Biometals (2010) 23:727–737
DOI 10.1007/s10534-010-9339-8

Escherichia coli ferredoxin-NADP$^+$ reductase and oxygen-insensitive nitroreductase are capable of functioning as ferric reductase and of driving the Fenton reaction

Kouji Takeda · Junichi Sato · Kazuyuki Goto · Takanori Fujita · Toshihiro Watanabe · Mitsuru Abo · Etsuro Yoshimura · Junichi Nakagawa · Akira Abe · Shinji Kawasaki · Youichi Niimura

Received: 13 October 2009 / Accepted: 8 April 2010 / Published online: 21 April 2010

Abstract Two free flavin-independent enzymes were purified by detecting the NAD(P)H oxidation in the presence of Fe(III)-EDTA and *t*-butyl hydroperoxide from *E. coli*. The enzyme that requires NADH or NADPH as an electron donor was a 28 kDa protein, and N-terminal sequencing revealed it to be oxygen-insensitive nitroreductase (NfnB). The second enzyme that requires NADPH as an electron donor was a 30 kDa protein, and N-terminal sequencing revealed it to be ferredoxin-NADP$^+$ reductase (Fpr). The chemical stoichiometry of the Fenton activities of both NfnB and Fpr in the presence of Fe(III)-EDTA, NAD(P)H and hydrogen peroxide was investigated. Both enzymes showed a one-electron reduction in the reaction forming hydroxyl radical from hydrogen peroxide. Also, the observed Fenton activities of both enzymes in the presence of synthetic chelate iron compounds were higher than their activities in the presence of natural chelate iron compounds. When the Fenton reaction occurs, the ferric iron must be reduced to ferrous iron. The ferric reductase activities of both NfnB and Fpr occurred with synthetic chelate iron compounds. Unlike NfnB, Fpr also showed the ferric reductase activity on an iron storage protein, ferritin, and various natural iron chelate compounds including siderophore. The Fenton and ferric reductase reactions of both NfnB and Fpr occurred in the absence of free flavin. Although the k_{cat}/K_m value of NfnB for Fe(III)-EDTA was not affected by free flavin, the k_{cat}/K_m value of Fpr for Fe(III)-EDTA was 12-times greater in the presence of free FAD than in the absence of free FAD.

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

Kouji Takeda and Junichi Sato contributed equally to this research and are co-first authors.

Electronic supplementary material The online version of this article (doi:10.1007/s10534-010-9339-8) contains supplementary material, which is available to authorized users.

K. Takeda · J. Sato · K. Goto · T. Fujita · S. Kawasaki · Y. Niimura (✉)
The Department of Bioscience, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan
e-mail: niimura@nodai.ac.jp

T. Watanabe · J. Nakagawa
Department of Food Science and Technology, Tokyo University of Agriculture, Hokkaido, Japan

M. Abo · E. Yoshimura
Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

A. Abe
Department of Internal Medicine, Nephrology Division, University of Michigan, Ann Arbor, MI, USA

Abbreviations

NTA Nitrilotriacetic acid

DTPA Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid

Introduction

Iron is an abundant element in the earth's crust, and most organisms use iron for their living. Aerobic organisms use iron for the processes of intracellular respiration, carry of oxygen, nitrogen fixation and photosynthesis. Fe^{2+} is used to generate energy in aerobic organisms. In addition, Fe^{2+} is highly reactive: Fe^{2+} transfers an electron to hydrogen peroxide produced from the oxygen metabolic pathway in aerobic organisms, then produces a hydroxyl radical through the Fenton reaction that causes damage to proteins, lipids and DNA, and results in cell death. It is thought that most intracellular iron exists as Fe^{3+} to keep from triggering the Fenton reaction. Therefore, when the Fenton reaction occurs, the Fe^{3+} must be reduced to Fe^{2+}. In some in vitro Fenton systems, superoxide was shown to be capable of reducing free iron (Brawn and Fridovich 1981; Lesko et al. 1980; McCord and Day 1978). However, it is not likely that the intracellular concentration of superoxide is high enough to contribute in that way (Imlay and Fridovich 1991; Tyler 1975). Other candidate reductants such as thiols, α-ketoacids, and NAD(P)H are all abundant inside cells, and each of these can reduce Fe^{3+} in vitro (Imlay and Linn 1988; Rowley and Halliwell 1982; Winterbourn 1979). However, it is still not conclusive whether these candidates would function as predominant reductants in vivo.

In *E. coli*, the Fenton reaction has been shown to take place through the reduction of Fe^{3+} by reduced free flavin generated by flavin reductase in a hyper-reductive environment when respiration is blocked in the bacteria (Woodmansee and Imlay 2002). Our previous study showed 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 Fe^{3+}, free FAD, and hydrogen peroxide (Takeda et al. 2007). Furthermore the DrgA protein showed the free flavin-independent Fenton activity although the Fenton activity was significantly amplified by the addition of free flavin (Takeda et al. 2007). Thus there might be not only free flavin-dependent Fenton system (no activity shown without free flavin) but also the free flavin-independent Fenton system (the activity shown without free flavin) in bacteria. In addition, this suggested that the Fe^{2+}-dependent Fenton reaction in *E. coli* may be evoked by protein(s) that processes the Fenton reaction via free flavin-independent system such as the DrgA protein.

