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Neutron and X-ray Crystal Analysis of a Bilin Reductase PcyA

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We determined the neutron crystal structure of PcyA in complex with BV. This is the first study of the neutron structure of a ferredoxin-dependent bilin reductase (FDBR) family member, revealing the protonation state of BV and the surrounding residues. We found that two forms of BV, neutral BV and protonated BVH^+, were coupled with the two conformation/protonation states of the essential residue Asp105. Further, His88 and His74 near BV were singly protonated and were connected with an intervening hydronium ion. To our knowledge, this is the third example of a hydronium ion in a protein structure. Neutron analysis also revealed how X-ray irradiation of the PcyA-BV crystal altered the structure of the PcyA-BV complex. It should be noted that the present structure provides a structural basis for the reaction mechanisms of other FDBRs.

1 Introduction

Bilin compounds are fundamentally important for oxygenic photosynthetic organisms because they are utilized as pigments for photosynthesis (phycobilins) and photoreceptors (phytochromobilin). Red algae and cyanobacteria use phycobilisomes, complexes comprised of phycobiliproteins, to harvest photon energy, and these organisms adapt to light quality and nutrient conditions by changing the components of the phycobilisome. Phycocyanobilin:ferredoxin oxidoreductase (PcyA) catalyzes two of the steps of two-proton-coupled two-electron reduction of biliverdin IXα (BV), a heme catabolite, to phycocyanobilin. Revealing the protonation states in the active site of PcyA and the substrate BV is important for understanding this unique reaction mechanism; however, previous structural analyses were unsuccessful in determining the location of hydrogen atoms in PcyA because X-rays are not strongly diffracted by hydrogen atoms.

2 Experiment

2.1. Crystallization

The crystallization procedure and conditions were performed to grow the large crystals necessary for neutron crystallography as follows: Purified PcyA was concentrated to 120 mg/mL by centrifugation using an Amicon Ultra centrifugal. The PcyA-BV complex was prepared by incubating the concentrated PcyA and BV overnight on ice. The crystal for neutron diffraction was obtained by the sitting drop vapor diffusion method at 293 K from a 20 μL drop of the PcyA-BV complex solution and 20 μL of the reservoir solution containing 200 mM NaCl, 1.4 M ammonium sulfate, and 50 mM MES (pH 5.9). These procedures were performed under dark conditions except for a small green spotlight. The crystal was grown to approximately 2.5 mm × 1.8 mm × 0.6 mm in 1 month.

2.2. Neutron diffraction experiment. The crystal was soaked in 50 μL of 50 mM MES buffer (pD 6.3) containing 200 mM NaCl and 1.4 M ammonium-d8 sulfate (D8, 98%; Cambridge Isotope Laboratories Inc.) prior to neutron diffraction. The soaking solution was exchanged three times in 3 weeks. Time-of-flight (TOF) neutron diffraction data were collected at BL03 iBIX at the Materials and Life Sciences Experimental Facility (MLF) of the Japan Proton Accelerator Research Complex (J-PARC, Tokai, Japan) at room temperature

under dark conditions. A total of 24 data sets were collected using a wavelength of 3.0 to 5.6 Å with a detector distance of 490 mm. Exposure time for each data set was 9 hours at 300 kW. The TOF neutron data were indexed, integrated, scaled, and processed with STARGazer. The same crystal was used for X-ray diffraction experiment at room temperature.

2.3. X-ray diffraction experiment. X-ray diffraction data from the same crystal were collected using an ADSC Quantum270 CCD detector at NE3A at room temperature. The wavelength of the synchrotron radiation, transmittance, and slit size were 1.0 Å, 10%, and 0.05 mm (vertical) × 0.05 mm (horizontal), respectively. The sample-to-detector distance and oscillation range were 150.7 mm and 1.0°, respectively. In an effort to minimize the amount of damage due to radiation, the position of the crystal during irradiation was changed for each shot. Data were integrated, merged, and processed with HKL-2000 software.

2.4. X-ray intensity data, structure, and absorption spectra from independent crystals with high-dose and low-dose synchrotron irradiation at 100 K. The crystals were soaked in a crystallization solution containing 25% (v/v) glycerol, and flash frozen in liquid N_2. X-ray intensity data were collected at 100 K using synchrotron radiation (λ = 1.0 Å) from BL5A. The oscillation angle, exposure time, slit size, and sample-to-detector distance were 1°/frame, 1.0 sec, 0.2 mm (v) × 0.2 mm (h), and 175.8 mm, respectively. X-rays were attenuated by the insertion of an aluminum filter. The transmittances of the X-rays were 30% (thickness of Al = 0.3 mm) and 1.0% (thickness of Al = 1.2 mm) for high- and low-dose data, respectively. The number of images in each data set was 135.

3 Results and Discussion

In this study, we succeeded in obtaining a very large crystal of the PcyA-BV complex and soaked it into the deuterated solution to exchange the hydrogen atoms in the crystal with deuterium atoms. As a result, neutron diffraction intensity data were able to be collected. We have determined the neutron crystal structure of the PcyA-BV complex at room temperature at a 1.95 Å resolution. The wild-type PcyA-BV structure, by avoiding X-ray-induced photo reduction, also revealed the existence of the axial water molecule. These results, including hydrogen localizations, will provide crucial information for elucidating the unique catalytic mechanisms of PcyA [1].

We found two protonation states in the PcyA-BV complex. One is BVH^+ and $Asp105^-$, in which four pyrroles of BV were protonated and Asp105 was deprotonated. The other is the combination of the neutral BV and neutral Asp105. In this state, an axial water is hydrogen-bonded to the BV A-ring pyrrole.

His88 Nδ was protonated to form a hydrogen bond with the lactam O atom of the BV A-ring. His88 and His74 were linked by hydrogen bonds via H_3O^+. These results imply that Asp105, His88, and the axial water molecule contribute to proton transfer during PcyA catalysis.

At first, we modeled the water molecule on the neutron-scattering length density map between His88 and His74 in a similar manner to a previous X-ray crystallographic study [2]. We found residual neutron-scattering length densities close to the His88 Nδ and Nε atoms. Nδ-protonated His88 was nicely fitted to the residual density, whereas the residual density near the Nε atom was significantly far enough from the Nε atom to be modeled as a hydrogen atom. The density is out of the imidazole plane, although a proton bound to the Nε atom should be in the plane. From these facts, the density close to His88 Nε was not likely corresponded to the H atom covalently bound to the His88 Nε atom. The water molecule was regarded as a hydronium ion (actually D_3O^+) (Fig. 1).

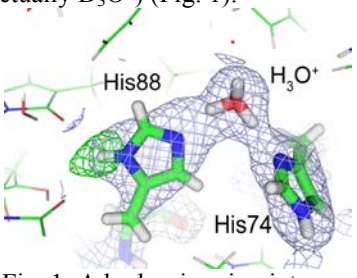

Fig. 1: A hydronium ion intervening His88 and His74

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Research Achievements

1. This work was broadcasted on NHK Mito local news.

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