxime were determined with X-ray crystallography. The structures of the active site in complex with cefoxitin and cefotaxime are shown in figures 4 and 5, respectively.
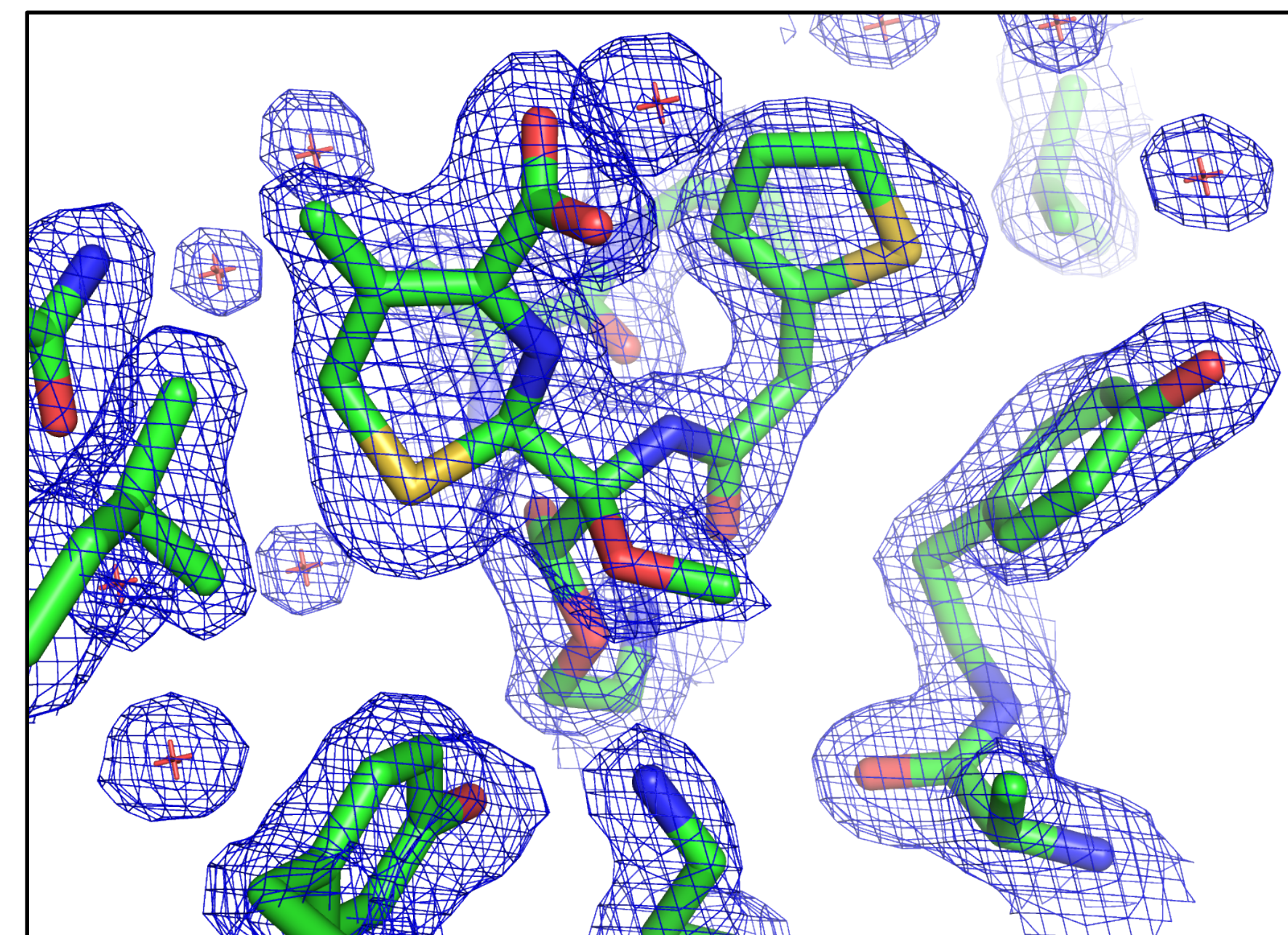