In this study, we investigated the flavin-independent Fenton reaction system in *E. coli*. Both free flavin-dependent and free flavin-independent Fenton activities were found in *E. coli* cell extracts. Two free flavin-independent enzymes were purified by detecting the NAD(P)H oxidation in the presence of Fe(III)-EDTA and *t*-butyl hydroperoxide from *E. coli* cell extracts. In addition, these enzymes turned out to be NfnB and Fpr, showing the catalytic activities of ferric reduction. The characterization of these enzymes and their involvement in the Fenton reaction and ferric reduction are presented.

Materials and methods

Materials

In general, chemicals were purchased from Wako (Japan). Fe(III)-EDTA, NTA, DTPA were purchased from Dojindo (Japan). Enterobactin was purchased from EMC microcollections GmbH (Germany). Ferrichrome, deferoxamine, transferrin and ferritin were purchased from Sigma (USA).

Cell culture and preparation of cell-free extracts

Escherichia coli JM109 was grown at 37°C in LB medium under conditions of vigorous aeration by jar-fermenter (0.5 volume of air/volume of medium/min, 300 rpm). After the absorbance at 660 nm reached around 2.5, the cells were harvested and then suspended in three times volume of 50 mM sodium phosphate (pH 7.0, 194 ml) of wet weight (64.8 g) of the cell pellet and stored at −80°C. The cells were thawed and passed through a high Pressure homogenizer (RANNIE 7.30VH MINILAB, APV, USA) at 10,000 psi and then sonicated for 15 min at 4°C. Phenylmethylsulfonyl fluoride (final concentration,

2 mM) was added to the suspension before and after the passage through the high Pressure homogenizer, and after sonication. The resultant suspension was centrifuged at 9,780×g_{max} (Avanti HP-25 centrifuge, Beckman, rotor type 25.5) for 30 min at 4°C to remove unbroken cells and then was ultracentrifuged at 186,010×g_{max} (XL-100K centrifuge, Beckman, rotor type 45Ti) for 2 h at 4°C. The supernatant (218 ml) was used as cell free extracts.

Enzyme purification

All purification steps were done below 4°C. Ammonium sulfate was added to the cell-free supernatant (218 ml) and adjusted to 2.24 M. The pH of the supernatant was adjusted to 8.0 with 2.8% ammonium solution. After stirring for 20 min, the precipitation was removed by centrifugation at 63,988×g_{max} (Avanti HP-25 centrifuge, Beckman, rotor type 25.5) for 30 min. The supernatant (230 ml) was subjected to a Toyopearl Butyl-650S (Tosoh, Japan) column (3.2 × 52.0 cm) pre-equilibrated with 50 mM sodium phosphate (pH 8.0) containing 2.24 M ammonium sulfate at a flow rate 1.5 ml/min. Unbound protein was eluted with 5 column volumes of the same buffer. The bound protein was eluted with a linear gradient with 4.4 column volumes of ammonium sulfate from 2.24 to 0 M and additionally with 5 column volumes of 0 M ammonium sulfate. The enzyme activities were found between 0.83 and 0.71 M and between 0.42 and 0.32 M ammonium sulfate as two major peaks. The initial peak was NADPH specific fraction and the following peak was both NADH and NADPH specific fraction. The active fractions were separately pooled and named fraction A (NADPH specific) and B (NADH and NADPH specific) by following the order. The fraction A (97 ml) and B (92 ml) were dialyzed three times against 10 l of 10 mM sodium phosphate (pH 8.0). After the dialysis, each dialysate was adjusted to pH 8.0 with 0.1 M sodium hydroxide and centrifuged at 63,988×g_{max} (Avanti HP-25 centrifuge, Beckman, rotor type 25.5) for 30 min. The supernatants from A (97 ml) and B (92 ml) were subjected separately to a DEAE Sepharose Fast Flow (GE Healthcare Bio-Sciences, USA) column (3.3 × 23.5 cm) pre-equilibrated with 10 mM sodium phosphate (pH 8.0) at a flow rate 2.5 ml/min. Unbound protein was eluted with 5 column volumes of the same buffer, and the enzyme was eluted with a linear gradient with 5 column volumes of NaCl (0–250 mM). Each active fraction from A (20 ml) and B (15 ml) was pooled, and dialyzed three times against 5 l of 10 mM sodium phosphate (pH 8.0). After dialysis, each dialysate was adjusted to pH 8.0 with 0.1 M sodium hydroxide and centrifuged at 17,360×g_{max} (MX-150, TOMY) for 10 min. The resultant supernatants (A (20 ml) and B (15 ml)) were separately subjected on a POROS HQ-H (Applied biosystems, Japan) column (1.0 × 10.0 cm) pre-equilibrated with 10 mM sodium phosphate (pH 8.0). Unbound protein was eluted with 4 column volumes of the same buffer, and the enzyme was eluted with a linear gradient 100 column volumes of NaCl (0–250 mM). Each active fraction from A and B was pooled, concentrated by an Apolo membrane (cut-off size 10 kDa, Orbital Bioscience) and dialyzed against 50 mM sodium phosphate (pH 7.0). The purity and molecular weight of the enzyme were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). To determine the N-terminal amino acid sequences of proteins, the proteins were electro-transferred to a polyvinylidene difluoride membrane and the sequences were determined by a protein sequencer (model 492, Applied Biosystems).

Enzyme assay

Stoichiometry of the Fenton reaction

Stoichiometry and confirmation of the products of the Fenton reaction were determined under anaerobic conditions as described before (Takeda et al. 2007). The quantitative analysis of hydrogen peroxide and hydroxyl radical were done as described before (Gutteridge 1987; Sedewitz et al. 1984).

