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Increasing the electron-transfer ability of *Cyanidioschyzon merolae* ferredoxin by a one-point mutation – A high resolution and Fe-SAD phasing crystal structure analysis of the Asp58Asn mutant

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ABSTRACT

Cyanidioschyzon merolae (Cm) is a single cell red algae that grows in rather thermophilic (40–50 °C) and acidic (pH 1–3) conditions. Ferredoxin (Fd) was purified from this algae and characterized as a plant-type [2Fe–2S] Fd by physicochemical techniques. A high resolution (0.97 Å) three-dimensional structure of the CmFd D58N mutant molecule has been determined using the Fe-SAD phasing method to clarify the precise position of the Asn58 amide, as this substitution increases the electron-transfer ability relative to wild-type CmFd by a factor of 1.5. The crystal structure reveals an electro-positive surface surrounding Asn58 that may interact with ferredoxin $NADP^+$ reductase or cytochrome *c*.

1. Introduction

Cyanidioschyzonmerolae (Cm) is a single cell red algae found in a hot spring near Napoli, Italy. It grows well in rather thermophilic (40–50 °C) and acidic (pH 1–3) conditions. This algae is thought to be one of the most ancient eukaryotes [1]. Therefore, it seems to be one of the best organisms for studying 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 thermostable ferredoxin D58N mutant from Cm has been determined and compared with the structure of the wild-type CmFd [2]. Furthermore, in order to clarify the structural and surface elements of the D58N mutant, its surface electrical potential was compared with that of the wild-type protein, as this region affects essential interactions with electron acceptor proteins. A slight difference between the three-dimensional structures of these proteins was also determined by an Fe-SAD (Single-wavelength Anomalous Dispersion of iron atom) phasing method that utilized no prior structural information regarding the wild-type molecule.

2. Materials and methods

2.1. Protein expression and purification

Recombinant *Cyanidioschyzon merolae* Ferredoxin (CmFd, 1–97 residues) was subcloned from a full-length vector into a pTTQ18 vector (Amersham Pharmacia Biotech, Uppsala, Sweden) using standard cloning protocols. The protein was expressed in *Escherichia coli* JM83. The harvested cells were suspended in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0 with 1 mg/ml lysozyme, 5 μg/ml deoxyribonuclease I, 5 μg/ml ribonuclease A, and 10 mM $MgCl_2$. Next, a cell-free extract was obtained by freeze–thawing several times followed by centrifugation at 15,000×*g* for 30 min.

The cell-free extract was applied onto a DEAE-Sepharose column (2.5 × 30 cm) equilibrated with 50 mM Tris–HCl, pH 8.0, 0.15 M NaCl. The ferredoxin was eluted with a linear gradient of 0.15–0.5 M NaCl, and then ammonium sulfate was added to a final concentration of 2.5 M. The solution was loaded onto a HiLoad 26/10 Phenyl Sepharose high performance column (GE Healthcare) equilibrated with 50 mM Tris–HCl, pH 8.0, 0.2 M NaCl, 2.5 M ammonium sulfate, and ferredoxin was eluted with a negative linear gradient of 2.5–0 M ammonium sulfate. The ammonium sulfate concentration of the pooled peak fractions was raised to 2.5 M, and this solution was applied to a DEAE-TOYOPEARL 650S column (TOSOH) equilibrated with 50 mM Tris–HCl, pH 8.0, 1 M NaCl, 2.5 M ammonium sulfate and eluted in a 2.5–0 M linear gradient of ammonium sulfate. Pooled peak fractions were again brought to 2.5 M ammonium sulfate, applied to a TSKgel Phenyl-5PW (TOS-

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Table 1
Data collection and refinement statistics (molecular replacement).

