A Copper Metallochaperone for Photosynthesis and Respiration Reveals Metal-specific Targets, Interaction with an Importer, and Alternative Sites for Copper Acquisition*

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A bacterial two-hybrid assay revealed interaction between a protein now designated bacterial Atx1 and amino-terminal domains of copper-transporting ATPases CtaA (cellular import) and PacS (thylakoid import) but not the related zinc (ZiaA) or cobalt (CoaT) transporters from the same organism (Synechocystis PCC 6803). The specificity of metallochaperone interactions coincides with metal specificity. After reconstitution in a N₂ atmosphere, bacterial Atx1 bound 1 mol of copper mol⁻¹, and apoPacSN acquired copper from bacterial-Atx1. Copper was displaced from Atx1 by p-(hydroxymercuri)phenylsulfonate, indicative of thiol ligands, and two cysteine residues were obligatory for two-hybrid interaction with PacSN. This organism contains compartments (thylakoids) where the copper proteins plastocyanin and cytochrome oxidase reside. In copper super-supplemented mutants, photooxidation of cytochrome oxidase reside. In copper super-supplemented mutants, photooxidation of cytochrome oxidase was greater in Δatx1ΔctaA than in ΔctaA, showing that Atx1 contributes to efficient switching from iron in cytochrome c₆ to copper in plastocyanin for photosynthetic electron transport. Cytochrome oxidase activity was also less in membranes purified from low [copper]-grown Δatx1 or ΔpacS, compared with wild-type, but the double mutant Δatx1ΔpacS was non-additive, consistent with Atx1 acting via PacS. Conversely, activity in Δatx1ΔctaA was less than in either respective single mutant, revealing that Atx1 can function without the major copper importer and consistent with a role in recycling endogenous copper.

There is essentially no free copper in the cytoplasm of Saccharomyces cerevisiae (1), and it is anticipated that this applies to other cells. It has become apparent that copper metallochaperones assist in the delivery of copper ions to target proteins (such as superoxide dismutase) or to target compartments, encouraging advantageous copper-protein partnerships while inhibiting others en route (reviewed in Refs. 2–4). In S. cerevisiae cells, copper is delivered to cytosolic superoxide dismutase via CCS (5–8), to mitochondria via COX17 (9, 10) and to the Golgi via ATX1 (11, 12). It is feasible that the lack of intracellular compartments in most prokaryotes (with the crucial exception of most cyanobacteria) circumvents a requirement for a network of copper metallochaperones (2), although Enterococcus hirae contains an ATX1-like protein, CopZ, which is implicated in efficient copper perception by the transcriptional regulator CopY (13). Recent studies have allowed the formulation of hypotheses about how copper is released from metallochaperones, but it is less clear how and/or where copper metallochaperones acquire copper.

Cyanobacteria are believed to occupy an evolutionary transition in the use of copper, the photosynthetic generation of dioxygen having liberated this element from inorganic sulfides and made it available for uptake and recruitment in emergent proteins (14). Photosynthetic electron transport occurs at internal thylakoid membranes in higher plant chloroplasts and in most cyanobacteria including Synechocystis PCC 6803. It is possible to monitor copper trafficking to this internal compartment (with substantial copper requirements) in vivo in some of these organisms (15). Within the thylakoid lumen is the soluble copper protein plastocyanin, which shuttles electrons between membranous photosystems (reviewed in Ref. 16). Despite the pivotal role of plastocyanin in the primary conversion of light to chemical energy within the biosphere, it has been largely neglected in studies of the intracellular trafficking of copper by metallochaperones. Thylakoid membranes of Synechocystis PCC 6803 are also a site of respiratory electron transport (17), and copper must be supplied to the CuA and the intramembranous CuB, sites of cytochrome oxidase.

The target for ATX1 in S. cerevisiae is CCC2 (12), a Golgi-localized variant P-type ATPase (18), often termed CPx-type (19) or P₁-type (20). Copper-transporting CPx-type ATPases have been described in bacteria, including cyanobacteria (21, 22), Synechocystis PCC 6803, CtaA and PacS, both of which are required for efficient switching to the use of copper in plastocyanin rather than heme iron in cytochrome c₆ for photosynthetic electron transport (15). Disruption of ctaA also reduced the total amount of copper cell⁻¹, whereas disruption of pacS conferred copper sensitivity. The presence of two CPx-type ATPases, one contributing to copper import as well as one contributing to copper compartmentalization, makes this an attractive model in which to study the action of any putative ATX1-like copper metallochaperone in relation to understanding the mechanisms of copper acquisition and release. This
organism contains two additional CPx-type ATPases, ZiaA and CoaT, which are known to be expressed in response to and required for growth in (elevated) zinc (27) and cobalt (29), respectively, highlighting questions about how structurally related transporters discern and select different metals from a common cytosol. Metallochaperones have not previously been documented in an organism containing CPx-type ATPases with differing metal specificities.

