X-Ray and Neutron Joint Refinement Displays Shared Proton and Keto Form of Quinone Cofactor in Copper Amine Oxidase

In recent neutron crystallography, protein structures have been modeled by joint refinement using X-ray as well as neutron diffraction data. Here, we report the neutron crystal structure of a well-studied enzyme, copper amine oxidase, as an example of joint refinement. High-resolution X-ray diffraction data to 1.14 Å resolution was collected at Photon Factory under a nitrogen gas cryostream (100 K). As a result, we succeeded in observing key structures such as a shared proton and a keto form of topaquinone cofactor. This enzyme structure shows that X-ray diffraction data is indispensable for reliable analysis and plays an essential role in neutron protein crystallography.

X-rays are scattered by electrons, while neutrons are scattered according to their interaction with atomic nuclei. Whereas the X-ray scattering factors are proportional to the number of electrons in the atom, the scattering length for neutrons varies depending on the nucleus of the atom or its isotope: e.g. hydrogen (H) and deuterium (D) atoms provide negative and positive scattering length, respectively. The scattering lengths of H and D are comparable to that of carbon atoms, so the remarkable advantage is that H and D can be clearly observable in the neutron crystal structure analysis of proteins. To be precise, X-ray analysis provides information about the electron cloud, whereas neutron analysis provides information about the atomic coordinates. In fact, the position of H atoms is the most valuable information obtained in the neutron crystallography of proteins. In the neutron crystallography of proteins, recently most structures have been refined by joint refinement, in which both X-ray and neutron diffraction data are used by the refinement program PHENIX [1]. In this study, we report an example of our joint refinement analysis of a copper amine oxidase [2]. High-resolution X-ray diffraction data also plays an essential role in model-building.

Table 1: Statistics of X-ray diffraction data collection.

Beamline	PF, BL-5A
Wavelength (Å)	1.0
Space group	C2
Cell dimensions (Å, °)	a = 157.55, b = 61.78, c = 92.23, β = 112.13
Resolution range (Å)	50.00 - 1.14
(outer shell)	(1.16 - 1.14)
Rint	0.069 (0.801)
I/σ(I)	46.6 (2.00)
Completeness (%)	99.4 (99.9)
Multiplicity	5.2 (3.5)
Observed reflections	1526790
Unique reflections	296297 (14901)

The enzyme of copper amine oxidase catalyzes the oxidative deamination of various primary amines to produce the corresponding aldehydes, hydrogen peroxide, and ammonia. This study targeted its recombinant enzyme derived from *Arthrobacter globiformis* (AGAO), which consists of a homodimer of 70 kDa subunits, each containing Cu^{2+} ions and protein-derived quinone cofactor, topaquinone (TPQ). Here, diffraction data of the same AGAO crystal to the resolution of 1.14 Å was collected at BL-5A in Photon Factory (PF) [**Table 1**], after neutron diffraction data had been collected at BL03 iBIX in J-PARC to 1.72 Å resolution. When both X-ray and neutron diffraction data are used to refine the coordinates of one model, an advantage of performing joint refinement is that the number of parameters obtained in the experiment can be increased for the refined coordinates. Accordingly, the addition of X-ray diffraction data can complement the experimental parameters and

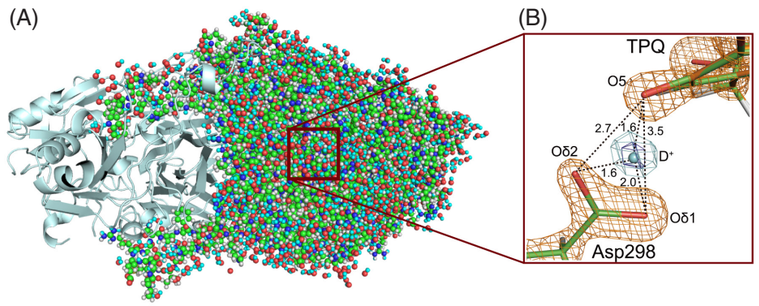

Figure 1: Neutron/X-ray crystal structure of amine oxidase. **(A)** The dimer structure of AGAO is shown using a space-filling model for one monomer and a ribbon model for the other; hydrogen [white], deuterium [cyan], carbon [green], oxygen [red], nitrogen [blue], and sulfur [yellow] atoms are indicated. **(B)** A triply shared proton between the cofactor and the catalytic base. $F_o - F_c$ omit electron density maps, calculated without contributions of TPQ and Asp298, are shown in orange at 8.0 σ. $F_o - F_c$ neutron omit maps, calculated without contributions from the delocalized deuteron, are drawn in dark blue [4.9 σ] and cyan [4.2 σ] with respective distances [Å].

