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Neutron structural studies to elucidate the reaction mechanism of phytochromobilin synthase, HY2

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1 Introduction

Phytochromobilin synthase HY2, a ferredoxin-dependent bilin reductase (FDBR), is an enzyme that utilizes electrons supplied by ferredoxin to site-specifically reduce biliverdin (BV) to produce phytochromobilin. In order to elucidate the reaction mechanism in detail, we planned to conduct neutron crystal structure analysis, in which hydrogen atoms can be observed. However, the crystals of HY2(WT)-BV had needle-like shapes and were too small to obtain neutron diffraction intensity data, thus first, the M89R mutant was prepared by replacing Met89 with arginine to improve the packing of the molecules in the crystal. In addition, the HY2(M89R+4CS) mutant, in which four cysteine residues were replaced with serine residues to remove dimer formation during purification, was prepared and the good crystallization conditions were searched.

2 Experiment

The HY2(M89R+4CS) mutant obtained by the expression system in *E. coli* was purified and titrated with substrate BV to estimate the appropriate amount of BV required for complex formation and to obtain the HY2(M89R+4CS)-BV complex. Crystallizations of the complex were carried out using the sitting drop vapor diffusion method.

3 Results and Discussion

As results of neutron diffraction experiments (test measurements) using these crystals, diffraction spots of approximately 3-5 Å were successfully observed in both room- and cryo-temperature measurements. Since even larger and better-quality crystals are needed for neutron structural analysis, in parallel, we prepared the mutants that have the potentials to produce larger crystals. We screened new crystallization conditions using four types of C-terminal deletion mutants, in which the deleted regions are not present in other FDBRs and are expected to cause structural instability. Then, we succeeded in obtaining thicker crystals of the M89R+4CS (56-335) mutant in complex with BV than before [HY2(M89R+4CS)-BV]. X-ray diffraction experiments on these crystals yielded diffraction intensity data corresponding to a resolution of 1.55 Å (Table 1), allowing us to determine the space group was $P6_1$ and the lattice constants were a, b = 80.48 Å and c = 90.05 Å. Then, the structure was successfully determined. However, the structure of BV in this mutant was changed compared to that that in WT, and this was thought to be caused by the additional glycerol as a cryoprotectant because electron density for glycerol was found near BV. Therefore, we carried out the X-ray diffraction experiments again for crystals obtained under the condition without glycerol (Table 1). As a result, even without glycerol, the conformations of BV varied in different crystals. Taken together, it is suggested that HY2 can produce phytochromobilin correctly from BV which has several conformations.

Table 1: X-ray intensity data statistics

Date	2022/7/7	2023/11/24	2023/12/26
Crystal	HY2(M89R+4CS)-BV	M89+4CS(56-335)-BV with cryo-protectant	M89+4CS(56-335)-BV without cryo-protectant
Beamline	BL-1A	BL-1A	BL-17A
Style	Full automatic	Remote	Full automatic
Resolution (Å)	43.24-1.50 (1.53-1.50)	45.02-1.55 (1.58-1.55)	44.95-1.41 (1.43-1.41)
Space group	$P2_12_12_1$	$P6_1$	$P6_1$
Cell parameters a, b, c (Å)	42.49, 56.77, 129.71	80.48, 80.48, 90.05	80.98, 80.98, 89.90
$I/\sigma(I)$	16.3 (2.1)	19.9 (3.0)	19.7 (2.2)
Completeness (%)	100.0 (99.8)	99.0 (98.0)	100.0 (100.0)
Mosaicity (º)	0.132	0.100	0.104
R_{merge}	0.072 (0.902)	0.063 (0.795)	0.060 (0.639)

$CC_{1/2}$	0.998 (0.730)	0.999 (0.853)	0.998 (0.917)

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