The cytosolic N-terminal region of CCC2 contains two domains that form a structure (βαβαβα ferredoxin-like fold) similar to the small (73 amino acids) soluble ATX1, with CCC2 and ATX1 possessing complementary acidic and basic surfaces (30). Both proteins contain the motif MXXCXX (X represents any amino acid) required for metal binding and implicated in the formation of bridged heterodimeric sites during copper transfer from ATX1 to CCC2. The thermodynamic gradient for copper transfer in vitro from ATX1 to an isolated amino-terminal domain of CCC2 is shallow (31), raising questions about whether copper transfer is vectoral in vivo and, if so, how? Open reading frame (ORF)1 sss2857 from the fully sequenced genome of Synechocystis PCC 6803 (32) encodes a 64-amino acid polypeptide with similarity to the amino-terminal region of PacS (22%), the amino-terminal region of CtaA (14%), and ATX1 (22%) and containing the motif CXXC but with no associated methionine residue (Fig. 1). Several features (described above) suggest that this could be a valuable organism for studying copper metallochaperones, and the initial aim of this research was therefore to establish whether or not the product of sss2857 interacts with amino-terminal regions of PacS and/or CtaA to shuttle copper to the thylakoid. Our data support this, and sss2857 is designated atx1 although the target compartment, the proteins supplied, and the metal-binding motif are distinct from eukaryotic ATX1.

We describe the production of mutants of Synechocystis PCC 6803 deficient in atx1, alone and in combination with pacS or ctaA. Their phenotypes (i) show that the action of atx1 is positive with respect to copper-dependent thylakoid redox processes, (ii) show that CtaA is not obligatory for Atx1 function, and (iii) are consistent with Atx1 acting solely in the same pathway as PacS. We report in vitro analyses of the copper binding properties of Synechocystis PCC 6803 Atx1 and its capacity to exchange copper with the amino-terminal region of PacS. We have exploited a bacterial two-hybrid system to show in vivo interaction between Synechocystis PCC 6803 Atx1 and the amino-terminal region of PacS and a requirement for the CXXC motif of Atx1 for such interaction. An in vivo interaction between Atx1 and the amino-terminal region of CtaA was also detected. Optional copper acquisition from the importer would imply reversal of the vector for transfer between Atx1 and the different cytosolic N-terminal regions of the two ATPases. Comparative modeling of these domains therefore provides insight into the mechanisms that can facilitate transfer. Finally, we report a lack of in vivo interaction between Atx1 and amino-terminal regions of ZiaA and CoaT. This is the first example of discriminatory target recognition by a metallochaperone coinciding with discriminatory specificity of transport by CPx-type ATPases.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, DNA Manipulations, and Southern Analyses—**

Synechocystis PCC 6803 was grown either in liquid BG-11 medium (which contained 0.3 μM copper), on medium C plates, or using GM-11-C that lacked copper as a micronutrient element or BG-11-FC that also lacked micronutrient iron but contained 0.2 μM copper (15). Cells were transformed to antibiotic resistance as described by Hagmann and Zuther (33). Escherichia coli strains JM101, SURE, BL21, and BacterioMatch™ (Strategene) were grown in Luria-Bertani medium (34). DNA manipulations were performed as described by Sambrook et al. (34). Genomic DNA was purified from Synechocystis PCC 6803 using a protocol previously described for plant cell cultures but excluding CaCl2 (35). Aliquots (10 μg) of DNA were used as restriction endonucleases, resolved by agarose gel electrohoresis, transferred to nylon filters, and washed (after probing) to a stringency of 0.5× SSC, 0.1% (w/v) SDS at 65 °C (34). The sequences of all PCR and QuikChange (Strategene) products were validated by sequence determination (ABI Prism 377 DNA sequence analyzer and Applied Biosystems 3800 Molecular Biology Work station for sample preparation and data analysis) using primers 5′-GTAATCGTAGACTTACGCAG-3′ and 5′-GCTACTATTCCGTCATAGG-3′

