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Purification of ferredoxin-NADP$^+$ oxidoreductase from thermophilic cyanobacterium *Synechococcus elongatus*.

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Introduction

Ferredoxin-NADP$^+$ oxidoreductase (FNR, EC 1.18.1.2.) is an enzyme containing an FAD as an electron-transfer component and catalyzes electron transfer between ferredoxin and NADP$^+$/NADPH. The mature FNR proteins, purified and characterized from higher plants, show the molecular masses in a range of 33 kDa to 36 kDa. Cyanobacterial FNR proteins, having the molecular masses in a range of 31.5-36 kDa, are remarkably homologous to higher plant FNRs with FAD- and NADP$^+$-binding domain.

In spite of the similarity in structural and biochemical features of these FNRs, three cyanobacterial *petH* genes (*Synechococcus* PCC 7002, Schluchter and Bryant 1992; *Anabaena variabilis* PCC7119, Fillat et al. 1993 and *Synechocystis* PCC6803, van Thor et al. 1998) encode a protein being composed of three domains with a molecular mass of about 45 kDa. The first, N-terminal domain is unique in cyanobacteria and similar to the 9-kDa phycocyanin-associated linker polypeptide CpcD. The N-terminal domain of cyanobacterial FNR is considered to function as anchor of FNR onto phycobilisome. The two domains of C-terminal side correspond to the FAD- and NADP-binding domains of higher plant FNR protein. It has been reported that many cyanobacterial phycobilisomes contain substoichiometric amount of polypeptide with masses of approximately 45-50 kDa (Tandeau de Marsac and Cohen-Bazire 1977, Yamanaka et al. 1978). However, it is remained to be explicit whether the FNR with CpcD-like domain is a dominant form in living cells.

We describe here the purification of *S. elongatus* FNR protein as the 45 kDa form retained CpcD-like domain, and the state of FNR *in vivo.*

Material and Methods

Culture of S. elongatus.

The thermophilic unicellular cyanobacterium *S. elongatus* was generously obtained form Dr. Hirano of Toray Co. Ltd. and semicontinuously and aseptically grown at 52 ºC ℃ in the inorganic medium under the light intensity of 100 $\mu E \cdot m^{-2} \cdot sec^{-1}$. The culture was continuously bubbled by air containing 5% (v/v) CO_2. Cells were harvested by centrifugation at the late-exponential growth phase and stored at –80 ºC until use.

Purification of FNR protein

Cells were suspended in buffer A (50mM Tris-HCl (pH 8.0), 0.1mM EDTA, 0.1mM PMSF, 0.1mM 2-mercaptoethanol), and then disrupted by sonication, followed by twice treatments with 80 % chilled acetone. Proteins extracted with buffer A at 4 ºC overnight was fractionated by salting-out with ammonium sulfate saturation (30-40 %). The

precipitates were dissolved in buffer B (20mM Tris-HCl, pH 8.0, 1 mM EDTA). After extensive dialysis against buffer B, the dialysate was applied to a DEAE-cellulose column equilibrated with buffer B. Proteins were eluted with a linear gradient system of 50 to 400 mM NaCl. Fractions showing DCPIP reduction activity were collected, and dialyzed against 10mM potassium phosphate buffer (pH 7.0). The dialysate was applied to hydroxyapatite column equilibrated with 10mM potassium phosphate (pH7.0). The hydroxyapatite column was washed with 20mM potassium phosphate, and then the absorbed proteins were eluted with a linear gradient system of 20 to 300mM potassium phosphate.

S. elongatus FNR partially purified by salting-out and DEAE-cellulose chromatography, was subjected to TOYOPEARL HW50S gel filtration chromatography with a buffer system (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA). Fractions showing DCPIP reduction activity were collected, and dialyzed against 20 mM Tris-HCl, pH 8.0, 1 mM EDTA. The dialysate was applied to mono Q column equilibrated with 20 mM Tris-HCl, pH 8.0, and 1 mM EDTA. After washing with 5 ml of 40 mM NaCl, 20 mM Tris-HCl, pH 8.0, and 1 mM EDTA, proteins were eluted with a linear gradient system of 40 to 500 mM NaCl. The fractions containing DCPIP reduction activity were collected, and subjected to mono Q chromatography again.

Assay for FNR activity

The diaphorase activity of FNR was measured by DCPIP reduction at ambient temperature, using extinction coefficient of 22 $mM^{-1}cm^{-1}$ at 600 nm.

Analytical techniques

For immunoblot analysis, proteins were electrophoresed through SDS-PAGE, then transfered electrophoretically to PVDF membrane and incubated with antibodies raised against radish leaf FNR. Bound antibodies were visualized with protein A-conjugated horseradish peroxidase and ECL Western blotting detection reagents (Amersham).

