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Purification, crystallization and preliminary X-ray analysis of ferredoxin isolated from thermophilic cyanobacterium *Mastigocladus laminosus*

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Ferredoxins are soluble iron–sulfur proteins that are involved in numerous electron-transfer reactions. Plant-type ferredoxins, which carry a single [2Fe–2S] cluster, serve as the electron acceptors of Photosystem I. The ferredoxin from the thermophilic cyanobacterium *Mastigocladus laminosus* has unique thermostable properties. The isolated protein is active at temperatures of higher than 338 K. The gene encoding the ferredoxin from *M. laminosus* was subcloned into an expression vector and overproduced in *Escherichia coli*. The recombinant protein was purified to near-homogeneity and crystallized using the hanging-drop method. Thin needle-like crystals were grown in several crystallization conditions but were unsuitable for X-ray analysis owing to poor scattering. In order to obtain better diffracting crystals, ferredoxin was purified directly from *M. laminosus* cells. Single crystals were obtained using 30% PEG 4000, 0.32 *M* $Mg(NO_3)_2$, 20 m*M* Tris–HCl pH 8.2. These crystals diffracted to 1.25 Å resolution using synchrotron radiation and were found to belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 28.45, b = 50.93, c = 110.91 Å. The asymmetric unit was found to contain two ferredoxin molecules, with a corresponding V_M of 1.9 $Å^3$ Da^{-1} and a solvent content of 34%. The present study indicates that overcoming the poor diffraction of crystals obtained from recombinant protein can be achieved by producing crystals from protein purified from the native host.

1. Introduction

Ferredoxins (Fds) are soluble iron–sulfur proteins present in bacteria, plants and mammalian cells that are involved in numerous electron-transfer reactions. Fds are characterized by their prosthetic/functional groups, *i.e.* one or two iron–sulfur clusters, [4Fe–4S], [3Fe–4S] or [2Fe–2S], according to which they are classified. The Fds in plants, algae and photosynthetic bacteria carry a single [2Fe–2S] cluster. Each iron in the cluster is coordinated by four sulfur ligands: two inorganic sulfurs that bridge the irons and two other sulfurs that are coordinated by cysteines. The four S atoms are arranged in an approximately tetrahedral arrangement around each iron. The vicinity of the cluster is highly conserved in various Fds (Knaff & Hirasawa, 1991; Barker *et al.*, 1992; Holden *et al.*, 1994). The typical oxidation–reduction midpoint potential (E_m) value of various Fds is approximately −0.42 V (Cammack *et al.*, 1977), making the reducing form of the proteins one of the strongest soluble reductants found in nature. The reduction of plant-type ferredoxin (Fd) is accomplished by the Photosystem I reaction centre during oxygenic photosynthesis. In addition to the photosynthetic electron transfer from water to Fd, which requires both Photosystem I and Photosystem II, Fd also participates in a cyclic electron flow that involves only Photosystem I. Hence, plant-type Fds serve as the electron donor in various important reactions such as $NADP^+$ reduction, where FNR (Fd-NADP reductase) is the active enzyme, carbon assimilation, nitrite reduction for nitrogen assimilation, sulfite reduction, glutamate synthesis and thioredoxin reduction for metabolism regulation (Knaff & Hirasawa, 1991). The structure of this class of Fds is well characterized and the sequences of more than 70 plant, algae and cyanobacteria Fds have been determined.

Fd isolated from the thermophilic cyanobacterium *Mastigocladus laminosus* has been shown to possess thermostable properties, with maximal activity at 338 K (Hase *et al.*, 1978). *M. laminosus* Fd, like other thermostable proteins, is intriguing. The factors determining thermostability in various proteins are continually being assessed (Scandurra *et al.*, 1998). The thermostability of the Fd from *M. laminosus* was discovered more than 20 years ago (Hase *et al.*, 1978), yet the reasons for this thermostability are still an enigma.

