A Periplasmic Iron-binding Protein Contributes toward Inward Copper Supply*

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Periplasmic substrate binding proteins are known for iron, zinc, manganese, nickel, and molybdenum but not copper. Synechocystis PCC 6803 requires copper for thylakoid-localized plastocyanin and cytochrome oxidase. Here we show that mutants deficient in a periplasmic substrate binding protein FutA2 have low cytochrome oxidase activity and produce cytochrome c6 when grown under copper conditions (150 nM) in which wild-type cells use plastocyanin rather than cytochrome c6. Anaerobic separation of extracts by two-dimensional native liquid chromatography followed by metal analysis and peptide mass-fingerprinting establish that accumulation of copper-plastocyanin is impaired, but iron-ferredoxin is unaffected in ΔfutA2 grown in 150 nM copper. However, recombinant FutA2 binds iron in preference to copper in vitro with an apparent Fe(III) affinity similar to that of its paralog FutA1, the principal substrate binding protein for iron import. FutA2 is also associated with iron and not copper in periplasm extracts, and this Fe(III)-protein complex is absent in ΔfutA2. There are differences in the soluble protein and small-molecule complexes of copper and iron, and the total amount of both elements increases in periplasm extracts of ΔfutA2 relative to wild type. Changes in periplasm protein and small-molecule complexes for other metals are also observed in ΔfutA2. It is proposed that FutA2 contributes to metal partitioning in the periplasm by sequestering Fe(III), which limits aberrant Fe(III) associations with vital binding sites for other metals, including copper.

Oxygenic phototrophs have exceptional requirements for metals such as iron, copper, and manganese within the photosynthetic machinery (1, 2). There is interest in understanding how metal ions partition to the correct cellular destinations in all organisms (3), including to the approximately one-third of proteins that need metals (4). The periplasm is a key location for metal partitioning. Bacterial ATP binding cassette (ABC)-type importers include substrate binding proteins which may be free within the periplasm or anchored to the outer face of the plasma membrane. It is anticipated that these proteins assist in loading metal ions onto the correct importers. There is similarity in the metal-binding sites of several substrate binding proteins that contribute toward the import of distinct metals, such as manganese versus zinc, and subtle differences in second coordination spheres may be crucial for metal selectivity (5). It has also been noted that the nature of interactions between substrate binding proteins and their respective metal transporters may make a key contribution to metal specificity (5).

In the cyanobacterium Synechocystis PCC 6803 substrate binding proteins are well defined for manganese and zinc, MntC and ZnuA, respectively (5–8). Two further substrate binding proteins, FutA1 and FutA2 (9, 10), are thought to act as ferric-binding proteins. Mutants in futA1 are 63% deficient in iron accumulation, and futA1.futA2 double mutants are 95% deficient. However, futA2 single mutants only show a 16% reduction in iron accumulation (9, 10). Cyanobacteria, unlike most other bacteria, have known cytoplasmic copper enzymes. Cyanobacterial thylakoids contain plastocyanin that transfer electrons from cytochrome b₅₆₅ to photosystem I. Cryoelectron micrographs (11, 12) and periplasm-targeted TorA-green fluorescent protein (13) have established that thylakoids are discrete compartments that are not contiguous with the periplasm. In the cyanobacteria-related chloroplasts of higher plants (14, 15) and some algae, electron flow through copper within plastocyanin is obligatory for the conversion of light into chemical energy, whereas some cyanobacteria and other algae can replace plastocyanin with heme-iron containing cytochrome c₅₅₃, when copper becomes limiting (16–21). Cyanobacterial thylakoids also contain a caa₃-type cytochrome oxidase (22), which requires three atoms of copper per molecule. Synechococcus PCC 7942 has two copper transporting P-type ATPases, one of which, PacS, is located at the thylakoid membrane (23), and the other, CtaA, imports copper at the plasma membrane (24). Copper is supplied to thylakoid enzymes in Synechocystis PCC 6803 via the actions of CtaA and PacS plus a copper metallochaperone that is called AtxA1 or ScAtxA1 (25–29). It is hypothesized that by being passed via ligand exchange across the cytosol, the specificity of metallochaperone-transporter interactions keeps copper away from binding sites for other metals (30). This model requires that copper partitions onto the correct transport and trafficking pathway at the plasma membrane.

The present study was initiated because a screen indicated that FutA2 may bind copper or might interact in a copper-dependent manner with a copper-protein. We, therefore, investigated the possibility that FutA2 contributes to copper supply for thylakoids and confirmed that ΔfutA2 mutants do have phenotypes similar to those that had previously established roles for CtaA, PacS, and AtxA1 (25, 26) in inward copper supply.