**Insertional Inactivation of atx1 to Generate Single and Double Mutants—** Synechocystis PCC 6803 genomic DNA was used as a template for PCR with primers 5′-GGAAGCTTCTTTACGCAG-3′ and 5′-GCTACTATTCCGTCATAGG-3′ to amplify a fragment, 2.2 kb, which included ORF sss2857 (atx1), which was ligated to pGEMT (Promega) to create pGEMT-SSR. Subsequently primers 5′-CAATACCTTGATCACAGCAGTGTGCGCCGCG-3′ and 5′-GGCCACAGGGTCAACAGGATCGGATATCGTGGGTACAGTTAGTTG-3′ were used to introduce an EcoRV site within atx1 (via QuikChange (Strategene) site-directed mutagenesis according to the manufacturer’s protocol), into which was ligated the chloramphenicol acetyl transferase gene to generate pIN-ATX1.

Synechocystis PCC 6803 was transformed to chloramphenicol resistance following incubation with pIN-ATX1 transformants selected on solid medium containing 7.5 μg ml−1 chloramphenicol prior to growth in liquid medium. Interruption of atx1 by insertion of the chloramphenicol acetyl transferase gene in all copies of the Synechocystis PCC 6803 chromosome was confirmed by Southern analysis using HinCII-digested DNA and probing with a 32P-labeled fragment of atx1 to identify a diagnostic 2.26-kb fragment (1.76 kb in the wild type). An identical procedure was used to inactivate atx1 in existing mutants (15) deficient in pacS or ctaA, except that the solid selective medium contained 25 μg ml−1 kanamycin in addition to chloramphenicol, whereas 50 μg ml−1 kanamycin was added to liquid cultures of these strains.

**Membrane Isolation and Assays of Cytochrome Oxidase Activities—** Logarithmically growing cultures were subcultured on alternate days (to ~ 1 × 10^6 cells ml⁻¹) for 7 days (to standardize growth rates). Total membranes were prepared, and cytochrome oxidase assays performed as described previously (15, 36) except that all assays were performed using freshly prepared membranes and freshly reduced cytochrome c. All comparisons relative to experiments performed on a single day with equivalent trends having been observed in separate experiments minimize errors of interpretation resulting from day-to-day variability in these assays that possibly arise from variation in the efficiency of reduction of the substrate and/or changes in temperature.

**Single Turnover Cytochrome Kinetics—** Measurements of cytochromes f plus c₈ were made by analyses of flash-induced (xenon flashlamp, 15-microfarad capacitor at 1000 V, 6-μs half-peak width, filtered with RG625 glass filters and delivered by two 10-mm diameter lightpipes to both sides of the sample cuvette) absorbance changes (matrix deconvoluted at 554 nm) in whole cells as described previously (15, 37, 38). Cells deficient in ctaA were grown in BG-11-C medium supplemented with either 0.6 or 0.8 μM copper because we have previously established (15) that such super-supplementation partly restores use of plastocyanin, increasing the likelihood of detecting phenotypes attributable to any impairment of copper trafficking to plastocyanin via Atx1. In all analyses the size of the transients did not increase on successive flashes, indicating that there was sufficient P700 to cause full photooxidation of cytochromes f plus c₈ plus plastocyanin with a single flash. Hence, the 20-replicate average of the first flash transient is shown in the figures.

**Cloning, Production, and Quantification of Recombinant Atx1—** Synechocystis PCC 6803 genomic DNA was used as template for PCR with primers 5′-GTATCATTTCATATGACTATTCAACTAACT-3′ and 5′-GGAAATCCGGTCACGTCTGCCACGTTGACAC-3′. The amplified fragment of DNA containing the atx1 ORF was ligated to pGEM-T prior to subcloning into the Ndel/EcoRI sites of pET29a to create pETATX1. Recombinant protein was generated in E. coli (BL21) exposed to copper (1 mM). Lysates (50 ml) were applied to Sephadex G-25 (2.5 × 50 cm), and 25 ml fractions (5 ml) eluted in 25 mM Tris-HCl, pH 7.0 were eluted in total copper protein and for copper by atomic absorption spectrophotometry. Pooled copper peak fractions were applied to Q-Sepharose and sequentially eluted with 25 mM Tris-HCl, pH 7.0, followed by 0.7 M NaCl, 25 mM Tris-HCl, pH 7.0. Fractions were again analyzed for copper and protein and copper-containing fractions desalted on Sephadex G-25 in 25 mM Tris-HCl, pH 7.0. A single prominent band of the anticipated size was

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1 The abbreviations used are: ORF, open reading frame; PMPS, p-(hydroxymercuri)phenylsulfonate.
detected by PAGE, and the amino-terminal ten residues of sequence (Beckman LF 3000 protein sequencer) confirmed the identity of the purified protein. A further aliquot was hydrolyzed and analyzed for amino acid composition (Alta Bioscience) to allow calibration of colorimetric estimations of AtxA1 using Coomassie Blue R250 (correction factor = 2.46 compared with bovine serum albumin).