Figure 4: Active site of AmpC N152G in an acyl-intermediate complex with cefoxitin. Blue cages represent electron density contoured at 1σ. Image and subsequent images were produced with Pymol 1.3.
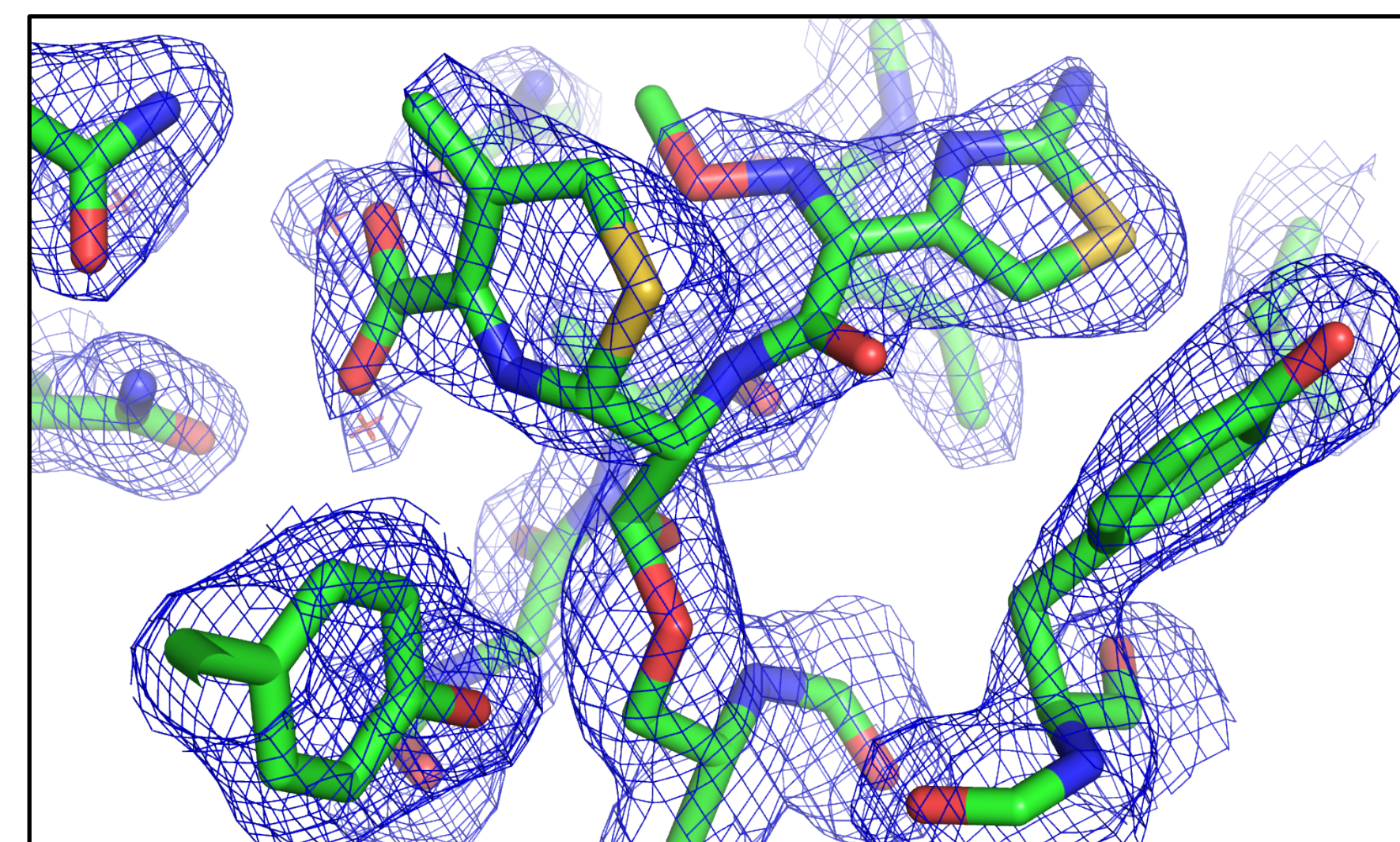

Figure 5: Active site of AmpC N152G in an acyl-intermediate complex with cefotaxime. Blue cages represent electron density contoured at 1σ.

