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Cyanidioschyzon merolae ferredoxin: A high resolution crystal structure analysis and its thermal stability

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ABSTRACT

Cyanidioschyzon merolae **(Cm) is a single-cell red alga that grows under moderately thermophilic (40–50 °C), acidic (pH 1–3) conditions. We purified a Cm ferredoxin (Fd) that was characterized as a plant-type [2Fe–2S] Fd by physicochemical techniques. X-ray crystallography revealed that the overall three-dimensional structure of CmFd was highly similar to, but slightly different from, the [2Fe–2S] Fd from *Spinacia oleracea*, whose growth temperature is 15–20 °C. Therefore, slight structural differences, including non-covalent-bond number and amino acid sequence, may underlie the differential thermostabilities of the plant-type Fds.**

1. Introduction

Cyanidioschyzon merolae (Cm), a single-cell red alga with a diameter of 1.5–2.0 μm, was isolated from a hot spring near Napoli, Italy. Cm grows well in moderately thermophilic (40–50 °C), acidic (pH 1–3) areas. As alga may represent one of the most ancient eukaryotic life forms [1], Cm is therefore very well suited to the study of the origin of eukaryotic cells.

Photosynthetic organisms are known to contain [2Fe–2S] ferredoxins (Fds) that act as electron transfer proteins in photosynthesis. In this study, the three-dimensional structure of a moderately thermostable Fd from Cm was characterized and compared with a *Spinacia oleracea* Fd (SoFd) containing a [2Fe–2S] cluster [2]. In order to elucidate the structural elements underlying the thermal stability of CmFd, we compared the numbers of non-covalent bonds and amino acid sequences in CmFd and other [2Fe–2S] Fds from photosynthetic organisms with various preferred growth temperatures.

2. Materials and methods

2.1. Protein expression and purification

Recombinant *C. merolae* ferredoxin (CmFd, residues 1–97) was subcloned from the full-length vector into vector pTTQ18 (Amersham Pharmacia Biotech, Uppsala, Sweden) using standard cloning protocols. The protein was expressed in *Escherichia coli* JM83 grown at 37 °C in Luria–Bertani medium supplemented with 50 μg/ml ampicillin. Cells were induced at OD_{600} = 0.3 with 1 mM isopropyl β-D-thiogalactoside, and incubation was continued overnight at 37 °C. The harvested cells were suspended in 10 mM Tris–HCl/1 mM EDTA [pH 8.0] plus lysozyme, deoxyribonuclease I, ribonuclease A, and 10 mM $MgCl_2$. Several freeze-thaw cycles disrupted the cells, and the resulting suspension was centrifuged at 15 000×*g* for 30 min to obtain cell-free extract.

The cell-free extract was loaded onto a DEAE-Sepharose column (2.5 × 30 cm) equilibrated with 50 mM Tris–HCl [pH 8.0]/0.15 M NaCl. After washing the column with the same buffer, the Fd was eluted with a linear gradient of 0.15–0.5 M NaCl in 50 mM Tris–HCl [pH 8.0]. Ammonium sulfate was added to the eluate to a final concentration of 2.5 M, and the solution was loaded onto a HiLoad 26/10 Phenyl Sepharose high-performance column (GE Healthcare,

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Uppsala, Sweden) equilibrated with 50 mM Tris–HCl [pH 8.0]/0.2 M NaCl/2.5 M ammonium sulfate. Fd was eluted with a negative linear gradient of 2.5–0 M ammonium sulfate. After raising the ammonium sulfate concentration to 2.5 M, the sample was loaded onto a DEAE-TOYOPEARL 650S column (TOSOH) equilibrated with 50 mM Tris–HCl [pH 8.0]/1 M NaCl/2.5 M ammonium sulfate. Fd was eluted using a negative linear gradient of 2.5–0 M ammonium sulfate in the same buffer. Finally, after again raising the ammonium sulfate concentration to 2.5 M, the sample was loaded onto a TSKgel Phenyl-5PW (TOSOH) column equilibrated with 50 mM Tris–HCl [pH 8.0]/0.2 M NaCl/2.5 M ammonium sulfate, and Fd was eluted with a negative linear gradient (2.5–0 M) of ammonium sulfate. Fd purity was determined via native polyacrylamide gel electrophoresis [3]. A protein assay was accomplished using a dye-binding assay [4].

