Thermodynamics of copper and zinc distribution in the cyanobacterium *Synechocystis* PCC 6803

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Edited by Harry B. Gray, California Institute of Technology, Pasadena, CA, and approved May 27, 2011 (received for review February 2, 2011)

Copper is supplied to plastocyanin for photosynthesis and cytochrome *c* oxidase for respiration in the thylakoids of *Synechocystis* PCC 6803 by the membrane-bound P-type ATPases CtaA and PacS, and the metallochaperone Atx1. We have determined the Cu(I) affinities of all of the soluble proteins and domains in this pathway. The Cu(I) affinities of the trafficking proteins range from 5×10^{16} to 5×10^{17} M^{-1} at pH 7.0, consistent with values for homologues. Unusually, Atx1 binds Cu(I) significantly tighter than the metal-binding domains (MBDs) of CtaA and PacS ($CtaA_N$ and $PacS_N$), and equilibrium copper exchange constants of approximately 0.2 are obtained for transfer to the MBDs. Dimerization of Atx1 increases the affinity for Cu(I), but the loop 5 His61 residue has little influence. The MBD of the zinc exporter ZiaA ($ZiaA_N$) exhibits an almost identical Cu(I) affinity, and Cu(I) exchange with Atx1, as $CtaA_N$ and $PacS_N$, and the relative stabilities of the complexes must enable the metallochaperone to distinguish between the MBDs. The binding of potentially competing zinc to the trafficking proteins has been studied. $ZiaA_N$ has the highest Zn(II) affinity and thermodynamics could be important for zinc removal from the cell. Plastocyanin has a Cu(I) affinity of 2.6×10^{17} M^{-1}, 15-fold tighter than that of the Cu_A site of cytochrome *c* oxidase, highlighting the need for specific mechanisms to ensure copper delivery to both of these targets. The narrow range of Cu(I) affinities for the cytoplasmic copper proteins in *Synechocystis* will facilitate relocation when copper is limiting.

copper homeostasis | metallochaperones | zinc homeostasis | cyanobacteria

Up to 50% of enzymes bind metals yet in most cases it is currently not understood how the correct metal is incorporated into the right protein. Metal-binding selectivity can be controlled by dedicated metallochaperones (1–10), which deliver the metal to cellular destinations, and by metal availability at the site of protein folding (11). Copper is an essential trace element involved in key processes such as iron uptake, the removal of superoxide radicals, and electron transfer (12). Uncontrolled reactivity, and the ability to bind at sites for other metals (11, 13), makes copper toxic and there is general agreement that labile copper pools do not exist in the cytosol (4–6, 11, 14, 15). Metallochaperones are used to deliver copper to targets via ligand exchange reactions (2, 5, 6), with protein interactions thought to enhance the kinetics and specificity of copper transfer (4, 5, 15, 16). The importance of Cu(I) affinities for the trafficking of copper in humans has recently been highlighted (15). An overarching thermodynamic driving force exists from glutathione to the copper storage protein metallothionein. As in related studies, mainly also in eukaryotes (17–23), shallow thermodynamic gradients are found between the copper metallochaperone Atx1 (HAH1 in humans) and the metal-binding domains (MBDs) of copper-transporting P-type ATPases (15). Zinc is a potentially competing metal for copper and its cellular levels are also carefully controlled (24–27). Zinc is required by many more proteins than copper and in the absence of any known cytoplasmic metallochaperones (28) the mechanism of delivery of this metal to targets is not well understood (27, 28).

Cyanobacteria are rare examples of prokaryotes with a cytoplasmic requirement for copper and offer a relatively simple system for investigating the homeostasis of this metal in a primary producer. The cell biology of cyanobacterial copper proteins and their compartmentalization, particularly in *Synechocystis* PCC 6803, has therefore received considerable attention (11, 29–37). Cyanobacteria have a need for copper in their thylakoids for the type 1 copper site of plastocyanin, which functions in photosynthetic electron transfer, and the Cu_A and Cu_B centers of cytochrome *c* oxidase, the terminal electron acceptor of aerobic respiration. How copper is distributed in the thylakoids between these two targets is not known, but delivery to this location is dependent on a cytosolic pathway (Fig. 1*A*) involving the two membrane-bound P-type ATPases CtaA and PacS and the soluble metallochaperone Atx1 (31, 32). It was originally proposed, on the basis of in vivo experiments, that copper is imported into the cytoplasm by CtaA and exported to the thylakoids by PacS (30, 31). However, recent in vitro data indicate that both CtaA and PacS export copper from the cytoplasm, albeit with different rates (37), and it has been suggested that CtaA could be located at the thylakoid membrane (10). Atx1 interacts in two-hybrid assays with the N-terminal MBDs of CtaA and PacS ($CtaA_N$ and $PacS_N$) (32), and Atx1 and $PacS_N$ form a detectable complex in vitro in the presence of Cu(I) (34). The related P-type ATPase ZiaA is involved in zinc export, although Cu(I) displaces Zn(II) from its MBD ($ZiaA_N$) (33). $ZiaA_N$ does not form a complex with Atx1 that is detectable by two-hybrid assays (33) or NMR (38). It has recently been suggested that Atx1 may bind Zn(II) in vivo (39).