Fenton reaction activity, ferric reductase activity and flavin reductase activity

Oxidation of 150 μM NAD(P)H was measured anaerobically at 340 nm at 25°C in a reaction mixture containing free flavin and recombinant NfnB or Fpr for flavin reductase activity, and Fe(III) complexes and recombinant NfnB or Fpr 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 oxidized NAD(P)H per min.

Both NfnB and Fpr are flavoprotein. The molar extinction coefficients for NfnB bound FMN at 445 nm and for Fpr bound FAD at 450 nm were determined to be 12.7 and 9.45 mM^{-1} cm^{-1}, respectively, by the method of Ohnishi et al. (1995). In Tables 1, 3, 4 and 5, the protein concentrations based on these molar extinction coefficients were used to determine the specific activity (U/mg protein) of the corresponding enzyme.

Substrate specificity

Substrate specificity was examined under anaerobic condition. NAD(P)H solution (final concentration 150 μM, in 50 mM sodium phosphate (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 for NfnB and Fpr, respectively. The concentration of each substrate was fixed at 100 μM each, except for Fe(III)-EDTA (115 μM). A 1,4-benzoquinone 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 at 340 nm. Reduction of cytochrome c, DCIP, ferricyanide and nitrofurazone was measured at 550, 600, 420 and 400 nm, respectively.

Steady-state kinetics

The values of K_m and k_{cat} for Fe(III)-EDTA, FAD and FMN were determined from Lineweaver–Burk plots of the kinetic data obtained at 25°C at various substrate concentrations in 50 mM sodium phosphate (pH 7.0) containing 200 μM NAD(P)H. The consumption of NAD(P)H was monitored with a spectrophotometer at 340 nm (Hitachi U-3310). To determine the k_{cat} values for NfnB and Fpr, the molecular weight predicted from the deduced entire amino acid sequence for each enzyme was used. The molecular weights for NfnB and Fpr were 25,135 and 28,981, respectively. 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 NfnB and Fpr

We cloned the genes of *NfnB* and *Fpr* from *E. coli*. *E. coli* JM109 DNA fragments containing the open reading frames of NfnB and Fpr were amplified by the polymerase chain reaction (PCR) using the forward primer, 5′-aaa gaa ttc cat cat cat cat cat cat atg gat atc att tct gtc-3′ and the reverse primer, 5′-tag agc tca tta cac ttc ggt taa g-3′ for *NfnB* and 5′-aaa gaa ttc cat cat cat cat cat cat atg gct gat tgg gta ac-3′ and the reverse primer, 5′-ttg agc tct tac cag taa tgc tcc g-3′ for *Fpr*. The forward primer was designed to introduce six His Tags following an *Eco*R I site, and the reverse primer contained a *Sac* I site as underlined.

The PCR products were 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 NfnB and Fpr were done at 4°C and monitored by SDS-PAGE. The cell pellet (20 g of wet weight) was suspended in 60 ml of 50 mM sodium phosphate

Table 1 NAD(P)H oxidoreductase activities responsible for the Fenton reaction in the dialyzed cell-free extracts

Electron donor	NAD(P)H oxidoreductase activities responsible for the Fenton reaction (mU/mg protein)		
	FAD	FMN	No addition
NADH	69.2 ± 3.2	91.4 ± 2.5	44.2 ± 1.3
NADPH	88.7 ± 0.6	110.6 ± 0.3	87.7 ± 6.7

The NAD(P)H oxidoreductase activities responsible for the Fenton reaction were determined by measuring the difference of NAD(P)H consumption at 340 nm in a 50 mM sodium phosphate (pH 7.0) in the presence and absence of *t*-butyl hydroperoxide at 30°C. The reaction mixture contained 115 μM Fe(III)-EDTA, 15 μM flavin and 1 mM *t*-butyl hydroperoxide. Specific activity is expressed as enzyme activity per mg of protein. Values represent the mean of duplicate determinations with variations indicated

(pH 7.0). The cell suspension was treated with 20 mg DNase for 30 min at 4°C. Each cell suspension was thawed and passed through a French Pressure cell (Aminco) twice at 24,000 Ib/in^2, and then sonicated for 4 min by a probe-type sonicator (Branson). Phenylmethylsulfonyl 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 63,988$\times g_{max}$ (Avanti HP-25 centrifuge, Beckman, rotor type 25.5) for 20 min to remove unbroken cells. After centrifugation, the supernatant was ultracentrifuged at 186,010$\times g_{max}$ (XL-100K centrifuge, Beckman, rotor type 45Ti) for 2 h. The supernatant (74 ml) was dialyzed twice against 5 l of 50 mM sodium phosphate (pH 7.0) containing 300 mM NaCl. The dialysate was subjected to a TALON (TAKARA) column for purification of His-tagged protein (2.2 × 5.3 cm) pre-equilibrated with 50 mM sodium phosphate (pH 7.0) containing 300 mM NaCl at a flow rate 1 ml/min. Unbound protein was eluted with 5 volumes of the same buffer. The enzyme was eluted by stepwise with 50, 100, 150 and 300 mM imidazole from the column. The fractions eluted with 50 mM imidazole and 100 mM imidazole contained Fpr (80 ml) and NfnB (100 ml), respectively, and were dialyzed three times against 6 l of 50 mM sodium phosphate (pH 7.0). After dialysis, the dialysate was concentrated to 37 ml for Fpr and 25 ml for NfnB by a membrane filter (cut off size 20 kDa, Advantec). The purity and molecular weight of the enzyme were determined (Supplementary Fig. S2) as described above.

