Iron-Binding Activity of FutA1 Subunit of an ABC-type Iron Transporter in the Cyanobacterium Synechocystis sp. Strain PCC 6803

Hirokazu Katoh, Natsu Hagino and Teruo Ogawa
Bioscience Center, Nagoya University, Chikusa, Nagoya, 464-8601 Japan

The futA1 (slr1295) and futA2 (slr0513) genes encode periplasmic binding proteins of an ATP-binding cassette (ABC)-type iron transporter in Synechocystis sp. PCC 6803. FutA1 was expressed in Escherichia coli as a GST-tagged recombinant protein (rFutA1). Solution containing purified rFutA1 and ferric chloride showed an absorption spectrum with a peak at 453 nm. The absorbance at this wavelength rose linearly as the amount of iron bound to rFutA1 increased to reach a plateau when the molar ratio of iron to rFutA1 became unity. The association constant of rFutA1 increased to reach a plateau when the molar ratio of iron to rFutA1 became unity. The association constant of rFutA1 for iron in vitro was about 1x10⁹. These results demonstrate that the FutA1 binds the ferric ion with high affinity. The activity of iron uptake in the ΔfutA1 and ΔfutA2 mutants was 37 and 84%, respectively, of that in the wild-type and the activity was less than 5% in the ΔfutA1ΔfutA2 double mutant, suggesting their redundant role for binding iron. High concentrations of citrate inhibited ferric iron uptake. These results suggest that the natural iron source transported by the Fut system is not ferric citrate.

Key words: Cyanobacterium — FutA1 — Iron transporter — Periplasmic binding protein — Synechocystis sp. PCC 6803.

Abbreviations: ABC, ATP-binding cassette; GST, Glutathione S-transferase; rFutA1, recombinant FutA1; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PBS, phosphate-buffered saline.

Introduction

Low levels of iron accessible by microorganisms are recurring nutrient stress conditions in both marine and fresh water oxic ecosystems and limit the primary production of phytoplankton in vast regions of the world’s ocean (Straus 1994, Butler 1998). Iron deficiency causes a variety of physiological and morphological changes in cyanobacteria. These include a loss of light-harvesting phycobilisomes (Guikema and Sherman 1983), changes in the spectral characteristics of chlorophyll (Guikema and Sherman 1983, Pakrasi et al. 1985), decrease of thylakoids (Sherman and Sherman 1983), and replacement of iron-containing cofactors with non-iron cofactors such as ferredoxin with flavodoxin (Laudenbach et al. 1988).

In the cyanobacterium Synechocystis sp. strain PCC 6803, the acquisition of ferric iron occurs mainly by an ABC-type iron transporter composed of subunit proteins encoded by four fut genes, futA1 (slr1295), futA2 (slr0513), futB (slr0327), and futC (ssl878) (Katoh et al. 2000, Katoh et al. 2001). Based on the sequence homology, the Fut system is related to the Sfu, Hit and Fbp systems in Serratia (Angerer et al. 1990), Haemophilus (Sanders et al. 1994) and Neisseria (Chen et al. 1993, Nowalk et al. 1994), respectively. Mutants inactivated for futB or futC, which encode inner membrane-bound or membrane-associated subunits, respectively, showed poor growth in iron-free BG-11 medium and low activity of ferric iron transport. The double mutant lacking both futA1 and futA2, which encode periplasmic binding proteins, showed the same phenotype as ΔfutB and ΔfutC single mutants. However, single mutants, ΔfutA1 and ΔfutA2 did not show the phenotype similar to that of ΔfutB and ΔfutC mutants. Thus the Fut system has an unusual composition of having two kinds of periplasmic binding proteins, FutA1 and FutA2. The iron-binding properties of these proteins and the form of iron transported by the Fut system have not been clarified. In order to solve these problems, we attempted to express futA1 and futA2 in Escherichia coli, and obtained soluble recombinant FutA1 fused to GST (rFutA1). We report in this paper the iron-binding properties of rFutA1 and the form of iron transported by the Fut system.

Results

Expression and purification of a recombinant FutA1 (rFutA1) protein

The region of signal peptide (37 amino acids in the N-terminal) in the FutA1 polypeptide was predicted using the PSORT program (Nakai and Kanehisa 1991). Truncated FutA1 lacking the predicted signal peptide was expressed in E. coli as a 60-kDa protein fused to GST by addition of IPTG (Fig. 1, lane 1). The protein was collected in the soluble fractions (lane 2) and was purified by Glutathione Sepharose 4B resin (lane 3). Trials to obtain recombinant FutA2 protein in soluble fractions by the same method were unsuccessful. The rFutA2 protein was unsolubilized even in the presence of 1% Triton. Therefore, further analysis was abandoned.