By overlaying the two structures, we can compare the orientations of the two drugs. One obvious difference is the orientation of the C_4 carboxylate, shown in figure 6. We see that the C_4 carboxylate group on the dihydrothiazine ring of the cefotaxime structure hydrogen bonds to waters, while the cefoxitin structure shows a flip of the ring and the carboxylate now bonds to a water and Gln120.
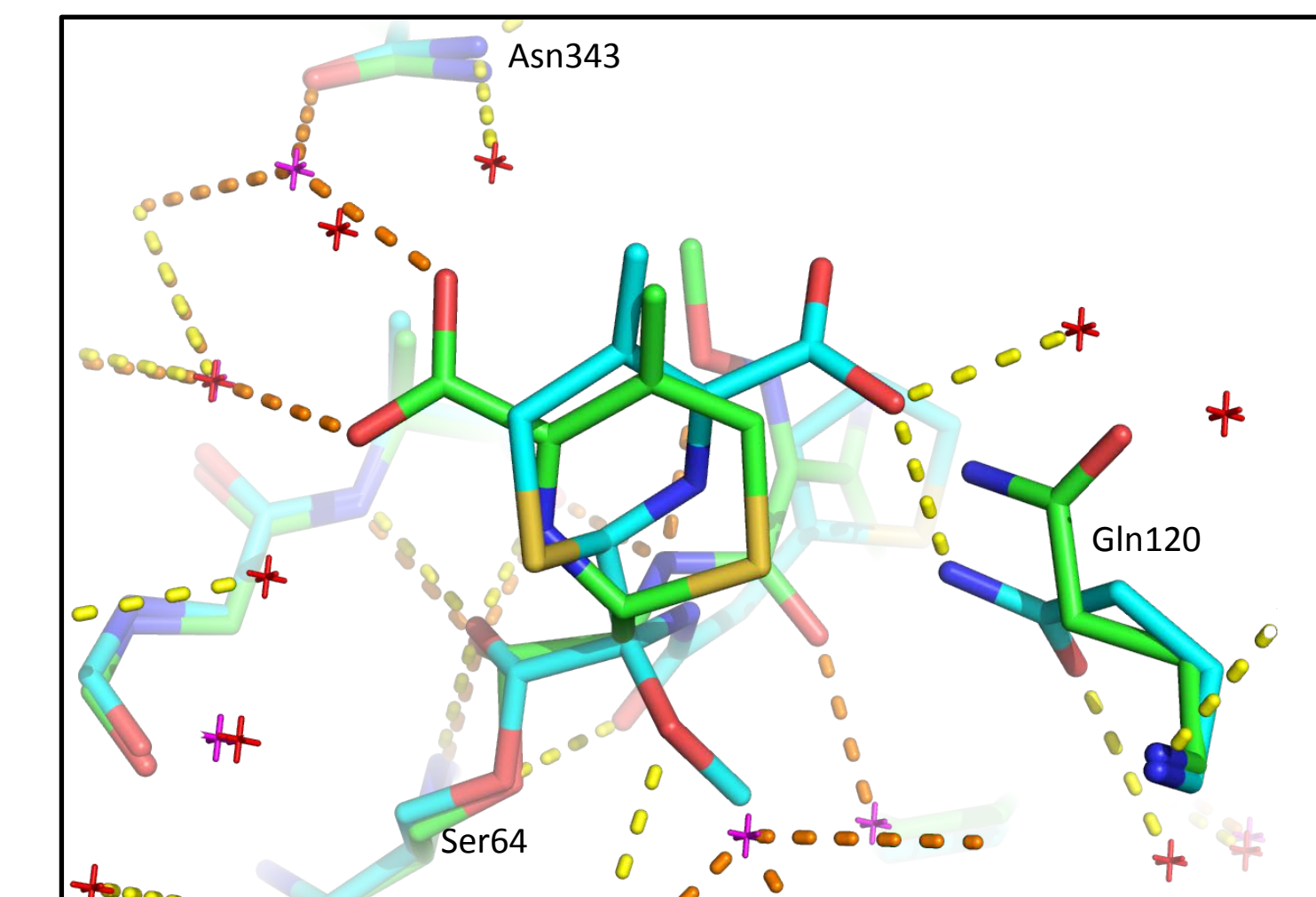

Figure 6: Overlay of AmpC N152G acylated to cefoxitin (cyan) or cefotaxime (green), focusing on C_4 carboxylate. Red waters and yellow hydrogen bonds are associated with the cefoxitin structure, while magenta waters and orange hydrogen bonds are associated with the cefotaxime structure.

