Full Paper

Free flavins accelerate release of ferrous iron from iron storage proteins by both free flavin-dependent and -independent ferric reductases in *Escherichia coli*

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Junichi Satoh,^{1,†} Shinya Kimata,^{1,†} Shota Nakamoto,¹ Tatsuya Ishii,¹ Eisuke Tanaka,¹ Sayuri Yumoto,¹
Kouji Takeda,² Etsuro Yoshimura,³ Yu Kanasaki,⁴ Taichiro Ishige,⁴ Keisuke Tanaka,⁴ Akira Abe,⁵
Shinji Kawasaki,¹ and Youichi Niimura^{1,*}

¹ Department of Bioscience, Tokyo University of Agriculture, Japan

² Education course, Tokyo University of Agriculture, Japan

³ The Open University of Japan, Japan

⁴ Nodai Genome Research Center, Tokyo University of Agriculture, Japan

⁵ Department of Ophthalmology, Sapporo Medical University, Japan

Ferredoxin NADP⁺ oxidoreductase (Fpr) and oxygen-insensitive NAD(P)H nitroreductase (NfnB) are purified from *Escherichia coli* JM109 (*E. coli* JM109) as a predominant free flavin-independent ferric reductase. In the present study, we prepared natural iron storage proteins, *E. coli* ferritin A (FtnA) and bacterioferritin (Bfr), to show the effective ferrous iron release from these proteins by Fpr and NfnB in the presence of free flavins. Fpr and NfnB showed flavin reductase activity for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and riboflavin, and their ferrous iron release activities were positively associated with the catalytic efficiencies (k_{cat}/K_m) for individual flavins. The ferrous iron release activity of *E. coli* cell-free extracts was affected by flavin reductase activity of the extracts. The Butyl TOYOPEARL column chromatography of the extracts, on the basis of NAD(P)H-dependent flavin reductase activity, resulted in the separation of six active fractions containing Fpr, NfnB, NAD(P)H-quinone oxidoreductase (QOR), flavin reductase (Fre) or alkyl hydroperoxide reductase subunit F (AhpF) as major components. Like Fpr and NfnB, recombinant QOR, Fre, and AhpF showed flavin reductase activity and ferrous iron release activity in the presence of free flavins, indicating an association of flavin reductase activity with ferrous iron releasing

activity. Taken together, both free flavin-dependent and free flavin-independent ferric reductases in *E. coli* require free flavins to mediate an electron transfer from NAD(P)H to ferric iron in the iron storage proteins for the effective ferrous iron release.

Key Words: *Escherichia coli*; ferric reductase; ferritin; flavin reductase; free flavin; iron; iron storage protein

Introduction

Iron is an essential metal in biological processes such as DNA synthesis, intracellular respiration, nitrogen fixation, and photosynthesis, in which it is utilized as a cofactor for iron-binding proteins. It is believed that iron-binding proteins may incorporate iron in the form of ferrous ion. Iron generally, however, exists in its ferric form under normal aerobic conditions. Thus, the reduction of ferric iron to ferrous iron is an essential reaction for the utilization of iron *in vivo*.

There are two types of ferric reductase reactions: a reaction using free flavin (non-protein-bound flavin), and a reaction independent of free flavin (Fontecave et al., 1994). Free flavin-dependent ferric reductases have been reported in many organisms (Coves and Fontecave, 1993; Coves et

*Corresponding author: Youichi Niimura, Department of Bioscience, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan.

Tel: 81-3-5477-2761 Fax: 81-3-5477-2764 E-mail: niimura@nodai.ac.jp

[†]These authors contributed equally to this paper.

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al., 1993a; Eschenbrenner et al., 1994; Halle and Meyer, 1992a, b; Huyer and Page, 1989; Kimata et al., 2018; Le Faou and Morse, 1991; Mazoch et al., 2004; Moody and Dailey, 1985; Sato et al., 2010; Vadas et al., 1999). Also, free flavin-independent ferric reductase activity has been found in *E. coli* (Takeda et al., 2010) and *Synechocystis* sp. PCC6803 (*Synechocystis*) (Sato et al., 2011). We purified ferredoxin NADP⁺ oxidoreductase (Fpr) and oxygen-insensitive NAD(P)H nitroreductase (NfnB) from *E. coli* (Takeda et al., 2010) and Fpr from *Synechocystis* as free flavin-independent ferric reductases (Sato et al., 2011). The purified *E. coli* Fpr and NfnB showed free flavin-independent ferric reductase activity for ferric iron compounds bound to synthetic chelators, such as Fe(III)-EDTA. However, their activities for natural ferric iron transporter protein (transferrin) or ferric iron storage protein (ferritin) were much lower than those for the ferric iron compounds bound to synthetic chelators (Takeda et al., 2010).