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Recombinant FutA1, expressed as a glutathione S-transferase fusion protein, is known to bind Fe(III) in a 1:1 metal-protein complex (9), but similar studies with FutA2 proved unsuccessful because the protein was not soluble. Refolded heterologously expressed FutA2 binds Fe(III) with similar affinity to FutA1. Furthermore, FutA2 was found to bind iron in preference to copper in vitro.

To resolve the apparent contradiction between the in vivo phenotypes of ΔfutA2 cells, which support a role in copper homeostasis, and the in vitro metal binding properties of recombinant FutA2, which appear not to, metal binding to FutA2 was investigated in vivo. A comparison was made between two-dimensional native liquid chromatography profiles of the major soluble iron and copper pools in periplasm extracts of wild-type cells and ΔfutA2. These analyses first demonstrated that FutA2 does bind iron rather than copper in vivo but also established that additional copper as well as iron and several other metals accumulate in the periplasm of ΔfutA2. Furthermore, the nature of the periplasm-copper complexes (proteins and small molecules) differs in these mutants compared with wild type. Analogous profiles of extracts prepared from entire cells under anaerobic conditions identify plastocyanin disulfonic acid (BCS) for 1 h. Gels were attached using a UV-visible spectrum was recorded (25 °C) on a spectrophotometer (AAS; Thermo Electron Corp.) using protein concentration was determined with an M Series atomic absorption spectrophotometer (PerkinElmer Life Sciences). The molecular weight of purified FutA2 was determined by MALDI-TOF mass spectrometry.

Iron was removed from FutA2 by incubating with a 3000-fold molar excess of sodium citrate in 25 mM Tris, pH 7.5, and 200 mM NaCl at room temperature for 24 h. The protein was exchanged using a centrifugal filtration device (Vivaspin, 10-kDa cutoff) into 5 mM Tris, pH 7.5, plus 2 mM citrate and then into 5 mM Tris, pH 7.5. This procedure removed >95% of the iron from FutA2.

**EXPERIMENTAL PROCEDURES**

Diagonal Two-dimensional PAGE—Synechocystis PCC 6803 was cultured under constant light conditions at 28 °C in liquid BG11 (31) or BG11-C medium (26). Native gels were made to 10% (w/v) acrylamide containing 7% (v/v) Rhinohide gel strength additive (Molecular Probes). First-dimension tube gels were prepared in 2.4 × 4.0 × 160-mm siliconized glass tubes (Bio-Rad) to ~100 mm, topped with 100 mm KCl, 1 mm dithiothreitol, 0.5 mm CuSO4, 1 mm PMSF, via freeze-grinding in liquid nitrogen. The crude lysate was centrifuged (20,000 × g, 20 min, 4 °C), and an aliquot (100 μl containing ~200 μg of total protein) of the supernatant was resolved by PAGE (tube gel) in Tris/glycine buffer, pH 8.3, then stored at −20 °C. After slow thawing, gels were extruded under water pressure and equilibrated with or without 10 mM bathocuproine disulfonic acid (BCS) for 24 h. The gels were attached using 1% (w/v) agarose to 200 × 160 × 0.75-mm second-dimension slab gels (Bio-Rad) containing 1 mM BCS where necessary. Proteins were resolved by electrophoresis in Tris/glycine buffer containing 250 μM BCS where necessary. Proteins were visualized using Coomassie G250 (Invitrogen) and scanned using a transmission scanner (GE Healthcare). Trypsin digests of purified gel bands were analyzed using a Voyager-DE (ABI) matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer. Peptide mass fingerprints were analyzed using the Mascot search tool (matrixscience.com).

Membrane Isolation, Cytochrome Oxidase Assays, and Analysis of Cytochrome c6—Membrane isolation and cytochrome oxidase assays were performed as described previously (25). For cytochrome c6 analysis, a 1-liter culture of each strain at equivalent cell density was harvested by centrifugation and washed in 100 mM Tris, pH 6.8, and cells were suspended in 100 mM Tris, pH 6.8, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF and lysed with glass beads. The lysate was centrifuged (20,000 × g, 20 min, 4 °C), and the supernatant was analyzed for protein content using Coomassie plus reagent (Pierce). SDS was added to 0.1% (w/v) to 100 μg of each protein sample then resolved on 18% (w/v) acrylamide SDS-PAGE. Staining for heme was performed as described elsewhere (32). Analyses of the effects of copper on growth were performed as described previously (25).

