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Synechocystis ferredoxin-NADP$^+$ oxidoreductase is capable of functioning as ferric reductase and of driving the Fenton reaction in the absence or presence of free flavin

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Abstract We purified free flavin-independent NADPH oxidoreductase from *Synechocystis* sp. PCC6803 based on NADPH oxidation activity elicited during reduction of *t*-butyl hydroperoxide in the presence of Fe(III)-EDTA. The N-terminal sequencing of the purified enzyme revealed it to be ferredoxin-NADP$^+$ oxidoreductase (FNR_S). The purified enzyme reacted with cytochrome *c*, ferricyanide and 2,6-dichloroindophenol (DCIP). The substrate specificity of the enzyme was similar to the known FNR. DNA degradation occurring in the presence of NADPH, Fe(III)-EDTA and hydrogen peroxide was potently enhanced by the purified enzyme, indicating that *Synechocystis* FNR_S may drive the Fenton reaction. The Fenton reaction by *Synechocystis* FNR_S in the presence of natural chelate iron compounds tended to be considerably lower than that in the presence of synthetic chelate iron compounds. The *Synechocystis* FNR_S is considered to reduce ferric iron to ferrous iron when it evokes the Fenton reaction. Although *Synechocystis* FNR_S was able to reduce iron compounds in the absence of free flavin, the ferric reduction by the enzyme was enhanced by the addition of free flavin. The enhancement was detected not only in the presence of natural chelate iron compounds but also synthetic chelate iron compounds.

Keywords Ferredoxin-NADP$^+$ oxidoreductase · Ferric reductase · Fenton reaction

Abbreviations

DCIP	2,6-Dichloroindophenol
DTPA	Diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid
FNR	Ferredoxin-NADP$^+$ oxidoreductase
NTA	Nitrilotriacetic acid

Introduction

Iron is an abundant element in the earth's crust, and is essential for most organisms for their living. Aerobic

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organisms use iron for the processes of intracellular respiration, oxygen transportation, DNA synthesis, nitrogen fixation and photosynthesis. The reduction of ferric iron to ferrous iron is an essential reaction in the utilization of iron in vivo, and ferrous iron is used to generate energy in aerobic organisms. In addition, ferrous iron is highly reactive and it transfers an electron to hydrogen peroxide produced in the course of oxygen metabolism in aerobic organisms. Subsequently it produces a hydroxyl radical through the Fenton reaction that may cause damage to proteins, lipids and DNA, and result in cell death (Imlay et al. 1988; Imlay and Linn 1988). It is thought that most intracellular iron exists as ferric iron in order not to trigger the Fenton reaction.

In *E. coli*, the Fenton reaction has been shown to take place through the reduction of ferric iron in the presence of reduced free flavin generated by flavin reductase in a hyper-reductive environment when respiration is blocked in the bacteria (the Fenton reaction by free flavin-dependent ferric reduction) (Woodmansee and Imlay 2002). Photosynthetic organisms utilize light energy to produce oxygen by themselves during photosynthetic process. Thus, unnecessary radiation of light energy induces hyper-reduced conditions in the organisms and as a result, an accumulation of intracellular reactive oxygen species (ROS) occurs in them. We reported that free reduced flavin generated by DrgA protein of the *Synechocystis* sp. PCC6803, a prokaryote capable of photosynthesis and categorized as an oxygen photosynthetic bacterium, causes the Fenton reaction in the presence of ferric iron, free FAD, and hydrogen peroxide (the Fenton reaction by free flavin-dependent ferric reduction) (Takeda et al. 2007). In addition, we found that DrgA protein reduces ferric iron compounds and modestly drives the Fenton reaction in the absence of free flavin (J.S., unpublished result). Furthermore we purified the enzymes driving the Fenton reaction in the absence of free flavin from *E. coli* JM109. As a result two enzymes were identified, ferredoxin-$NADP^+$ reductase (Fpr (hereafter called *E. coli* FNR)) and oxygen-insensitive nitroreductase (NfnB) (Takeda et al. 2010). These enzymes reduce iron compounds in the absence of free flavin, and then drive the Fenton reaction. *E. coli* FNR shows a wider range of reactivity for synthetic and natural iron compounds and higher enzymatic activity than NfnB. The ferric reductase activity of these enzymes in the absence of free flavin exceeds that of DrgA protein. In the present study we newly purified an enzyme showing the free flavin-independent Fenton reaction activity from *Synechocystis* sp. PCC6803. The purified enzyme was different from DrgA protein.

Materials and methods

Materials

Unless otherwise stated, chemicals were purchased from Wako (Japan). Fe(III)-EDTA, NTA, DTPA were purchased from Dojindo (Japan). Ferrichrome, deferoxamine, transferrin and ferritin were purchased from Sigma (USA).

Cell culture and preparation of cell-free extracts

Synechocystis sp. PCC6803 cell culture and preparation of cell-free extracts were done as described previously (Takeda et al. 2004).

Enzyme purification

All purification steps were done below 4°C, and then the Fenton reaction activity was assayed by monitoring the NADPH oxidation for each active fraction. The cell-free extracts from 30.7 g wet cells were treated with streptomycin (final concentration 2%) to remove nucleic acids and stirred for 30 min on ice. Then the supernatant (120 ml) was centrifuged at 186,000g_{max} (XL-100 K centrifuge, Beckman, rotor type 45Ti) for 2 h at 4°C. After centrifugation, the supernatant (149 ml) was supplied with 1.14 M ammonium sulfate. The pH of the supernatant was adjusted to 7.0 with 2.8% ammonium solution and stirred for 30 min. After centrifugation at 64,000g for 30 min, the supernatant (160 ml) was subjected to a Butyl Toyopearl (Tosoh, Japan) column (3.5 cm $\varphi \times 22.0$ cm) pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 1.14 M ammonium sulfate at a flow rate 1.0 ml/min. Unbound protein was eluted with five column volumes of the same buffer. The bound protein was eluted with linear gradients with two column volumes of ammonium sulfate from 1.14 to 0.77 M, followed by five column volumes of ammonium sulfate from 0.77 to 0 M at a flow rate 2.5 ml/min.