Cloning, Production, and Quantification of Recombinant PacS N—Synechocystis PCC 6803 genomic DNA was used as template for PCR with primers 5'-GAAGGGCCGCGCAAT-GACTTTCACTAATCTG-3' with 5'-GAAGATTC-TCTCATACCCCGTTACAAATTGCCGA-3' (the latter annealing to DNA 3' of the atx 1 stop codon). The amplified fragment of DNA containing codons 1-95 encoding the entire amino-terminal region of PacS (Fig. 1), was ligated to pGEM-T prior to subcloning into the Ndel/EcoRI sites of pET29a to create pETPACSN. Recombinant protein was generated in E. coli (BL21) exposed to copper (1 mM). Lysates (1.5 ml) were applied to Sephadex G-75 (1.5 x 20 cm), and fractions were eluted in 50 mM potassium phosphate buffer, pH 7.0. Analysis of the total protein. Pooled low molecular weight protein fractions were applied to contiguous columns of Q-Sepharose and SP-Sepharose eluted with 50 mM potassium phosphate buffer, pH 7.0. A single prominent band of the anticipated size was detected by PAGE. An aliquot of protein was hydrolyzed and analyzed for amino acid composition (Alta Bioscience) to allow calibration of colorimetric estimation of PacS N (correction factor = 0.8 compared with bovine serum albumin). Attempts to overexpress CtaAN have to date produced low yields of soluble protein precluding further analyses.

Preparation of Apo and Copper-bound Recombinant Proteins—Proteins were incubated with 10 mM dithiothreitol, transferred to a N₂ atmosphere chamber, and fractionated on Sephadex G-25 equilibrated in and eluted with hydrochloric acid, pH 2.0. If metallated protein was required, recovered material was exposed to a 2-fold molar excess of copper prior to the addition of 0.5 mM potassium phosphate buffer (pH 7.0) to return the pH to 7.0. The sample was further fractionated on Sephadex G-25 equilibrated in and eluted with 50 mM potassium phosphate buffer, pH 7.0. If an apo protein was required, the same procedure was used but without the addition of copper to the protein at pH 2.0.

Metal Transfer—The method was an adaptation of that used to investigate metal transfer between S. cerevisiae AtxA1 and CceA2 (31). Aliquots (total volume of 0.5 ml in 50 mM potassium phosphate, pH 7.0) of apoPacSN or copper-Atx1 were applied to Q-Sepharose (1-ml column) equilibrated with 50 mM potassium phosphate, pH 7.0, and eluted (0.5 ml fractions) with 5 ml of the same buffer, followed by 5 ml of 1 M NaCl. All procedures were performed in a N₂ atmosphere chamber using syringes to manually load and elute the column. Identical aliquots of apoPacSN and copper-Atx1 from the same two preparations on the same day) were mixed (total volume again 0.5 ml) and similarly incubated with reductant (10 mM dithiothreitol). The protein was applied to Sephadex G-25 (1.5 x 20 cm), and fractions were eluted in 50 mM potassium phosphate buffer, pH 7.0 analyzed for total protein. If an apo protein was required, recovered material was exposed to a 2-fold molar excess of copper prior to the addition of 0.5M potassium phosphate buffer. 

RESULTS

The Product of ORF ssr2857, AtxA1, Binds One Copper Atom via Thiol Ligands—To test whether ssr2857 encodes an ATX1-like copper-protein, the gene was expressed in E. coli cells grown in medium supplemented with 1 mM copper at the time of protein production. Copper was co-purified with recombinant AtxA1 of Synechocystis PCC 6803. Copper was displaced from the purified protein by PMPS (data not shown) consistent with thiol coordination via the sole pair of cysteine residues (Fig. 1). The amount of AtxA1-associated copper was substoichiometric and variable in different preparations. In a N₂ atmosphere chamber, however, demetallation, reconstitution with excess Cu⁺, and removal of unbound metal by gel filtration on Sephadex G-25 recovered 30.2 nmol of AtxA1 with 29.9 nmol of associated copper as determined by atomic absorption spectrophotometry.
pendent switching to the use of plastocyanin in Synechocystis interactions within a bacterial (E. coli PCC 7942) and contributes toward copper-deporter, PacS. PacS resides at the thylakoid membrane in 6803 Atx1 is the amino-terminal region of the copper trans-