The reduction of ferric iron in iron storage proteins leads to the release of ferrous iron (Niederer, 1970). Some biological low molecular weight reductants such as thiols, ascorbate, and reduced free flavins are known to be involved in the release of ferrous iron from iron storage proteins (Melman et al., 2013; Sirivech et al., 1974). Especially, reduced free flavins are effective at releasing ferrous iron from the iron storage proteins and can be enzymatically generated from its oxidized form using NAD(P)H as a reducing reagent (Sirivech et al., 1974). In our previous study, it was found that Fpr and NfnB purified from *E. coli* as free flavin-independent ferric reductases have flavin reductase activity (Takeda et al., 2010). Thus, these findings suggested the possibility that Fpr and NfnB accelerate the release of ferrous iron from iron storage protein in the presence of free flavin. Conversely, *Pseudomonas aeruginosa* Fpr is capable of releasing ferrous iron from iron storage proteins without free flavin (Weeratunga et al., 2009; Yao et al., 2011).

In this study, we investigated the release of ferrous iron from *E. coli* ferritin A and bacterioferritin by *E. coli* Fpr and NfnB in the presence or absence of free flavin. Furthermore, the same release reaction by *E. coli* enzymes, other than Fpr and NfnB, with flavin reductase activity was examined.

Materials and Methods

Materials. In general, chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan). Bathophenanthroline-disulfonic acid, disodium salt was purchased from Dojindo (Kumamoto, Japan).

Cell culture. *Escherichia coli* strain JM109 (*E. coli* JM109) was grown at 37°C in LB medium with vigorous aeration. *E. coli* strain BL21 DE3 and *E. coli* JM109 harboring expression plasmids were aerobically grown at 37°C in LB medium containing 50 µg/mL ampicillin.

Purification of recombinant NfnB and Fpr. Recombinant NfnB (rNfnB) and recombinant Fpr (rFpr) were overexpressed and purified from *E. coli* JM109, as described previously (Takeda et al., 2010).

Cloning, expression, and purification of recombinant FtnA, Bfr, and AhpF. We cloned the genes encoding ferritin A (*ftnA*), bacterioferritin (*bfr*), and alkyl hydroperoxide reductase subunit F (*ahpF*) from *E. coli* JM109. *E. coli* DNA fragments containing open reading frames were amplified by polymerase chain reaction (PCR) using the forward primers 5'-caccatgctgaaaccagaaatgat-3' (*ftnA*), 5'-caccatgaaaggtgatactaaagt-3' (*bfr*), and 5'-caccatgctcgacacaaatatgaaaac-3' (*ahpF*) and the reverse primers 5'-ttagttttgtgtgtcgcagg-3' (*ftnA*), 5'-tcaaccttcttcgcggatct-3' (*bfr*), and 5'-ttatgcagttttgtgcgaatc-3' (*ahpF*). The forward primer contained the sequence "cacc" at the 5' end (underlined). These four bases are necessary for the insertion of intended DNA fragments into the expression plasmid vector pET101D-TOPO.

We used Champion pET101 Directional TOPO expression kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) to generate *E. coli* BL 21 DE3 that overexpresses rFtnA, rBfr, or rAhpF. The PCR products containing each open reading frame were subcloned into the pET101D-TOPO vector, according to the manufacturer's instructions. The overexpression of the recombinant protein in *E. coli* BL 21 DE3 was induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

All purification steps for recombinant proteins were performed at 4°C. The cell pellets for rFtnA (34 g wet cell) and rBfr (35 g wet weight) were suspended in 3-times their volume of 50 mM Tris-HCl buffer (pH 7.5). The cell pellet for rAhpF (34 g wet weight) was suspended in 3-times its volume of 50 mM sodium phosphate buffer (pH 7.0). The cell suspension was treated with DNase at one-thousandth the weight of the cell pellets in gram, for 30 min at 4°C. Each cell suspension was passed through a French Pressure cell (FA078A from SLM-AMINCO; Thermo Fisher Scientific, Inc., Waltham, MA, USA) twice at 24,000 lb/in². Phenylmethylsulfonyl fluoride (final concentration, 2 mM) was added to the suspensions before and after passage through the French Pressure cell. The suspensions were centrifuged at 64,000 × g for 20 min to remove unbroken cells. After centrifugation, the supernatants were treated with streptomycin (final concentration, 3%) to remove nucleic acids, stirred for 30 min on ice, and ultracentrifuged at 186,000 × g for 2 h. After centrifugation, ammonium sulfate was added to the supernatants of rFtnA (154 mL), rBfr (118 mL), and rAhpF (170 mL) to give final concentrations of 1.51 M, 2.65 M, and 1.51 M, respectively. The pH of the cell-free extracts was adjusted to 7.0 using 2.8% ammonium solution, and the solutions were stirred for 30 min. After centrifugation at 64,000 × g for 30 min, the supernatants of rFtnA (176 mL), rBfr (126 mL), and rAhpF (184 mL) were applied to a Butyl TOYOPEARL 650S (Tosoh, Tokyo, Japan) column (3.2 cmø × 38.0 cm) pre-equilibrated with 50 mM Tris-HCl (pH 7.5) for rFtnA and rBfr or 50 mM sodium phosphate (pH 7.0) for rAhpF, containing the same concentration of ammonium sulfate as each supernatant (1.51 M for rFtnA, 2.65 M for rBfr, and 1.51 M for rAhpF) at a flow rate of 1.0 mL/min. The column was washed with 5 column volumes of the same buffer. The bound proteins were eluted using a linear gradient with 5.9 column volumes of ammonium sulfate from 2.65 to 0 M for rBfr and