The enzyme activities were recovered in fractions between 0.77 and 0.53 M, and between 0.36 and 0.21 M, as two major peaks. The initial peak turned out to be free-flavin dependent enzyme fraction and the second peak was free-flavin independent enzyme fraction. The free-flavin independent enzyme fraction was used in the following steps. The pooled fraction (122 ml) was dialyzed three times against 5 l of 50 mM sodium phosphate buffer, pH 7.0. The dialysate was centrifuged at 64,000g_{max} (Avanti HP-25 centrifuge, Beckman, rotor type 25.5) for 20 min. The supernatant was subjected to a DEAE Sepharose Fast Flow (GE Healthcare, UK) column (3.3 cm φ × 23.5 cm) pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0) at a flow rate 2.0 ml/min. Unbound protein was eluted with five column volumes of the same buffer, and the enzyme was eluted with linear gradients with two column volumes of NaCl from 0 to 100 mM, followed by six column volumes of NaCl from 100 to 300 mM at a flow rate 4.0 ml/min. The enzyme activities were recovered in fractions between 100 and 160 mM NaCl as a single peak. The active fractions were pooled (123 ml), and dialyzed three times against 4 l of 50 mM sodium phosphate buffer (pH 7.0). After dialysis, the dialysate was centrifuged at 64,000g_{max} (Avanti HP-25 centrifuge, Beckman, rotor type 25.5) for 20 min. The supernatant (124 ml) was subjected on a POROS HQ-H (Life Technologies, Japan) column (1.0 cm φ × 10.0 cm) pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0). Unbound protein was eluted with ten column volumes of the same buffer, and the enzyme was eluted with a linear gradient 50 column volumes of NaCl (0 to 300 mM). The active fractions between 77 and 178 mM NaCl were pooled (140 ml) and concentrated to 5 ml with an Apollo membrane (cut-off size 9 kDa, Orbital Bioscience). Subsequently, the concentration of sodium chloride in this enzyme solution was diluted by the addition of 15 ml of 50 mM sodium phosphate buffer (pH 7.0), and the solution was concentrated to 6 ml. This operation was repeated six times. Subsequently, the resultant enzyme solution (6 ml) diluted into 30 ml by the addition of 50 mM sodium phosphate buffer (pH 7.0) was subjected on a Red Sepharose (Life Technologies, Japan) column (1.1 cm φ × 21 cm) pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0). Unbound protein was eluted with five column volumes of the same buffer. The enzyme was eluted with a linear gradient of 12 column volumes of NaCl (0 to 500 mM). The active fractions were pooled (72 ml), and concentrated to 1 ml with an Apollo membrane (cut-off size 9 kDa, Orbital Bioscience). Subsequently, the concentration of sodium chloride in this enzyme solution was diluted by the addition of 19 ml of 50 mM sodium phosphate buffer (pH 7.0), and the solution was concentrated to 1 ml. This operation was repeated five times. The resultant enzyme solution (1 ml) was centrifuged at 17,400g_{max} (MX-150, TOMY) for 10 min and used as the purified enzyme. The purity and molecular weight of the enzyme were estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli 1970). To determine the N-terminal amino acid sequences of protein, the protein was electrotransferred to a polyvinylidene difluoride membrane and the sequence was determined with a protein sequencer (model 492, Applied Biosystems).

Enzyme assay

Fenton reaction activity, ferric reductase activity and flavin reductase activity

Oxidation of NAD(P)H was measured anaerobically at 340 nm at 25°C in a reaction mixture containing free flavin and recombinant FNR for flavin reductase activity, and Fe(III) complexes and recombinant FNR for ferric reductase activity (Takeda et al. 2007). The Fenton reaction was initiated by the addition of 200 μM hydrogen peroxide into the same reaction mixture (Takeda et al. 2007).

The molar extinction coefficients of NADH and NADPH at 340 nm are 6.22 and 6.20 mM^{-1} cm^{-1}, respectively. One unit activity was defined as 1 μmol of oxidaized NAD(P)H per min. The molar extinction coefficient of FNR was determined by resolving flavins from the protein and quantitating free flavins. Spectra of the protein in 50 mM sodium phosphate buffer (pH 7.0) were obtained, and the flavins were then released by the addition of 0.1% SDS (final concentration) at room temperature for 30 min. The molar extinction coefficient for FNR bound flavins at 459 nm was determined to be 8.99 mM^{-1} cm^{-1} using molar extinction coefficients of FAD at 450 nm (ε = 11.3 mM^{-1} cm^{-1}) and FMN at 445 nm (ε = 12.5 mM^{-1} cm^{-1}). The molar extinction coefficient of FNR was used to determine enzyme activity (U/mg protein).

Substrate specificity

Substrate specificity was examined under anaerobic condition. NAD(P)H solution (final concentration 150 μM, in 50 mM sodium phosphate buffer (pH 7.0)) was pre-incubated at 25°C in a micro black-cell. NAD(P)H oxidation was measured at 340 nm. Individual substrate was added into the cell and incubated till the base line at 340 nm reached constant. The enzyme reaction was initiated by adding the purified enzyme to the mixture. NADH and NADPH were used as an electron donor. The concentration of each substrate was 100 μM except for cytochrome *c* (50 μM) and Fe(III)-EDTA (115 μM). A FAD reductase activity, FMN reductase activity, Fe(III)-EDTA reductase activity and the Fenton activity in the presence of Fe(III)-EDTA and 200 μM hydrogen peroxide were determined by measuring absorbance at 340 nm. Reduction of cytochrome *c*, DCIP and ferricyanide was measured at 550, 600 and 420 nm, respectively. The molar extinction coefficients of cytochrome *c* at 550 nm, DCIP at 600 nm and ferricyanide at 420 nm are 18.5, 20.6 and 1.0 mM^{-1} cm^{-1}, respectively.

Steady-state kinetics

The values of K_m and k_{cat} for Fe(III)-EDTA and ferric citrate were determined from Lineweaver–Burk plots of the kinetic data obtained at various concentrations of substrates in 50 mM sodium phosphate buffer (pH 7.0) containing 200 μM NADPH at 25°C. The consumption of NADPH was monitored with a spectrophotometer at 340 nm (Hitachi U-3310). To determine the k_{cat} values for FNR, the molecular weight predicted from the deduced entire amino acid sequence was used. The molecular weight for FNR was 34,795. The Michaelis–Menten constants were determined by nonlinear regression analysis with Enzyme Kinetics Module 1.3 (Sigma Plot 11; SYSTAT Software, Chicago, IL).

