*Neutron crystallography of copper-containing amine oxidase in the wide pH (pD) range*

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## 1. Introduction

Copper amine oxidase (CuAO) catalyzes the oxidative deamination of various primary amines to produce the corresponding aldehydes, hydrogen peroxide, and ammonia. All CuAOs so far characterized structurally are the homodimers of a 70–95-kDa subunit, each containing a $Cu^{2+}$ ion and a protein-derived quinone cofactor, topaquinone (TPQ). It is well known that the cofactor TPQ is post-translationally generated from a specific Tyr residue in the polypeptide chain through a self-catalytic mechanism only requiring molecular oxygen and $Cu^{2+}$ ion (Fig. 1). We have studied the molecular mechanisms of TPQ generation and amine oxidation with CuAO from the soil bacterium *Arthrobacter globiformis* (AGAO) through the X-ray crystallography and in-solution kinetic analysis. Recently, we determined the AGAO crystal structure in the oxidative form by neutron and X-ray joint refinement at 1.72 Å and at pD 7.4 (*PNAS* 2020, 117, 10818-10824). We can specify the proton positions of the active site's important residues in the determined structures, including the TPQ cofactor. Although it is believed that a para quinone part of TPQ has a planar structure, we found that TPQ has a nonplanar and distorted structure. In addition, a part of TPQ takes a keto form that is equilibrated with a normal enol form.

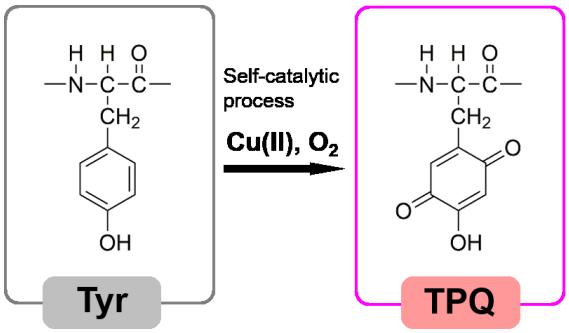



Fig. 1 Self-catalytic TPQ biogenesis

Our study aims to understand the reaction mechanisms of AGAO based on the protonation states of the active-site residues, which are experimentally determined by neutron crystallography at several pHs. We especially focus on TPQ and the side chain carboxyl group of Asp298 that indicates abnormal p*K*a, 7.5. A previous study for the structure at pH 7.4 showed that Asp298 is involved in the interaction with TPQ and the presence of the keto form. The distinct protonation state of Asp298 at pH 6.0, where Asp298 should be completely protonated, would have an effect on the TPQ situation. The protonation state of the active-site residues/TPQ and proton transfer within them is key to understanding the catalytic mechanism of the enzyme.

## 2. Experiment

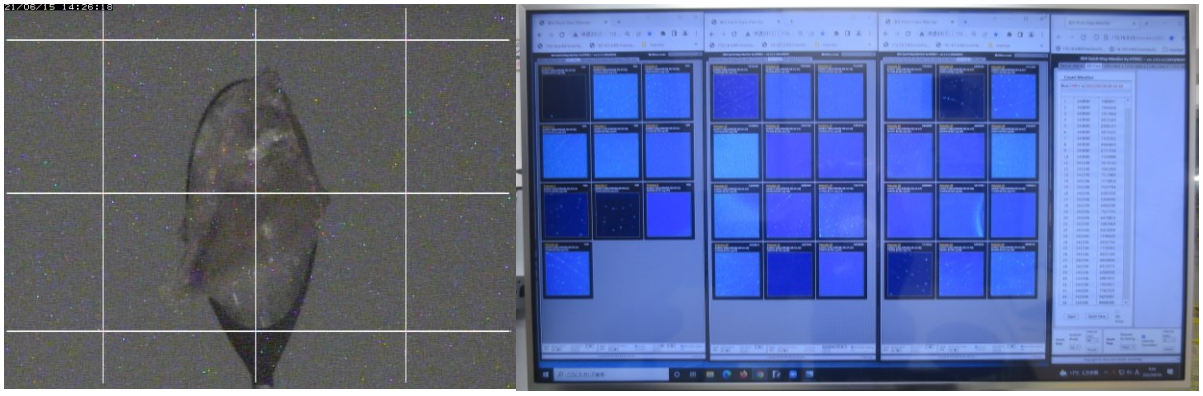
The large size of AGAO crystals (typically about 10 mm, 3, 5 x 2 x 1 mm) are prepared at 16 °C with the crystallization buffer (1.05 M potassium-sodium tartrate in 25 mM HEPES buffer, pH 7.4) in the dialysis buttons. To change pH of the AGAO crystals and exchange dissociable hydrogen atoms with deuterium, the buttons containing the crystals are first dialyzed against 1.05 M potassium-sodium tartrate, 25 mM MES buffer, pH 6.0 overnight and then against deuterated 1.05 M potassium-sodium tartrate, 25 mM MES $D_2O$ buffer, pD 6.0 for at least two weeks. Finally, the crystals are dialyzed against the cryo D2O buffer (3.5 M malonate buffer, pD 6) at ambient temperature overnight for cryoprotection. The AGAO crystals are picked up with a cryo loop and freeze-trapped under the cryo stream at about 100 K. Thus, obtained crystals are stocked in liquid nitrogen and are transported to J-PARC.

The AGAO crystals are first used for assessments of crystal quality. A time-of-flight (TOF) diffraction image of 30 min exposure was taken at BL03 (iBIX) in J-PARC for each crystal that was mounted on a goniometer at 100K under a cryo stream. The best crystal was applied for the full set measurement among several crystals.

Full set data measurement for neutron diffraction of the AGAO crystal was performed at 100 K from 2022/6/5 to 2022/6/15. A total of 35 sets of images by 6 h exposure ($T_0$ = 463000) were done for the above period. The beam power was 767 kW. After collecting neutron diffraction data, the same crystal was used for subsequent X-ray diffraction experiments.

## 3. Results

Total 20 crystals were applied to the test diffraction measurements on 2021/6/15, 2022/02/15, and 2022/06/05. The crystal (ID: AopD6_6, volume: 3.62 $mm^3$) was selected in terms of the resolution and shape of the identified diffraction spots. Fig. 2A and 1B show the image of the crystal and the TOF diffraction image in the full-set data measurement. Many diffraction spots with clear shapes were observed at about 1.8 Å resolution. Now we are optimizing the parameters for the image processing using *STARGazer*. As for X-ray diffraction data, we plan to collect it with the same crystal used for the neutron diffraction measurements at BL-5A in Photon factory.



A B

Fig. 2 Photo image (A) and TOF diffraction (B) of the AGAO crystal

## 4. Conclusion

We successfully obtained the neutron diffraction data for AGAO at pD 6.0 at about 1.80 Å resolution in the present study. After obtaining the X-ray diffraction data, we conduct X-ray- and neutron-joint refinement using *Phenix*. We expect that the proton coordinates of the structure determined shed further insight on the mechanism of the cofactor biogenesis and amine oxidation of AGAO.