2.2. Measurements of thermal stability and stability in acidic and alkaline media

Fd thermal stability was measured by incubating the protein in 50 mM Tris–HCl [pH 8.0]/0.2 M NaCl for 24 h at 20, 30, 40, 50, 60, 70, 80, 90, or 100 °C. The electron transfer activity remaining in the *S. oleracea* Fd-NADP$^+$ reductase system was assessed via a pig cytochrome *c* reduction assay [5]. Fd stability at various pH levels was measured by incubating 2.4 mg/ml Fd solutions between pH 1 and 13 for 24 h at 25 °C. Solution pH was adjusted as follows: 200 mM KCl/HCl (for pH 1.3 and 2.5), 200 mM glycine/HCl (for pH 3.0), 200 mM citric acid/NaOH (for pH 4.0 and 5.0), 200 mM potassium monohydrogen phosphate/HCl (for pH 6.0, 7.0, and 8.0), 200 mM glycine/NaOH (for pH 9.0, 10.0, and 10.7), potassium monohydrogen phosphate (for pH 11.4), and 200 mM KCl/NaOH (for pH 12.5). The absorbance at 425 nm was measured after incubation.

2.3. Ultraviolet (UV)–visible, electron paramagnetic resonance (EPR), and resonance Raman (RR) spectra

UV–visible absorption spectra were recorded at 25 °C on a UV/VIS Spectrophotometer V-530 (JASCO) or a Thermo Fisher Scientific Nano Drop ND-1000. Low-temperature EPR spectra were measured at 10 K as described previously [6]; the concentration of Fd was 2 mg/ml. Low-temperature RR spectra were recorded at 77 K with an Ar ion laser at 448.0 nm as described elsewhere [7] with a Fd concentration of ~20 mg/ml.

2.4. Crystallization and data collection

CmFd crystals suitable for data collection were obtained at 298 K via hanging-drop vapor diffusion. A 2.0 µl aliquot of 15 mg/ml protein solution in 50 mM Tris–HCl buffer [pH 8.0] was mixed with an equal volume of reservoir solution (3.3 M ammonium sulfate/100 mM Tris–HCl [pH 8.0]). The crystals grew to full size ($0.1 \times 0.05 \times 0.02$ mm) after 3 weeks. The crystals were soaked in cryoprotectant (50 mM Tris–HCl [pH 8.0]) and 20% glycerol for a few seconds and then immersed in liquid nitrogen. The X-ray diffraction data were collected from beamlines BL44XU (DIP6040 imaging plate detector) at Spring-8. Data were processed using HKL2000 and SCALEPACK [8].

2.5. Structure determination and refinement

Protein structure was determined by molecular replacement using the program CCP4:MOLREP [9]. *S. oleracea* ferredoxin (SoFd, Protein Data Bank entry 1a70), which has 62.1% sequence identity to CmFd at the amino acid level, was used as an initial search model. All refinements were carried out with CCP4:REFMAC5 [10]. The structure was visualized and modified using Coot [11], and model geometry was analyzed with PROCHECK [12].

3. Results and discussion

3.1. Purification

Fd purification was performed via four sequential column chromatographies to yield a highly purified Fd preparation. Native

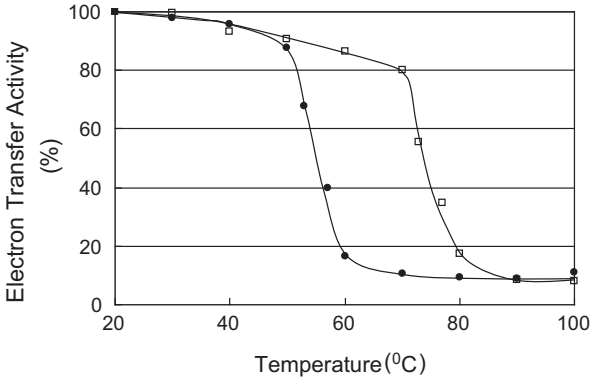

Fig. 1. Thermal stability of *C. merolae* ferredoxin and *S. oleracea* ferredoxin. Both ferredoxins were incubated at each temperature for 30 min and the electron transfer activity was measured in a spinach ferredoxin-NADP$^+$ reductase system.