Cloning, expression, and purification of recombinant FNR

We cloned the gene of FNR (*petH*) from *Synechocystis* sp. PCC6803. *Synechocystis* DNA fragment containing the open reading frame, *petH*, was amplified by the polymerase chain reaction (PCR) using the forward primer, 5′-acg aat tca cca tga caa caa ccc cca aag-3′ and the reverse primer, 5′-act cta gat tag tag gtt tcc acg tgc c-3′ for FNR. The forward primer and the reverse primer contained an *Eco*R I site and a *Xba* I site, respectively, as underlined.

The PCR product was subcloned into the pTrc99A vector for transformation of *E. coli* strain JM109. IPTG-induced recombinant protein was purified.

All steps of the purification procedure of recombinant FNR were done at 4°C, and monitored by SDS–PAGE and measuring the Fenton reaction activity. The cell pellet (35 g of wet weight) was suspended in 105 ml of 50 mM sodium phosphate buffer (pH 7.0). The cell suspension was treated with 35 mg DNase for 30 min at 4°C, thawed and passed through a French Pressure cell (SLM-Aminco) twice at 24,000 Ib/in^2, and then sonicated for 4 min by a probe-type sonicator (Branson). Phenylmetylsulfonyl fluoride (final concentration, 2 mM) was added to the suspension before and after the passage through the French Pressure cell, and after sonication. The suspension was centrifuged at 64,000g_{max} (Avanti HP-25 centrifuge, Beckman, rotor type 25.5) for 20 min to remove unbroken cells. After centrifugation, the supernatants were treated with streptomycin (final concentration 3%) to remove nucleic acids and stirred for 30 min on ice, and was centrifuged at 186,000g_{max} (XL-100 K centrifuge, Beckman, rotor type 45Ti) for 2 h. After centrifugation, the supernatant (118 ml) was supplied with 2.65 M ammonium sulfate. The pH of the supernatant was adjusted to 7.0 with 2.8% ammonium solution and stirred for 30 min. After centrifugation at 64,000g for 30 min, the supernatant (126 ml) was subjected to a Butyl Toyopearl (Tosoh, Japan) column (3.2 cm φ × 38.0 cm) pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 2.65 M ammonium sulfate at a flow rate 1.0 ml/min. Unbound protein was eluted with five column volumes of the same buffer. The bound protein was eluted by a linear gradient with 5.9 column volumes of ammonium sulfate from 2.65 to 0 M, and additionally with two column volumes of 0 M ammonium sulfate at a flow rate 2.5 ml/min. The pooled fraction (188 ml) between 1.32 and 1.14 M ammonium sulfate was dialyzed three times against 6 l of 50 mM sodium phosphate buffer, pH 7.0. After dialysis, the dialysate was centrifuged at 64,000g_{max} (Avanti HP-25 centrifuge, Beckman, rotor type 25.5) for 20 min. The dialysate was subjected to a DEAE Sepharose Fast Flow (GE Healthcare, UK) column

(2.4 cm φ × 56.0 cm) pre-equilibrated with a 50 mM sodium phosphate buffer (pH 7.0) at a flow rate 2.0 ml/min. Unbound protein was eluted with five column volumes of the same buffer, and the enzyme was eluted by a linear gradient with 5.9 column volumes of NaCl from 0 to 300 mM, and additionally with two column volumes of 300 mM NaCl at a flow rate 4.0 ml/min. The active fraction (242 ml) pooled between 125 mM and 150 mM NaCl was dialyzed three times against 7 l of 50 mM sodium phosphate buffer (pH 7.0). After dialysis, the dialysate was centrifuged at 64,000g_{max} (Avanti HP-25 centrifuge, Beckman, rotor type 25.5) for 20 min. The supernatant (146 ml) was subjected on a Red Sepharose (Life Technologies, Japan) column (1.1 cm φ × 21 cm) pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0) at a flow rate 1.0 ml/min. Unbound protein was eluted with five column volumes of the same buffer, and the enzyme was eluted with a linear gradient of six column volumes of 0 to 500 mM NaCl. The active fraction between 0 and 230 mM NaCl was pooled (114 ml) and concentrated to 4 ml with an Apollo membrane (cut-off size 9 kDa, Orbital Bioscience). Subsequently, the concentration of sodium chloride in this enzyme solution was diluted by the addition of 16 ml of 50 mM sodium phosphate buffer (pH 7.0), and the solution was concentrated to 4 ml. This operation was repeated eight times. The resultant enzyme solution (4 ml) was centrifuged at 17,400g_{max} (MX-150, TOMY) for 10 min and used as the purified enzyme. The purity and molecular weight of the enzyme were estimated by SDS–PAGE (Laemmli 1970).

DNA degradation in Fenton reaction

DNA degradation was measured under anaerobic conditions as described before (Takeda et al. 2007). The recombinant FNR protein (9.4 μg), Fe(III)-EDTA (final conc., 5.8 μM), NADPH (final conc., 100 μM) and 3.2 μg pBR322 DNA were used in the reaction.

Results and discussion

Purification of the NADPH oxidoreductase driving the Fenton reaction

We reported that DrgA protein purified from *Synechocystis* sp. PCC6803 functions as ferric reductase, and drives the Fenton reaction in the presence of free FAD (Takeda et al. 2007). At the time we obtained the purified DrgA protein by monitoring the Fenton reaction activity in the presence of free flavin. Although the purified DrgA protein evoked the Fenton reaction in the absence of free flavin, the activity was very weak.