Production and Analysis of Refolded Recombinant FutA2—Escherichia coli BL21 (DE3) containing pET29aFutA2trunc (a pET29a derivative harboring the futA2 gene without its signal sequence) recovered from 4 liters of culture were disrupted by sonication. An insoluble fraction that contained FutA2 was re-suspended in 400 ml of 6 M urea in 50 mM Tris, pH 7.5, and incubated at 4 °C for 1 h and centrifuged, and the supernatant was diluted 17-fold with 50 mM Tris, pH 7.5, over 80 h at 4 °C with slow stirring. Any precipitate that formed was removed by centrifugation followed by a further rapid 3-fold dilution of the supernatant in 50 mM Tris, pH 7.5 (~120 mM urea). The extract was incubated at 4 °C overnight with DEAE-Sepharose (GE Healthcare) equilibrated in 50 mM Tris, pH 7.5, with stirring. Bound proteins were subsequently eluted with 50 mM Tris, pH 7.5, containing 500 mM NaCl and exchanged into 50 mM Tris, pH 7.5, by dialysis. FutA2 was subsequently purified on a HiTrap Q HP anion exchange column and gel filtration (Superdex75). To ensure FutA2 was fully loaded with iron, protein was incubated with 4 eq of FeCl3 overnight. Purified FutA2 (>95% as judged by a 12.5% w/v SDS-PAGE gel) gave an A280/A450 ratio of <1.15 and a yield of ~10 mg/liter of cell culture. Iron concentration was determined with an M Series atomic absorption spectrophotometer (AAS; Thermo Electron Corp.) using protein in 5 mM Tris, pH 7.5. To determine the molar extinction coefficient, a UV-visible spectrum was recorded (25 °C) on a A35 spectrophotometer (PerkinElmer Life Sciences). The molecular weight of purified FutA2 was determined by MALDI-TOF mass spectrometry.

³ The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; AAS, atomic absorption spectrometer; BCS, bathocuproine disulfonic acid; ICP-MS, inductively coupled plasma mass spectrometry; HPLC, high performance liquid chromatography.
Preparation of Isolates from Synechocystis PCC 6803 Containing FutA2—Periplasmic extracts were prepared essentially as described previously (33) from a 2-liter culture of wild-type Synechocystis PCC 6803 grown in BG11 medium. Cells were harvested (6000 × g, 20 min, 4 °C), washed in 50 mM Tris, pH 7.5, and resuspended in 0.5 M sorbitol, 50 mM Tris, pH 7.5. EDTA was added to 1 mM and incubated at room temperature with occasional mixing for 10 min. After centrifugation (30,000 × g, 20 min, 4 °C), the cells were suspended in ice-cold double-deionized H2O and centrifuged, and a clear supernatant fraction, representing the periplasmic extract, was recovered. PMSF and Tris, pH 7.8, were added to 1 and 20 mM, respectively, before loading onto a 1-ml HiTrap Q HP column at 1 ml min⁻¹ at 4 °C. The column was washed with 20 mM Tris, pH 7.8, 10 mM NaCl, eluted with 250 mM NaCl. The eluted fraction was resolved on a Sephadex G-50 size exclusion column (GE Healthcare) in 20 mM Tris, pH 7.8, 50 mM NaCl, and 1-ml fractions were collected every min. Fractions were analyzed by SDS-PAGE (Bio-Rad), and a ∼36-kDa band was excised and identified by MALDI-TOF-MS mass-fingerprinting as FutA2 (13 sequences matched, 60% sequence coverage). Fractions containing FutA2 were concentrated using a Centricon YM-3 ultrafiltration device (Amicon). Metal analysis was performed with furnace AAS with Zeeman correction.

Two-dimensional Native Liquid Chromatography of Periplasm Extracts—Wild-type and ∆futA2 cells (∼2 × 10¹¹) were used to prepare periplasmic extracts as outlined above, with minor modifications. The EDTA concentration was reduced to 10 μM followed by an additional sorbitol wash to remove residual traces of EDTA. PMSF and Tris, pH 8.8, were added to 1 and 20 mM, respectively, before loading onto a 1-ml HiTrap Q HP column at 0.5 ml min⁻¹ and 4 °C. The column was washed with 20 mM Tris, pH 8.8, and then eluted with increasing concentrations of NaCl. A single peak fraction (1 ml) was collected containing proteins eluted at each concentration of NaCl, and the column was washed (−5 ml) before application of the next buffer. An aliquot (200 μl) of each peak fraction was subjected to high pressure size exclusion chromatography on a TSK SW3000 column (Tosoh Biosciences) in 10 mM Tris, pH 7.5, 50 mM NaCl, at 0.5 ml min⁻¹, collecting fractions every 1 min. Fractions were analyzed for copper by furnace AAS in initial profiles, but subsequently fractions were analyzed for multiple metals with high sensitivity using inductively coupled plasma mass spectrometry (ICP-MS; Thermo Electron Corp., X series with autosampler).