15 of the CXXC motif and transfer to CCC2 is thought to involve ligands from MXCXXC motifs of both partners; these are required for a detectable S. cerevisiae two-hybrid interaction using ATX1 and the isolated domain of CCC2 (41). To further test our proposed analogy between bacterial Atx1 and yeast ATX1, both cysteine 12 and cysteine 15 of Synechocystis PCC 6803 Atx1 were converted to serine by site-directed mutagenesis, and the bacterial two-hybrid interaction with PacSN was reanalyzed. There was indeed no detectable interaction between Atx1(C12/15S) and PacSN (Fig. 2A, second panel).

Atx1 Interacts with the Amino-terminal Region of CtaA in a Bacterial Two-hybrid Assay—By analogy to ATX1 in S. cerevisiae, a most likely candidate partner for Synechocystis PCC 6803 Atx1 is the amino-terminal region of the copper transporter, PacS. PacS resides at the thylakoid membrane in Syn-echococcus PCC 7942 (22) and contributes toward copper-dependent switching to the use of plastocyanin in Synechocystis PCC 6803 (15). It is now possible to analyze protein-protein interactions within a bacterial (E. coli) cell (BacterioMatch™, Stratagene), and therefore this method has been used to identify partners for Atx1. Fig. 2A shows greatly enhanced β-galactosidase activity when Atx1 and the amino-terminal region of PacS (PacSN) were used as target and bait within this system compared with cells in which one or both partner(s) was/were absent (data not shown). The magnitude of activity was similar to that detected with Atx1 and PacSN (Fig. 2B). Activity was similar to controls when the Atx1 mutant C12S/C15S was used in conjunction with CtaA (data not shown).

Atx1 Does Not Interact with the Amino-terminal Regions of ZiaA or CoaT in a Bacterial Two-hybrid Assay—The yeast genome encodes two deduced CPx-type ATPases, but there is no evidence that either of these ATPases handles metals other than copper. Synechocystis PCC 6803 therefore provides an opportunity to investigate possible contributions of metallochaperones to metal specificity. Does bacterial Atx1 solely interact with the amino-terminal domains of the copper transporters, or can it interact with the equivalent regions of all four CPx-type ATPases in this organism? There was no detectable increase in β-galactosidase activity when Atx1 and the amino-terminal region of either ZiaA or CoaT was used within the bacterial two-hybrid system compared with cells in which one or both partner(s) was/were absent (data not shown). Fig. 2B shows that the magnitude of activity generated by the interaction of Atx1 with either PacSN or CtaAN is substantially greater than that obtained using either ZiaAN or CoaTN.

The Amino-terminal Region of PacS Can Acquire Copper from Atx1 in Vitro—The observed in vivo interaction between bacterial Atx1 and PacSN encourages a hypothesis that the former donates copper to the latter. We have therefore tested whether or not copper can transfer from Atx1 to the amino-terminal region of PacSN in vitro. Atx1 associated strongly with the Q-Sepharose anion exchange matrix whereas PacSN was not retained (Fig. 3, upper panels). Fractionation of in vitro metallated copper-Atx1 confirmed co-migration of ~1.0 mol equivalent of copper and conversely an absence of copper associated with in vitro demetallated apoPacSN. Following co-incubation of two further, similar aliquots of the same preparations of apoPacSN and copper-Atx1, the amount of copper associated with Atx1 declined and, most importantly, copper became associated with apoPacSN. This provides evidence in support of copper trafficking from Atx1 to PacS. It remains formally possible that in vitro these two proteins form a stable heterodimer that dissociates upon chromatography, with copper then partitioning to either partner. It is presumed that dissociation and metal transfer occurs in vivo and is somehow driven in the direction of PacS.