In the present study, we found the activity driving the Fenton reaction in the absence of free flavin in *Synechocystis* cell-free extract. To identify the presumed enzyme driving this Fenton reaction, we monitored the NAD(P)H oxidation in the presence of Fe(III)-EDTA and *t*-butyl hydroperoxide plus or minus free flavin. In the early step of enzyme purification, the cell-free extracts were subjected to Butyl Toyopearl chromatography. Two main active fractions were separated by the chromatography. One fraction showed free flavin-dependent activity, while another fraction showed free flavin-independent activity. Western blotting analysis using by anti-DrgA protein antibody revealed that the free flavin-dependent fraction possessed an immuno-reactive protein with the antibody but the free flavin-independent fraction did not (data not shown). These results suggest that there is an enzyme, different from DrgA protein, driving the Fenton reaction in the absence of free flavin in *Synechocystis*.

Table 1 summarizes the results of the purification procedure. A six-step procedure was employed, and the enzyme activity driving the Fenton reaction in the absence of free flavin was monitored by NADPH oxidation throughout the purification. The purified enzyme appeared as a single band of molecular mass of 34 kDa as estimated by SDS–PAGE (Supplementary data, Fig. 1), and the overall recovery of the activity was 2.25% (Table 1). The N-terminal amino acid sequence of the 34 kDa protein was determined to be TMTTTPKEKKADDIPVNIYRPKTP, and it turned out to be identical to the sequence of ferredoxin-$NADP^+$ oxidoreductase (FNR) from *Synechocystis* sp. PCC6803 (NP_441779 accession number in the GeneBank). Although the enzyme activity, the gene and protein expression and the localization of *Synechocystis* FNR have been well-characterized (van Thor et al. 1998, 1999, 2000; Cassan et al. 2005; Thomas et al. 2006; Korn et al. 2009), little is known about the catalytic property of FNR for iron compounds. To investigate the property of FNR for iron compounds the recombinant FNR was prepared.

Table 1 Purification of enzyme catalyzing NAD(P)H-dependent *t*-butyl hydroperoxide reducing activity driving the Fenton reaction

	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Fold	Yield (%)
Cell-free extracts	2138.9	43657	0.020	1	100
Ammonium sulfate	925.0	23360	0.025	1.25	53.5
Butyl Toyopearl	274.9	6173.2	0.023	1.15	14.1
DEAE Sepharose	144.7	5194.4	0.15	7.5	11.9
HQ-H	95.2	2373.7	0.10	5.0	5.43
Red Sepharose	4.44	980.9	0.91	45.5	2.25

The NAD(P)H oxidoreductase activities responsible for the Fenton reaction was determined by measuring the difference of NAD(P)H consumption at 340 nm in a 50 mM sodium phosphate (pH 7.0) at 25°C in the presence and absence of *t*-butyl hydroperoxide. The reaction mixture contained cell-free extracts, 150 μM NAD(P)H, 115 μM Fe(III)-EDTA, and 1 mM *t*-butyl hydroperoxide. Specific activity is shown as enzyme activity per mg of protein. Experimental details are described in the "Materials and methods" section

The gene coding for *Synechocystis* FNR subcloned into an expression vector (pTrc99A) was introduced into *E. coli* JM109. IPTG-induced recombinant FNR protein was purified (Supplementary data, Fig. S1). The purification of the recombinant FNR from *E. coli* extracts was almost consistent with that of native FNR form *Synechocystis*, and the overall recovery of the activity was 52.3%. We decided to use the recombinant protein in the subsequent experiments as the substrate specificity (data not shown) and physicochemical character (Fig. S1) of recombinant FNR were the same as that of native FNR.

Characterization of FNR protein

FNR is a FAD-bound flavoprotein and a monomeric protein (Bianchi et al. 1993; Serra et al. 1995; Carrillo and Ceccarelli 2003; Ceccarelli et al. 2004). Recombinant *Synechocystis* FNR showed an absorption spectrum characteristic of flavoprotein (Fig. 1). Furthermore, the flavin coenzyme released from the FNR by hot methanol treatment (Takeda et al. 2007) was identified as flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) by HPLC analysis. The ratio of FAD to FMN was 7.5 to 1 (data not shown). The absorbance at 459 nm was markedly decreased by the addition of 0.3 and 0.6 mM NADPH under anaerobic conditions, indicating that the flavins bound to FNR were reduced (Fig. 1). The purified recombinant protein showed a single band of molecular mass of 34 kDa on SDS–PAGE (Fig. S1). By contrast the molecular mass of the intact enzyme was estimated to be 60 kDa by gel filtration (data not shown), indicating that *Synechocystis* FNR may form as a dimer in vivo. *Synechocystis* sp. PCC6803 has been known to have two FNR isofomes (FNR_L, *ca.* 46 kDa and FNR_S, *ca.* 34 kDa), which are encoded by the same gene (Thomas et al. 2006). FNR_L contains a phycobilisome (PBS) binding domain in the N-terminal region and is involved in NADPH production through its association with PBS (Schluchter and Bryant 1992; van Thor et al. 1999). It has been reported that FNR_S results from proteolytic degradation of the N-terminal domain of FNR_L and does not bind to PBS (Nakajima et al. 2002).

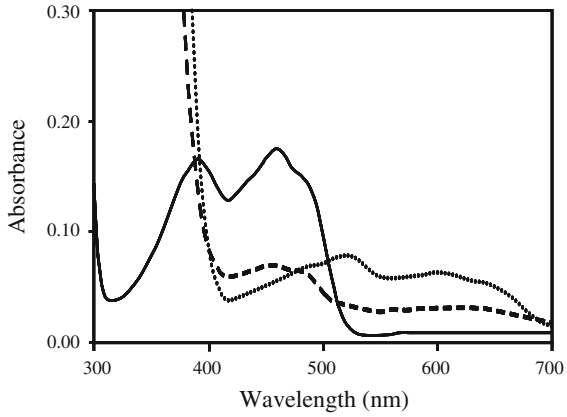

Fig. 1 Absorption spectra of recombinant FNR_S and recombinant FNR_S reduced by NADPH. Absorption spectra of recombinant FNR_S (56.0 μM) (*solid line*) and recombinant FNR_S after anaerobic reduction with 0.3 mM NADPH (*dash line*) and 0.6 mM NADPH(*dotted line*) in a 50 mM sodium phosphate buffer, pH 7.0, at 25°C

Table 2 Comparison of substrate specificity of recombinant FNR_S in the presence of NADH or NADPH