	Crystal 1 D58N
Data collection	
Space group	$P2_12_12_1$
Cell dimensions	
a, *b*, *c* (Å)	34.03, 46.53, 66.14
α, β, γ (°)	90.00, 90.00, 90.00
Resolution (Å)	0.97 (1.00–0.97)[a]
R_{sym} or R_{merge}	0.066 (0.604)
I/I	23.2 (2.6)
Completeness (%)	96.1 (94.4)
Redundancy	12.53 (6.19)
Refinement	
Resolution (Å)	26.97–0.97
No. reflections	57345
R_{work}[b]/R_{free}[c]	0.1517/0.1700
No. atoms	
Protein	736
Ligand/ion	4
Water	88
B-Factors	
Protein	13.21
Ligand/ion	9.09
Water	27.26
R.m.s. deviations	
Bond lengths (Å)	0.026
Bond angles (°)	2.445

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

OH) column equilibrated with 50 mM Tris–HCl, pH 8.0, 0.2 M NaCl, 2.5 M ammonium sulfate and eluted with a negative linear gradient (2.5–0 M) of ammonium sulfate.

2.2. Assay for electron transfer activity

Electron transfer activity measurements of the ferredoxins were accomplished using a cytochrome *c* reduction assay system with NADPH and ferredoxin-NADP$^+$ reductase [3]. The thermal stability of the ferredoxins was measured in 50 mM Tris–HCl, pH 8.0, 0.2 M NaCl by incubating the ferredoxins at 20, 30, 40, 50, 60, 70, 80, 90 and 100 °C for 30 min before measuring their activity. The stability of the ferredoxins at various pH values was tested by incubating them for 24 h at 25 °C in fourteen different buffers from pH 1.3 to 13.0 before measuring their activity.

2.3. Crystallization and data collection

Crystals of CmFd D58N suitable for data collection were obtained at 298 K using a hanging-drop vapor diffusion technique. A 2.0 µl aliquot of protein solution at a concentration of 15 mg/ml in 50 mM Tris–HCl buffer, pH 8.0 was mixed with an equal volume of a 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 using a Rigaku VariMax-RAPID system. The diffraction data were integrated and scaled using the CrystalClear package (Rigaku).

2.4. Structure determination and refinement

The structure was determined by a method that exploited the single-wavelength anomalous dispersion (SAD) due to the Fe atom in the molecule using the program CCP4:Crank [4]. All refinements were carried out using CCP4:REFMAC5 [5]. The structure was visualized and modified using Coot [7] The model geometry was analyzed using PROCHECK [6].

3. Results and discussion

3.1. Purification

The purification via four sequential column chromatographies produced a highly purified ferredoxin preparation. The purity of the preparation was tested via native-PAGE, and a single band was detected [8,9].

3.2. Crystallization and X-ray crystal structure analysis

In order to analyze slight structural differences between wild-type CmFd and the D58N mutant, crystallization and high-resolution analysis of the three-dimensional structure of CmFd-D58N was performed. The size of the crystal obtained was $0.1 \times 0.05 \times 0.02$ mm. The crystals of CmFd-D58N belong to the

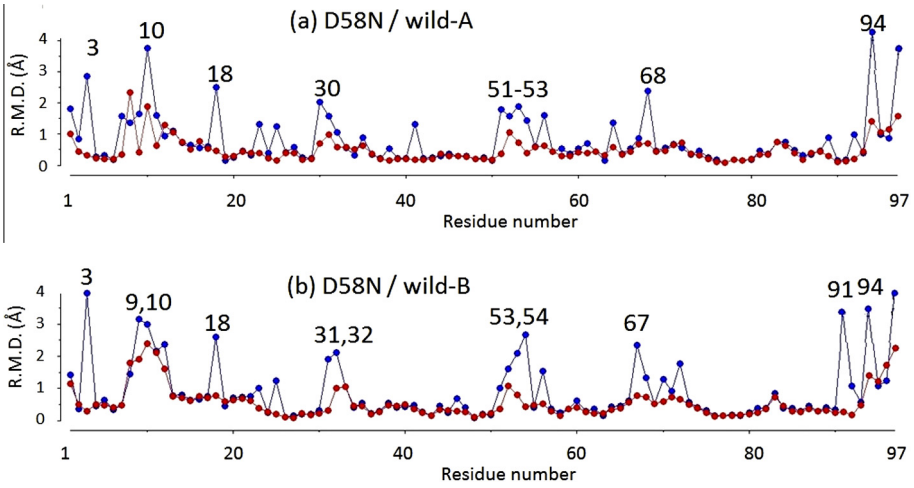

Fig. 1. Comparison of root mean-square distances of peptide chains between CmFd D58N (this study) and wild-type proteins A or B. Red circles are main chain, blue circles are side chain deviations for (A) wild-type protein A and (B) wild-type protein B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