Two-dimensional Native Liquid Chromatography of Anaerobic Whole Cell Extracts—Anaerobic extracts were prepared from ∼1 × 10¹¹ cells via freeze-grinding in liquid nitrogen. Ground cells were thawed in an anaerobic chamber, and all subsequent steps were conducted under a nitrogen atmosphere. PMSF and Tris, pH 8.8, were added to 1 and 50 mM, respectively, and the cellular lysate was clarified via successive centrifugations (6000 × g, 15 min, 4 °C). An aliquot containing 7 mg of protein of the soluble extract was loaded onto a 1-ml HiTrap Q HP column and eluted with increasing NaCl. An aliquot of each ion exchange fraction was further resolved by size exclusion chromatography as described previously. Ion exchange and high pressure size exclusion chromatography were performed as above but under nitrogen followed by ICP-MS metal analysis.

The proportion of cells still intact after freeze-grinding was tested for two extractions using a Coulter counter and found to be 40 and 37%. The proportion of extracts containing 7 mg of protein loaded onto ion exchange was 0.26 (±0.03), and the data in Fig. 9 has been corrected accordingly.

RESULTS

Identification of FutA2 in Chelate-PAGE and Copper-related Phenotypes of ∆futA2—In an attempt to screen for proteins with readily exchangeable copper sites in Synechocystis PCC 6803, cell extracts were resolved using diagonal, chelate, native two-dimensional polyacrylamide gel electrophoresis (two-dimensional PAGE) similar to that used by others to identify calcium-binding proteins (34). Separate aliquots of extracts were resolved by native PAGE with and without the copper chelator BCS added after the first dimension by soaking the tube gel in 10 mM BCS and including 1 mM BCS in the second dimension gel. The properties of a subset of proteins with readily exchangeable copper should become altered after exposure to the chelator, causing them to migrate differently in b versus a. ApcA (allophycocyanin) and PsbV (cytochrome c₅₅₃) were controls identified by mass-fingerprinting to test whether or not they were distinct from adjacent bands of interest.

FIGURE 1. Diagonal chelate gels of Synechocystis PCC 6803 proteins. a, a section of diagonal native PAGE using 100 μl (∼200 μg) of total protein extracted from cultures grown in BG-11 medium. Native proteins align along a diagonal due to use of the same separation criteria in both first and second dimensions. b, a section of diagonal native PAGE of the same extract used in a but with the copper chelator BCS added after the first dimension by soaking the tube gel in 10 mM BCS and including 1 mM BCS in the second dimension gel. The properties of a subset of proteins with readily exchangeable copper should become altered after exposure to the chelator, causing them to migrate differently in b versus a. ApcA (allophycocyanin) and PsbV (cytochrome c₅₅₃) were controls identified by mass-fingerprinting to test whether or not they were distinct from adjacent bands of interest.

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### Table 1

| Oxidase activity | nmol of cytochrome c mg⁻¹ protein min⁻¹ |
|------------------|--------------------------------------|
| Wild type        | 7.17 (±0.20)                         |
| ΔctaA            | 4.42 (±0.75)                         |
| ΔfutA2           | 2.40 (±0.80)                         |

sequence coverage) were also affected by BCS. slr0729p was expressed in *E. coli* and purified by a combination of anion exchange (HiTrap Q HP) and size exclusion chromatography (Superdex 75) but did not bind either monovalent or divalent copper (data not shown). It is possible that slr0729p interacts with a copper-protein, but this was not pursued.

It was considered plausible that FutA2 may have a role in copper uptake at the plasma membrane; because it is a deduced periplasmic substrate binding protein, because ΔfutA2 cultures have only slight impairment of iron uptake, unlike ΔfutA1 cultures (9, 10), and because related proteins that contribute toward the import of other metals are well characterized in this organism (5–7). A transient (from 48 to 72 h) decline in optical density at 540 nm was detected in ΔfutA2 cultures grown in BG11-C medium (data not shown), which has no added copper (9), rather than BG11, which contains 300 nm copper. This could arise due to impaired growth, altered morphology, or changes in pigment composition of ΔfutA2 upon depletion of endogenous copper.

Membranes isolated from ΔctaA and ΔpacS mutants have less cytochrome oxidase activity than wild type (26). The most severe single phenotype was observed in ΔctaA. Membranes isolated from ΔfutA2 mutants also have low cytochrome oxidase activity (Table 1). The phenotype of ΔfutA2 cells was at least as severe as ΔctaA (Table 1).