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Sequence comparison between the *M. laminosus* and mesophilic Fds indicates a high identity between the proteins. There are only 13 and 15 amino-acid alterations between the thermophilic *M. laminosus* Fd and the Fds from *Anabaena* 7120 and *Aphanothece sacrum*, respectively. The three-dimensional crystal structures of 11 plant-type and cyanobacterial Fds have been determined to high resolution; however, none of them is of a thermostable Fd. Two structures of a thermostable Fd from the cyanobacterium *Synechococcus elongatus* (Baumann *et al.*, 1996; Hatanaka *et al.*, 1997) have been determined by nuclear magnetic resonance (NMR) techniques, yet the interpretations of the factors determining the thermostability were inconclusive. As a first step towards structure determination of *M. laminosus* Fd and elucidation of the structural components which induce thermostability, we report the purification, crystallization and preliminary X-ray analysis of this protein.

2. Bacterial overexpression and purification of recombinant Fd

The gene encoding the Fd (*petF*) was isolated from *M. laminosus* (He *et al.*, 1998) and subcloned as a C-terminal hexahistidine fusion into the expression vector pET20b (Novagen). The recombinant protein (rFd) was overproduced in *Escherichia coli* strain BL21 (DE3) (Novagen). The transformed cells were grown in TB medium containing 10 m*M* $FeCl_3$ and 100 µg ml^{-1} ampicillin (selective marker) at 310 K until the absorption at 600 nm reached 0.6. 1 m*M* IPTG was then added to induce Fd expression. 5–6 h after induction, cells were harvested and pelleted by centrifugation at 277 K for 10 min at 6000 rev min^{-1} using a Sorvall GSA rotor. The pellet was resuspended in lysis buffer (20 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl) and broken in a French press at 13 MPa at 277 K. After centrifugation (17 000 rev min^{-1} at 277 K for 15 min in a Sorvall SS34 rotor), the supernatant was loaded onto a 50 ml Ni–agarose column. The column was washed with three column volumes of a solution containing 2 *M* NaCl and 20 m*M* Tris–HCl pH 8.0. The protein was eluted with 10 m*M* histidine, 50 m*M* NaCl and 20 m*M* Tris–HCl pH 8.0. The elution pool (0.6 g protein) was loaded onto a Q-Sepharose HR 16/20 anion-exchange column (Pharmacia) using Äkta Explorer. Increasing the NaCl concentration in 20 m*M* Tris–HCl pH 8.0 buffer stepwise to 0.45 *M* NaCl induced Fd elution. The protein pool was concentrated using a Centricon-10 (Amicon) and injected onto a Sephacryl S100 HK 26/100 gel-filtration column (Pharmacia) equilibrated in 100 m*M* NaCl, 0.02% NaN_3, 20 m*M* Tris–HCl pH 8.0. The same buffer was used to purify the Fd; SDS gel electrophoresis followed by native gel electrophoresis was used to analyze the purity of the isolated Fd. The latter indicated that the protein had been purified to near-homogeneity. The primary structure of the recombinant Fd (rFd) was then analyzed and verified *via* mass-spectroscopic methods. The purified protein was concentrated to ~12 mg ml^{-1} and used for crystallization.