Table 1
Crystallographic data and refinement statistics of *C. merolae* ferredoxin.

X-ray source	Spring-8 BL44XU
Detector	DIP6040
Wavelength (A)	0.9
Crystal-to-detector distance (mm)	200
Exposure time (s)	6
Data collection temperature (K)	100
No. of crystals per image	1/210
Space group	$P2_1$
Unit cell parameters	
a (A)	36.586
b (A)	54.922
c (A)	42.935
β (°)	96.32
Resolution range (A)	36.36–1.18
No. of total reflections	229 984
No. of unique reflections	50 687
$\langle I/(I)\rangle$	22.72
completeness (%)	96.8 (78.1)[a]
R_{merge} (%)	6.8 (33.9)[a]
Redundancy	4.3 (3.4)[a]
No. of molecules per asymmetric unit	2
V_M (A^3/Da)	1.94
Resolution range (A)	36.36–1.18
Sigma cutoff (F)	0.0
R_{cryst}[b] (%)	17.7
R_{free}[c] (%)	19.5
No. of atoms	
No. of protein atoms	1472
No. of prosthetic atoms	8
No. of water molecules	100
Average B-factor	
All	11.27
Proteins	18.986
Prosthetic group	13.092
Water molecules	25.302
Rmsd for bonds (A)	0.032
Rmsd for angles (deg)	2.55
Ramachandran plot (%)	
Most favored	96.8
Additional allowed	3.2
Disallowed	0.0

[a] Values in parentheses are for the highest-resolution shell.
[b] R_{cryst} was calculated from the working set (95% of the data).
[c] R_{free} was calculated from the test set (5% of the data).

polyacrylamide gel electrophoresis revealed a single protein band in the Fd preparation.

3.2. Thermal stability and stability at various pH levels

CmFd thermal stability experiments (Section 2.2) indicated that CmFd is more stable than SoFd by approximately 20 °C (Fig. 1), a difference that likely reflects differences in the optimum growth temperatures of the organisms. CmFd is also more stable than SoFd in both acidic conditions (pH 2.5–6.0) and alkaline conditions (pH 10.5–12.5).

3.3. Iron–sulfur cluster

The UV–visible absorption spectra, low-temperature EPR spectra, and low-temperature RR spectra were measured to identify the iron–sulfur cluster in CmFd, and the spectra were compared with those of SoFd. The overall shapes of the three CmFd spectra were similar, but slightly different from the SoFd spectra. The peaks in the UV–visible region were a maximum of 2 nm different, the RR spectra exhibited 4 cm^{-1} differences at most, and the *g*-values in the EPR spectra were maximally 0.004 different. These observations indicate that CmFd contains a plant-type [2Fe–2S] cluster, but that small structural differences from SoFd exist in or near that Fe–S cluster.

3.4. Crystallization and structure analysis

We carried out high-resolution X-ray crystallography to analyze the slight three-dimensional structural differences between CmFd and SoFd (Table 1). CmFd crystals belong to the tetragonal space group $P2_1$, with unit-cell parameters a = 36.586 A, b = 54.922 A,