**Recombinant FutA2 Avidly Binds Ferric Ions in Preference to Copper**—Multiple constructs generated only insoluble protein, consistent with the work of others (9). These constructs included full-length, 30-residue truncation, 31-residue truncation, 34-residue truncation (in pET29a), and glutathione S-transferase fusion (in pGEX-6P-2). Expression was tested under different isopropyl 1-thio-β-D-galactopyranoside concentrations, at different temperatures, and in *E. coli* BL21 tuner cells (Novagen). An unsuccessful attempt was also made to export FutA2 to the *E. coli* periplasm using an *E. coli* TorA_TAT signal sequence fusion (in pT7.5, a gift from Tracy Palmer, JIC, Norwich, UK). Recombinant insoluble FutA2 was, therefore, refolded from urea and purified as a red-colored protein (molecular mass, 35,002 Da compared with a theoretical value of 34,998 Da). The UV-visible spectrum (Fig. 2a) exhibits a maximum at 450 nm with a molar absorption coefficient of 6000 M⁻¹ cm⁻¹, which is reminiscent of ferric-FutA1 (9) and ferric-binding proteins from pathogenic bacteria (35, 36). The properties of the 450-nm band identify it as arising from a ligand-to-metal charge transfer transition consistent with one or more Tyr ligands as for FutA1. Iron rather than copper was detected by AAS in aliquots of FutA2, as purified (without deliberate iron addition). Iron is a minor contaminant of urea (±0.0005%), but at 6 M urea this represents one plausible iron source. The affinity of FutA2 for Fe(III) was estimated, by analogy to other ferric binding proteins (34), via competition with citrate. A citrate concentration of 20.8 mM (620-fold molar excess relative to FutA2) removed 50% of the Fe(III) (Fig. 2, a and b), giving an apparent dissociation constant ~6-fold tighter than for Fe(III)-FutA1 using the same method (9).

The UV-visible spectrum of apo-FutA2 incubated with 10 eq of Cu(II) gave a feature at 660 nm (Fig. 2c) of similar intensity to Cu(II) and buffer alone (not shown). Incubation with ~1 eq of Fe(III) in the presence of 10 eq of Cu(II) restored the feature at 450 nm, confirming that FutA2 binds Fe(III) in preference to Cu(II) (Fig. 2c). Subsequent fractionation by size exclusion chromatography on Sephadex G-25 followed by metal analysis via ICP-MS similarly shows iron and only trace amounts of...
FutA2 Is Associated with Iron in Periplasm Extracts—The identification of FutA2 by chelate-PAGE (Fig. 1) coupled with copper-related phenotypes of ΔfutA2 cells (Table 1) support the notion that FutA2 may be a periplasmic copper-protein, whereas in vitro studies (Figs. 2 and 3) show preferential iron binding to recombinant FutA2. What metal associates with FutA2 in the Synechocystis PCC 6803 periplasm? Isolates containing FutA2 (identified by peptide mass-fingerprinting) recovered from periplasm extracts of Synechocystis PCC 6803 by a combination of ion exchange chromatography (HiTrap Q HP eluted with 250 mM NaCl), ultrafiltration (Centricon YM-10), and size exclusion chromatography (TSK SW3000) contained copper as detected by AAS. However, the copper:protein stoichiometry varied (1:4, 1:4, 1:10, 1:2, 1:0.3) in replicate isolates, and impurities were sometimes visible on SDS-PAGE when large amounts of isolate were resolved. Repeated comparisons of the hypothetical Cu(II)-FutA2 pool in wild-type versus ΔfutA2 gave variable results due to the sensitivity of copper detection by AAS at the relatively low metal concentrations in these periplasm extracts. Periplasm extracts from wild-type and ΔfutA2 were, therefore, resolved by two-dimensional native liquid chromatography (ion exchange and gel filtration), and fractions were analyzed by ICP-MS to simultaneously quantify multiple metals and achieve greater sensitivity (Fig. 4). Analogous profiles were obtained with three replicate extracts of each genotype. Fractions were further resolved by SDS-PAGE, and regions of the profile containing FutA2 in wild-type extracts are shown (Fig. 5). Arrows denote the bands containing FutA2 (20 sequences matched, 76% sequence coverage). Both copper and iron co-elute with FutA2 from the 300 mM NaCl fraction, but notably, copper is displaced by one fraction and, importantly, only iron co-elutes with FutA2 from the 200 mM NaCl fraction (Fig. 5). Copper appears to be bound to an unknown protein that partly co-purifies with FutA2, and this is confirmed by retention of the copper pool, but not the iron pool, in ΔfutA2 (Figs. 4 and 5). The variable copper-protein stoichiometries previously determined in FutA2 isolates is presumed to have resulted from varying degrees of separation of FutA2 and the unknown periplasmic copper-protein. FutA2 binds iron in the periplasm (Figs. 4 and 5).