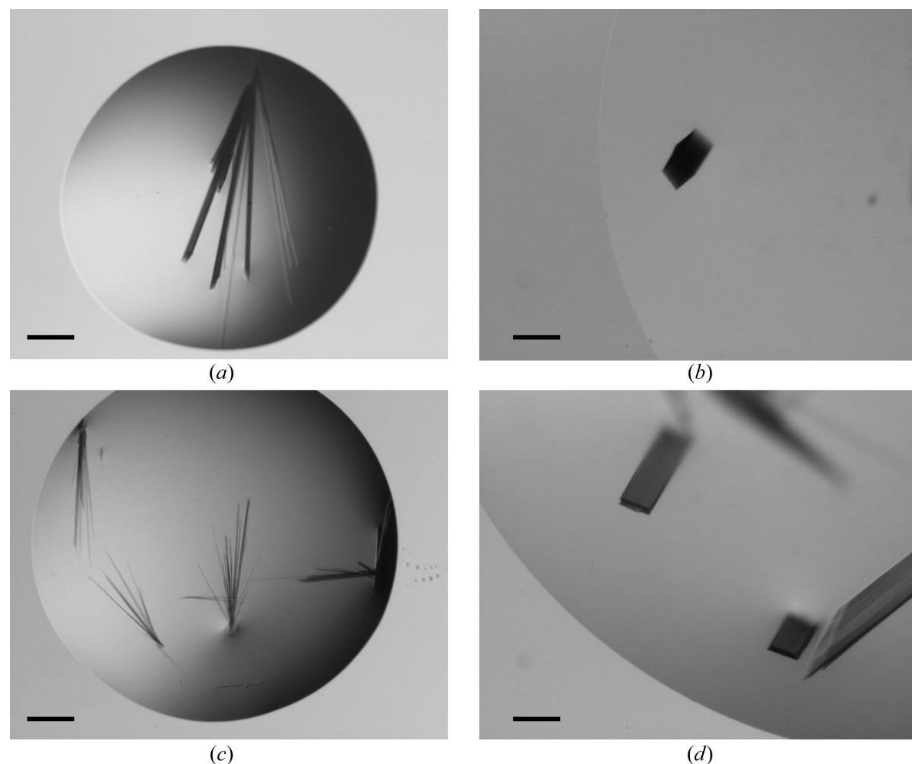

Figure 1
(*a*) and (*b*) Crystals of *M. laminosus* Fd isolated from native cyanobacterial cells. (*c*) Crystals of rFd initially grew as thin needle-like clusters; subsequent optimization of the crystallization conditions led to the production of thin plate-like single crystals (*d*). The scale bars in (*a*) and (*c*) represent 0.25 mm and in (*b*) and (*d*) 0.1 mm.

3. Isolation and purification of native Fd

M. laminosus cells were grown at 333 K in medium D of Castenholz at pH 8.2 (Castenholz, 1969) with constant stirring as previously described in Binder & Bachofen (1979). The cultures were bubbled with water-saturated air supplemented with 5% CO_2. The cells were illuminated with white light at an intensity of approximately 0.01 J cm^{-2} s^{-1}. Cells grown in 10 l carboys for 3–4 d were allowed to settle after aspiration of the growth medium and 1–2 l of cells were pelleted by centrifugation at 277 K for 10 min at 9000 rev min^{-1} in a Sorvall GSA rotor. The pellets (from six carboys; ~14 g) were resuspended in a solution containing 20 m*M* Tris–HCl pH 8.0 and a mixture of protease inhibitors: 1 m*M* phenyl methane sulfonyl fluoride, 1 m*M* benzamidine and 1 m*M* aminocaproic acid. The suspended cells were then broken in a Bead-Beater chamber (Biospec products) in the presence of 0.1 mm diameter glass beads at 277 K for nine cycles of 30 s each with intervals of 10 min. The homogenate was separated from the beads, which were then washed repeatedly with the same resuspending solution. Unbroken cells, membrane and residual beads were removed from the soluble fraction by centrifugation for 15 min at 17 000 rev min^{-1} in a Sorvall SS34 rotor. The fraction containing Fd was pelleted from the resulting supernatant by fractionation at 55 and 95% $(NH_4)_2SO_4$ and centrifugation for 15 min at 10 000 rev min^{-1} in a Sorvall GSA rotor. The final pellet was resuspended in 20 m*M* Tris–HCl buffer pH 8.0 containing 0.02% NaN_3. From these initial purification steps, the Fd-containing fraction (usually 12 mg protein) was used in successive ion-exchange and size-exclusion/gel-filtration chromatography purification steps as described above for the rFd.

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Table 1
Synchrotron data-collection statistics.

Values in parentheses refer to the highest resolution shell (1.27–1.25 Å).

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	
a	28.45
b	50.93
c	110.91
Resolution range (Å)	40–1.25
No. of reflections	44557
Redundancy	6.5
Data completeness (%)	98.0 (96.0)
R_{sym}† (%)	6.5 (24.7)
$\langle I/\sigma(I)\rangle$	18.9 (4.3)

† $R_{sym} = \sum |I - \langle I\rangle| / \sum I$.