Cytochrome Oxidase Activity Is Less in Membranes Purified from Δatx1 Synechocystis PCC 6803—Evidence that bacterial Atx1 interacts with PacSN suggests a role in the supply of copper to thylakoidal proteins. To test this assumption, mutant Synechocystis PCC 6803(atx1) was obtained following integration of pIN-ATX1, which contains ORF ssr2857 (atx1) inter-
ruptured by the chloramphenicol acetyl transferase gene. Southern analysis confirmed integration via a double homologous recombination event at the atx1 locus and segregation of cells containing the antibiotic resistance gene in all copies of the chromosome. Hereinafter the strain is called Δatx1. There was indeed a small but statistically significant decline in cytochrome oxidase activity in membranes isolated from Δatx1 compared with wild type when cells were cultured in BG-11-C medium (Fig. 4A).

ctaA and atx1 Are Additive, but pacS and atx1 Are Non-additive with Respect to Cytochrome Oxidase Activity—A most obvious hypothesis is that CtaA, Atx1, and PacS act in a linear sequence to supply copper to the thylakoid. Double mutants were therefore used to test for epistasis. *Synechocystis* PCC 6803(atx1,ctaA) and *Synechocystis* PCC 6803(atx1,pacS) were generated via integration of pIN-ATX1 into the genome of kanamycin-resistant single mutants (15) *Synechocystis* PCC 6803(ctaA) and *Synechocystis* PCC 6803(pacS). The single transporter mutants are called ΔctaA and ΔpacS hereinafter. Southern analysis confirmed integration via a double homologous recombination event at the atx1 locus, and segregation of cells containing the antibiotic resistance gene in all copies of the chromosome in both strains, which are hereinafter called Δatx1ΔctaA and Δatx1ΔpacS. Membranes isolated from ΔpacS contain significantly less cytochrome oxidase activity than either wild type or Δatx1 after growth in BG-11-C medium. Significantly, cytochrome oxidase activity in membranes from the double mutant Δatx1ΔpacS was no less than the lower of the single mutants, ΔpacS (Fig. 4A). Thus, there is no evidence that Atx1 can influence cytochrome oxidase activity without PacS. We previously reported a decline in cytochrome oxidase activity in membranes isolated from ΔctaA grown in BG-11-C (15). Here we report that activity in membranes from the double mutant Δatx1ΔctaA was significantly less than either of the respective single mutants (Fig. 4B), showing that Atx1 can still function in the absence of CtaA.

**Fig. 3.** Copper transfer from copper-Atx1 to apoPacSN. Protein (open squares) and copper (closed circles) concentrations of fractions eluted from Q-Sepharose in the absence (first 5 ml) or presence (second 5 ml) of 1 M NaCl. Controls demonstrate that apoPacSN does not bind to the column (top) whereas copper-Atx1 does bind (middle). Identical second aliquots of the same two preparations of copper-Atx1 and apoPacSN were mixed and then chromatographed (bottom).

**Fig. 4.** Cytochrome oxidase activity in purified membranes and kinetics of photooxidation of cytochrome *c*6 in single and double mutants. Cytochrome oxidase activity was determined using total membranes purified from each of the genotypes after growth in BG-11-C medium. Each analysis was performed in triplicate in each experiment, and each panel represents an individual experiment performed on a single occasion; some day-to-day variability in reaction rates was observed. A, cytochrome oxidase activity is lower in purified membranes from single mutants Δatx1 and ΔpacS but no lower in the double mutant Δatx1ΔpacS. B, cytochrome oxidase activity is lower in purified membranes from Δatx1ΔctaA than in either respective single mutant, C, kinetics of photooxidation and re-reduction of cytochrome *c*6 in copper-supersupplemented Δatx1ΔctaA and Δatx1. Light-induced absorbance change in intact cells was deconvoluted at 554 nm for cytochrome *c*6 plus f (ΔΔ) in response to a 6-μs pulse of actinic light (coincident with the drop in ΔΔ) in cells grown in medium supersupplemented with 0.8 μM copper. The magnitude of the decrease in ΔΔ and hence the relative amount of photooxidation of cytochrome *c*6 is given on the left of each trace. The subsequent rise corresponds to re-reduction of cytochrome *c*6 by the cytochrome *b*6 complex. Equivalent data were obtained in two further experiments performed on separate occasions with separate cultures (not shown) and also in a triplicated experiment with cells super-supplemented with 0.6 μM copper (not shown). The decrease in ΔΔ was always greater, and the rate of return swifter, in cells deficient in atx1 and ctaA (bottom) compared with cells deficient in ctaA alone (top).