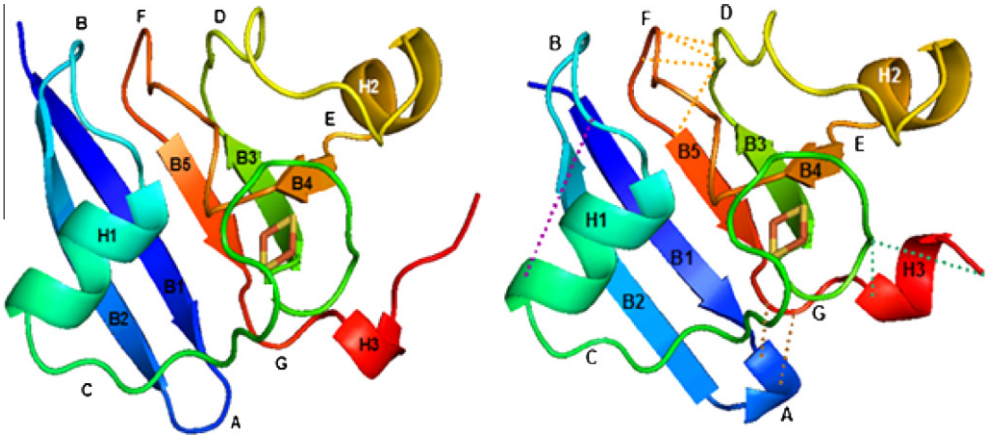

Fig. 2. Comparison of three-dimensional structure of *C. merolae* ferredoxin (right) with *S. oleracea* ferredoxin (left). Symbols are: H plus number for helix; B plus number for beta-structure; simple A–G for turn. The dotted lines indicate the hydrogen bonds which are not in the *S. oleracea* ferredoxin but also in the *C. merolae* ferredoxin.

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S. oleracea          AAYKVTLVT-PTG-NVEFQCPDDVYILDAAEEEGIDLPYSCRAGSCSSCAG:49
C. fusca             --YKVTLKT-PSG-EETIECPEDTYILDAAEEAGLDLPYSCRAGACSSCAG:47
Synechocystis sp.    ASYTVKLIT-PDG-ESSIECSDDTYILDAAEEAGLDLPYSCRAGACSTCAG:49
Anabaena 7120        ATFKVTLINEAEGTKHEIEVPDDEYILDAAEEQGYDLPFSCRAGACSTCAG:51
S. platensis         ATYKVTLINEAEGINETIDCDDDTYILDAAEEAGLDLPYSCRAGACSTCAG:51
M. laminosus         ATYKVTLINEAEGLNKTIEVPDDQYILDAAEEAGIDLPYSCRAGACSTCAG:251
C. merolae           -MYKIQLVNQKEGIDVTIQCAGDQYILDAAEEEGVDLPYSCRAGACSTCAG:50
                      1         10         20         30         40         50

S. oleracea          -KLKTGSLNQDDQSFLDDDQIDEGWVLTCAAYPVSDVTIETHKKEELTA-  :97
C. fusca             -KVESGEVDQSDQSFLDDAQMGKGFVLTCVAYPTSDVTILTHQEAAL--Y  :94
Synechocystis sp.    -KITAGSVDQSDQSFLDDDQIEAGYVLTCVAYPTSDCTIETHKEEDL     :96
Anabaena 7120        -KLVSGTVDQSDQSFLDDDQIEAGYVLTCVAYPTSDVVIQTHKEEDL--Y  :98
S. platensis         -TITSGTIDQSDQSFLDDDQIEAGYVLTCVAYPTSDCTIKTHQEEGL--Y  :98
M. laminosus         -KLISGTVDQSDQSFLDDDQIEAGYVLTCVAYPTSDCVIETHKEEEL     :297
C. merolae           -KLVKGSVDQSDQSFLDEDQISKGFILTCVAYPTSDCVIQTHQEEAL--Y  :97
                                60         70         80         90
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Fig. 3. Amino acid sequence alignments of ferredoxins from seven photosynthetic organisms. Abbreviations are *S. oleracea*: *Spinacia oleracea*; *C. fusca*: *Chlorella fusca*; *S. platensis*: *Spirulina platensis*; *M. laminosus*: *Mastigocladus laminosus*; *C. merolae*: *Cyanidioschyzon merolae*.

and $c = 42.935$ A, $\alpha = \gamma = 90°$, and $\beta = 96.32°$. Since the molecular mass of CmFd is 10.7 kDa, it is assumed that there are two monomers per asymmetric unit and four monomers in a unit cell, resulting in a Matthews coefficient (V_M) of 1.94 A^3/Da and a solvent content of 36.08%.