Expression of NfnB and Fpr in iron limited culture

E. coli strain K-12 BW25113 was inoculated to 3 ml LB medium and cultured for about 6.5 h at 37°C. Then, we inoculated the *E. coli* to 3 ml Davis medium containing K_2HPO_4 (7 g/l), KH_2PO_4 (2 g/l), $MgSO_4{\cdot}7H_2O$ (0.1 g/l), $(NH_4)_2SO_4$ (1 g/l), sodium citrate$\cdot 2H_2O$ (0.5 g/l), glucose (2 g/l) and 1 ppm Fe(III)-EDTA and pre-cultured for 13.5 h at 35°C. Finally, a total of 500 µl of the cultured cell suspension was transferred to 50 ml Davis medium containing different iron concentrations (12.2 ± 0.1, 1.16 ± 0.03, 0.106 ± 0 and 0.01 ± 0 ppm) that determined by Simultaneous Multi-element Analysis Atomic Absorption Spectrophoto-meter (Model Z-9000, HITACHI) and then cultured for 5 h at 37°C. After cultivation, 2 ml of each medium was centrifuged at 17,360$\times g_{max}$ for 5 min at 4°C (MX-150, TOMY) and the pellet of the cells was stored at −80°C before use.

Protein extracts from each pellet were prepared as described previously with a slight modification (Kawasaki et al. 2004). Twelve–fifteen micrograms of protein per lane was loaded on 15% SDS–polyacrylamide gel and electrophoresed by the method of Laemmli (Laemmli 1970). The proteins were electro-transferred to a polyvinylidene difluoride membrane (NIPPON Genetics) using a semidry transfer apparatus (NA-1512, NIHON EIDO). The membrane was treated with 5% skim milk in 10 mM sodium phosphate buffer (pH 7.2) containing 150 mM NaCl (PBS buffer) for 1 h at room temperature and then incubated with anti-Fpr rabbit polyclonal antibody or anti-NfnB rabbit polyclonal antibody (Takara) at 4°C overnight. After the incubation, the membrane was washed 3 times with 0.5% (v/v) Tween-20 in PBS buffer for 5 min at room temperature and then washed with PBS buffer for 5 min at room temperature. The membrane was incubated with donkey anti-rabbit IgG antibody conjugated with horseradish peroxidase (Roche) as described above. We detected the protein band in the presence of Lumi Blotting Substrate (Roche) by Lumi-Imager (Mannheim Boehringer).

Results

Cell free NAD(P)H oxidoreductase activity driving the Fenton reaction

We investigated the Fenton reaction by measuring *t*-butyl hydroperoxide reducing activity using dialyzed cell-free extracts, NADH or NADPH (as electron donor), FAD or FMN (as free flavin), and Fe(III)-EDTA (as iron compound). The dialyzed cell-free extracts supplemented with NADH and NADPH showed the highest Fenton activity in the presence of free flavin in the reaction mixture. Furthermore the sizable Fenton activity was detected in the absence of free flavin (Table 1). In the cell extracts supplemented with NADH, the Fenton activity in the absence of free flavin was almost half of that in the presence of free flavin. On the contrary, in the cell

extracts supplemented with NADPH, the Fenton activity was similarly detected in either presence or absence of free flavin. These results suggested that there is a flavin-independent system driving the Fenton reaction in *E. coli*.

Purification of the NAD(P)H oxidoreductase driving the Fenton reaction

To identify the presumed enzyme driving the free flavin-independent Fenton reaction (the activity shown without free flavin), the protein(s) were purified from *E. coli* by detecting the NAD(P)H oxidation in the presence of Fe(III)-EDTA and *t*-butyl hydroperoxide. Table 2 summarizes the various steps of the protocol developed for the enzyme purification. A four-step procedure was employed. In the Butyl Toyopearl chromatography, the Fenton activity was eluted as two major peaks. The initial fraction of the chromatography required NADPH as an electron donor for the Fenton reaction. The following fraction required NADH or NADPH for the reaction. The enzymes purified from the initial fraction and the following fraction appeared as single proteins of molecular masses of 30 and 28 kDa, respectively, estimated by SDS polyacrylamide gel electrophoresis (Supplementary Fig. S1). The N-terminal amino acid sequence of the 30 kDa protein was determined to be ADWVTGKVTKVQNWTDALFS, and it turned out to be identical to that of ferredoxin-$NADP^+$ reductase (Fpr; AAA23805 accession number in the GeneBank). In addition, the N-terminal amino acid sequence of the 28 kDa protein was determined to be MDIISVALKRHSTKAFDASK, and it turned out to be identical to that of oxygen insensitive nitroreductase (NfnB; accession number AAC43263 in the GeneBank).

The overall recovery of the activity was 1.1% for Fpr and 3.3% for NfnB (Table 2). Furthermore, we confirmed that the ferric reductase activity was co-eluted with the Fenton activity in both Butyl Toyopearl chromatography and DEAE Sepharose Fast Flow chromatography (data not shown).

