The Making of an Enzyme-in-Action Movie

3-Hydroxyanthranilate-3,4-dioxygenase (HAO) is a critical, non-heme iron enzyme involved in tryptophan-kynurenine metabolism and NAD^+ biosynthesis; it activates molecular oxygen to cleave an aromatic substrate. Its unstable, acyclic product, 2-amino-3-carboxymuconic semialdehyde (ACMS), is either metabolized to acetoacetate through a series of downstream enzymes, or auto-cyclizes to quinolinic acid (QUIN). All kingdoms of life use QUIN as a universal precursor for *de novo* biosynthesis of NAD^+, and in mammals specifically, QUIN is formed by the non-enzymatic decay of the product of the HAO-catalyzed reaction. Overproduction of QUIN is associated with neurodegenerative and other undesired diseases. As a result, HAO has long been recognized as a potential drug target.[1] Despite the early discovery and biological significance of HAO, its catalytic mechanism and how it balances downstream catalytic biomachinery versus non-enzymatic QUIN production has remained elusive.

Major hindrances to further understand the catalytic and regulatory mechanism of HAO are its fast turnover (25 s^{-1}), lack of intermediates, and undefined product conformation. The exact conformation of ACMS has not been characterized due to its short lifetime and a large number of possible conformers. To overcome these challenges, the highlighted work combined single-crystal spectroscopies and x-ray crystallography to probe changes in the active site and the overall conformation of HAO during *in crystallo* catalysis. The enzyme was first crystallized and then incubated with its aromatic substrate under anaerobic conditions. Subsequently, crystals of enzyme-substrate complex were exposed to oxygen for different times to interrogate any possible intermediates. The accumulation of catalytic intermediate in the crystalline state was identified using the integrated single-crystal UV-vis spectroscopy system at SSRL BL9-2. As shown in Fig. 1, substrate-bound HAO crystals exhibited a λ_{max} at 327 nm. Upon exposure to oxygen for 2, 3, 5, 7, 9 min or longer exhibited features distinct from those of the substrate-enzyme complex, suggesting specific intermediates populate at these time points. Lastly, substrate-bound crystals mostly converted to product-bound form with a λ_{max} at 374 nm. This result indicated that the crystals are catalytically active and the reaction rate in the crystalline state is more than 10,000-fold slower than in solution. More importantly, the considerably slower catalysis and evidence of accumulating intermediates provide a promising platform to further structurally characterize the reaction intermediates and enzyme-bound product. Furthermore, investigations of *in crystallo* HAO reaction using single-crystal electron paramagnetic resonance spectroscopy also support significantly slower turnover and detects the presence of a radical intermediate during catalysis.

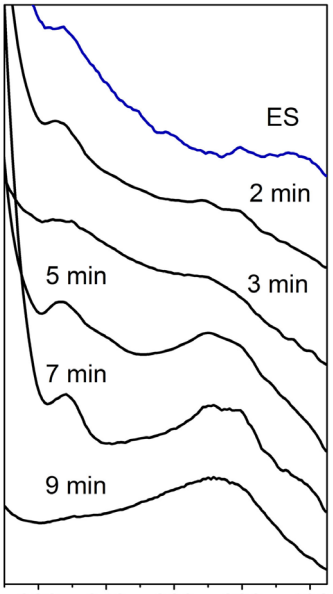

Figure 1. Representative absorption spectra of time-resolved *in crystallo* reaction obtained from single-crystal UV-vis microspectroscopy.

Next, x-ray diffraction was employed to determine the atomic structure of each intermediate (confirmed by *in-situ* UV-vis spectroscopy) as well as associated active site loop movements.[2] Besides substrate monodentate and bidentate bound structures, structures of intermediates after oxygen activation were also identified and characterized, including superoxo, alkylperoxo, seven-membered lactone, and two enol tautomers of the unstable dioxygenase product (Fig. 2). The seven-membered, mono-oxygenated lactone intermediate presumably corresponds to the radical species detected by EPR spectroscopy and supports a catalytic mechanism with stepwise incorporation of oxygen, providing a utility for future medicinal

chemistry efforts. The finding of two enol tautomers unveils an unexpected isomerase activity upon product formation, which helps to understand how a metabolic enzyme competes with a non-enzymatic reaction to control the product outcome for distinct biological needs. A total of 7 high-resolution intermediate structures allows for a step-by-step visualization of the catalytic cycle and protein dynamics during catalysis (see the Enzyme in Action Movie in PNAS Figures & SI). Overall, these results reveal a detailed dioxygenase mechanism along with potential isomerization activity that fine-tunes product profiling and affects the production of QUIN at a junction of the metabolic pathway.

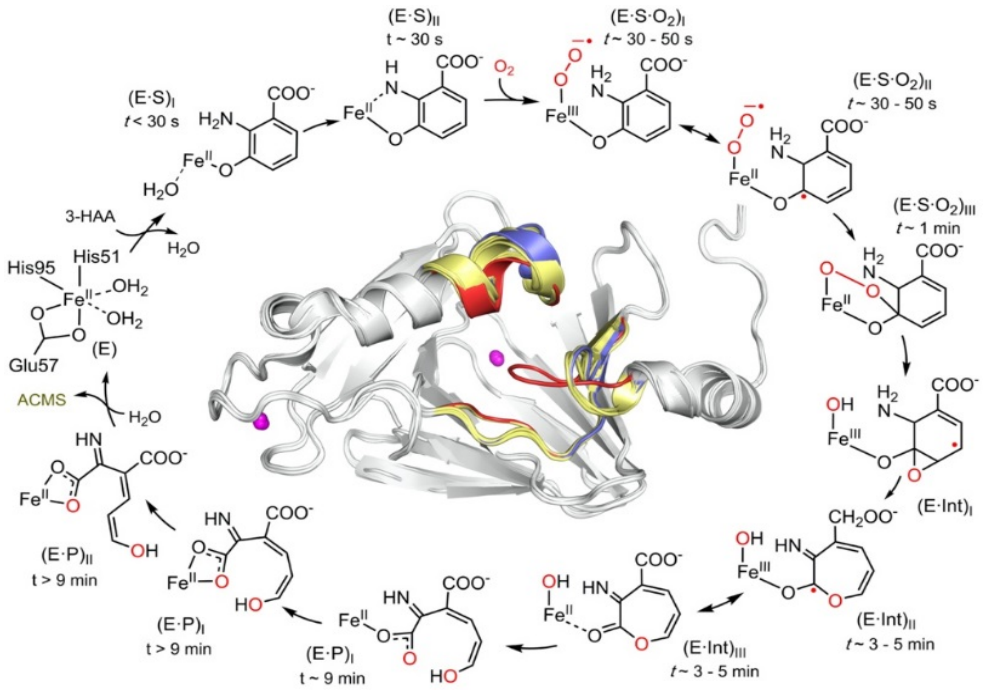

Figure 2. HAO catalytic cycle defined by the characterized intermediates.

References

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2. Y. Yang, F. Liu and A. Liu. "Adapting to Oxygen: 3-Hydroxyanthranilate 3,4-dioxygenase Employs Loop Dynamics to Accommodate Two Substrates with Disparate Polarities", *J. Biol. Chem*., **293**, 10415 (2018)

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