Electron acceptor	Enzyme activity (U/mg protein)	
	NADH	NADPH
Cytochrome C	N.D.	0.39 ± 0.01
DCIP	N.D.	35.27 ± 0
Ferricyanide	N.D.	35.28 ± 1.42
FAD	N.D.	6.00 ± 0.03
FMN	N.D.	5.92 ± 0.09
Fe(III)-EDTA	N.D.	0.31 ± 0.01
Fenton reaction	0.44 ± 0.04	3.07 ± 0.06

Experimental details are described in the "Materials and methods" section. Oxidation of 150 μM NAD(P)H was measured in the presence of an electron acceptor. Specific activity is shown as enzyme activity per mg of purified recombinant protein. Values represent the mean of duplicate determinations, and variations are indicated

N.D. not detected (less than 0.01 U/mg protein)

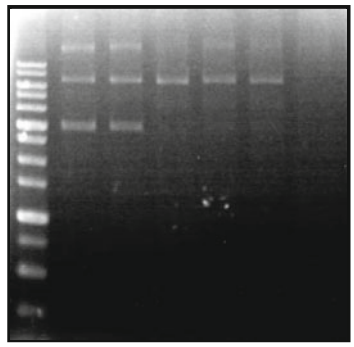

Fig. 2 DNA degradation was measured under anaerobic conditions as described previously (Takeda et al. 2007). Recombinant FNR_S (9.4 μg), Fe(III)-EDTA (final conc., 5 μM) and 3.2 μg pBR322 DNA was used

Recently, Thomas et al. proposed that *Synechocystis* FNR_S results from an internal translation initiation, not from proteolysis of FNR_L (Thomas et al. 2006). The present amino acid sequence and SDS–PAGE analyses indicate that the FNR purified from *Synechocystis* was a truncated form missing the 1-111 amino acids of N-terminal domain of FNR_L i. e. the present FNR purified from *Synechocystis* should be FNR_S. Hereafter we call the purified FNR FNR_S. FNR has been known to react with ferredoxin, flavodoxin, cytochrome *c*, ferricyanide and DCIP using NADPH as an electron donor (Onda et al. 2000; Wan and Jarrett 2002; Morsy et al. 2008). As summarized in Table 2, the present *Synechocystis* FNR_S showed the activity not only for the known substrates including cytochrome *c*, ferricyanide, DCIP, FAD and FMN but also for Fe(III)-EDTA.

DNA degradation

DNA has been known to be degraded by the hydroxyl radical generated in the Fenton reaction (Imlay et al. 1988; Imlay and Linn 1988). Using NADPH as an electron donor, we examined DNA degradation of pBR322 plasmid in the presence of Fe(III)-EDTA and hydrogen peroxide in the reaction mixture. The reaction resulted in complete degradation of the DNA (Fig. 2, lane 6), indicating that *Synechocystis* FNR_S drove the Fenton reaction.

Ferric reductase and peroxide reducing activities driving the Fenton reaction

We found that *Synechocystis* FNR_S drives the Fenton reaction in the presence of Fe(III)-EDTA and hydrogen peroxide as substrates. The Fenton reaction activity of *Synechocystis* FNR_S with various iron compounds was summarized in Table 3A. The Fenton reaction activity of the FNR for natural chelators and synthetic chelators varied between 0.11 and 1.22 U/mg protein and between 1.37 and 4.88 U/mg protein, respectively. Thus, the activity was much lower for natural chelate iron compounds than for synthetic chelate iron compounds, suggesting that the Fenton reaction driven by FNR proceeds more efficiently by using synthetic chelate iron compounds than natural chelate iron compounds as substrates.

When the Fenton reaction takes place, ferric iron must be reduced to ferrous iron. We investigated ferric reductase activity of the FNR for various iron compounds. The FNR reacted not only with synthetic chelate iron compounds such as Fe(III)-EDTA and Fe(III)-DTPA, but also with natural chelate iron compounds such as ferric ammonium citrate and the iron storage protein, ferritin. The ferric reductase activity of the FNR for natural chelators (0.40–0.59 U/mg protein) was seemed to be lower than that for synthetic chelators (0.31–2.40 U/mg

Table 3 Effects of different iron compounds on the ferric reductase activities and NADPH oxidoreductase activities driving the Fenton reaction in the absence and the presence of free flavin

Iron compounds	Enzyme activity (U/mg protein)	
	Ferric reductase activity	Fenton reaction
A (in the absence of free flavin)		
Natural chelate iron compounds		
Ferric citrate	N.D.	0.30 ± 0.04
Ferric ammonium citrate	0.40 ± 0.01	1.22 ± 0.11
Fe(III)-Ferrichrome	N.D.	0.16 ± 0.03
Fe(III)-Deferoxamine	N.D.	0.11 ± 0.01
Synthetic chelate iron compounds		
Fe(III)-nitrilotriacetic acid	N.D.	1.37 ± 0.05
Fe(III)-EDTA	0.31 ± 0.03	2.18 ± 0.05
Fe(III)-DTPA	2.40 ± 0.09	4.88 ± 0.21
Natural iron transporter protein		
Transferrin from bovine	N.D.	N.D.
Natural iron storage protein		
Ferritin from horse spleen[a]	0.59 ± 0.13	0.33 ± 0.01
Iron compounds	Enzyme activity (U/mg protein)	
	Ferric reductase activity	Fenton reaction
B (in the presence of FAD)		
Natural chelate iron compounds		
Ferric citrate	1.59 ± 0.14	3.35 ± 0.03
Ferric ammonium citrate	2.20 ± 0.07	3.60 ± 0.52
Fe(III)-Ferrichrome	1.12 ± 0.02	2.80 ± 0.09
Fe(III)-Deferoxamine	0.75 ± 0.06	1.78 ± 0.14
Synthetic chelate iron compounds		
Fe(III)-nitrilotriacetic acid	1.16 ± 0	13.73 ± 0.14
Fe(III)-EDTA	3.06 ± 0.29	8.99 ± 0.16
Fe(III)-DTPA	5.35 ± 0	11.92 ± 0
Natural iron transporter protein		
Transferrin from bovine	0.38 ± 0	0.75 ± 0.10
Natural iron storage protein		
Ferritin from horse spleen[a]	0.33 ± 0.12	0.18 ± 0.06

