Combining X-Ray and Neutron Crystallography with in Silico DFT Analyses of Low-Potential [4Fe-4S] Ferredoxin

Ferredoxin (Fd) is a small protein with a metal cofactor called an iron-sulfur (Fe-S) cluster that functions in a variety of electron transfer reactions in cells. In this study, we determined the neutron and X-ray structures of Fd from *Bacillus thermoproteolyticus* (*Bt*Fd) and experimentally clarified the hydrogen-bonding network containing [4Fe-4S] clusters. Density functional theory calculations revealed that the protonation state of Asp64 has a significant influence on the electron orbitals of the Fe-S cluster. The spectroscopic and electrochemical experiments supported the results of the DFT. This is the first identification of the plausible switch for redox regulation of ferredoxin in nature.

In living organisms, various proteins assist in redox reactions, some of which contain clusters of iron and sulfur (Fe-S clusters, **Fig. 1(a)**) [1], and these Fe-S clusters play an important function in the transfer of electrons between proteins. Ferredoxin (Fd) is a small acidic protein with a metal cofactor called an iron-sulfur (Fe-S) cluster that functions in a variety of electron transfer reactions in cells. Microbial types of Fd have [4Fe-4S]-type clusters and cover a wide range of redox potentials (-700 to +500 mV) (**Fig. 1(b)**). Our group has previously determined the high-resolution (0.92 Å resolution) structure of Fd by X-ray crystallography [2], revealing the fine structure of the Fe-S cluster and its surrounding environment. However, many unknowns remain regarding the structural factors that promote stable electron transfer and the mechanism that maintains the potential itself. In this study, to apply the density functional theory (DFT), we attempted to determine the exact hydrogen-bonding network by combining X-ray and neutron crystallography.

Neutron crystallography needs large crystals to obtain clear diffraction spots, therefore we applied the dialysis method as a crystallization of *Bt*Fd. Giant crystals were obtained in a couple of weeks in 0.1 M MES (pH 6.5–7.0) containing 200 mM NaCl, 81.5 – 89 % saturated ammonium sulfate (**Fig. 1(c)**). X-ray diffraction data from the same crystal used for neutron crystallography were collected using an Pilatus3 S2M detector at AR-NW12A under room temperature.

Prior to the neutron diffraction experiment, the crystal was soaked in deuterium reagent. Time-of-flight (TOF) neutron diffraction data were collected at BL03 iBIX in a Japan Proton Accelerator Research Complex (J-PARC, Tokai, Japan) at room temperature.

The 3D structure of ferredoxin was determined by joint refinements of X-ray and neutron data, and the exact positions of atoms including hydrogen around the Fe-S cluster were experimentally determined at 1.5 Å resolution (**Fig. 2(a)**) [3]. The overall structure was nearly identical to the previously reported cryogenic X-ray structure at 0.92 Å resolution [1], with a rms deviation of 0.16 Å for Cα atoms when all residues were superimposed with least-squares fitting. Although the neutron-scattering length density map clearly shows the [4Fe-4S] cluster, the density for sulfur atoms was very low compared with most of the other atoms including the iron atoms (**Fig. 2(b)**). This is a reasonable result because the sulfur atom has a smaller neutron-scattering length. The hydrogen atoms around the [4Fe-4S] cluster were visualized, and the exact directions of hydrogen bonds could be determined (**Fig. 2(b)**). Based on the exact positions of the hydrogen atoms, the electronic structure around the Fe-S cluster was calculated by DFT method. We found, for the first time, that the lowest unoccupied molecular orbital (LUMO) derived from the Fe-S are distributed not only around the Fe-S cluster but also to Asp64 at a distance of more than 10 Å away from the cluster (**Fig. 2(c)**). Interestingly, the distribution of the LUMO to Asp64 was observed only in the absence of a hydrogen atom on the carboxy group in the side chain of Asp64 (-COO-), while in the presence of a hydrogen atom (-COOH), the electrons were distributed only around the Fe-S cluster.

Since the rate of electron transfer to oxygen

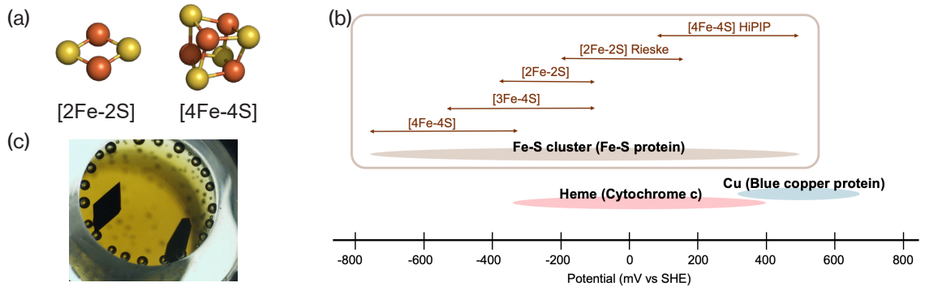

Figure 1: (a) The structures of Fe-S clusters. The yellow and brown ball indicated the sulfur and iron atom, respectively. **(b)** Typical redox potential range of various metal cofactors. **(c)** The large crystals of *Bt*Fd in the dialysis button.