Iron, Copper, and Other Metals Accumulate in Periplasm—The magnitude of periplasm iron pools other than Fe(III)-FutA2 show an increase in ΔfutA2 relative to wild type (Fig. 4). Additional low molecular weight copper pools also appear in ΔfutA2 (Fig. 4). The total amount of iron and copper in each of three independent pairs of profiles was consistently greater in the mutant with ~3-fold (4.1 ± 1.4 versus 1.4 ± 0.5 × 10^6) and ~4-fold (3.1 ± 0.1 versus 0.8 ± 0.1 × 10^6) mean increases, respectively. An increase in low molecular weight copper and iron complexes is evident in master profiles (supplemental Figs. S1 and S2).
Changes in the periplasmic manganese, cobalt, and zinc profiles are also evident (Fig. 6). The total amount of manganese and cobalt, but not zinc, in each of three independent pairs of profiles was consistently greater in the mutant (supplemental Fig. S2). It is notable that \( /H9004^{futA2} \) cells accumulate additional, unknown, high \( M_r \) protein(s) that co-migrate with iron (Fig. 4).

In addition, a brown-colored compound washed from the surface of \( /H9004^{futA2} \) cells in buffer containing sorbitol and 1 mM EDTA before the release of periplasmic contents by osmotic shock, and some pigmentation was also evident in the periplasm extracts. The UV-visible spectrum for this compound has maxima at 263, 327, 361, and 420 nm, and it was detected in three independent extracts from \( /H9004^{futA2} \) but not wild type (Fig. 7). Size exclusion chromatography on Sephadex G-25 indicates a molecular mass in the region of 1 kDa (data not shown).

Copper-dependent Switching to Plastocyanin Is Impaired in \( /H9004^{futA2} \)—Although FutA2 binds iron preferentially in vitro and in vivo, \( /H9004^{futA2} \) show a proportionally greater increase in copper than in iron, within periplasm protein and small-molecule complexes. The \( \Delta futA2 \) mutants were, therefore, tested for additional copper-related phenotypes to oppose or support the deduction that reduced cytochrome oxidase activity was a function of impaired inward copper supply. Copper-dependent switching from use of iron in heme-containing cytochrome \( c_6 \) to copper in plastocyanin for photosynthetic electron transport can be monitored by staining PAGE gels for soluble hemoproteins (32). No cytochrome \( c_6 \) staining was detected using proteins from wild-type \( \text{Synechocystis} \) grown in medium containing 300 nm copper, slight staining with 150 nm copper, and intense staining with no added copper (data not shown). Both \( \Delta ctaA \) and \( \Delta futA2 \) continued to accumulate high levels of cytochrome \( c_6 \) which appears as a doublet band (38), relative to wild type when grown in 150 nm copper (Fig. 8). The same amount of total protein was loaded onto each track as is evident from the similar intensity of the faint upper purple band of phycocyanin.

Major Soluble Iron and Copper Pools of \( /H9004^{futA2} \) and Wild Type—To directly investigate the consequence of loss of FutA2 on supply of iron and copper, total cell extracts from wild-type and \( \Delta futA2 \) cells grown in 150 nm copper were prepared under

![FIGURE 5. FutA2 is associated with iron rather than copper in periplasm extracts.](image)

![FIGURE 6. Manganese, cobalt, and zinc in protein and small-molecule complexes from the periplasm of \( \text{Synechocystis} \) and \( \Delta futA2 \).](image)
Synechocystis PCC 6803 cultures grown in 150 nM copper were resolved by type, in three independent extracts (chromatography). A single major protein co-migrating with the copper-only pool was achieved using two HPLC columns (Fig. 9). This decline in plastocyanin-associated copper (from 0.51 ± 0.22 to 0.09 ± 0.01 × 10^5 copper atoms/cell) and also in total copper in the major soluble pools (from 5.2 ± 1.8 to 2.3 ± 1.0 × 10^5 copper atoms/cell) was detected in profiles from three replicate extracts. Total copper recovered in periplasm protein and small-molecule complexes represents 1.5 and 13.6% of that detected in complexes extracted anaerobically from total cells in wild-type and ΔfutA2 profiles, respectively, when the scales in Fig. 9 were set to similar sensitivity to Fig. 4. It is noted that these values exclude metal associated with membrane proteins, and also the yields obtained by osmotic shock and by freeze-grinding are likely to be different.