Characterization of NfnB and Fpr proteins

Chemical stoichiometry of the Fenton reaction

The chemical stoichiometry of hydrogen peroxide reductase activity of NfnB and Fpr proteins in the presence of NAD(P)H and Fe(III)-EDTA was investigated. From a mass balance, we estimated that in the enzymatic reaction of NfnB, 168 μM of hydrogen peroxide was reduced by consuming 97 μM of NADH, generating 170 μM of hydroxyl radical as final product. Also we estimated that in the enzymatic

Table 2 Purification of enzyme catalyzing NAD(P)H-dependent *t*-butyl hydroperoxide reducing activity responsible for the Fenton reaction

	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification index	Yield (%)
NADPH (Fpr)					
Cell-free extracts	7478	120	0.016	1.0	100
Butyl Toyopearl	158.6	19.1	0.12	7.5	16
DEAE Sepharose	18.1	2.72	0.15	9.4	2.3
HQ-H	0.03	1.3	43.3	2706.3	1.1
NADH (NfnB)					
Cell-free extracts	7477	135	0.018	1.0	100
Butyl Toyopearl	138	18.3	0.13	7.2	13.6
DEAE Sepharose	N.D.	4.8	–	–	3.6
HQ-H	0.14	4.5	32.1	1783	3.3

The NAD(P)H oxidoreductase activities responsible for the Fenton reaction were determined by measuring the difference of NAD(P)H consumption at 340 nm in a 50 mM sodium phosphate (pH 7.0) in the presence and absence of *t*-butyl hydroperoxide at 30°C. 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 expressed as enzyme activity per mg of protein

N.D. not detected. – Not available. Experimental details are described in the "Materials and methods" section

Table 3 Comparison of substrate specificity of native NfnB and Fpr with that of recombinant NfnB and Fpr in the presence of NADH or NADPH

Electron acceptor	Enzyme activity (U/mg protein)	
	Native	Recombinant
NfnB		
Nitrofurazone	8.82 ± 0.52	14.7 ± 0.4
1,4-Benzoquinone	159.7 ± 1.2	211.9 ± 18.9
Ferricyanide	525.9 ± 5.8	549.0 ± 23.7
Fe(III)-EDTA	16.8 ± 0.1	16.3 ± 0.3
Fenton reaction	151.8 ± 4.7	127.8 ± 2.5
Fpr		
Cytochrome C	0.23 ± 0.01	0.55 ± 0.20
DCIP	0.88 ± 0.09	3.06 ± 0.03
Ferricyanide	10.49 ± 0	17.6 ± 0.38
Fe(III)-EDTA	3.33 ± 0.24	5.78 ± 0.09
Fenton reaction	35.41 ± 4.23	36.00 ± 0.29

Experimental details are described in the "Materials and methods" section. Oxidation of 150 μM NADH or NADPH was measured in the presence of an electron acceptor. NADPH and NADH were used for Fpr and NfnB, respectively. Specific activity is expressed as enzyme activity per mg of purified native or recombinant protein. Values represent the mean of duplicate determinations with variations indicated

reaction of Fpr, 140 μM of hydrogen peroxide was reduced by consuming 87 μM of NADPH, generating 144 μM of hydroxyl radical as final product. Thus, in both cases, the chemical stoichiometry of the reaction can be formulated as follows.

$$2H_2O_2 + NAD(P)H + H^+ \rightarrow 2OH^\bullet + NAD(P)^+ + 2H_2O \quad (\text{one - electron reduction})$$

Substrate specificity of native and recombinant enzymes

As summarized in Table 3, NfnB and Fpr showed similar substrate specificity as previously reported (Bianchi et al. 1993; McIver et al. 1998; Wan and Jarrett 2002; Zenno et al. 1996a, b). In the presence of NADH, the specific activity of endogenous NfnB was also comparable to that of recombinant NfnB. In the presence of NADPH, the specific activity of endogenous Fpr was comparable to that of recombinant Fpr. Therefore, we used recombinant Fpr and NfnB proteins in the subsequent experiments.

Ferric reductase and peroxide reducing activities driving the Fenton reaction

We detected ferric reductase activity of recombinant NfnB and Fpr using various iron compounds (Table 4).

The activity of ferric reductase of NfnB was detected for synthetic chelators but not for natural chelators. The enzyme showed the highest activity for Fe(III)-EDTA (16.86 U/mg protein) and also modest activity for the iron transport protein transferrin (0.33 U/mg protein).

On the other hand, the activities of ferric reductase of Fpr for both natural chelators and synthetic chelators were detected. The highest activities for natural and synthetic chelators were observed in use of ferric citrate (1.35 U/mg protein) and Fe(III)-DTPA (11.73 U/mg protein), respectively. Interestingly, Fpr acts on the iron transport protein, transferrin (0.38 U/mg protein), and the iron storage protein, ferritin (0.99 U/mg protein), as well as small molecular weight chemicals.

In the Fenton reaction using hydrogen peroxide as substrate, Fpr showed much higher activity for synthetic chelators than for natural chelators. The highest activities of Fpr for natural and synthetic chelators were observed in use of ferric citrate (4.11 U/mg protein) and Fe(III)-EDTA (30.22 U/mg protein), respectively. The Fenton reaction of Fpr with hydrogen peroxide catalyzed by natural chelate iron compounds seems to follow roughly the order of the corresponding pM (Table 4).

Taken together, it was suggested that Fpr is a type of reductase different from NfnB.

Kinetic parameters for Fe(III)-EDTA

We measured the K_m values of NfnB for NADH and Fpr for NADPH. The K_m value for NADH was 4.0 μM in the presence of 115 μM Fe(III)-EDTA and NADPH was 1.8 ± 0.1 μM in the presence of 150 μM Fe(III)-EDTA. Thus, we used 200 μM NAD(P)H (saturated concentration) to examine kinetic parameters for Fe(III)-EDTA. The k_{cat}/K_m values of NfnB and Fpr for Fe(III)-EDTA were $8.41 \pm 0.45 \times 10^4$ and $7.63 \pm 0.53 \times 10^4$ M^{-1} s^{-1}, respectively, in the absence of free flavin (Table 5).