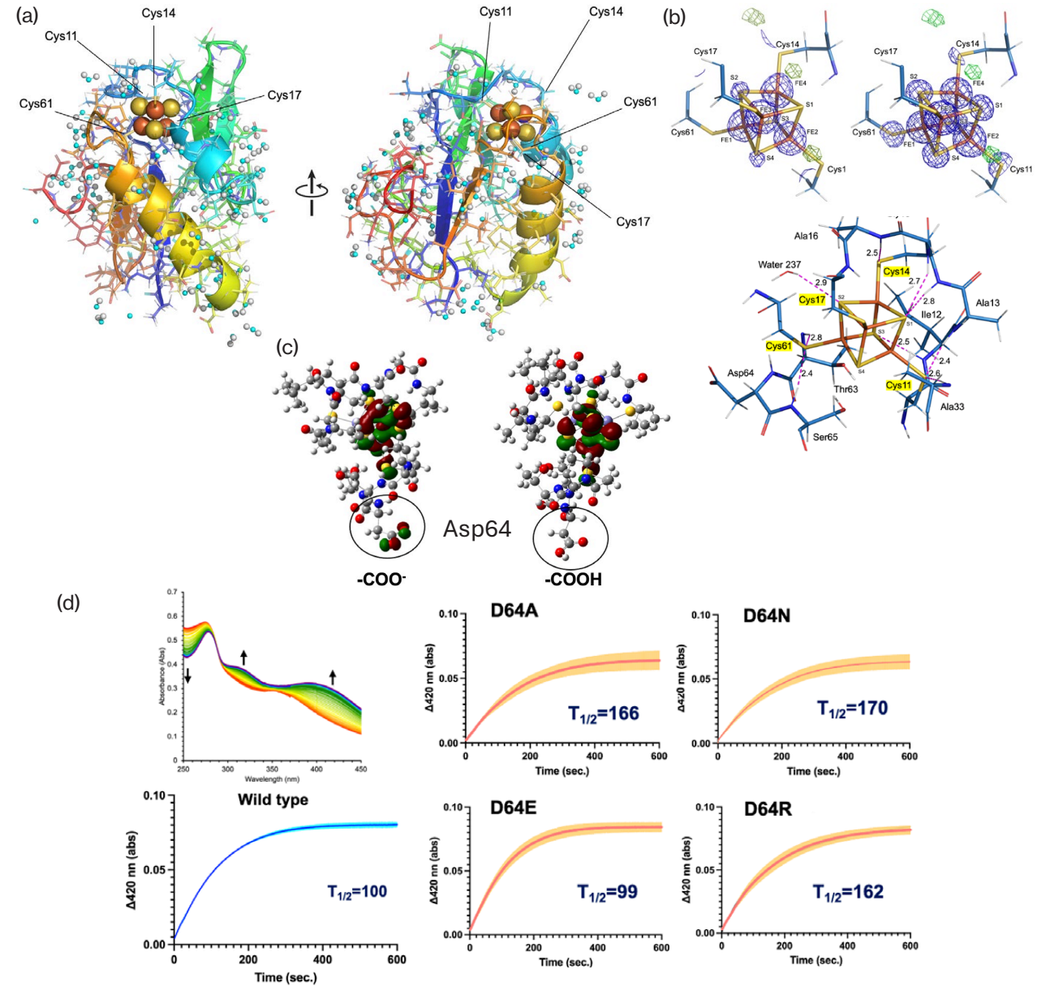

Figure 2: (a) Overall neutron structure of *Bt*Fd. The [4Fe-4S] cluster and water molecules are shown as a space-filling model. White and cyan balls indicate hydrogen (deuterium) and oxygen atoms, respectively. **(b)** The neutron-scattering length density map and X-ray electron density map around the [4Fe-4S] cluster. Hydrogen bonds with the [4Fe-4S] cluster and its ligand Sγ atoms of cysteines. **(c)** Distribution of LUMOs. The red and green colors indicate positive and negative phases, respectively. **(d)** Time-dependent changing of UV-vis absorption spectrum of wild-type *Bt*Fd and its mutants by air oxidation. All figures in **Fig. 2** are from reference [3] (CC BY 4.0).

depends on the potential, the electron behavior calculated by DFT is assumed to be reflected in the oxidation rate of the cluster in air *Bt*Fd has completely different UV-visible spectra in the reduced and oxidized states (**Fig. 2(d)**). Evaluation of the oxidation rate revealed that, compared to the wild type, the neutralizing mutation of the Asp64 side chain resulted in an extremely slow oxidation rate (**Fig. 2(d)**).

In this study, we elucidated for the first time in the world the existence of a “nano-switch mechanism” in which the presence or absence of a single hydrogen atom in the aspartic acid side chain changes the electronic state of the Fe-S cluster. We also revealed that this nano-switch mechanism was conserved in archaeal Fd, thus this mechanism is believed to be widely used in the biological world.

REFERENCES

[1] D. C. Johnson, D. R. Dean, A. D. Smith and M. K. Johnson, *Annu. Rev. Biochem.* **74**, 247 (2005).
[2] K. Fukuyama, T. Okada, Y. Kakuta and Y. Takahashi, *J. Mol. Biol.*, **315**, 1155 (2002).
[3] K. Wada, K. Kobayashi, I. Era, Y. Isobe, T. Kamimura, M. Marukawa, T. Nagae, K. Honjo, N. Kaseda, Y. Motoyama, K. Inoue, M. Sugishima, K. Kusaka, N. Yano, K. Fukuyama, M. Mishima, Y. Kitagawa and M. Unno. *eLife*, 10.7554/eLife.102506.2 (2024).

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