In most organisms Atx1 and the MBDs of copper and zinc transporters possess highly similar ferredoxin-like (βαββαβ) folds with a conserved CXXC metal-binding motif on loop 1/α1 (8, 35). It is assumed that in vivo these proteins all bind a single Cu(I) ion, with high affinity (18, 20–23, 40), via a two-coordinate site involving the Cys ligands (2, 5, 6, 8, 35, 40). A residue on loop 5 approaches the Cu(I) site in all Atx1s and MBDs, can tune the pK_a of one of the Cys ligands (22), and has been suggested to play a role in copper transfer (10, 22, 34, 41, 42). In certain cyanobacterial Atx1s this residue is unusually a His, compared to the Tyr typically found in prokaryotic Atx1s (35, 41). This His61 residue is in the second-coordination sphere of the metal in a crystal structure of a *Synechocystis* Atx1 dimer (35), but is thought to be a Cu(I) ligand in a solution dimer (43, 44). His61 helps to stabilize dimeric Atx1 (35). The corresponding residue is a Phe in $CtaA_N$ and a Tyr in $PacS_N$ and $ZiaA_N$.

In this work we have determined the Cu(I) affinities of the copper-trafficking and cytoplasmic target proteins [plastocyanin and the Cu_A site of cytochrome *c* oxidase (Cu_A-CCO)] and also $ZiaA_N$ from *Synechocystis*. The influence of Atx1 dimerization and the residue on loop 5 on Cu(I) affinity have been measured.

Author contributions: C.D. designed research; A.B. performed research; A.B. and C.D. analyzed data; and C.D. and A.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1101448108/-/DCSupplemental.

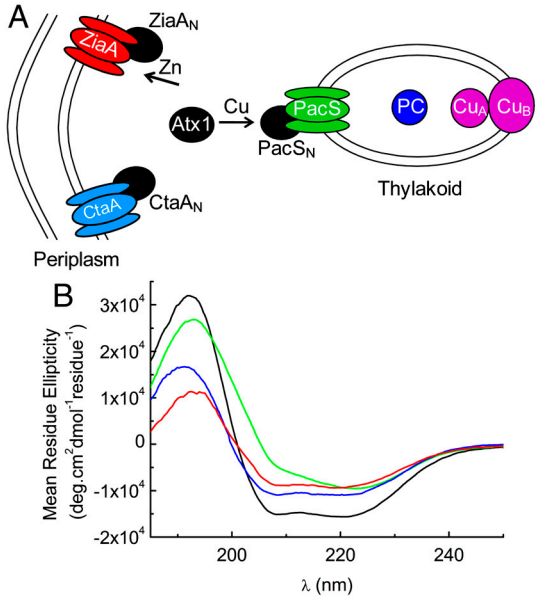

Fig. 1. The *Synechocystis* PCC 6803 copper and zinc proteins that have been studied. (*A*), CtaA and PacS are two copper-transporting ATPases and ZiaA a zinc exporting ATPase, Atx1 is the copper metallochaperone, PC represents plastocyanin, and Cu_A and Cu_B are the copper sites of cytochrome *c* oxidase. CtaA is shown on the plasma membrane as originally suggested (31), although it may be located on the thylakoid membrane (10). Some PacS may be present on the plasma membrane (36). (*B*), Far-UV CD spectra (20 °C) of WT apo-Atx1 (black line), apo-$PacS_N$ (green line), apo-$CtaA_N$ (blue line), and apo-$ZiaA_N$ (red line) measured in 150 mM potassium phosphate pH 7.0, except for $CtaA_N$ (67 mM potassium phosphate pH 8.0), as described in the *SI Text* (22).