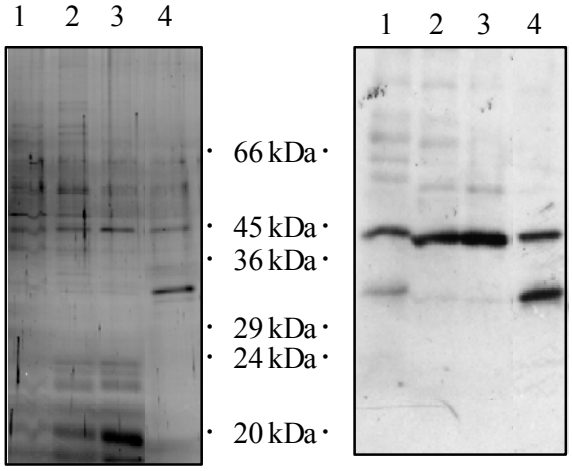

Fig. 1. Purification of *S. elongatus* FNR. Left panel: Samples corresponding with 20 µg protein were loaded in each lane. The gel was stained by silver staining. Right panel: Immunoblot analysis with antisera raised against radish leaf FNR. Samples corresponding with 20 mU DCPIP reduction activity were loaded in each lane. : Lane 1, crude extract: Lane 2, 30-40 % ammonium sulfate fraction: Lane 3, DEAE-cellulose chromatography: Lane 4, hydroxtapatite chromatography.

Results and discussion

A strongly immunoreactive species with molecular mass of 45 kDa was observed in the crude extract (Fig. 1, lane 1), suggesting that *S. elongatus* FNR in a form of 45 kDa was dominant *in vivo*. The apparent molecular mass of *S. elongatus* FNR on SDS-PAGE was 45 kDa in the fraction partially purified by salting-out and DEAE-cellulose chromatography, indicating that *S. elongatus* FNR was not proteolytically cleaved and retained CpcD-like domain (Fig. 1, lanes 2 and 3). The fraction was dark-blue, and contained low-molecular weight proteins with molecular masses of 15-20 kDa, which were considered to be subunits of phycobiliproteins. When hydroxyapatite chromatography were carried out in the absence of protease inhibitors, phycobiliproteins were removed from FNR, but FNR was proteolytically cleaved and produced a form of 34 kDa (Fig. 1, lane 4).

The FNR fraction partially purified by salting-out and DEAE-cellulose chromatography, was subjected to TOYOPEARL HW50S gel filtration chromatography. The active fractions were collected, and applied to Superose 12 gel filtration chromatography. The apparent molecular mass of *S. elongatus* FNR on Superose12 gel filtration chromatography, however, was ca. 78 kDa (Fig. 2), larger than that on SDS-PAGE.

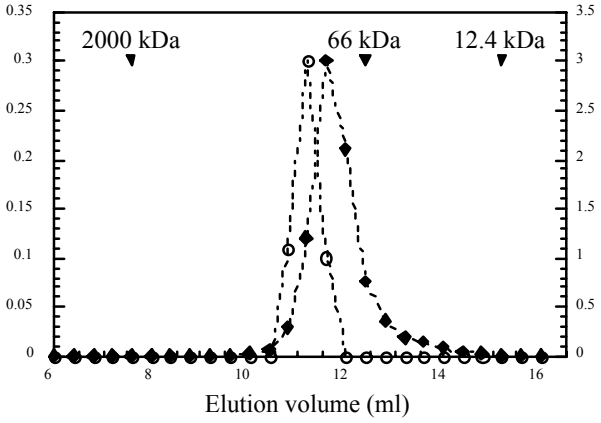

Fig. 2. Elution profile of *S. elongatus* FNR on Superose 12 gel filtration chromatography. The FNR fraction purified by salting-out, DEAE-cellulose chromatography and TOYOPEARL HW50S gel filtration chromatography, was subjected to Superose 12 gel filtration chromatography with a buffer system (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA). FNR and phycobiliproteins were monitored by DCPIP reduction activity (○) and absorbance at 615 nm (♦).

The N-terminal region of *S. elongatus* FNR with molecular mass of 45 kDa was similar to CpcD phycocyanin associated linker polypeptide (data not shown), which was considered to function as anchor of FNR on phycobilisome. These results suggested that *S. elongatus* FNR partially purified by TOYOPEARL HW50S gel filtration chromatography, was associated with phycobiliprotein.

For further purification, the FNR fraction partially purified by TOYOPEARL HW50S chromatography, was subjected to repetitive mono Q chromatography. Almost all of phycobiliproteins was removed from the FNR fractions by mono Q chromatography, but the FNR fraction contained still a small amount of phycobiliproteins. When the FNR fractions was subjected to Superose12 gel filtration chromatography, the apparent molecular mass of FNR was ca. 78 kDa (data not shown). The molecular mass of FNR on SDS-PAGE was 45 kDa in the fraction, indicating that FNR retained CpcD-like

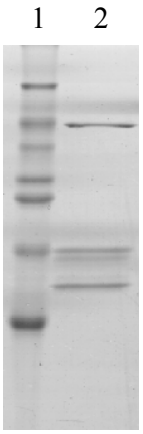

Fig. 3. *S. elongatus* FNR associating with phycobiliproteins. The sample corresponding with 5 mU DCPIP reduction activity was loaded in lane 2. Molecular weight marker was loaded in lane 1 (molecular weight values are as follows from upper to lower: 66, 45, 36, 29, 20 and 14)

domain (Fig. 3). Polypeptides with molecular mass of ca. 20 kDa and 16 kDa were found on SDS-PAGE in the fraction (Fig. 3). These polypeptides were considered to be subunits of phycocyanin because the fraction showed an absorption maximum at 615 nm. These results indicated that *S. elongatus* FNR was associated with phycocyanin, even after phycobilisome was disassembled.

From these observation described above, it was indicated that *S. elongatus* FNR was associated with phycocyanin, even after disassembly of phycobilisome, and that *S. elongatus* FNR was proteolytically cleaved after FNR was dissociated from phycocyanin. The association of *S. elongatus* FNR with phycocyanin might hinder FNR from being attacked by protease(s).

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