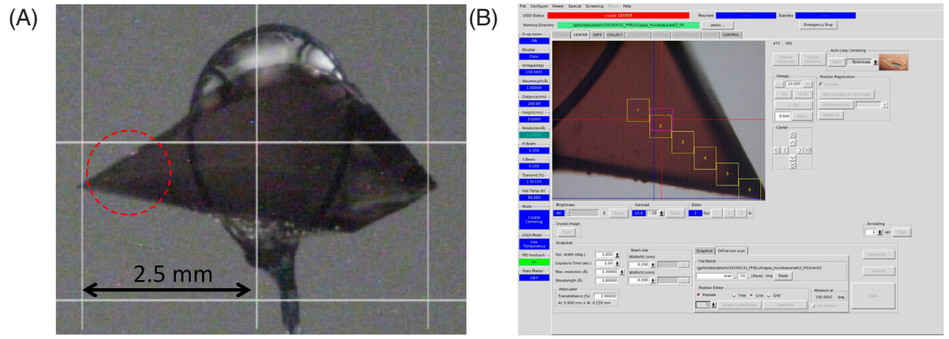

Figure 2: Photographs of the crystal used for data collection. **(A)** The crystal mounted at BL03 iBIX in J-PARC. The dotted red circle shows the area used for X-ray data collection at PF. **(B)** Snapshot of PC display in the X-ray diffraction data collection at BL-5A. The red and yellow squares in the center of the crystal photo correspond to the size of 0.2 × 0.2 mm.

reduce the model bias. Furthermore, it is possible to add contrast to the density maps depending on the type of atom, since the scattering length maps of specific atoms in neutron data are different from electron density maps calculated from X-ray data. Because the positions of carbon, nitrogen, oxygen, and sulfur atoms appear to be more precisely determined by high-resolution X-ray, neutron data can contribute to determining the position of H or D effectively. In other words, determination of the precise positions for non H/D atoms with X-ray data can also facilitate positional refinement of covalently connected H/D atoms. For example, an interesting shared proton was observed between the side chain carboxylate of Asp298 and O5 of TPQ in AGAO [**Fig. 1**]. The positions of the three oxygen atoms forming direct interactions with the shared proton are constrained by high-resolution X-ray crystallography. This made it possible to discuss H or D positions more reliably. In addition to information on protonation, the neutron structure of AGAO demonstrated that the novel distorted structure of TPQ, the keto form, is contained in the active site and takes the equilibrium state with the enolate form having a flat ring. These keto/enolate structures of TPQ, together with the protonation state of the active-site residues, are the key to understanding enzymatic functions.

On the other hand, it is important to consider the collection of X-ray diffraction data at the beamline. In neutron crystallography, large crystals must be prepared in general. In this study, an extremely large crystal with a size of 5 mm was prepared, and diffraction data of both X-rays and neutrons were collected with the same crystal [**Fig. 2**]. The temperature was set to 100 K under a nitrogen gas cryostream. From measurements under the frozen conditions, the sample could be maintained in the stable state even though the X-ray and neutron diffraction measurements take a long time, and it is also possible to collect high-resolution diffraction data. Notably, we have succeeded in determining the neutron crystal structure for a large protein with a molecular weight of about 140 kDa. In principle, the diffraction data of X-rays and neutrons should be planned to have the same conditions of the same crystal for joint refinement. Even if a large crystal used for neutron analysis is apparently single, X-ray diffraction spots caused by slight distortion of the crystal may be observed when an X-ray with a width of about 0.2 mm is irradiated at PF beam lines. Actually, such unfavorable diffraction spots were observed in this measurement for the large AGAO crystal with the beam irradiated to a thick part of the crystal center. Therefore, the direction of the crystal was arranged by bending the wire of the cryoloop under the cryostream to collect a data set derived only from a thin part away from the crystal. The positions of the detectors could be greatly separated from the crystal in the beam line hatch of BL-5A. This apparatus arrangement was useful, enabling us to change the direction of the crystals manually.

As described above, it has become possible to collect high-quality X-ray diffraction data from an extremely large crystal leading to high-resolution neutron crystal structure with joint refinement. X-ray diffraction data is also a prerequisite for the neutron crystallography of proteins. This result is largely attributable to the beamlines at PF.

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BEAMLINE

BL-5A

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