Many flavin reductases show ferric reductase activity (Coves and Fontecave 1993; Filisetti et al.

Table 4 Effect of different iron compounds on the ferric reductase activities and NAD(P)H oxidoreductase activities driving the Fenton reaction

Iron compounds	Enzyme activity (U/mg protein)	
	Ferric reductase activity	Fenton reaction
NfnB		
Natural chelate iron compounds		
Ferric citrate	N.D.	0.10 ± 0
Fe(III)-ferrichrome	N.D.	0.02 ± 0
Fe(III)-deferoxamine	N.D.	N.D.
Ferric enterobactin	0.015 ± 0	0.022 ± 0
Synthetic chelate iron compounds		
Fe(III)-NTA	0.60 ± 0.03	14.21 ± 0.29
Fe(III)-EDTA	16.86 ± 0.13	127.8 ± 2.5
Fe(III)-DTPA	7.81 ± 0.40	29.87 ± 0.53
Natural iron transporter protein		
Transferrin from bovine	0.33 ± 0	1.60 ± 0.02
Natural iron storage protein		
Ferritin from horse spleen[a]	N.D.	N.D.
Fpr		
Natural chelate iron compounds		
Ferric citrate	1.35 ± 0.01	5.46 ± 0.02
Fe(III)-ferrichrome	0.51 ± 0.02	2.02 ± 0.03
Fe(III)-deferoxamine	0.70 ± 0.03	0.83 ± 0.02
Ferric enterobactin	0.014 ± 0	0.045 ± 0
Synthetic chelate iron compounds		
Fe(III)-NTA	1.05 ± 0.01	22.14 ± 0.91
Fe(III)-EDTA	5.78 ± 0.09	36.00 ± 0.28
Fe(III)-DTPA	11.73 ± 0.26	23.16 ± 0.48
Natural iron transporter protein		
Transferrin from bovine	0.38 ± 0.01	2.23 ± 0.08
Natural iron storage protein		
Ferritin from horse spleen[a]	0.99 ± 0.04	0.93 ± 0

Experimental details are described in the "Materials and methods" section. Oxidation of 150 μM NAD(P)H was measured anaerobically at 340 nm at 25°C in a reaction mixture containing Fe(III) complexes and recombinant NfnB or Fpr in the absence of free flavin for ferric reductase activity, and the same reaction mixture was used by the addition of 200 μM hydrogen peroxide for the Fenton reaction. NADH and NADPH were used for NfnB and Fpr, respectively. The final concentrations of the ferric citrate, Fe(III)-ferrichrome, Fe(III)-deferoxamine, ferric enterobactin, Fe(III)-NTA, Fe(III)-EDTA, Fe(III)-DTPA and Transferrin were 100, 167, 167, 30, 180, 115, 180 and 100 μM, respectively. Specific activity is expressed as enzyme activity per mg of purified recombinant NfnB or Fpr protein

N.D. not detected (less than 0.01 U/mg protein). Values represent the mean of duplicate determinations with variations indicated

[a] Reaction mixture contained 0.24 mg/ml ferritin

2005; Fontecave et al. 1987, 1994; Pierre et al. 2002). Therefore, we measured the k_{cat} and K_m values of individual enzyme for Fe(III)-EDTA in the presence of free flavin (Table 5). At first, the K_m values of NfnB for FMN and Fpr for FAD were determined in the presence of saturated NAD(P)H. The K_m values of NnfB for FMN and of Fpr for FAD were 106.1 ± 18.5 and 42.9 ± 2.3 μM, respectively. For the determination of kinetic parameters of Fe(III)-EDTA, the reaction assay of NfnB contained 50 μM free FMN. Higher FMN concentrations interfered with the measurement of the activity. As shown

Table 5 Kinetic parameters for Fe(III)-EDTA of NfnB and Fpr in the presence or absence of free flavin

Substrate	K_m for substrate (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
NfnB[a]			
In the absence of FMN	539.4 ± 55.8	49.8 ± 2.01	8.41 ± 0.44 × 10^4
In the presence of FMN[b]	621.7 ± 218.1	62.3 ± 10.2	10.78 ± 2.14 × 10^4
Fpr[a]			
In the absence of FAD	39.6 ± 4.6	3.0 ± 0.14	7.63 ± 0.53 × 10^4
In the presence of FAD[c]	3.64 ± 0.25	3.53 ± 0.06	9.72 ± 0.05 × 10^5

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

[a] Recombinant Fpr and NfnB were used

[b] In the presence of 50 μM FMN

[c] In the presence of 150 μM FAD

in the Table 5, FMN did not have so much effect on the K_m or k_{cat} of NfnB. The k_{cat}/K_m value of NfnB for Fe(III)-EDTA was 10.78 ± 2.13 × 10^4 M^{-1} s^{-1} in the presence of free FMN. For the Fpr, we used 150 μM free FAD to examine the Fpr kinetic parameter for Fe(III)-EDTA in the presence of free FAD. The K_m of Fpr for Fe(III)-EDTA was greatly lowered (11-fold decrease) by the addition of FAD in the reaction mixture but not the k_{cat}. The k_{cat}/K_m value of Fpr for Fe(III)-EDTA was 9.72 ± 0.05 × 10^5 M^{-1} s^{-1} in the presence of free FAD.