from 1.51 to 0 M for rFtnA and rAhpF, followed by 2 column volumes of 0 M ammonium sulfate at a flow rate of 5.0 mL/min. The fractions of rFtnA (410 mL), rBfr (375 mL), and rAhpF (270 mL) were dialyzed 3 times against 10 L of 50 mM sodium phosphate buffer (pH 7.0). After dialysis, the dialysate was centrifuged at $64,000 \times g$ for 20 min. The supernatant of rFtnA (510 mL), rBfr (475 mL), and rAhpF (360 mL) were applied to a DEAE Sepharose Fast Flow column (GE Healthcare, Little Chalfont, UK; $2.4 \text{ cm} \phi \times 56.0 \text{ cm}$) pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.5) for rFtnA and rBfr, and with 50 mM sodium phosphate buffer (pH 7.0) for rAhpF at a flow rate of 2.0 mL/min. After washing the column with 5 column volumes of the same buffer, the enzymes were eluted with 5.9 column volumes of linear gradient of NaCl concentration from 0 to 300 mM, followed by 2 column volumes of 300 mM NaCl, at a flow rate of 4.0 mL/min. The fractions of rFtnA (500 mL) and rAhpF (90 mL) were pooled. For rBfr, the fraction (320 mL) was dialyzed 3 times against 10 L of 50 mM sodium phosphate buffer (pH 7.0) for further purification processes. The dialysate was centrifuged at $64,000 \times g$ for 20 min, and then a portion of the supernatant (175 mL) was applied to a hydroxyapatite column (Bio-Rad Laboratories, Inc., Hercules, USA; $1.1 \text{ cm} \phi \times 21 \text{ cm}$) pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.5) at a flow rate of 1.0 mL/min. After washing the column with 5 column volumes of the same buffer, the proteins were eluted with a linear gradient of 0 to 800 mM potassium phosphate.

The active fractions of rFtnA, rBfr (200 mL), and rAhpF were concentrated to 20 mL using ultrafiltration (ADVANTEC® cut-off size 10 kDa; Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and an Apollo membrane (cut-off size 9 kDa; Orbital Bioscience, Topsfield, MA, USA). Subsequently, the concentration of NaCl in this protein solution was reduced to less than 1 mM by adding 50 mM Tris-HCl buffer (pH 7.5) for rFtnA and rBfr, and 50 mM sodium phosphate buffer (pH 7.0) for rAhpF. The resulting protein solutions were centrifuged at $17,400 \times g$ for 10 min and used as the purified proteins. The purity of the proteins was determined using SDS-PAGE (Supplementary Fig. S1(A)) (Laemmli, 1970).

Purification of recombinant Fre and QOR. The proteins rFre and rQOR were expressed as $6 \times \text{His}$ -tagged enzymes from the ASKA clones JW3820 and JW4169 (NBRP, *E. coli* Strain National BioResource Project), and purified using a TALON metal affinity resin (Takara Bio Inc., Tokyo, Japan), as described elsewhere (Kitagawa et al., 2005). The protein amount in rFre and rQOR solutions was determined by Bradford assay. The molar concentrations of these enzyme solutions were calculated using the protein amount and the molecular mass (rFre, 28,971 g/mol; rQOR, 32,463 g/mol) that was estimated based on the value of the intact proteins from the UniProt database (Fre, entry ID: P0AEN1; QOR, entry ID: P39315) (<https://www.uniprot.org/>).

Loading rFtnA and rBfr with iron. To use rFtnA and rBfr as substrates, ferric iron was introduced into these proteins as follows. A $0.75 \mu\text{M}$ iron storage protein (FtnA, $\epsilon_{280} = 26.7 \text{ mM}^{-1} \text{ cm}^{-1}$; Bfr, $\epsilon_{280} = 27.6 \text{ mM}^{-1} \text{ cm}^{-1}$) was

mixed with $90 \mu\text{M}$ ferrous ammonium sulfate in 50 mM HEPES-NaOH (pH 7.0) at 25°C for 1 h under aerobic conditions. Holo-iron storage proteins were then applied to desalting column (Zeba spin; Thermo Fisher Scientific Inc.) to remove extra irons located outside of the proteins. The iron content of collected iron storage proteins was determined using bathophenanthroline ($\epsilon_{535} = 22,400 \text{ M}^{-1} \text{ cm}^{-1}$) as a ferrous iron chelator. About 97% and 83% of the mixed ferrous iron was incorporated in the ferric form to rFtnA and rBfr, respectively (Supplementary Fig. S2).

Release of iron from rFtnA and rBfr in the presence or absence of free flavins.