[a] Reaction mixture contained 0.24 mg/ml ferritin

Experimental details are described in the "Materials and methods" section. In the absence of free flavin (A) or the presence of 80 μM FAD (B), oxidation of 150 μM NADPH was measured at 340 nm at 25°C in a reaction mixture containing Fe(III) complexes and recombinant FNR_S protein for ferric reductase activity, and the same reaction mixture was used with the addition of 200 μM hydrogen peroxide for the Fenton reaction. The final concentrations of the ferric citrate, ferric ammonium citrate, Fe(III)-ferrichrome, Fe(III)-deferoxamine, Fe(III)-NTA, Fe(III)-EDTA, Fe(III)-DTPA and transferrin were 100, 100, 167, 167, 180, 115, 180 and 100 μM, respectively. Specific activity is shown as enzyme activity per mg of purified recombinant FNR_S protein. Values represent the mean of duplicate determinations, and variations are indicated

N.D. not detected (less than 0.01 U/mg protein)

protein). We observed a similar tendency in the ferric reductase activity of *E. coli* FNR in the absence of free flavin (Takeda et al. 2010).

Ferric reductase activity of *E. coli* FNR is markedly amplified by the addition of the free flavin although the FNR shows the ferric reductase activity in the absence

of free flavin (Takeda et al. 2010). We examined the ferric reductase activity and the Fenton reaction activity of *Synechocystis* FNR_S using various iron compounds in the presence of free flavin. When ferric citrate and Fe(III)-EDTA were used as substrates in the presence of various concentrations of free flavin, FAD, the ferric reductase activity by *Synechocystis* FNR_S was saturated at 80 μM of FAD. Thus, 80 μM free FAD was used in the following study. The addition of FAD resulted in an increase of the ferric reductase activity for synthetic chelate iron compounds and natural chelate iron compounds (Table 3B). Furthermore, *Synechocystis* FNR_S reacted with siderophores (Fe(III)-ferrichrome and Fe(III)-deferoxamine) in the presence of free flavin, but the FNR_S didn't show the ferrisiderophore reductase activity in the absence of free flavin (Table 3A, B). FhuF of *E. coli* could reduce siderophore in the absence of free flavin (Matzanke et al. 2004). These observations suggest that the reactivity of *Synechocystis* FNR_S for ferrisiderophores is different from that of FhuF.

The Fenton reaction by *Synechocystis* FNR_S was also enhanced by the addition of free FAD (Table 3B). The activity was higher for synthetic chelate iron compounds than for natural chelate iron compounds in the presence of free flavin as observed in the absence of free flavin (Table 3A). Our previous study showed that the Fenton reaction activity of *E. coli* FNR driven by natural chelate iron compounds in the absence of free flavin seems to follow roughly the order of the corresponding pM (Takeda et al. 2010). We observed similar Fenton reaction activities in *Synechocystis* FNR_S as with *E. coli* in that the reaction is driven by natural chelate iron compounds in the absence of free flavin.

Kinetic parameters of ferric reductase activity for Ferric citrate and Fe(III)-EDTA

Although a large number of ferric reductases require free flavins for reduction of iron compounds, some enzymes show free flavin-independent ferric reductases activity (Coves and Fontecave 1993; Filisetti et al. 2005; Fontecave et al. 1987, 1994; Mazoch et al. 2004; Pierre et al. 2002; Sato et al. 2010; Schröder et al. 2003; Takeda et al. 2007, 2010; Yeom et al. 2009).

The k_{cat}/K_m values for ferric citrate and Fe(III)-EDTA of *Synechocystis* FNR_S in the absence of free flavin were 1.59×10^3 and 1.67×10^3 M^{-1} s^{-1}, respectively (Table 4). Anusevičius et al. reported that the FNR of *Anabaena* sp. PCC 7119 show ferric reductase activity with NADPH and Fe(III)-EDTA as an electron donor and substrate, respectively (Anusevičius et al. 1997, 2005). The k_{cat}/K_m value of *Synechocystis* FNR_S for Fe(III)-EDTA was similar to that of *Anabaena* FNR (2.22×10^3 M^{-1} s^{-1}) (Anusevičius et al. 2005). As shown in Table 3 and 4, *Synechocystis* FNR_S ferric reductase activity was enhanced by the addition of free FAD. The k_{cat}/K_m values of the FNR for ferric citrate and Fe(III)-EDTA in the presence of free FAD were 4.10×10^4 and 1.25×10^5 $M^{-1}s^{-1}$, respectively.

We already reported that *E. coli* FNR and NfnB purified from *E. coli* function as free flavin-independent ferric reductases (Takeda et al. 2010). The k_{cat}/K_m

Table 4 Kinetic parameters for Fe(III)-EDTA and Fe(III)-citrate of FNR_S in the absence or presence of free flavin

Substrate	K_m for substrate (μM)	k_{cat} (s^{-1})	K_{cat}/K_m (M^{-1} s^{-1})
Fe(III)-EDTA			
in the absence of FAD	576.2 ± 104.0	0.95 ± 0.10	$1.67 \pm 0.12 \times 10^3$
in the presence of FAD[a]	16.8 ± 3.4	2.00 ± 0.17	$1.22 \pm 0.15 \times 10^5$
Fe(III)-citrate			
in the absence of FAD	160.0 ± 35.1	0.24 ± 0	$1.59 \pm 0.35 \times 10^3$
in the presence of FAD[a]	27.6 ± 5.21	1.11 ± 0.07	$4.10 \pm 0.51 \times 10^4$

[a] in the presence of 80 μM FAD

Experimental details are described in the "Materials and methods" section. Recombinant FNR_S was used. Oxidation of 200 μM NAD(P)H was measured in the presence of an electron acceptor. Values represent the mean of duplicate determinations, and variations are indicated

value of NfnB for Fe(III)-EDTA is not affected in the presence of free flavin. By contrast the k_{cat}/K_m value of *E. coli* FNR for Fe(III)-EDTA is enhanced by the addition of free FAD. The enhancement of the ferric reductase activity by the addition of free flavins occurs similarly to FprA and FprB of *Pseudomonas putida* (*P. putida*) (Yeom et al. 2009). These observations suggest that there are two types of free flavin-independent ferric reductases: (1) The activity is not enhanced by the addition of free flavin. 2) The activity is amplified by the addition of free flavin. *Synechocystis* ferredoxin-$NADP^+$ oxidoreductase must be categorized as type 2-free flavin-independent ferric reductase similar to ferredoxin-$NADP^+$ reductases purified from *E. coli* and *P. putida*.