Expression levels of NfnB and Fpr proteins in the various iron limited culture

To examine the protein expression level of NfnB and Fpr in *E. coli*, we cultured *E. coli* in various concentrations of iron (Fe(III)-EDTA). Western blot analysis showed that the level of NfnB was mostly constant over the wide range of limited conditions (Fig. 1). On the contrary, the level of Fpr was significantly increased in *E. coli* cultured in lower iron concentrations (Fig. 1).

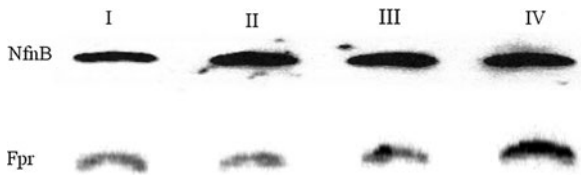

Fig. 1 Expression of NfnB and Fpr proteins in the iron limited culture. Experimental details are described in the "Materials and methods" section. Western blots show the protein expression level of NfnB and Fpr in *E. coli* cultured under various iron concentrations; 12.2 ± 0.1 ppm, *Lane I*; 1.16 ± 0.03 ppm, *Lane II*; 0.106 ± 0 ppm, *Lane III*; 0.017 ± 0 ppm, *Lane IV*

Discussion

Our previous study showed that *Synechocystis* DrgA protein functioning as nitroreductase and ferric reductase is capable of driving the Fenton reaction via free flavin-independent system. The Fenton reaction was markedly enhanced by the DrgA protein in the presence of free flavin (Takeda et al. 2007). The Fenton reaction driven via free flavin-dependent system has been shown in *E. coli* (Woodmansee and Imlay 2002). However, it was uncertain whether the Fenton reaction is driven by flavin-independent system in *E. coli*.

In this study, we found both free flavin-dependent and free flavin-independent Fenton reaction activities in *E. coli* cell-free extracts. Two free flavin-independent enzymes, showing a one-electron reduction of hydrogen peroxide in the presence of Fe(III)-EDTA, were purified from *E. coli*. When the Fenton reaction occurs, the ferric iron must be reduced to ferrous iron. There are two types of ferric reductase reaction: a reaction using free flavin, and a reaction independent of free flavin. Although most of the ferric reductases require free flavin for ferric reduction, FerB of *Paracoccus denitrificans* (*P. denitrificans*), DrgA protein of *Synechosystis*, ActVB of *Streptomyces coelicolor* (*S. coelicolor*), FprA and FprB of

Pseudomonas putida (*P. putida*) can reduce ferric iron to ferrous iron without free flavin. The k_{cat}/K_m values of the ferric reductase reaction from *P. denitrificans* FerB, *Synechosystis* DrgA protein, *S. coelicolor* ActVB, *P. putida* FprA and FprB for Fe(III)-EDTA in the absence of free flavin are 1.0×10^2, 3.67×10^4, 9.33×10^4, 1.32×10^6 and 1.17×10^6 M^{-1} s^{-1}, respectively (Filisetti et al. 2005; Mazoch et al. 2004; Takeda et al. 2007; Yeom et al. 2009). The present study showed that the k_{cat}/K_m value for Fe(III)-EDTA of NfnB, 8.41×10^4 M^{-1} s^{-1}, in the absence of free flavin is almost the same as that of Fpr, 7.63×10^4 M^{-1} s^{-1}. However, the k_{cat} and K_m values of NfnB are 13–16 times greater than those of Fpr. The k_{cat}/K_m values for Fe(III)-EDTA of *E. coli* Fpr and NfnB are between the values determined for *P. denitrificans* FerB and *P. putida* Fprs.

The ferric reductase activity for Fe(III)-EDTA of *Synechosystis* DrgA is significantly amplified by the addition of free flavin (Takeda et al. 2007). The k_{cat}/K_m values of *Synechosystis* DrgA for this ferric reduction in the presence and absence of free flavin are 10.9×10^6 and 3.67×10^4 M^{-1} s^{-1}, respectively. Therefore, we examined effects of free flavin on the k_{cat} and K_m values of *E. coli* NfnB and Fpr for Fe(III)-EDTA. The k_{cat} and K_m values of NfnB for Fe(III)-EDTA in the presence of free FMN are similar to those in the absence of free FMN (Table 5). Thus, free FMN has only a small effect on the k_{cat}/K_m value of NfnB for Fe(III)-EDTA, indicating that the catalytic efficiency of NfnB is not affected by free FMN. On the other hand, the K_m of Fpr was greatly affected by the addition of free FAD but not the k_{cat} (Table 5). The k_{cat}/K_m value of Fpr for Fe(III)-EDTA in the presence of free FAD was 9.72×10^5 M^{-1} s^{-1}, which is similar to those of *P. putida* FprA and FprB, indicating that the catalytic efficiency of Fpr is 12-times greater in the presence of free FAD than in the absence of free FAD. Taken together, these results indicate that both NfnB and Fpr act as a flavin-independent ferric reductase in the absence of free flavin but Fpr shows flavin-dependent ferric reductase activity in the presence free FAD. In the presence of free flavin, the reaction mechanism in ferric reduction by Fpr might be different from that of NfnB.