The release of ferrous iron was detected using ferrozine ($\epsilon_{562} = 27,900 \text{ M}^{-1} \text{ cm}^{-1}$) as a ferrous iron chelator (Stookey, 1970). Solutions mixed with holo-iron storage proteins and free flavins (oxidized forms, solution A) were aerated with argon gases for 30 min. Free flavins were omitted from solution A when a flavin-free solution was prepared. Solutions mixed with NADH or NADPH and ferrozine (solution B) were also bubbled with argon gas for 30 min. Then, 0.5 mL of solution B was mixed with 1 mL of solution A using gas-tight syringes, to begin the reaction. After 500 s, the enzyme solutions were added. The reaction was monitored for more than 1 h. The final rFtnA and rBfr concentrations were $0.5 \mu\text{M}$ in the reaction mixture. The final concentration of ferric iron stored in rFtnA and rBfr was $58.1 \pm 2.1 \mu\text{M}$ and $49.7 \pm 3.1 \mu\text{M}$, respectively. The rFtnA, rBfr, and rAhpF molar concentrations were calculated using the molar extinction coefficients of the proteins, while the rFre molar concentrations were calculated using the amounts and molecular mass of the proteins (26,242 g/mol). The rFtnA and rBfr extinction coefficients were determined to be $\epsilon_{280} = 26.7 \text{ mM}^{-1} \text{ cm}^{-1}$ and $\epsilon_{280} = 27.6 \text{ mM}^{-1} \text{ cm}^{-1}$, respectively, by colorimetric quantification of amino acids labeled with ninhydrin reagent after protein hydrolysis. The labeled amino acids were detected by absorbance at 440 nm (proline) or 570 nm (the others) by using the amino acid analyzer L-8900 (Hitachi, Tokyo, Japan). This amino acid analysis was performed by Toray Research Center, Inc. (Tokyo, Japan). The molar extinction coefficient of rAhpF was determined to be $\epsilon_{450} = 12.9 \text{ mM}^{-1} \text{ cm}^{-1}$ by spectral analysis of free FAD, which was released from rAhpF protein following addition of 0.1% SDS (Ohnishi et al., 1995). Ferrous iron release was monitored by an absorbance at 562 nm using a spectrophotometer (U-3310 from Hitachi, Tokyo, Japan).

Enzyme assays. Assays for flavin reductase activity in purified rFpr, rNfnB, rQOR, rFre, and rAhpF were measured anaerobically at 25°C in 50 mM HEPES-NaOH (pH 7.0) containing $150 \mu\text{M}$ NAD(P)H and $100 \mu\text{M}$ flavins. Assays for flavin reductase activity in partially purified enzyme or cell-free extracts were performed aerobically at 25°C in 50 mM sodium phosphate (pH 7.0) containing $150 \mu\text{M}$ NAD(P)H and $150 \mu\text{M}$ free flavins. Assays for ferric reductase activity in purified rQOR, rFre and rAhpF were performed anaerobically at 25°C in 50 mM HEPES-NaOH (pH 7.0) containing $150 \mu\text{M}$ NAD(P)H and $100 \mu\text{M}$ Fe(III)-EDTA with or without $10 \mu\text{M}$ Rf for rQOR and rFre, or $10 \mu\text{M}$ FAD for rAhpF. In both enzyme activity assays, NAD(P)H consumption was monitored by the

absorbance at 340 nm (NADH, $\epsilon_{340} = 6,220 \text{ M}^{-1} \text{ cm}^{-1}$; NADPH, $\epsilon_{340} = 6,200 \text{ M}^{-1} \text{ cm}^{-1}$) using a spectrophotometer (U-3310 from Hitachi). We added rFpr, rNfnB, rQOR, rFre, and rAhpF at 1.3, 3.8, 45.6, 4.1, and 2.1 μg , respectively, to the 1.5 mL reaction mixtures. A unit (U) of enzyme activity was defined as the amount of enzyme that oxidized 1 μmol NAD(P)H to NAD(P)⁺ per minute. The turnover number of the purified enzymes was calculated from the unit of enzyme activity using their theoretical molecular masses, which were obtained from the UniProt database (Fpr (entry ID: P28861), 27,751 g/mol; NfnB (entry ID: P38489), 23,905 g/mol; AhpF (entry ID: P35340), 56177 g/mol; rFre and rQOR, described above).

Steady-state kinetics. Flavin reductase activity of rFpr, rNfnB, rFre and rAhpF was measured anaerobically at 25°C in 50 mM HEPES (pH 7.0) containing 200 μM NAD(P)H and various concentrations of FAD, FMN, or Rf (1.6–300 μM). The activity was calculated as the decrease in absorbance at 340 nm, which corresponds to the NAD(P)H oxidation. The values of K_m and k_{cat} for FAD, FMN and Rf were determined by nonlinear regression analysis of the initial reaction velocity versus substrate concentration with the Michaelis-Menten equation, using SIGMAPLOT version 11 (Systat Software, San Jose, CA, USA).

Purification of enzymes with flavin reductase activity from *E. coli* cell-free extract. All purification steps were carried out at 4°C. *E. coli* cell-free extracts were prepared from 75 g wet cells, as described previously (Takeda et al., 2010). The soluble fraction was obtained from the cell-free extracts after ultracentrifugation and streptomycin treatment as described above (the section of purification of FtnA, Bfr, and AhpF). Ammonium sulfate precipitation was conducted by adding solid ammonium sulfate to the supernatant, giving a final concentration of 1.51 M (200 g/L), and the solution was stirred for 20 min. The solution was centrifuged at $48,380 \times g$, and the supernatant was applied to a Butyl-TOYOPEARL 650S column equilibrated with 50 mM sodium phosphate buffer containing 1.51 M ammonium sulfate (pH 7.0). After washing the column with the same buffer, enzymes were eluted using 2,000 mL of ammonium sulfate gradient from 1.51 to 0 M. The six active fractions (fr. A, B, C, D, E, F) with flavin reductase activity were pooled.