One other major change between the two structures is the orientation of the amide which is on the R_1 sidechain, shown in figure 7. In the cefotaxime structure, the carbonyl of the amide points out toward residue-152 and hydrogen bonds to a water and Gln120. In the structure of AmpC bound to cephalothin, which is a good substrate for AmpC, the carbonyl is seen in a similar position and it also hydrogen bonds with Asn152[7]. Additionally, we see the nitrogen of the amide hydrogen bonding with the main-chain nitrogen of Ala318, as is also seen in the cephalothin structure. But in the cefoxitin structure, we find that the carbonyl has rotated away from Gln120 and towards Ser64, and it now hydrogen bonds with the main-chain nitrogen of Ser64. The nitrogen shows no hydrogen bonds. This different conformation may be the reason behind the slower activity of AmpC against cefoxitin.
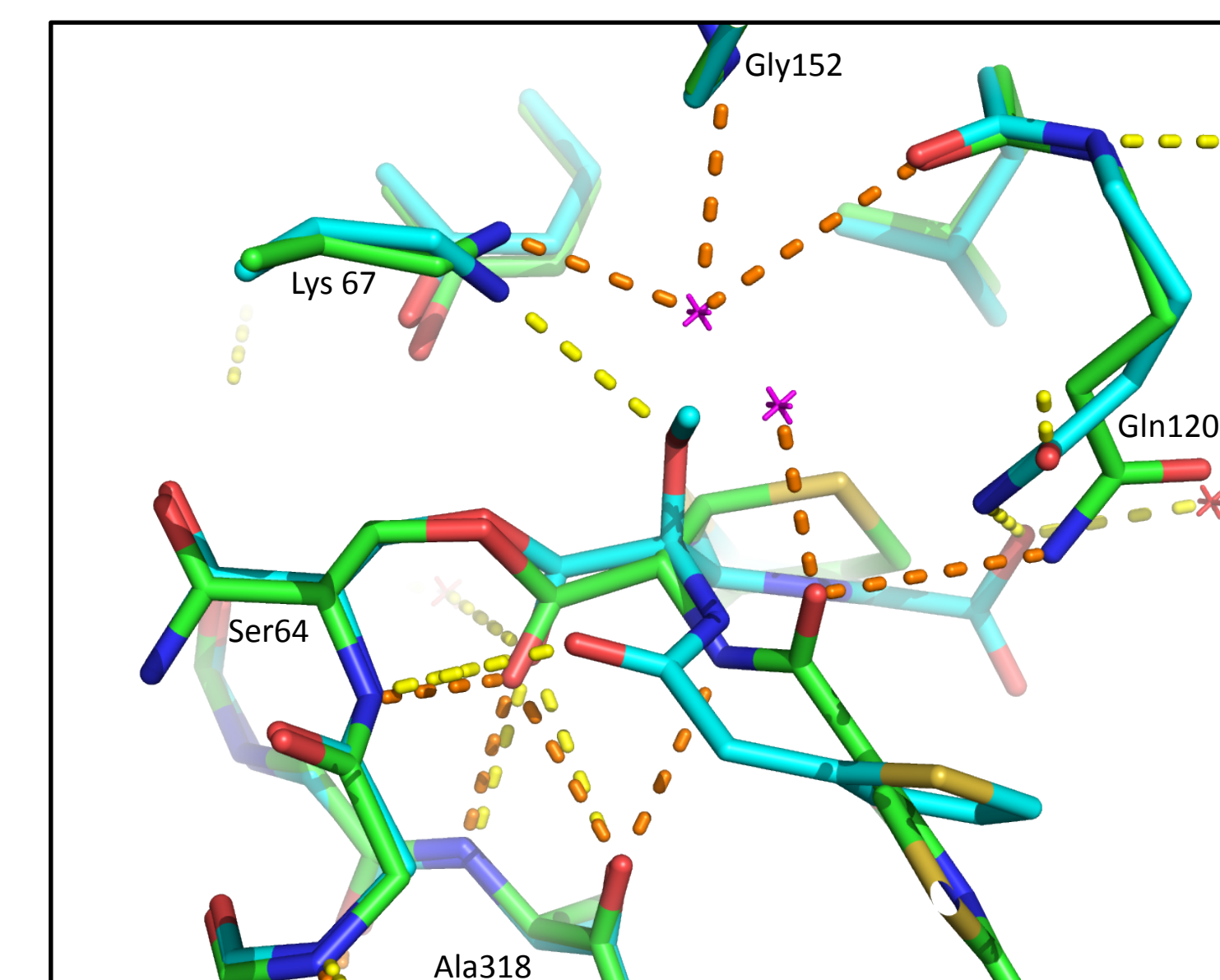

Figure 7: Overlay of AmpC N152G acylated to cefoxitin or cefotaxime, focusing on the amide of R_1 side chain. Coloring is the same as in figure 6.

Conclusions and Future Work

- Mutations at residue 152 in AmpC decrease the binding affinity of three different β-lactam drugs

- These mutations increased the catalytic activity of AmpC against cefotaxime and oxacillin and decreased activity against cefoxitin.

- The crystal structures of AmpC N152G acylated to cefoxitin and cefotaxime were used to infer possible reasons behind the different rates of hydrolysis of the two drugs, which include rotation of the R_1 amide and flipped dihydrothiazine ring, moving the C_4 carboxylate.

Future work includes determining the structure of AmpC with oxacillin, as well as taking additional measurements of kinetic constants in order to obtain more precise values.

Acknowledgments

7. Beadle, B. M., Trehan, I., Focia, P. J. & Shoichet, B. K. (2002). *Structure*, 10, 413–424.