**Photooxidation of Cytochrome *c*6 Is Greater and Re-reduction Faster in Δatx1ΔctaA Compared with ΔctaA in Copper-supersupplemented Media**—The observations that Atx1 from *Synechocystis* PCC 6803 binds copper and is required for normal levels of cytochrome oxidase activity support a role as a copper metallochaperone in the supply, either directly or indirectly, of this enzyme. Cytochrome oxidase has been detected at both the plasma membrane and at the thylakoid membrane in this organism (43, 44) although it is unclear whether it is active at the plasma membrane. Photosynthetic electron transport occurs exclusively at the thylakoid (43). Some cyanobacteria and green algae (Ref. 45 and citations therein) adapt an inadequate copper supply by exploiting heme iron in cytochrome *c*6 as an alternative to copper in plastocyanin for shuttling electrons inside the thylakoid lumen (from one membranous photosystem to the other). Does Atx1 contribute to efficient switching from cytochrome *c*6 to plastocyanin?

Photosynthetic electron flow through cytochrome *c*6 can be
monitored in intact cells as the decrease in absorbance deconvoluted at 554 nm upon exposure to a pulse of actinic light (46) (in the dark the pool of cytochrome $c_6$ is largely reduced). At increasing [copper] there is an inverse relationship between the magnitudes of photooxidation of cytochrome $c_6$ and plastocyanin, with less of the former and more of the latter (15, 45, 46). We previously established that in medium containing 0.2 $\mu$M copper, photooxidation of cytochrome $c_6$ is greater in both Δ$ctaA$ and Δ$pacS$ compared with wild-type cells; this correlates with an increase in cytochrome $c_6$ and a decrease in plastocyanin transcript abundance (15). Super-supplementation of Δ$ctaA$ with 0.8 $\mu$M copper partly reversed the phenotype, presumably because of copper acquisition via other (perhaps nonspecific) metal transporters, partly restoring the use of plastocyanin (15). Here we have examined photosynthetic electron transport in Δ$ataA$ cells because the phenotype attributable to $atax1$ with respect to cytochrome oxidase activity (Fig. 4B) was most severe in a Δ$ataA$ background. Fig. 4C shows that the magnitude of the decrease in ΔA deconvoluted at 554 nm was greater in Δ$ata1$Δ$ataA$ compared with Δ$ataA$, implying impaired trafficking of copper to thylakoidal plastocyanin in cells that do not contain AtxA. Equivalent trends were observed in three independent experiments. In addition, it was noted that re-reduction of cytochrome $c_6$ was substantially faster in cells deficient in $atax1$. This is consistent with impaired activity of thylakoidal cytochrome oxidase because cytochrome $c_6$ can either donate electrons to photosystem I or to cytochrome oxidase, and the absence of the latter leads to the accumulation of a larger pool of reduced electron donors for cytochrome $c_6$.

**Δ$ata1$ Is Hypersensitive to Low Iron**—It was speculated that a greater dependence upon cytochrome $c_6$ rather than plastocyanin for photosynthetic electron transport in Δ$ata1$ could confer a greater dependence on iron. Iron deficiency generated by subculture in medium containing the iron chelator deferoxamine mesylate slowed the growth of both wild-type and Δ$ata1$ cells (compare y-axes on the two panels of Fig. 5). Mutants deficient in AtxA were even more sensitive to low iron than wild-type cells; this was observed in two further replicate experiments (not shown). Note that the errors on Fig. 5 are too small to show above and below the data points. There was no significant difference in copper tolerance of Δ$ata1$ compared with wild type (data not shown).

**DISCUSSION**

Several lines of evidence (Figs. 2–5) support a role for *Synechocystis* PAC 6803 AtxA in the delivery of copper via PaccS to the thylakoid, providing copper for proteins involved in photosynthetic and respiratory electron transport. It remains to be reported whether or not analogous proteins target copper to thylakoidal plastocyanin in plant chloroplasts. That $atax1$ is required for optimal cytochrome oxidase activity in a Δ$ataA$ background (Fig. 4) implies that AtxA can acquire copper from other locations, although it clearly does also interact with CtaA (Fig. 2). It is proposed that bacterial AtxA, and by analogy some eukaryotic copper metallochaperones, contribute toward the efficient reuse of cell copper by scavenging from weak cytosolic sites. Structural models of bacterial AtxA, PacSC, and CtaA suggest mechanisms of ligand exchange, which, though subtly different, provide support to those proposed for copper release from eukaryotic ATX1. The absence of any detectable interaction between AtxA and the amino-terminal regions of ZiaA and CoaT (Fig. 2) illustrates a contribution of protein-protein interactions in determining metal specificity, i.e. which metals go to which locations within a cell.