Absorption spectra of deferreted and iron-saturated rFutA1 protein

The absorption spectrum of deferreted rFutA1 protein did not show any peak in the visible region between 340 and
Iron-binding protein of *Synechocystis*

Iron-binding properties of rFutA1

There was a linear relationship between the amount of iron added to the solution containing deferrated rFutA1 and the absorbance at 453 nm until the molar ratio of ferric chloride to deferrated rFutA1 became unity (Fig. 3). This indicates that absorbance at this wavelength is an accurate measure of the ferration state of this protein and that 1 molecule of rFutA1 is capable of incorporating 1 molecule of ferric ion. The iron chelator citrate effectively removed iron from ferrated rFutA1 (Fig. 4). The concentration of citrate required to remove 50%
Iron-binding protein of *Synechocystis* 825

of the iron from ferrated rFutA1 was 6.5 mM, which represents a 100-fold molar excess of citrate over rFutA1. The relative affinity of rFutA1 for iron estimated by using the association constant of citrate for iron (1×10^17, Crichton 1990) was about 1×10^19. Thus, ferric ion is the ligand that FutA1 protein binds to, supporting the role of this protein as a periplasmic binding subunit of the ABC-type ferric ion transporter in *Synechocystis* PCC 6803. There remains a possibility that additional GST region in rFutA1 affects the relative affinity of FutA1 protein for iron.

Fig. 5 shows protein sequence alignment of FutA1, FutA2 and HitA. Deduced amino acid sequences of FutA1 and FutA2 of *Synechocystis* and HitA of *H. influenzae* were aligned. Residues identical to those in HitA are shown in reverse type. Four residues (His 9, Glu 57, Tyr 195, and Tyr 196) involved in iron binding in HitA are represented by sharp symbols above the sequence (Bruns et al. 1997).

**Discussion**

Bacterial ABC transporter possesses a periplasmic binding protein that has high affinity to its specific ligand (Higgins et al. 1990, Tam and Saier 1993). The ABC-type iron transporter encoded by the *fit* genes possesses two periplasmic binding proteins, FutA1 and FutA2 (Katoh et al. 2001). We demonstrated in this study that FutA1 binds iron without organic iron chelator such as siderophore or citrate. This is the first finding of the presence of high affinity Hit/Fbp-type iron-acquisition system in photosynthetic microorganisms. A recombinant FutA1 (rFutA1) was successfully obtained as a soluble protein. Ferric ion bound to this protein gave a typical absorption spectrum with a peak at 453 nm, which was about 30 nm lower than the peak position of Fe^{3+} bound to HitA. Structural analysis of HitA suggested that four amino acid residues, His 9, Glu 57, Tyr 195, and Tyr 196 are involved in binding iron in the FutA proteins. The difference in absorption maxima between Fe^{3+}-HitA and Fe^{3+}-rFutA1 may be attributed to the substitution of...
Glu 57 in HitA to Val in FutA1 or to the presence of GST region in FutA1.

The natural iron source for the Fut system is unclear. In the case of Fbp system, free ferric iron is passed across the outer membrane through the transferrin receptor protein (TbpA), which shows similar characteristics to the siderophore receptors (Irwin et al. 1993, Anderson et al. 1994). However, Synechocystis PCC 6803 does not possess a homologue of tbpA. Furthermore, our previous results indicated that simultaneous inactivation of four genes homologous to siderophore receptor genes had no effect on the uptake of iron and there has been no report on the production of siderophore by Synechocystis PCC 6803. In E. coli, citrate strongly enhanced the uptake of iron because this organism possesses an inducible ferric dicitrate transporter encoded by fecA, B, C, D, and E (Frost and Rosenberg 1973, Staudenmaier et al. 1989). Ferric citrate passes across the outer membrane through the FecA protein, localized in the outer membrane. Synechocystis does not possess a homologue of fecA, although the homologues of fecB, C, D, and E have been identified in its genome (Kaneko et al. 1996). Inactivation of fecB or fecE did not decrease the activity of ferric iron uptake (Katoh et al. 2001). Furthermore, citrate competitively inhibited the binding of iron to rFutA1 in vitro and the uptake of ferric iron in vivo regardless of culture conditions of the cells (Fig. 4, 6). Taken together, Synechocystis is unable to take up iron in the form of Fe3+-citrate under the experimental conditions used in this study. The negative effects of citrate on iron uptake were also observed on enb mutants of Salmonella typhimurium which were blocked in the biosynthesis of enterobactin, an iron chelator that was secreted by the wild-type bacteria when they were grown on low iron media (Pollack et al. 1970). The inhibitory effect of citrate on iron uptake in Synechocystis suggests that ferric citrate transport system is not functioning, presumably due to the absence of fecA. Alternatively, fecBCDE genes might encode a transporter for another molecule.

The FutA2 protein plays an essential role in ferric iron acquisition by ΔfutA1 mutant. Citrate inhibited the uptake of ferric iron by this mutant, suggesting that the form of iron bound to FutA2 is not ferric citrate. The deduced protein sequence of FutA2 is very similar to FutA1 and three amino acid residues of HitA functioning to bind ferric ion are conserved in these FutA proteins. These results imply that the ligand for FutA2 is also ferric iron, although this assumption should be confirmed by spectral analyses of FutA2 protein.