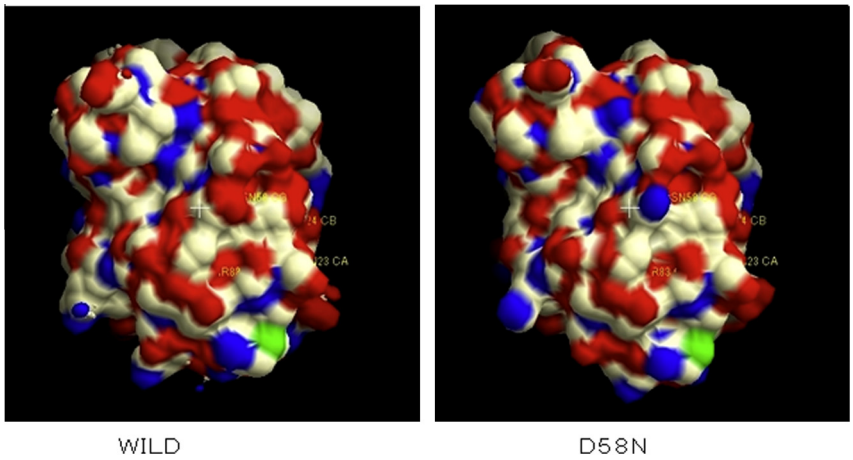

Fig. 2. Comparison of electrostatic potentials of the CmFd D58N (this study) and wild-type CmFd surfaces. Red, blue, and white represent negative, positive and uncharged surfaces, respectively. Left is wild-type and right is D58N. The molecular models were produced using the program CueMol ver. 2.0 (http://cuemol.sourceforge.jp/en/). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 34.02Å, b = 46.53 Å, and c = 66.14 Å and $\alpha = \beta = \gamma = 90°$. Because the molecular mass of CmFd is 10.7 kDa, we assumed there was one monomer per asymmetric unit and thus 4 monomers per unit cell, resulting in a Matthews coefficient (V_M) of 2.45 $Å^3$ Da^{-1} and a solvent content of 49.75%.

The structure analysis was performed by single wavelength anomalous dispersion (SAD) method exploiting the Fe-atoms in the molecule, and a structural model was built using initial phases from the Fe-SAD. The refined CmFd-D58N model has an R_{factor} of 15.1% (R_{free} = 17.0%) from the native data to 0.97 Å resolution. The current refined model includes residues 1–97 and a [2Fe–2S] prosthetic group in the protein. Crystallographic data and refinement statistics are listed in Table 1. The final coordinates and structure factors have been deposited in the Protein Data Bank (entries 3WCQ, RCSB096170).

3.3. Comparison of wild-type and CmFd-D58N structures

CmFd is a plant-type [2Fe–2S]cluster containing 97 amino acid residues and its wild-type structure has been determined (2, PDBID: 3AB5). Its secondary structure is comprised of two α-helixes (H2;25–31, H3;67–71), two 3_{10} helixes (H1;9–12, H4;93–96)and five β-sheets (B1;2–8, B2;13–19, B3;49–54, B4;74–76, B5;86–89. There exist six turns denoted turn A (20–24), B (32–48), C (55–66), D (72–73), E (77–85), and F (90–92). The long turn B maintains Fe atoms in the 2Fe–2S cluster, along with turn E (78Cys). While the wild-type CmFd crystal structure has two identical monomers in the asymmetric unit, the D58N mutant has only one monomer, whose three-dimensional structure is nearly identical to that of the wild-A or -B molecule except for 9–11(H1) main-chain and 3(B1), 9–11(H1), 18(B2), 31–32, 51–53(B3), 67–68(H3), 91–97 side-chain regions (Fig. 1).