Mutants deficient in PaccS are impaired in switching from the use of heme iron in cytochrome $c_6$ to the use of copper in plastocyanin for photosynthetic electron transport, which is thought to result from a loss of thylakoid copper import by PaccS (15). Here we also show loss of cytochrome oxidase activity in Δ$pacS$ (Fig. 4). The association of similar phenotypes with $atax1$ coupled with the observation that AtxA can interact *in vivo* with (Fig. 2) and transfer copper *in vitro* to (Fig. 3) PaccS supports a model in which AtxA1 delivers copper to PacS for thylakoid import (Fig. 6A). This model is analogous to the delivery of copper to Golgi-localized CCC2 by ATX1 in *S. cerevisiae*, even though one of the proteins indirectly supplied by AtxA1 in *Synechocystis* PAC 6803, cytochrome oxidase, is a target for the mitochondrial copper metallochaperone COX17 in *S. cerevisiae* (reviewed in Ref. 2). PacS appears to be the sole route for AtxA1-bound copper to reach cytochrome oxidase because the genes are not additive (Fig. 4). As noted by others (2), it remains to be reported whether or not the other analyzed bacterial copper metallochaperones, CpaZ, interacts *in vivo* with the amino-terminal regions of CPx-type ATPases, CopA and CopB. Where do copper metallochaperones acquire copper? Mutants deficient in CtaA accumulate less copper and, similar to ΔpaccS, show phenotypes associated with impaired copper supply to plastocyanin and cytochrome oxidase, consistent with CtaA acting as the principal copper importer at the plasma membrane (15). It is significant that CtaA is not obligatory for AtxA function (Fig. 4). This implies that AtxA scavenges copper from other sources, either in the cytosol or from secondary importers (Fig. 6B). An attractive hypothesis is that AtxA1 recycles endogenous copper, perhaps from degraded metalloproteins or otherwise associated with adventitious copper-binding sites in the cytosol (Fig. 6B). The severe phenotype of Δ$ata1$Δ$ataA$ would result from loss of both the principal importer and endogenous recycling of copper.

How is copper acquired and released from AtxA in *Synechocystis* PAC 6803, and does it optionally obtain copper from CtaA? The bacterial two-hybrid data might suggest the latter (Fig. 2). Pfahl et al. (47) proposed a model for copper transfer from ATX1 to CCC2 via bridged ATX1-CCC2 copper-binding sites. NMR structural analyses have subsequently shown that the first and final loops (loops 1 and 5) are the main sites for movement in copper-ATX1 upon contact with apoCCC2 (39). The metal-binding Cys residues are associated with loop 1, whereas loop 5 contains a conserved Lys in several eukaryotic ATX1 homologues and is also located close to the metal-binding site in the folded protein. It has been speculated that movement of this residue may trigger metal release from ATX1. Structural modeling of (ancestral) bacterial AtxA1, PacSC, and CtaA strengthens the hypothesis that loop 5 can influence copper transfer (Fig. 6). In bacterial AtxA1 the equivalent region to loop 5 contains a His residue that closely approaches the metal binding site when modeled on copper-ATX1, ideal for...
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contains Tyr-His in loop 5. A stable loop 5 of PacSN could favor ligand exchange to Tyr or His coincident with displacement of His from bacterial AtxA. The absence of known copper ligands in the loop 5 region of CtaA is consistent with reversal of the vector for metal transfer (donation to bacterial AtxA) (Fig. 6, F and G).

The amino-terminal region of ZiaA (111 amino acids) contains a single GMXCCXC motif (Fig. 1) within a domain that can be modeled to form a ferredoxin-like fold, followed by a region containing seven histidine residues arranged in HXH motifs. However, neither the amino-terminal region ZiaAN nor CtaAN (a 38-amino acid region that does not contain an obvious metal-binding motif) formed detectable two-hybrid interactions with AtxA (Fig. 2). The detected AtxA interactions are specific to the amino-terminal regions of the copper transporters (Fig. 6C). There is no free zinc in the cytosol of *E. coli* (48), and it is anticipated that the same is true for *Synechocystis* PCC 6803 and for cobalt, raising questions about what molecules interact with and supply metals ions to ZiaAN, and CtaAN. It is unclear how widespread metallochaperones are and how significant their associations are in defining which inorganic elements are acquired by metalloproteins in vivo.

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