Materials and Methods

Preparation of recombinant FutA1

A DNA fragment, carrying a truncated futA (slr1295 on "CyanoBase", http://www.kazusa.or.jp/cyano/) lacking 111 bases from the initiation codon, was amplified by PCR using primers containing EcoRI sites at their proximal ends. After digesting with EcoRI, the PCR product was cloned into the EcoRI site of the pGEX-2T expression vector (Amersham Pharmacia Biotech, U.K.). The resulting plasmid (pFutA1-gst) carried a chimeric gene which encodes a truncated FutA1 lacking the N-terminal 37 amino acids fused to glutathione S-transferase (GST). This plasmid was used to transform E. coli DH5α. Expression of the chimeric gene in the transformant was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). After a 2 h induction, E. coli carrying pFutA1-gst were harvested and re-suspended with ice-cold PBS. GST-FutA1 recombinant protein was isolated from the cells using GST purification Kit (Amersham Pharmacia Biotech, U.K.) according to the instruction manual. Briefly, cells were lysed using a sonic oscillator equipped with a conical horn of 10–5 mm diameter (Ohtake Co., Tokyo, Japan) at 20 W. Triton X-100 (20%) was added to the disrupted cells to a final concentration of 1%. After incubation for 30 min on ice with gentle agitation, the sample was centrifuged (12,000×g, 4°C, 10 min). The supernatant was transferred to a fresh tube, to which 1/50 volume of 50% slurry of Glutathione Sepharose 4B was added. The mixture was incubated at room temperature for 30 min with gentle agitation, then centrifuged and the supernatant was removed. After washing Glutathione Sepharose 4B pellet with PBS, the GST-FutA1 fusion protein was eluted from the matrix by incubating with Glutathione Elution Buffer at room temperature for 1 h.

Deferration/referration of rFutA1

Deferration and referration of rFutA1 were done according to the method used for Fbp in Neisseria (Nowalk et al. 1994). Solution containing purified rFutA1 (6 mg ml–1) was acidified by adding 0.1 volume of 0.1% acetic acid, and iron was chelated by adding 2,000-fold molar excess of sodium citrate (pH 8.0) over rFutA1. Excess citrate and iron-citrate were removed by using an Econo-10DG microbiorspin column (Bio-Rad) and fractions containing deferrated rFutA1 were collected in acid-washed tubes. Referration was accomplished by adding two-fold molar excess of FeCl3 to the solution containing 4.5 mg ml–1 (75 μM) apo-rFutA1 and 200 mM NaCl in 20 mM Tris-HCl (pH 8.0). Absorption spectra of solutions containing apo- and Fe3+-rFutA1 were measured using a UV/VIS spectrophotometer V-550 (Jasco, Tokyo, Japan).

Measurement of iron binding properties of rFutA1

FeCl3 (final concentrations between 0 and 150 μM) was added to a solution containing 4.2 mg ml–1 (70 μM) of deferrated rFutA1 and the mixture was incubated at room temperature for 15 min. Relative affinity of rFutA1 for iron in vitro, was estimated by the method employed for Fbp (Chen et al. 1993). Solution containing 3.9 mg ml–1 (65 μM) Fe3+-rFutA1 and 200 mM NaCl in 20 mM Tris-Cl buffer (pH 8.0) was mixed with sodium citrate (final concentrations between 0 and 130 mM) and was incubated at room temperature for 15 min. The amount of Fe3+-rFutA1 in the mixture was estimated from the absorbance at 453 nm.

Measurements of iron uptake

Uptake of iron by wild-type and mutant cells of Synechocystis sp. strain PCC 6803 was measured using the radioactive tracer 59FeCl3 (Amersham Pharmacia Biotech, U.K.) as described previously (Katoh et al. 2000, Katoh et al. 2001). Cells were pre-cultured in modified BG-11 medium (Stanier et al. 1971, Katoh et al. 2000) containing 1 μM FeCl3, with or without 100 μM sodium citrate. The reaction solution contained 1 μM 59FeCl3 and various concentrations of sodium citrate between 0 and 2 mM. Ferrozine (500 μM) was added to the reaction solution to inhibit ferrous iron uptake. Reaction was performed in the light at 700 μmol photons m–2 s–1 (400–700 nm). The light source was a 600 W halogen lamp (Cabinet Co., Tokyo). The gamma emission from 59Fe in the cells was measured by the Auto Well gamma system (model ARC-380; Aloka, Tokyo, Japan).
Protein analyses

Lysates of *E. coli* cells and proteins were suspended in the sample buffer used for SDS-PAGE and were heated at 95°C for 5 min. After gel electrophoresis in the buffer system of Laemmli (1970), polypeptides in the gel were stained with Coomassie Brilliant Blue. Concentration of rFutA1 was calculated from the absorbance at 280 nm. The extinction coefficient of rFutA1 was determined based on the number of tyrosine and tryptophan residues in the protein (Yin et al. 1988). Protein sequences were aligned using the DNASeqS program (Hitachi Software, Yokohama, Japan).

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References


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