The flavin-independent ferric reductase activity of NfnB was detected in the presence of synthetic chelate iron compounds but not in the presence of natural chelate iron compounds. On the other hand, Fpr reacted with various natural chelate iron compounds including the siderophore (Andrews et al. 2003; Braun 1985; Drechsel and Winkelmann 1997) and the iron storage protein (ferritin) to reduce ferric iron to ferrous iron. Thus Fpr may function as a ferric reductase in vivo. In iron metabolism, ferrous iron produced by ferric iron reduction is highly reactive and produces hydroxyl radicals that cause damage to cellular components via the Fenton reaction in the presence of hydrogen peroxide. It was found that the flavin-independent Fenton activity by Fpr is much lower in the presence of natural chelate iron compounds than in the presence of synthetic chelate iron compounds. Previously, we observed a similar tendency in the flavin-dependent Fenton reaction driven by *Synechocystis* DrgA (Takeda et al. 2007). The Fenton reaction associated with natural chelate iron compounds may be hard to occur in vivo. However, there is a possibility that Fpr and DrgA but not NfnB evoke hydroxyl radical generation from hydrogen peroxide associated with natural chelate iron compounds in vivo.

Fpr is a FAD-bound flavoprotein that reacts with ferredoxin, flavodoxin, cytochrome *c* and ferricyanide (Bianchi et al. 1993; McIver et al. 1998; Wan and Jarrett 2002). Furthermore, Fpr is induced by soxRS and hydrogen peroxide and concerned with the protection of oxidative stress (Giró et al. 2006; Krapp et al. 2002; Morimyo 1988; Pomposiello et al. 2001; Zheng et al. 2001). In this study, we discovered an appreciable increase in protein expression level of Fpr of *E. coli* at decreasing Fe(III) concentration. Although Fprs of *P. putida* show ferric reductase activities, they are induced by increasing Fe(III) concentration (Yeom et al. 2009). The expression of *E. coli* Fpr may be regulated by a different system from that of *P. putida* Fprs in iron metabolism. The *fpr* gene deleted mutant strains may be useful to better understanding for their growth and Fpr expression under iron depletion stresses.

Acknowledgments We thank K. Akiyoshi and K. Mizutani for their technical assistance.

References

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

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

Braun V (1985) The iron transport systems of *Escherichia coli*. In: Martonosi AN (ed) The enzymes of biological membranes. Plenum Press, New York, pp 617–652

Brawn K, Fridovich I (1981) DNA strand scission by enzymically generated oxygen radicals. Arch Biochem Biophys 206:414–419

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

Drechsel H, Winkelmann G (1997) Iron chelation and siderophores. In: Winkelmann G, Carrano CJ (eds) Transition metals in microbial metabolism. Harwood Academic, Amsterdam

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 J-L (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

Gutteridge JMC (1987) Ferrous-salt-promoted damage to deoxyribose and benzoate. The increased effectiveness of hydroxyl-radical scavengers in the presence of EDTA. Biochem J 243:709–714

Imlay JA, Fridovich I (1991) Assay of metabolic superoxide production in *Escherichia coli*. J Biol Chem 266: 6957–6965

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

Kawasaki S, Ishikura J, Watamura Y, Niimura Y (2004) Identification of O_2-induced peptides in an obligatory anaerobe, *Clostridium acetobutylicum*. FEBS Lett 571: 21–25

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

Lesko SA, Lorentzen RJ, Tso POP (1980) Role of superoxide in deoxyribonucleic acid strand scission. Biochemistry 19:3023–3028

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

McCord JM, Day ED Jr (1978) Superoxide-dependent production of hydroxyl radical catalyzed by iron-EDTA complex. FEBS Lett 86:139–142

McIver L, Leadbeater C, Campopiano DJ, Baxter RL, Daff SN, Chapman SK, Munro AW (1998) Characterisation of flavodoxin $NADP^+$ oxidoreductase and flavodoxin; key components of electron transfer in *Escherichia coli*. Eur J Biochem 257:577–585

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

Ohnishi K, Niimura Y, Hidaka M, Masaki H, Suzuki H, Uozumi T, Nishino T (1995) Role of cysteine 337 and cysteine 340 in flavoprotein that functions as NADH oxidase from *Amphibacillus xylanus* studied by site-directed mutagenesis. J Biol Chem 270:5812–5817

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

Rowley DA, Halliwell B (1982) Superoxide-dependent formation of hydroxyl radicals from NADH and NADPH in the presence of iron salts. FEBS Lett 142:39–41

Sedewitz B, Schleifer KH, Götz F (1984) Purification and biochemical characterization of pyruvate oxidase from *Lactobacillus plantarum*. J Bacteriol 160:273–278

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

Tyler DD (1975) Polarographic assay and intracellular distribution of superoxide dismutase in rat liver. Biochem J 147:493–504

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

Winterbourn CC (1979) Comparison of superoxide with other reducing agents in the biological production of hydroxyl radicals. Biochem J 182:625–628

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

Zenno S, Koike H, Tanokura M, Saigo K (1996a) Gene cloning, purification, and characterization of NfsB, a minor oxygen-insensitive nitroreductase from *Escherichia coli*, similar in biochemical properties to FRase I, the major flavin reductase in *Vibrio fischeri*. J Biochem 120:736–744

Zenno S, Koike H, Kumar AN, Jayaraman R, Tanokura M, Saigo K (1996b) Biochemical characterization of NfsA, the *Escherichia coli* major nitroreductase exhibiting a high amino acid sequence homology to Frp, a *Vibrio harveyi* flavin oxidoreductase. J Bacteriol 178:4508–4514

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