It has been assumed that *E. coli* FNR has role in the protection from oxidative stress because it is induced by superoxide and hydrogen peroxide, and regulated by *soxRS* (Bianchi et al. 1995; Giró et al. 2006; Krapp et al. 2002; Manchado et al. 2000; Morimyo 1988; Pomposiello et al. 2001; Zheng et al. 2001). We reported that *E. coli* FNR catalyzes ferric reduction and its protein expression level is increased upon reduction of Fe(III) concentration (Takeda et al. 2010). Razquin et al. stated that the *petH* gene that encodes FNR is induced under iron deficient conditions in heterocysts of *Anabaena* sp. PCC7120 (Razquin et al. 1996). By contrast the gene expression of *P. putida* FprA and FprB is induced by increasing Fe(III) concentration and both Fprs show ferric reductase activities (Yeom et al. 2009). Although the major role of FNR is known to catalyze the NADPH production in photosynthesis, some FNR_S show ferric reductase activity when catalyzing NADPH oxidation and are considered to be involved in various iron metabolisms in a variety of organisms.

The reduction of Fe^{3+} to Fe^{2+} is essential for iron metabolism (Andrews et al. 2003). We found two ferric reductases, FNR_S and DrgA protein, in *Synechocystis* sp. PCC6803. Although the ferric reductase activity of the FNR in the absence of free flavin is much higher than that of DrgA protein, both enzyme activities are amplified in the presence of free flavin. The k_{cat} values of these enzymes for Fe(III)-EDTA in the presence of free flavin are at the similar levels. It would now be necessary to determine free flavin content in vivo for better understanding whether FNR_S or DrgA protein is involved in the ferric reduction in *Synechocystis* sp. PCC6803.

References

Andrews SC, Robinson AK, Rodíguez-Quiñones F (2003) Bacterial iron homeostasis. FEMS Microbiol Rev 27:215–237

Anusevičius Z, Martínez-Júlvez M, Genzor CG, Nivinskas H, Carlos Gómez-Moreno, Cenas N (1997) Electron transfer reactions of *Anabaena* PCC 7119 ferredoxin:$NADP^+$ reductase with nonphysiological oxidants. Biochim Biophys Acta 1320:247–255

Anusevičius Z, Miseviciene L, Medina M, Martinez-Julvez M, Gomez-Moreno C, Cenas N (2005) FAD semiquinone stability regulates single- and two-electron reduction of quinones by *Anabaena* PCC7119 ferredoxin:$NADP^+$ reductase and its Glu301Ala mutant. Arch Biochem Biophys 437:144–150

Bianchi V, Reichard P, Eliasson R, Pontis E, Krook M, Jörnvall H, Haggård-Ljungquist E (1993) Escherichia coli ferredoxin $NADP^+$ reductase: activation of *E. coli* anaerobic ribonucleotide reduction, cloning of the gene (fpr), and overexpression of the protein. J Bacteriol 175:1590–1595

Bianchi V, Haggård-Ljungquist E, Ponti E, Reichard P (1995) Interruption of the ferredoxin (flavodoxin) NADP + oxidoreductase gene of *Escherichia coli* does not affect anaerobic growth but increases sensitivity to paraquat. J Bacteriol 177:4528–4531

Carrillo N, Ceccarelli EA (2003) Open questions in ferredoxin-NADP + reductase catalytic mechanism. Eur J Biochem 270:1900–1915

Cassan N, Lagoutte B, Setif P (2005) Ferredoxin-NADP + reductase. Kinetics of electron transfer, transient intermediates, and catalytic activities studied by flash-absorption spectroscopy with isolated photosystem I and ferredoxin. J Biol Chem 280:25960–25972

Ceccarelli EA, Arakaki AK, Cortez N, Carrillo N (2004) Functional plasticity and catalytic efficiency in plant and bacterial ferredoxin-NADP(H) reductases. Biochim Biophys Acta 6:155–165

Coves J, Fontecave M (1993) Reduction and mobilization of iron by a NAD(P)H: flavin oxidoreductase from *Escherichia coli*. Eur J Biochem 211:635–641

Filisetti L, Valton J, Fontecave M, Nivière V (2005) The flavin reductase ActVB from *Streptomyces coelicolor*: characterization of the electron transferase activity of the flavoprotein form. FEBS Lett 579:2817–2820

Fontecave M, Eliasson R, Reichard P (1987) NAD(P)H:flavin oxidoreductase of *Escherichia coli*. J Biol Chem 262:12325–12331

Fontecave M, Coves J, Pierre JL (1994) Ferric reductases or flavin reductases? Biometals 7:3–8

Giró M, Carrillo N, Krapp AR (2006) Glucose-6-phosphate dehydrogenase and ferredoxin-NADP(H) reductase contribute to damage repair during the soxRS response of *Escherichia coli*. Microbiology 152:1119–1128

Imlay JA, Linn S (1988) DNA damage and oxygen radical toxicity. Science 240:1302–1309

Imlay JA, Chin SM, Linn S (1988) Toxic DNA damage by hydrogen peroxide through the fenton reaction in vivo and in vitro. Science 240:640–642

Korn A, Ajlani G, Lagoutte B, Gall A, Setif P (2009) Ferredoxin:NADP + oxidoreductase association with phycocyanin modulates its properties. J Biol Chem 284:31789–31797

Krapp AR, Rodriguez RE, Poli HO, Paladini DH, Palatnik JF, Carrillo N (2002) The flavoenzyme ferredoxin (Flavodoxin)-NADP(H) reductase modulates NADP(H) homeostasis during the soxRS response of *Escherichia coli*. J Bacteriol 184:1474–1480