A small apparent decline in iron in one of the two major soluble pools shown in Fig. 9 was not statistically significant in the three replicates, and the total iron detected in the profiles of ΔfutA2 versus wild type was also similar (supplemental Fig. S3). The iron pool eluted in 1 M NaCl was fractionated to higher resolution using two HPLC columns, and fractions were analyzed by SDS-PAGE (Fig. 10). A single prominent band contained ferredoxin, as determined by polypeptide mass-fingerprinting, and co-migrates with iron (41% sequence coverage by two amino-terminal fragments) (Fig. 10). The ferredoxin iron pool appears unaltered in ΔfutA2 (Fig. 9e), and this is confirmed in profiles from three replicate extracts (supplemental Fig. S3) with mean values of 1.4 ± 0.2 and 1.8 ± 0.3 × 10^5 iron atoms/cell associated with ferredoxin in wild type and ΔfutA2, respectively.

**DISCUSSION**

Multiple independent lines of evidence establish that FutA2 acts in the supply of copper to thylakoid copper-proteins. In contrast to wild type, mutants lacking FutA2 have low cytochrome oxidase activity (Table 1), accumulate cytochrome c6 in the presence of copper (Fig. 8), and lose copper accumulation in plastocyanin (Fig. 9). The total amount of copper in the major soluble protein and small-molecule complexes analyzed under anaerobic conditions is less in ΔfutA2 cells, whereas conversely additional copper accumulates in soluble periplasmic protein and small-molecule complexes (Fig. 4). All of these data imply some role for FutA2 in the import of copper from the periplasm.

A simple model in which FutA2 binds copper in the periplasm and donates these ions to a copper-specific ABC importer is not supported by metal binding studies. Recombinant FutA2 avidly binds ferric ions (Fig. 2) in preference to copper (Figs. 2 and 3) with an apparent affinity for iron comparable with that of the principal ferric-binding protein for iron import, FutA1 (9). Furthermore, FutA2 associates with iron rather than copper in extracts from the *Synechocystis* PCC 6803 periplasm (Figs. 4 and 5).

By generating profiles of the principal soluble periplasm protein and small-molecule complexes simultaneously for multiple metals (Figs. 4 and 6), it has been possible to observe more than simply the loss of iron-FutA2 in osmotic shock extracts from ΔfutA2 cells. Interaction between iron homeostasis and the
homeostasis of copper and other metals in the periplasm is evident (Figs. 4 and 6). Additional atoms of iron per cell are also detected in soluble periplasmic proteins and small-molecule complexes in the mutant. Periplasmic substrate binding proteins of ABC importers involved in metal acquisition may fulfill metallochaperone-like functions in this compartment. Loss of such a soluble ferric chaperone activity in \( \text{H9004}^{futA2} \) provides a plausible explanation for the observed phenotypes. In this model ferric ions out-compete other metals from their bona fide binding sites in the \( \text{H9004}^{futA2} \) periplasm, including those normally involved in the import of copper at the plasma membrane. Fe(III) binds very tightly to some proteins and small organic molecules (such as some siderophores and indeed FutA2), whereas Fe(II) is much less competitive. The Irving-Williams series, extended to include Cu(I) as well as Cu(II) (cited in Ref. 39), predicts that copper will tend to out-compete divalent metals, including iron in its ferrous form, from cytoplasmic protein metal binding sites. However, in the less reducing environment of the periplasm, ferric ions are expected to be especially competitive, readily forming associations with organic molecules. Metallochaperones are thought to restrain copper from erroneous protein associations in the cytoplasm, and it is proposed that FutA2 performs an analogous function for ferric ions in the periplasm. FutA2 is expected to donate iron to an ABC importer, and this makes a substantive contribution to iron import in \( \Delta \text{futA1} \) cells (10). Future studies will test for homeostasis of copper and other metals in the periplasm.

FIGURE 9. Mutants in futA2 do not accumulate copper in plastocyanin but do accumulate iron in ferredoxin. a, extracts (7.0 mg) of soluble proteins prepared from wild-type cells grown in 150 nM copper under anaerobic conditions were resolved by anion exchange chromatography followed by high pressure size-exclusion chromatography then assayed for iron (red), copper (blue), and manganese (green). Contour intervals are \( 2.5 \times 10^4 \) atoms of iron, \( 1 \times 10^4 \) atoms of copper, and \( 1 \times 10^4 \) atoms of manganese/cell. Note the reduced sensitivities of the contours used here relative to profiles of periplasm protein and small-molecule complexes in Fig. 4 in order to simplify these profiles. Similar profiles were obtained in three independent extracts, and supplemental Fig. S3 shows the reproducible features on a master profile. b, an aliquot of the ion exchange fraction eluted in 100 mM NaCl was further separated using two SW 3000 columns to give greater resolution, and fractions were analyzed for copper (blue) and manganese (green). c, fractions indicated with a bar in panel b were resolved by SDS-PAGE, proteins were visualized using Sypro Ruby, and the indicated bands were excised and identified as plastocyanin (Pc) via mass-fingerprinting. d, an analogous separation to panel b but using an extract from the \( \Delta \text{futA2} \) mutant. e, an analogous separation to panel a but using an extract from the \( \Delta \text{futA2} \) mutant. Similar profiles were obtained for each of three independent extracts (\( n = 3 \)), and supplemental Fig. S3 shows the reproducible features on a master profile.
the effects of FutA2 deletion on the inward supply of other metals in which periplasmic profiles were altered under metal-limiting conditions. The ability of iron chelators to restore copper supply in ΔfutA2 cells also warrants investigation.

