Integrative, multi-temperature, time-resolved crystallography provides insight into molecular kinetics and dynamics of the Class A β-lactamase hydrolysis mechanism

A. Prester[1], D. von Stetten[2], G. Gore[1], H. Rohde[1], H. Ginn[3], E. C. Schulz[1,4,5]

[1]University Medical Center Hamburg-Eppendorf (UKE), Hamburg, Germany. [2]European Molecular Biology Laboratory, Hamburg Site c/o DESY. [3]DESY, Hamburg, Germany. [4]Institute for Nanostructure and Solid State Physics, University of Hamburg, Hamburg, Germany. [5]Max-Planck-Institute for the Structure and Dynamics of Matter, Hamburg, Germany.

a.prester@uke.de and ec.schulz@uke.de

The emergence and spread of antibiotic resistance poses an increasing threat to public health. Of particular concern is the production of β-lactamases mostly by Gram-negative bacteria, as these enzymes have the ability to hydrolyse and inactivate the most important class of antibiotics, the β-lactams. Despite years of intensive research on β-lactamases, some aspects of the reaction mechanism remain controversial. Especially the question which residues are involved in the acylation mechanism to form the acyl-enzyme intermediate. Concurrently, there have been remarkable advances in the field of time-resolved serial crystallography (TRX), which allows the observation of molecular processes at atomic resolution, within millisecond time frames and at different temperatures [1,2,3,4].

Here we aimed to elucidate the catalytic mechanisms of the extended-spectrum Class A serine β-lactamase CTX-M-14 from *Klebsiella pneumoniae*. To this end, we applied a novel multi-temperature time-resolved crystallography approach, and recorded 28 crystal structures, that follow the turnover reaction at 20 °C, 30 °C, and 37 °C, respectively. After triggering the hydrolysis of piperacillin, we observed the formation of a Michaelis-Menten state, a covalent acyl-enzyme intermediate, and an enzyme product complex within time frames of 0.1 s – 300 s (Fig. 1). Significant differences to existing protein structures of inactive CTX-M mutant variant structures, highlight the advantages of TRX with wild-type enzymes at near-physiological temperatures.

In addition, we complement these time-resolved data with ultra-high resolution cryostructures of the stable intermediates (0.77 – 1.04 Å) using the WT and mutant variants of the enzyme, providing insight into protonation states during each step of the catalysis.

The mechanistic insights into serine β-lactamase-mediated hydrolysis presented here consolidate the current state of knowledge and thus contribute to a better understanding of the reaction mechanism, which is of central importance for a pressing infectious disease problem.

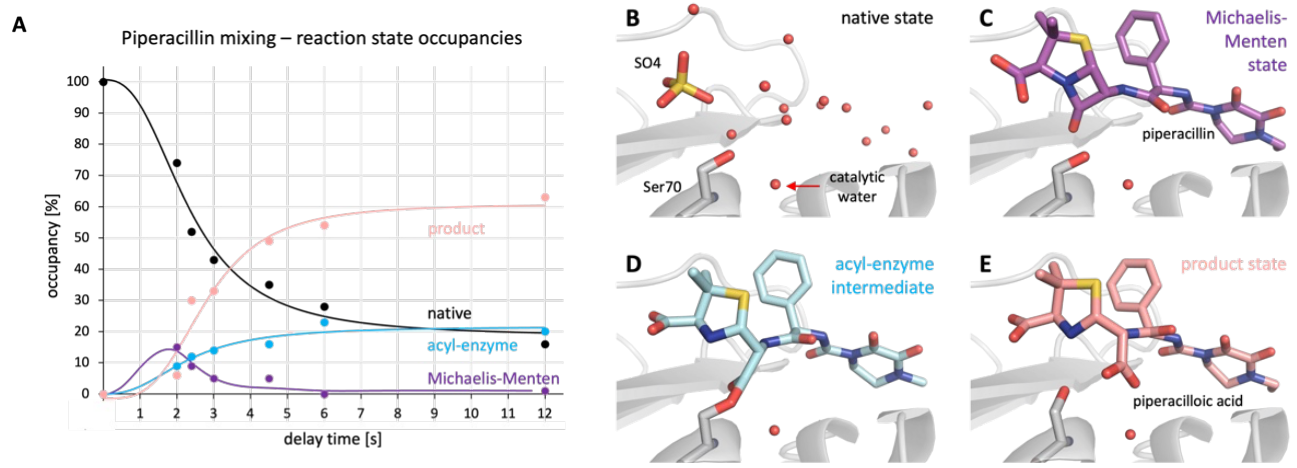

Figure 1. Observed reaction states during β-lactam hydrolysis reveal the (**B**) native state, (**C**) Michaelis-Menten state, (**D**) acyl-enzyme intermediate state, and the (**E**) product state of the wild type CTX-M-14 in complex with piperacillin. The occupancies of these states in the time-resolved datasets collected at 30°C are shown in (**A**), resembling the molecular kinetics of the turnover reaction.

[1] Brändén, G., & Neutze, R. (2021). Advances and challenges in time-resolved macromolecular crystallography. *Science*, 373(6558), eaba0954.

[2] Schulz, E. C., Mehrabi, P., Müller-Werkmeister, H. M., Tellkamp, F., Jha, A., Stuart, W., ... & Miller, R. D. (2018). The hit-and-return system enables efficient time-resolved serial synchrotron crystallography. *Nature methods*, 15(11), 901-904.

[3] Mehrabi, P., Schulz, E. C., Agthe, M., Horrell, S., Bourenkov, G., von Stetten, D., ... & Miller, R. D. (2019). Liquid application method for time-resolved analyses by serial synchrotron crystallography. *Nature methods*, 16(10), 979-982.

[4] Schulz, E. C., Prester, A., von Stetten, D., Gore, G., Hatton, C. E., Bartels, K., & Mehrabi, P. (2021). Probing the modulation of enzyme kinetics by multi-temperature, time-resolved serial crystallography. *bioRxiv*, 2021-11.