

High-resolution structural studies of NADH-cytochrome *b*<sub>5</sub> reductaseYu HIRANO<sup>1,2</sup>, Shigenobu KIMURA<sup>3</sup>, Kunio MIKI<sup>4</sup> and Taro TAMADA<sup>1,\*</sup><sup>1</sup>National Institutes for Quantum Science and Technology, Inage, Chiba 263-8555, Japan<sup>2</sup>The Japan Science and Technology Agency, Kawaguchi, Saitama, 332-0012, Japan<sup>3</sup>Ibaraki University, Hitachi, Ibaraki, 316-8511, Japan<sup>4</sup>Kyoto University, Sakyo-ku, Kyoto, 606-8502, Japan

### 1 Introduction

Many redox proteins utilize cofactors such as flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NADH) to conduct proton and hydride transfer reactions. Precise structural information including hydrogen atoms is required to understand the molecular mechanisms underlying the redox reactions. NADH-cytochrome *b*<sub>5</sub> reductase (b5R) has a role for preserving cellular redox balance and functions as an electron supplier for various metabolic processes. The redox reaction cycle of b5R includes the proton and hydride transfer steps via NADH and FAD cofactors [1]. We performed high-resolution structure analyses of porcine liver b5R by both X-ray and neutron crystallography to reveal the structural basis of the redox reaction [2]. Because X-ray diffraction data generally contain higher resolution structural information about heavy atoms (those other than hydrogen atoms), the combination with neutron diffraction data is expected to provide more precise information about atomic positions involving hydrogen atoms bound to heavy atoms.

### 2 Experiment

Crystallization experiments were performed by the sitting-drop vapor diffusion method under an anaerobic condition. Large crystals of the oxidized forms of b5R with the sizes of approximately 2.0 mm<sup>3</sup> were obtained in two pD (potential of deuterium) conditions at pD 6.5 and 7.5.

The X-ray diffraction data sets for joint X-ray/neutron refinement were collected with the crystals used for neutron diffraction data collection at BL-5A beamline. The wavelengths of X-rays were set to 0.75 Å (pD 6.5) and 0.76 Å (pD 7.5). A joint X-ray/neutron structure refinement was performed with the program *Phenix* [3].

### 3 Results and Discussion

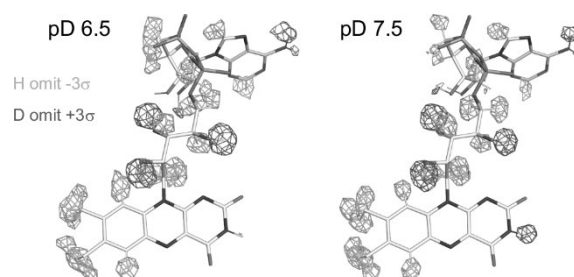
The X-ray and neutron diffraction data sets were obtained at 0.88 Å (pD 6.5) and 0.87 Å (pD 7.5) resolutions (Table 1). Hydrogen (H) and deuterium (D) atoms were included in the model during the X-ray/neutron joint refinement. Neutron scattering lengths are negative for H ( $-0.37 \times 10^{-12}$  cm) and positive for D ( $0.67 \times 10^{-12}$  cm). Thus, neutron-scattering-length-density map can differentiate H and D atom positions. The neutron  $F_o-F_c$  H/D omit map calculated at 1.45 Å (pD 6.5) and 1.50 Å (pD 7.5)

resolutions clearly shows all H/D atom positions bound to FAD (Fig. 1) and the H/D atom positions of the amino acid residues and solvent molecules involved in hydrogen-bonds with FAD for both pD conditions.

A structural difference between pD 6.5 and pD 7.5 was observed for the hydrogen-bond network from FAD to the protein surface. The side chain of His49 which is located in the hydrogen-bond network is in the double-protonated state at pD 6.5 and in the single-protonated state at pD 7.5. In addition, the stopped-flow UV-visible spectroscopy suggested that a histidine residue is involved in the b5R redox reaction cycle. The structural and spectroscopic studies indicated that the hydrogen-bond network is the proton pathway in b5R.

Table 1: Diffraction data statistics

	pD 6.5	pD 7.5
Resolution (Å)	50-0.88 (0.90-0.88)	50-0.87 (0.89-0.87)
Unit cell <i>a</i> , <i>b</i> , <i>c</i> (Å)	48.6, 72.2, 85.1	48.7, 72.3, 85.1
Completeness (%)	96.2 (91.7)	100.0 (99.9)
<i>R</i> <sub>sym</sub> (%)	6.9 (43.0)	5.6 (44.7)
<i>I</i> / $\sigma$ ( <i>I</i> )	55.0 (2.7)	68.4 (4.0)

Fig. 1:  $F_o-F_c$  neutron-scattering-length-density omit map of FAD.

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References

- [1] M. Yamada *et al.*, *JMB* **425**, 4295 (2013).
- [2] Y. Hirano *et al.*, submitted.
- [3] D. Liebschner *et al.*, *Acta D* **75**, 861 (2019).

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