Proteomic studies have shown that FutA2 is an abundant soluble protein within the periplasm of Synechocystis PCC 6803 (40). This is consistent with the intense FutA2 bands observed here (Fig. 5) and implies that FutA2 provides a large capacity for metal sequestration in this compartment. Other substrate binding proteins for ABC-type metal importers are associated with membranes via a variety of linkages. For example, in this organism ZnuA has a transmembrane helix versus plasma membranes (43, 44). Crucially, FutA1 has never been observed in soluble periplasmic extracts (40, 43). Conversely, as here, FutA2 was previously found in periplasm extracts released by cold osmotic shock (40), although it has also been detected in preparations of plasma membranes (41), thylakoid membranes (43, 44), and total soluble extracts (43, 45), the latter possibly including a subpopulation of folded FutA2 awaiting export via the TAT pathway. Cells deficient in FutA2 also accumulate a low $M_r$ pigmented compound at the cell surface (Fig. 7), and a role for this compound in metal chelation, perhaps acting as a sidophore that supplies FutA2 with iron, requires investigation. Its abundance in the periplasm of cells grown in iron-containing medium and lack of membrane association makes FutA2 suited to metal sequestration in this compartment by acting as a metallochaperone.

It is unclear why FutA2 was initially identified in chelate-PAGE (Fig. 1). Notably, the high abundance of FutA2 favors its detection. Copper was added to the extract in Fig. 1 and, therefore, may have only associated with a subpopulation of apo-FutA2 in vitro, although attempts to reconstitute recombinant Cu(II)-FutA2 do not strongly support this. BCS has a tight affinity for Cu(I) of $10^{-19}$ M (46) but a weak affinity for Cu(II), which may nonetheless be tighter than the Cu(II) affinity of FutA2. The affinity of BCS for Fe(III) is negligible (47), and although it is formally possible that at such high concentrations it removed iron from FutA2, this is unlikely. Finally, its detection in this assay could indicate that FutA2 interacts in a copper-dependent manner with a copper-protein. The abundant copper-protein complex that partly co-purifies with FutA2 (Fig. 5) would be one plausible candidate.

A more substantive contribution of FutA2 to copper supply, rather than iron supply in 150 nM copper medium, is evident from analyses of the major soluble metal pools in total cell extracts (Fig. 9). Intriguingly, these profiles reveal both of the soluble electron transport proteins that act on opposing sides of photosystem I, consistent with the notion that photosynthesis has a high demand for metals (1) in soluble as well as membrane-bound components. Plastocyanin donates electrons on the thylakoid luminal side of photosystem I, whereas ferredoxin accepts electrons on the cytoplasmic face in cyanobacteria. This ferredoxin contains a 2Fe–2S cluster, and its retention authenticates the anaerobic nature of these analyses. Moreover, when iron supply is limiting, Synechocystis PCC 6803 replaces ferredoxin with flavodoxin (37). Thus, there is dual reciprocal switching in this system, from plastocyanin in low copper and from ferredoxin in low iron. The profiles (Fig. 9), therefore, show that in 150 nM copper, ΔfutA2 switch from copper-plastocyanin but retain iron-ferredoxin, confirming a severe defect in copper supply rather than in iron supply.

Subtle variations in relative affinities for different metals rather than absolute affinities of periplasmic metal-binding proteins can mediate metal selectivity provided that the total numbers of ligands are in excess of the number of metal ions in this compartment. In conclusion, FutA2 is a periplasmic substrate binding protein that binds and sequesters iron but is required for normal inward supply of copper for copper enzymes. In the absence of FutA2, abnormal amounts of iron and copper accumulate in low $M_r$ proteins and other compounds in the periplasm. This suggests a model in which FutA2 sequesters Fe(III) to assist iron import and also to prevent these ions from competing for periplasmic binding sites for other metals, including the copper-binding sites of CtaA and/or other proteins that contribute toward plasma membrane copper-import.

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