The active fractions C, E, and F were applied to a DEAE sepharose Fast Flow column (GE Healthcare) in the second purification step. A large column (2.4 cm $\phi \times$ 56.0 cm) for fr. C and fr. F and a small column (2.0 cm $\phi \times$ 32.0 cm) for fr. E were used. The fractions C (150 mL), E (66 mL), and F (93 mL) were dialyzed 3 times against 10 L of 20 mM Tris-HCl buffer (pH 8.0), 50 mM Tris-HCl buffer (pH 8.5), and 50 mM HEPES buffer (pH 7.0), respectively. After centrifugation at $64,000 \times g$ for 20 min, the supernatants of fr. C (195 mL), fr. E (47 mL: this is a part of the sample—we accidentally lost some of it), and fr. F (115 mL) were applied to a DEAE Sepharose Fast Flow column equilibrated with 20 mM Tris-HCl (pH 8.0) for fr. C, 50 mM Tris-HCl (pH 8.5) for fr. E, and 50 mM HEPES (pH 7.0) for fr. F at a flow rate of 1.0 mL/min. After washing the column with the same buffer, the enzymes were

Fig. 1. Release of ferrous iron from rFtnA and rBfr by rFpr or rNfnB in the presence or absence of free flavins.

The release of ferrous iron from rFtnA and rBfr by rFpr or rNfnB in the presence or absence of 10 μM free flavins was measured under anaerobic conditions. The iron release from rFtnA by rFpr (A), from rBfr by rFpr (B), from rFtnA by rNfnB (C), and from rBfr by rNfnB (D) were observed. The reactions were performed in the presence of 400 μM ferrozine, 150 μM NADPH (A, B) or NADH (C, D), 0.03 μM enzymes, and 0.5 μM rFtnA or rBfr. The reaction was initiated by adding the enzyme, as indicated by the arrow. The reaction mixtures of rFtnA and of rBfr contained 60 and 50 μM ferric iron, respectively. The individual time course was plotted using the mean of three replicate experiments.

eluted with a linear gradient of 6.9 column volumes of NaCl concentration from 0 to 400 mM for fr. C, 11.6 column volumes of NaCl concentration from 0 to 400 mM for fr. E, and 6.3 column volumes of NaCl concentration from 0 to 300 mM for fr. F, at a flow rate of 4.0 mL/min. The active fraction of fr. C (200 mL), fr. E (78 mL), and fr. F (90 mL) were pooled and dialyzed 3 times against 10 L of 20 mM Tris-HCl (pH 8.0), 50 mM Tris-HCl (pH 8.5), and 50 mM HEPES (pH 7.0), respectively. After centrifugation at $64,000 \times g$ for 20 min, the dialysates of fr. C, fr. E, and fr. F were concentrated to 10 mL using an Apollo membrane (cut-off size 9 kDa, Orbital Bioscience). The concentrated fractions were centrifuged at $17,400 \times g$ for 10 min.

In the third step of purification, the supernatant of fr. C (3.8 mL) and fr. E (3.4 mL) were applied to a POROS HQ-10 column (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) pre-equilibrated with 20 mM Tris-HCl (pH 8.0) for fr. C and with 50 mM Tris-HCl buffer (pH 8.5) for fr. E, at a flow rate of 5.0 mL/min. In fr. C and fr. E, unbound proteins were eluted with 10 column volumes of the same buffer, and the enzymes were eluted with a linear gradient of 10 column volumes of NaCl concentration from 0 to 400 mM followed by 2 column volumes of 400 mM NaCl at a flow rate of 5.0 mL/min. The active fraction of fr. C was divided into two, and each fraction was pooled as fr. C1 (8 mL) and fr. C2 (6 mL). Fr. E (4 mL) was pooled. The supernatant of fr. F (2.7 mL) was applied to a gel filtration column Superdex 200 prep grade (GE Healthcare) pre-equilibrated with 50 mM HEPES (pH 7.0) containing 150 mM NaCl for fr. F, at a flow rate of 0.5 mL/min. Fr. F (90 mL) was concentrated to 10 mL using Apollo membrane (cut-off size 9 kDa, Orbital Bioscience). SDS-PAGE was performed using fr.

Table 1. Comparison of the observed rate constant of purified enzymes for ferrous iron release using rFtnA and rBfr in the presence or absence of free flavins.

