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Forty years of the structure of plant-type ferredoxin

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The X-ray structure of a [2Fe-2S]-type ferredoxin (Fd) from *Spirulina platensis*, solved by a collaborative group led by Profs Masao Kakudo, Yukiteru Katsube and Hiroshi Matsubara, was the first high-resolution structure of a plant-type Fd deposited in the Protein Data Bank. The main chain structure, comprising a [2Fe-2S] cluster ligated by four conserved cysteine residues, together with a molecular evolutionary study based on a series of amino acid sequence determinations, was reported in *Nature* in 1980. The consequent detailed crystallographic analysis, including crystallization, heavy atom derivatization, data collection, phase calculation and model building, was published by the same group in the *Journal of Biochemistry* in 1981. The pioneering X-ray analysis of *S. platensis* Fd at 2.5 Å resolution was a key milestone in structural research on the photosynthetic electron transport chain, informing related and challenging studies on other components of the photosynthetic electron transfer chain.

Keywords: X-ray crystallography; Protein Data Bank; photosynthesis; ferredoxin; electron transport.

Abbreviations: Fd, Ferredoxin; FNR, Fd-NADP$^+$ Reductase; PC, Plastocyanin; GaFd, gallium-substituted Fd; NMR, Nuclear Magnetic Resonance.

The plant-type ferredoxin (Fd) initially identified by Tagawa and Arnon (*1*) is widely distributed in photosynthetic organisms, from prokaryotic cyanobacteria to land plants. The active redox centre of Fd, a [2Fe-2S] cluster ligated by four conserved cysteines, has an extremely low redox potential of around −400 mV. The primary function of Fd in chloroplast is to transfer electrons from photosystem I to several Fd-dependent assimilatory enzymes such as Fd-NADP$^+$ reductase (FNR), sulfite reductase, nitrite reductase and Fd-dependent glutamate synthase. Among these, the photoreduction of NADP$^+$ to NADPH through FNR is the major system in chloroplast (Fig. 1).

A non-photosynthetic Fd has been also identified in root and fruit-cell plastids and characterized by Wada *et al.* (*2*). This non-photosynthetic Fd functions in a similar way to its photosynthetic counterpart, but is reduced by non-photosynthetic FNR in the oxidative pentose phosphate pathway instead of photosystem I. Compared with another copper-containing photosynthetic electron carrier protein, plastocyanin (PC), Fd has a relatively unique character. Whereas PC shuttles solely between cytochrome b_6f and photosystem I, Fd is reduced by either photosystem I or non-photosynthetic FNR and donates an electron to various Fd-dependent assimilatory enzymes. In trying to understand the molecular basis underlying these unique functions, the high-resolution X-ray structure of Fd was long awaited.

The main chain fold of the plant-type [2Fe-2S] structure was first reported in 1980 (*3*). About 25% of the residues were found to form β-structure, while 10% formed α-helix. A β-barrel-like structure was observed in the core; the arrangement of which is currently called a 'ubiquitin fold', although the crystal structure of ubiquitin was solved later (*4*). The structure of the [2Fe-2S] cluster was novel, comprising two iron atoms bridged by two inorganic sulfurs and tetrahedrally ligated by two Sγ atoms of cysteines. Three NH–S and one OH–S hydrogen bonds from the cluster-binding loop accommodate and stabilize this novel cluster in the structure; this arrangement is considered to be crucial to keep the redox potential of the cluster low.

Details of the X-ray crystallographic analysis of *Spirulina platensis* Fd crystals were published in the *Journal of Biochemistry* in 1981 (PDB ID: 4FXC, updated entry from 1FXC) (*5*). Brown thin-plate crystals were prepared by the dialysis method. The crystal data determined by precession photographs were space group $C222_1$ with unit cell dimensions of $a = 62.32$, $b = 28.51$, $c = 108.08$ Å and $\alpha = \beta = \gamma = 90°$. Uranyl derivative crystals were prepared by the soaking method. The diffraction intensities of the native and derivative crystals were measured on a Rigaku four-circle diffractometer equipped with a rotating anode X-ray generator using Ni-filtered Cu-$K\alpha$ radiation. The phase problem was solved by the isomorphous replacement method with anomalous information from the heavy atom derivative and native crystals. The atomic model was built physically by hand with a half-mirror device called a Richard box. The scale for manual model building was 2 cm/1 Å. All the charged groups were accessible to solvent molecules and the internal core was occupied by aliphatic nonpolar residues.

After the structure of *S. platensis* Fd was published, many other plant-type Fd structures followed: namely, Fd1 from *Aphanothece sacrum* at 2.2 Å in 1990 (1FXI); Fd1 from *Equisetum arvense* at 1.8 Å in 1994 (1FRR); Fd from

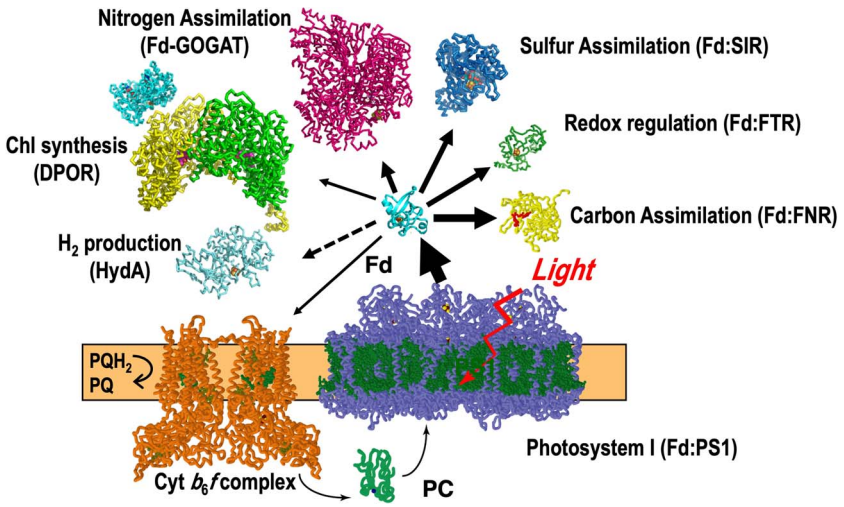

Fig. 1. Structural sketch of the protein network around Fd on the thylakoid membrane of cyanobacterium.