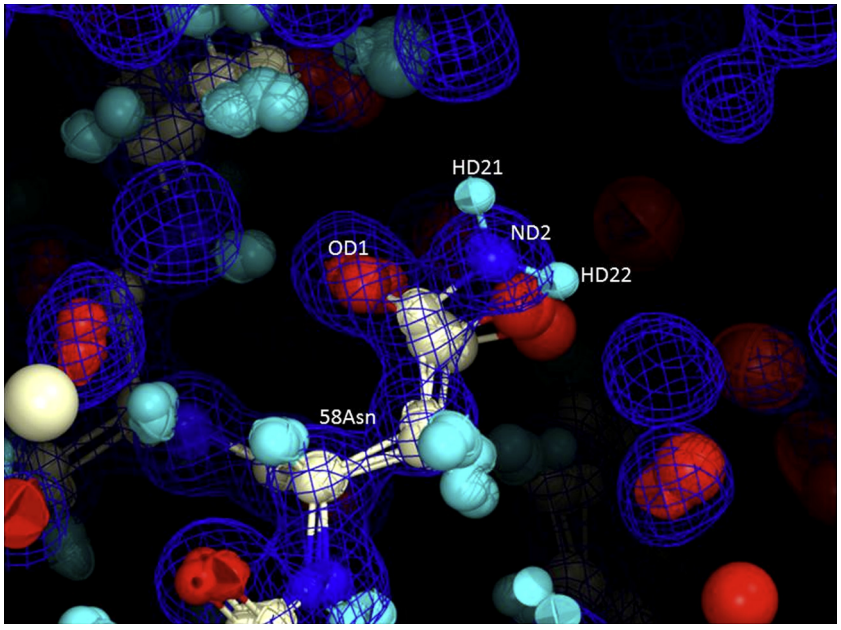

Fig. 3. Fourier $2F_o - F_c$ electron density map contoured at the 1.2σ level covering Asn58 of the D58N mutant, and Asp58 of wild-type A and B structures superimposed on the D58N molecule. The atoms are colored white for carbon, red oxygen, blue nitrogen and light-blue hydrogen. The molecular models were produced using the program CueMol ver. 2.0 (http://cuemol.sourceforge.jp/en/). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The D58N mutant has a better electron transfer ability than the wild-type CmFd in a NADPH- Ferredoxin $NADP^+$ Reductase (FNR) – CmFd – Cytochrome *c* electron carrying pathway [10], by a factor of 1.5. We have observed color exchange in an oxidized form of cytochrome *c* with a rate of ΔAbs/min. This means that electrons run through the cascade at a faster rate than wild Fd, but the interaction with FNR or cytochrome *c* is undefined.

When the surface electrical charges of these two CmFds were compared (Fig. 2), the D58N mutation was shown to create a positively charged surface region. This is to be expected for a change from a negatively charged to a neutral residue, but the positive charge could be induced by hydrogen atoms at the asparagine (–NH_2 or NH_3). This distinction could be important in explaining the electron transfer mechanism among CmFd and electron acceptors, therefore a bias-free model of this structure is needed. Fe-atoms of the Fe–S cluster in CmFd are appropriate atomic elements for phasing using Fourier transformation in a crystallographic model analysis with no prior structural information. In this work, high resolution (1.0 Å) data acquisition utilizing the anomalous dispersion effects of Fe atoms was highly suitable to generate an independent model without any structural preconceptions, especially regarding the D58N amide group. Hydrogen atoms were found at NH1 and NH2 instead of at –C = O or –COOH of D58 in the wild-type molecule (Fig. 3). These facts indicate the surface electrical charge of CmFd-D58N could be changed, thus improving the efficiency of electron transfer from this mutant protein to an electron acceptor.

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