	Observed-rate of iron release (min ⁻¹) ^a				
	Enzymes				
	rFpr	rNfnB	rQOR	rFre	rAhpF
FerritinA (FtnA)					
+ FAD	67±11	3±0	N.D. ^b	N.D.	81±25
+ FMN	97±6	4±1	1±0	7±3	7±2
+ Rf	69±8	8±1	4±2	31±14	42±2
– free flavin	N.D.	N.D.	N.D.	N.D.	N.D.
Bacterioferritin (Bfr)					
+ FAD	94±9	8±2	N.D.	N.D.	59±10
+ FMN	179±27	7±1	N.D.	28±10	6±2
+ Rf	95±10	7±1	N.D.	57±1	32±1
– free flavin	N.D.	N.D.	N.D.	N.D.	N.D.

^a The observed rate constants for ferrous iron release were calculated on the basis of the results in Figs. 1 and 3. Errors indicate standard deviation ($n = 3$).

^b N.D. = not detected.

Table 2. Kinetic parameters of *E. coli* rFpr, rNfnB, rFre and rAhpF for the reduction of different flavins by NADH or NADPH.

Enzymes	Substrates	K_m (μM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ s ⁻¹)
rFpr ^a	FAD	19.9±3.7	2.3±0.1	$1.2±0.3 \times 10^5$
	FMN	19.5±3.6	3.1±0.2	$1.6±0.5 \times 10^5$
	Rf	35.4±4.4	2.4±0.1	$0.7±0.2 \times 10^5$
rNfnB ^b	FAD	37.5±8.2	0.6±0.1	$1.7±0.5 \times 10^4$
	FMN	39.7±10.0	0.7±0.1	$1.8±0.5 \times 10^4$
	Rf	191.2±29.4	2.7±0.2	$1.4±0.7 \times 10^4$
rFre ^a	FAD	N.D. ^c	N.D.	N.D.
	FMN	8.3±0.5	2.3±0.1	$2.7±0.6 \times 10^5$
	Rf	6.2±0.6	7.7±0.2	$1.2±0.3 \times 10^6$
rAhpF ^b	FAD	63.0±5.1	14.8±0.5	$2.4±1.0 \times 10^5$
	FMN	106.9±30.6	2.2±0.3	$2.1±0.9 \times 10^4$
	Rf	300.0±48.0	9.3±0.9	$3.1±0.2 \times 10^4$

Experimental details are described in the Materials and Methods section. Oxidation of 200 μM NAD(P)H was measured anaerobically in the presence of various concentrations of free flavins from 1.6 to 300 μM.

Errors indicate standard deviation ($n = 3$).

^a Using 200 μM NADPH as an electron donor.

^b Using 200 μM NADH as an electron donor.

^c N.D. = not detected.

Table 3. Ferrous iron release activity of *E. coli* cell-free extracts in the presence or absence of free flavins.

	Ferrous iron release activity (μM/min/mg protein) ^a	
	NADH	NADPH
FerritinA (FtnA)		
+ FAD	7.2±0.2	0.4±0.2
+ FMN	2.7±0.3	3.3±0.2
+ Rf	5.1±0.6	5.4±0
– free flavin	N.D. ^b	N.D.
Bacterioferritin (Bfr)		
+ FAD	6.6±0.4	0.8±0.1
+ FMN	1.8±0.1	3.1±0.2
+ Rf	5.2±0.3	10.0±0.9
– free flavin	N.D.	N.D.

^a Ferrous iron release activity of *E. coli* cell-free extracts was calculated on the basis of the results in Supplementary Fig. S3. Errors indicate standard deviation ($n = 3$).

^b N.D. = not detected

Fig. 2. Separation of enzymes having flavin reductase activity using chromatography on Butyl TOYOPEARL 650S column.

The *E. coli* cell-free extracts were separated by a Butyl TOYOPEARL 650S column. The flavin reductase activity in the separated fractions was measured aerobically by the oxidation of NADH or NADPH in the presence of 150 μM free flavin. The activity is shown as the amount of enzyme that oxidized 1 μmol of NAD(P)H per 1 min (unit; U) per mL in each fraction. The flavin reductase activity was found in fractions A to F. The enzyme fractionation from the *E. coli* cell-free extracts was conducted in triplicate. Shown are the typical elution profiles of enzymes.

C1, fr. C2, fr. E, and concentrated fr. F, and the candidate enzymes were identified using LC-MS/MS (LC, Paradigm MS4 from Michrom BioResources Inc., Auburn, CA, USA; MS/MS, Thermo Finnigan LCQ Deca XP MAX from Thermo Fisher Scientific). Mass spectrometry data were used to identify proteins from the Swiss-Prot database.

Results and Discussion

Iron release from iron storage proteins in the presence of free flavin

In our previous study, the low level of ferric reductase activity of Fpr and NfnB on ferritin in horse spleen was

observed by measuring the oxidation of NADPH and NADH, respectively, in the absence of free flavins (Takeda et al., 2010). The ferric iron stored in iron storage proteins is reduced to ferrous iron, which is released from the proteins. In this study, to test the effective ferrous iron release from *E. coli* ferritin, the reaction by Fpr and NfnB was traced in the presence of free flavins. The direct ferrous iron release from recombinant ferritin A (rFtnA) or bacterioferritin (rBfr) (Figs. S1(A) and S2) was monitored using a colorimetric reagent, ferrozine, which chelates ferrous iron specifically.