4. Crystallization trials of Fd

Initial rFd crystallization assays were conducted using Hampton Research Crystal Screen I and II (Hampton Research, CA, USA) with the hanging-drop vapour-diffusion method at 293 K. Each drop described here and in subsequent experiments consists of 1 µl protein solution (12 mg ml^{-1} in 100 m*M* NaCl, 0.02% NaN_3, 20 m*M* Tris–HCl pH 8.0) and 1 µl reservoir solution. Numerous crystallization experiments resulted in precipitation, yet no crystals were obtained. We then examined several polyethyleneglycol (PEG) and ammonium sulfate grid screens. At this stage, we did not obtain any crystals of rFd and we therefore decided to pursue the crystallization of native Fd (nFd).

Crystallization assays for nFd were set up using similar conditions to those described for rFd. Initial crystals of Fd were obtained at 293 K in hanging drops with reservoir solution containing PEG 4000, Tris–HCl pH 8.0 and $MgCl_2$. Crystals were obtained as several clusters of needles when the reservoir solution contained 33–37% PEG 4000, 0.35–0.4 *M* $MgCl_2$, 20 m*M* Tris–HCl pH 8.0–8.5. In order to optimize the quality of the crystals, we screened different parameters such as temperature, drop size, divalent metal salts and the relative concentrations of the crystallization-mixture components. Single crystals were then obtained (Figs. 1*a* and 1*b*) at a temperature of 298 K within 3 d. The reservoir solution contained 30% PEG 4000, 0.32 *M* $Mg(NO_3)_2$, 20 m*M* Tris–HCl pH 8.2. The crystals diffracted to 1.9 Å resolution at room temperature but showed substantial diffraction decay after 12 h.

After the successful crystallization of nFd we decided to re-examine the crystallization assays for rFd. Initial crystallization trials were set up using the final crystallization conditions for nFd, yet only needle-like crystals appeared (Fig. 1*c*). We lowered the concentrations of the crystallization-mixture components [27% PEG 4000, 0.25 *M* $Mg(NO_3)_2$, 20 m*M* Tris–HCl pH 8.2], which resulted in thin plate-like crystals (Fig. 1*d*) after 4 d at 293 K. Nevertheless, these rFd crystals still exhibited poor diffraction quality and could not be analyzed.

5. Data collection and reduction

Initial diffraction data to a resolution of 1.9 Å were collected at 110 K on an R-AXIS IV++ image plate mounted on a belt-drive Rigaku RU-H3R rotating-anode generator with Max-Flux optics. Crystals were initially transferred to a cryoprotectant solution containing 40% PEG 4000, 0.3 *M* $Mg(NO_3)_2$, 20 m*M* Tris–HCl pH 8.2 for 1 min and then mounted on a cryoloop. Using synchrotron radiation at beamline ID14-1 of the European Synchrotron Radiation Facility (ESRF), Grenoble, France, diffraction extended to 1.25 Å. Crystals analyzed with synchrotron radiation were initially introduced into a cryoprotectant stabilizing solution in which the crystallization reservoir solution was supplemented with 40% PEG 4000. Data were collected at 100 K using an Oxford Cryosystems Cryostream device on a ADSC Quantum 4R CCD detector with an oscillation range of 0.5° and a radiation wavelength of 0.933 Å. Data were processed and scaled using the *HKL* package (Otwinowski & Minor, 1997). The asymmetric unit contained two molecules of Fd (MW 10.9 kDa), resulting in a crystal volume per protein mass (V_M) of 1.9 $Å^3$ Da^{-1} (Matthews, 1968) and a solvent content of 34%. We plan to determine the structure of *M. laminosus* Fd *via* molecular-replacement methods using available homologous Fd structures as search models. Crystal data-collection statistics are summarized in Table 1.

6. Conclusions and summary

Fd from the thermophilic cyanobacteria *M. laminosus* was overexpressed in *E. coli* in high yield and was purified to near-homogeneiety. However, no crystals could be obtained from this rFd. The availability of the cyanobacterial cell-culture system permitted direct isolation of Fd from the cells. The nFd purification resulted in relatively large amounts of highly pure protein. The latter yielded high-quality crystals from which high-resolution diffraction was obtained and a complete data set was collected using synchrotron radiation. When examining similar crystallization conditions using rFd, crystals were obtained but were not suitable for X-ray analysis. The approach reported here of protein isolation from the native cell rather than recombinant sources should be seriously considered when crystallization fails or when unsuitable crystals appear from a recombinant protein.

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