The CmFd refined model consists of two molecules (Mol-A, Mol-B), each including residues 1–97 and a [2Fe–2S] prosthetic group. An R_{factor} of 17.7% (R_{free} = 19.5%) was achieved from the native data up to 1.18 A resolution. The N-terminal amino acid was tentatively determined by the sequence similarity among plant-type [2Fe–2S] Fds, because the chloroplast signal sequence has not been identified. Final coordinates and structure factors were deposited in the Protein Data Bank (entries 3AB5, rcsb029037). Mol-B consists of amino acid residues and the Fe–S prosthetic group; in addition to the amino-acid residues and the prosthetic group, Mol-A contains a small molecule, probably O=C=C=O or HOC=COH, close to ^{67}Glu. Although Mol-A and Mol-B are slightly different, in the following section only Mol-B is used for comparison with Fds from other organisms.

We confirmed that CmFd is a plant-type [2Fe–2S] cluster-containing Fd with 97 amino acid residues. The secondary structure of the protein (Fig. 2) consists of two α-helices (H2:25–31 and H3:67–71), two 3_{10} helices (H1:9–12 and H4:93–96), and five β-sheets (B1:2–8, B2:13–19, B3:49–54, B4:74–76, and B5:86–89). B1 and B2 are anti-parallel around a hairpin loop. The sheet pairs B3/B5 and B3/B4 are antiparallel and act as a β-sheet wall, while B1 and B5 are parallel. This protein contains six turns (A:20–24, B:32–48, C:55–66, D:72–73, E:77–85, and F:90–92). In addition, the [2Fe–2S] cluster is coordinated by four sulfur atoms from residues Cys40, Cys45, Cys48, and Cys78.

3.5. Comparison of the three-dimensional structures of CmFd and SoFd

Although the overall structures of these two Fds are quite similar, turn A of CmFd contains five hydrogen bonds among four amino acid residues, while turn A in SoFd is composed of only two amino acid residues containing only two hydrogen bonds. It is possible that these structural differences contribute to the differences in thermal stability of the two Fds.

3.6. Structural comparison of CmFd with other [2Fe–2S] Fds

We compared the three-dimensional structure of CmFd with the solved structures of six plant-type [2Fe–2S] Fds: *Mastigocladus laminosus* (MlFd; growth temperature ~50 °C; Ref. [13]), *Anabaena* 7120 (~25 °C; Ref. [14]), *Synechocystis* sp. PCC 6803 (~30 °C; Ref. [15]), *Chlorella fusca* (~20 °C; Ref. [16]), *Spirulina platensis* (~25 °C; Ref. [17]) and SoFd (15–20 °C; Ref. [2]). The overall tertiary structures, the structures surrounding the [2Fe–2S] clusters, and most of the secondary structures of these seven Fds were quite similar

The number of ionic bonds in the seven Fds varied between four and eight. There seemed to be no relationship between the number of ionic bonds and the optimum growth temperature of the organisms, indicating that ionic bonds may not be responsible for the thermostability of these Fds. We were also unable to discern a connection between the number of hydrophobic interactions (50–65 in the seven Fds) and the optimum organismal growth temperature. However, the number of hydrogen bonds in the Fds isolated from organisms that grow at higher temperatures was greater than in organisms that grow at lower temperatures; the number of hydrogen bonds in the lower-temperature Fds ranged from 128 to 144, while the number in Fds grown at 42–50 °C (CmFd and MlFd) was 154 to 158.

A comparison of the amino acid sequences of the seven Fds revealed that in MlFd and CmFd, Gln23 has a hydrogen bond to Glu31, but no hydrogen bond appears in the other five Fds (Fig. 3). Decreased thermostability may therefore result from the mutation of Gln23 in CmFd to a non-hydrogen-bonding amino acid residue such as Ala, suggesting a strategy for engineering CmFd thermostability. Mutations at any of the other hydrogen-bond positions (Fig. 2B, dotted lines) may also affect the thermostability of CmFd, a prediction that we are currently in the process of confirming.

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