The release of ferrous iron from rFtnA and rBfr by rFpr and rNfnB was detected in the presence of 10 μM free

Table 4. Flavin reductase activity of *E. coli* rFpr, rNfnB, rQOR, rFre and rAhpF.

	Flavin reductase activity (turnover number, min ⁻¹) ^a				
	rFpr ^b	rNfnB ^c	rQOR ^b	rFre ^b	rAhpF ^c
FAD	75±8	36±2	N.D. ^d	N.D.	2129±107
FMN	100±6	31±10	<1	611±26	292±11
Rf	64±8	48±2	<1	1559±29	736±34

Experimental details of the enzyme activity are described in the Materials and Methods section. The activity was measured by oxidation of NADH or NADPH in the presence of 100 μ M free flavin and 50 mM HEPES buffer (pH 7.0). Errors indicate standard deviation ($n = 3$).

^a Using 100 μ M flavin as a substrate.

^b Using 150 μ M NADPH as an electron donor.

^c Using 150 μ M NADH as an electron donor.

^d N.D. = not detected.

flavins, while no ferrous iron release was observed in their absence (Fig. 1). In rFpr, flavin mononucleotide (FMN) was more effective in releasing ferrous iron than flavin adenine dinucleotide (FAD) and riboflavin (Rf). By contrast, rNfnB seemed to prefer Rf among the three to release ferrous iron from rFtnA. The observed rate constant of rFpr in the presence of Rf was over 8-fold higher than that of rNfnB (Table 1). We previously reported that rFpr and rNfnB showed flavin reductase activity (Takeda et al., 2010). The present kinetics study demonstrated that the k_{cat}/K_m values of rFpr for FAD, FMN and Rf were over 5-fold higher than those of rNfnB (Table 2). These results suggested that the effect of Fpr or NfnB on ferrous iron release is associated with their individual reducing efficiency for free flavins.

Purification of enzymes with flavin reductase and ferrous iron release activities from cell-free extracts

The flavin reductase activity in *E. coli* cell-free extracts was measured using NADH and NADPH as the electron donor and FAD, FMN, and Rf as the acceptor (150 μ M). The extracts showed different specificity for flavins between NADH and NADPH. The observed rate of flavin reduction using NADH increased in the order of FMN < Rf < FAD, whereas that using NADPH increased in the order of FAD < FMN < Rf (Supplementary Table S1). The ferrous iron release activity from rFtnA and rBfr by *E. coli* cell-free extracts was also examined with various combinations of electron donors and flavin. The release of ferrous iron from rFtnA and rBfr was observed only in the presence of free flavins (Supplementary Figs. S3(A)–(D)). The observed rate of ferrous iron release for NADH increased in the order of FMN < Rf < FAD (Table 3). By contrast, for NADPH, the observed rate increased in the order of FAD < FMN < Rf (Table 3). These results indicate that *E. coli* cell-free extracts require free flavins for the effective ferrous iron release, similar to rFpr and rNfnB, suggesting that the enzymes having flavin reductase activity in *E. coli* cell-free extracts are involved in the release of ferrous iron from FtnA and Bfr.

Flavin reductase (Fre) and NADPH-sulfite reductase (SiR) were purified from *E. coli* cell-free extracts as enzymes with flavin reductase activity (Coves et al., 1993b; Fontecave et al., 1987). In these studies, the purification of Fre and SiR was conducted in the presence of NADPH

Fig. 3. Release of iron from rFtnA and rBfr by rQOR, rAhpF, or rFre in the absence or presence of free flavins.

The release of ferrous iron from rFtnA and rBfr by rQOR, rAhpF or rFre in the presence or absence of 10 μ M free flavins was measured, in a combination of the iron storage proteins and the enzyme under anaerobic conditions. The iron release from rFtnA by rQOR (A), from rBfr by rQOR (B), from rFtnA by rAhpF (C), from rBfr by rAhpF (D), from rFtnA by rFre (E), and from rBfr by rFre (F) were investigated. The reactions were performed in the presence of 400 μ M ferrozine, 150 μ M NADH (C, D) or NADPH (A, B, E, F), 0.03 μ M enzymes and 0.5 μ M rFtnA or rBfr. The reaction was initiated by adding the enzyme, as indicated by the arrow. The reaction mixtures of rFtnA and of rBfr contained 60 and 50 μ M ferric iron, respectively. Data are presented as the mean of three replicate experiments.

Table 5. Comparison between flavin reductase activity and ferrous iron release activity of *E. coli* rFpr, rNfnB, rFre and rAhpF.

	rFpr	rNfnB	rFre	rAhpF
Flavin reductase activity (k_{cat}/K_m , M ⁻¹ s ⁻¹) ^a				
FAD	$1.2 \pm 0.3 \times 10^5$	$1.7 \pm 0.5 \times 10^4$	N.D. ^c	$2.4 \pm 1.0 \times 10^5$
FMN	$1.6 \pm 0.5 \times 10^5$	$1.8 \pm 0.5 \times 10^4$	$2.7 \pm 0.6 \times 10^5$	$2.1 \pm 0.9 \times 10^4$
Rf	$0.7 \pm 0.2 \times 10^5$	$1.4 \pm 0.7 \times 10^4$	$1.2 \pm 0.3 \times 10^6$	$3.1 \pm 0.2 \times 10^4$
Ferrous iron release activity (observed-rate of iron release, min ⁻¹) ^b				
FAD	94±9	8±2	N.D.	59±10
FMN	179±27	7±1	28±10	6±2
Rf	95±10	7±1	57±1	32±1

^a Data in Table 2 is cited.