Anabaena at 1.8 Å in 1997 (1QOB); Fd from *Anabaena* PCC 7119 at 1.17 Å in 1999 (1CZP); Fd from *Chlorella fusca* at 1.4 Å in 1999 (1AWD); Fd from *Synechocystis* sp. PCC 6803 at 1.8 Å in 2003 (1OFF); Fd from *Mastigocladus laminosus* at 1.25 Å in 2005 (1RFK); Fd2 from *E. arvense* at 1.2 Å in 2005 (1WRI); Fd from *Cyanidioschyzon melorae* at 1.18 Å in 2011 (3AB5); Fd from *Leptolyngbya boryana* at 1.76 Å in 2012 (3B2G); Fd1 from maize leaf at 1.7 Å in 2012 (3B2F); Fd from *Thermosynechococcus elongatus* at 1.5 Å in 2015 (5AUI); Fd2 from *Arabidopsis thaliana* at 2.34 Å in 2016 (4ZHO); Fd1 from *Solanum tuberosum* (potato) at 2.46 Å in 2016 (4ZHP); and Fd3 from maize root at 2.5 Å in 2017 (5H57). The resolution limits of X-ray structures that have been recently determined by using modern synchrotron sources are relatively higher as compared with those early structures; however, the strong X-ray irradiation of modern sources may cause partial reduction or radiation damage of the obtained structures (*6*). Ohnishi *et al*. made very thin plate crystals of *Chlamydomonas* Fd and measured the absorption spectra of these crystals before and after multiple different X-ray exposures. They confirmed that X-ray dose-dependent structural changes occur in Fd and, based on their observed absorption spectral changes, they estimated the minimum X-ray dose to maintain an oxidized redox state (*7*). The least X-ray-damaged Fd1 structure from *Chlamydomonas reinhardtii* was reported at 1.4 Å in *Journal of Biochemistry* in 2020 (6KUM).

Considering the molecular binding of electron carrier proteins, it may be readily anticipated that cytochrome b_6f and photosystem I each have a specific binding site for PC (Fig. 1). By contrast, it is more difficult to understand the Fd-binding mode of the many Fd-dependent proteins including photosystem I because the molecular size, prosthetic group and natural cellular abundance of these partners are so diverse. In addition, because the electron transfer complex formed by Fd is transient and the components are easily dissociated from each other, trials to crystallize the complexes formed between Fd and Fd-dependent enzymes are very challenging. In 2001, Kurisu *et al.* (*8*) succeeded in crystallizing a complex of Fd and FNR from maize leaf after carefully optimizing the ionic strength of the crystallization solution. The resultant X-ray structure of Fd:FNR at 2.59 Å resolution (1GAQ) revealed the spatial arrangement of each prosthetic group in the two proteins and showed the structural changes upon complex formation in both partners. This first complex structure of Fd and an Fd-dependent enzyme subsequently triggered X-ray structure determinations of other pairs of Fd complexes: namely, Fd:Fd-Thioredoxin Reductase at 2.4 Å (2PVG) and Fd:Fd-Thioredoxin Reductase:Thioredoxin at 3.0 Å in 2007 (2PUK); Fd:Sulfite Reductase at 2.08 Å in 2016 (5H92, 5H8V and 5H8Y); and Fd:Photosystem I from *T. elongatus* at 4.2 Å in 2018 (5ZF0).

X-ray analysis of the complex crystal between Fd and photosystem I mentioned above (*9*) was not easy, even under cryogenic conditions, probably due to partial reduction of Fd caused by strong X-ray exposure, resulting in a blob of electron density corresponding to bound Fd. Subsequently, Mutoh *et al.* introduced a gallium-substituted Fd (GaFd), which is never reduced and is permanently in the oxidized state. They determined the structure of GaFd from *Synechocystis* sp. PCC680 at 1.62 Å (5AUK) by X-ray crystallography and evaluated the equivalence of native Fd containing a [2Fe-2S] cluster and GaFd containing a [2Ga-2S] cluster by superposition of GaFd and native Fd (1OFF) based on C_α atoms and all atoms, resulting in rmsds of 0.286 and 0.549 Å, respectively (*10*). GaFd is also useful as a competitive inhibitor to identify Fd-dependent activity. Using this useful tool, Mignée *et al.* (*11*) measured the effects of Fd binding to photosystem I without reduction of Fd and Grabsztunowicz *et al.* (*12*) characterized the electron transport pathway of chromorespiration during flower development.

A remaining unanswered question regarding plant-type Fd is the dynamics of competitive Fd binding. At present, the accumulated structural knowledge is relatively limited to static rather than dynamic structures. For example, scientists are wanting to engineer a cyanobacterial model cell system to enhance the activity of Fd-dependent enzymes by tuning the electron transfer flow around Fd, but it is still difficult to design molecules artificially and

effectively for this purpose. In future studies, the dynamic behaviour of Fd should be studied in solution by NMR spectroscopy or isothermal calorimetry and also *in situ* by cryo-electron tomography of the thylakoid membrane at high resolution. A more integrated or hybrid approach towards Fd and Fd-dependent enzymes is eagerly awaited.

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Conflict of Interest

None declared.

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