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Manchado M, Michán C, Pueyo C (2000) Hydrogen peroxide activates the SoxRS regulon in vivo. J Bacteriol 182:6842–6844

Matzanke BF, Anemüller S, Schünemann V, Trautwein AX, Hantke K (2004) FhuF, part of a siderophore—reductase system. Biochemistry 43:1386–1392

Mazoch J, Tesařík R, Sedláček V, Kučera I, Turánek J (2004) Isolation and biochemical characterization of two soluble iron(III) reductases from *Paracoccus denitrificans*. Eur J Biochem 271:553–562

Morimyo M (1988) Isolation and characterization of methyl viologen-sensitive mutants of *Escherichia coli* K-12. J Bacteriol 170:2136–2142

Morsy FM, Nakajima M, Yoshida T, Fujiwara T, Sakamoto T, Wada K (2008) Subcellular localization of ferredoxin-NADP(+) oxidoreductase in phycobilisome retaining oxygenic photosysnthetic organisms. Photosynth Res 95:73–85

Nakajima M, Sakamoto T, Wada K (2002) The complete purification and characterization of three forms of ferredoxin-NADP(+) oxidoreductase from a thermophilic cyanobacterium *Synechococcus elongatus*. Plant Cell Physiol 43:484–493

Onda Y, Matsumura T, Kimata-Ariga Y, Sakakibara H, Sugiyama T, Hase T (2000) Differential interaction of maize root ferredoxin:NADP(+) oxidoreductase with photosynthetic and non-photosynthetic ferredoxin isoproteins. Plant Physiol 123:1037–1045

Pierre JL, Fontecave M, Crichton RR (2002) Chemistry for an essential biological process: the reduction of ferric iron. Biometals 15:341–346

Pomposiello PJ, Bennik MH, Demple B (2001) Genome-wide transcriptional profiling of the *Escherichia coli* responses to superoxide stress and sodium salicylate. J Bacteriol 183:3890–3902

Razquin P, Fillat MF, Schmitz S, Stricker O, Böhme H, Gómez-Moreno C, Peleato ML (1996) Expression of ferredoxin-NADP + reductase in heterocysts from *Anabaena* sp. Biochem J 15:157–160

Sato J, Takeda K, Nishiyama R, Fusayama K, Arai T, Sato T, Watanabe T, Abe A, Nakagawa J, Kawasaki S, Niimura Y (2010) *Chlorella vulgaris* aldehyde reductase is capable of functioning as ferric reductase and of driving the fenton reaction in the presence of free flavin. Biosci Biotechnol Biochem 74:854–857

Schluchter WM, Bryant DA (1992) Molecular characterization of ferredoxin-NADP + oxidoreductase in cyanobacteria: cloning and sequence of the petH gene of *Synechococcus* sp. PCC 7002 and studies on the gene product. Biochemistry 31:3092–3102

Schröder I, Johnson E, De Vries S (2003) Microbial ferric iron reductases. FEMS Microbiol Rev 27:427–447

Serra EC, Krapp AR, Ottado J, Feldman MF, Ceccarelli EA, Carrillo N (1995) The precursor of pea ferredoxin-NADP + reductase synthesized in *Escherichia coli* contains bound FAD and is transported into chloroplasts. J Biol Chem 270:19930–19935

Takeda K, Nishiyama Y, Yoda K, Watanabe T, Nimura-Matsune K, Mura K, Tokue C, Katoh T, Kawasaki S, Niimura Y (2004) Distribution of Prx-linked hydroperoxide reductase activity among microorganisms. Biosci Biotechnol Biochem 68:20–27

Takeda K, Iizuka M, Watanabe T, Nakagawa J, Kawasaki S, Niimura Y (2007) *Synechocystis* DrgA protein functioning as nitroreductase and ferric reductase is capable of catalyzing the Fenton reaction. FEBS J 274:1318–1327

Takeda K, Sato J, Goto K, Fujita T, Watanabe T, Abo M, Yoshimura E, Nakagawa J, Abe A, Kawasaki S, Niimura Y (2010) *Escherichia coli* ferredoxin-$NADP^+$ reductase and oxygen-insensitive nitroreductase are capable of functioning as ferric reductase and of driving the Fenton reaction. Biometals. doi:10.1007/s10534-010-9339-8

Thomas JC, Ughy B, Lagoutte B, Ajlani G (2006) A second isoform of the ferredoxin:NADP oxidoreductase generated by an in-frame initiation of translation. Proc Natl Acad Sci USA 103:18368–18373

van Thor JJ, Hellingwerf KJ, Matthijs HC (1998) Characterization and transcriptional regulation of the *Synechocystis* PCC 6803 petH gene, encoding ferredoxin-NADP + oxidoreductase: involvement of a novel type of divergent operator. Plant Mol Biol 36:353–363

van Thor JJ, Gruters OW, Matthijs HC, Hellingwerf KJ (1999) Localization and function of ferredoxin:NADP(+) reductase bound to the phycobilisomes of *Synechocystis*. EMBO J 18:4128–4136

van Thor JJ, Jeanjean R, Havaux M, Sjollema KA, Joset F, Hellingwerf KJ, Matthijs HC (2000) Salt shock-inducible photosystem I cyclic electron transfer in *Synechocystis* PCC6803 relies on binding of ferredoxin:NADP(+) reductase to the thylakoid membranes via its CpcD phycobilisome-linker homologous N-terminal domain. Biochim Biophys Acta 1457:129–144

Wan JT, Jarrett JT (2002) Electron acceptor specificity of ferredoxin (flavodoxin):$NADP^+$ oxidoreductase from *Escherichia coli*. Arch Biochem Biophys 406:116–126

Woodmansee AN, Imlay JA (2002) Reduced flavins promote oxidative DNA damage in non-respiring *Escherichia coli* by delivering electrons to intracellular free iron. J Biol Chem 277:34055–34066

Yeom J, Jeon CO, Madsen EL, Park W (2009) Ferredoxin-$NADP^+$ reductase from *Pseudomonas putida* functions as a ferric reductase. J Bacteriol 191:1472–1479

Zheng M, Wang X, Templeton LJ, Smulski DR, LaRossa RA, Storz G (2001) DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. J Bacteriol 183:4562–4570