^b Data of ferrous iron release from Bfr in Table 1 is cited.

^c N.D. = not detect.

and Rf. The present study showed that *E. coli* cell-free extracts have flavin reductase activity for FAD and FMN in the presence of NADH or NADPH (Table S1), suggesting that there are other enzymes with flavin reductase activity along with Fre and SiR in *E. coli*.

To identify the enzymes with flavin reductase activity

in *E. coli*, the fractionation of the cell-free extract was conducted by Butyl-TOYOPEARL 650S column chromatography. The flavin reductase activity was measured using NADH and NADPH as electron donors, and FAD, FMN, and Rf as acceptors (150 μ M). As a result, six active fractions (fr. A–F) were obtained (Fig. 2) and all of them showed flavin reductase activity (Table S1). These fractions also showed ferrous iron release activity from rFtnA and rBfr in the presence of free flavins (Supplementary Fig. S4). However, the same activity was not observed in the absence of free flavins (data not shown). As shown in Tables 1 and 2, rFpr and rNfnB showed flavin reductase and ferrous iron release activities. The western blot analysis using anti-Fpr and anti-NfnB antibodies against the six fractions revealed that fr. B and D contain Fpr and NfnB, respectively (data not shown). Compared with fr. D containing NfnB, other active fractions, fr. C, E, and F, showed higher activity and yield of the flavin reductase activity (Fig. 2; Table S1). Further purification of the enzymes with flavin reductase activity in fr. C, E, and F was conducted using additional column chromatography. As a result, modulator of drug activity B (MdaB, accession no. AAC76064) and N-ethylmaleimide reductase (NemA, accession no. AAC74722), alkyl hydroperoxide reductase subunit F (AhpF, accession no. AAC73707), and Fre and NAD(P)H-quinone oxidoreductase (QOR, accession no. AAC77168) were identified in fr. C, E, and F, respectively, by LC-MS/MS analysis.

Release of ferrous iron from iron storage proteins by rQOR, rFre, and rAhpF

To investigate the flavin reductase activity and the ferrous iron release activity of QOR, Fre and AhpF found in the major active fraction, we prepared the recombinant enzymes rQOR, rFre, and rAhpF (Fig. S1(B)). Similar to rFpr and rNfnB, rQOR, rFre, and rAhpF showed flavin reductase activity (Table 4), free flavin-dependent ferric reductase activity (Supplementary Table S2), and ferrous iron release activity (Fig. 3). However, the flavin reductase activity of rQOR was 500-fold lower than those of rFre and rAhpF (Table 4). In addition, the ferrous iron release activity of rQOR from rFtnA and rBfr was 8-fold lower than those of rFre and rAhpF (Table 1). Accordingly, to understand the relationship between flavin reductase activity and ferrous iron release activity, kinetic parameters for free flavins and the observed rate constant for ferrous iron release from rFtnA and rBfr of rFre and rAhpF were compared. The k_{cat}/K_m value of rFre for Rf was higher than that for FMN and FAD (Table 2). Among three tested free flavins, Rf was most effective in the ferrous iron release from rFtnA and rBfr by rFre (Table 1). By contrast, rAhpF used FAD as a better reductant to release ferrous iron than Rf and FMN (Table 1). In the presence of FAD, FMN or Rf, the order of the flavin reductase activity of rFre and rAhpF corresponded to their ferrous iron release activity. Thus, the flavin reductase activity of rFre and rAhpF showed a positive correlation with their respective ferrous iron release activity similar to rFpr and rNfnB.

As shown in Table 5, among four enzymes tested in this study, rFpr showed the highest observed rate constants for

ferrous iron release from rFtnA and rBfr in the presence of free flavins. Comparatively, Weeratunga et al. (2009) and Yao et al. (2011) reported that *Pseudomonas aeruginosa* Fpr (PaFpr) showed the release of ferrous iron from iron storage proteins in the absence of free flavin. The rate constant for ferrous iron release of Fpr in the presence of free flavin was approximately 3 orders of magnitude higher than that of PaFpr in the absence of free flavin.

Taken together, the present results suggest that free flavins are probably required for releasing the iron stored in ferritin in *E. coli*. Reduced flavins could serve as an efficient reductant for generating ferrous iron *in vitro*. Thus, it is important to determine the cellular free flavin content of *E. coli* to understand how the free flavins are involved in the release of ferrous iron from iron storage proteins. Recently, we established a quantitative analysis method for analyzing the composition and concentration of intracellular free flavins in *Amphibacillus xylanus* (Kimata et al., 2018). In the near future, the biological function of intracellular free flavins in the iron release mechanism and metabolism can be elucidated using this new method.

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Supplementary Materials

Supplementary figures and tables are available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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