We have also determined the Zn(II) affinities of Atx1, $ZiaA_N$, $CtaA_N$, and $PacS_N$. This analysis of copper delivery, along with the affinities of the trafficking sites for potentially competing zinc, gives insight into the factors that influence the homeostasis of these metals in a prokaryote and allows a comparison to be made with copper trafficking in other organisms.

Results

Protein Purification and Characterization. All purified proteins were verified by mass spectrometry (Table S1). His61Tyr Atx1 and wild type (WT), Tyr65His and Cys34Ser $PacS_N$ elute from a gel filtration column as monomers in both their apo and copper-loaded forms [*SI Text* and Table S2 (35)]. WT Atx1 with one equivalent of Cu(I) (Cu^I_1-protein) elutes as a dimer whilst the apo-protein is monomeric (35). Far-UV circular dichroism (CD) spectra show that WT and His61Tyr apo-Atx1, WT, Tyr65His, and Cys34Ser (reduced) apo-$PacS_N$ and apo-$ZiaA_N$ are folded (Fig. 1*B*, Fig. S1 *A* and *B*) with secondary structure compositions in agreement with those calculated from structures (35, 38) (Table S3). The Cu(I)-binding $IAC^{12}EAC^{15}$ loop has an almost identical structure in His61Tyr and WT Atx1 (35). Apo-$CtaA_N$ is partially unfolded at pH 7.0 [Cu(I) loading increases the α-helical content] but is folded at pH 8.0 (Fig. 1*B*, Fig. S1*C*, and Table S3).

Copper(I) Affinities of the Trafficking and Target Proteins. Very similar Cu(I) affinities are determined for $PacS_N$, $CtaA_N$, and $ZiaA_N$ at pH 7.0 ($\sim 7 \times 10^{16}$ M^{-1}) from competition experiments with the Cu(I) ligand bathocuproine disulfonate (BCS) (Table 1, Fig. 2 *A*–*D*, and Fig. S2 *A* and *B*). The Cu(I) affinity of WT Atx1 is tighter (Table 1), and is further increased by dimerization (*vide infra*). The Cu(I) affinity of His61Tyr Atx1 [monomeric (35)] is ~3-fold higher than those of the MBDs (Table 1, Fig. 2*E*, and Fig. S2*C*) and is similar to that determined for the monomeric WT protein (*vide infra*), consistent with His61 not being involved

Table 1. Cu(I) affinity constants (K_b values) for all the proteins studied plus the Cu(II) affinity of plastocyanin*

Protein	K_b (M^{-1})
WT Atx1	$(4.7 \pm 0.7) \times 10^{17,\dagger}$ (7.0)
	$(2.4 \pm 0.6) \times 10^{17,\dagger,\ddagger}$ (7.0)
	$(1.4 \pm 0.1) \times 10^{18,\S}$ (7.5)
	$(6.4 \pm 0.5) \times 10^{18,\S}$ (8.0)
His61Tyr Atx1	$(1.8 \pm 0.3) \times 10^{17}$ (7.0)
	$(3.7 \pm 0.2) \times 10^{17}$ (7.5)
	$(1.3 \pm 0.2) \times 10^{18}$ (8.0)
$CtaA_N$	$(6.4 \pm 0.7) \times 10^{16}$ (7.0)
	$(1.7 \pm 0.1) \times 10^{17}$ (7.5)
	$(8.7 \pm 0.9) \times 10^{17}$ (8.0)
$PacS_N$	$(7.8 \pm 0.7) \times 10^{16}$ (7.0)
	$(1.9 \pm 0.1) \times 10^{17}$ (7.5)
Tyr65His $PacS_N$	$(7.8 \pm 0.7) \times 10^{16}$ (7.0)
PacS95	$(7.5 \pm 0.5) \times 10^{16,\ddagger}$ (7.0)
$ZiaA_N$	$(6.5 \pm 1.0) \times 10^{16}$ (7.0)
Plastocyanin [Cu(I)]	$(2.6 \pm 0.5) \times 10^{17}$ (7.0)
Plastocyanin [Cu(II)]	$(4.8 \pm 1.9) \times 10^{14}$ (7.0)
Cu_A-CCO	$(1.7 \pm 0.7) \times 10^{16,\ddagger}$ (7.0)

*Measurements were performed at the pH value given in parenthesis, and in the presence of 200 mM NaCl. In a number of cases the influence of NaCl was checked and had almost no effect on the Cu(I) affinity. Almost all values are averages from titrations of apo-protein into $[Cu(BCS)_2]^{3-}$. Affinities usually within 20% were obtained from titrations of BCS into Cu(I)-protein.

†Affinities for monomeric WT Cu^I_1-Atx1.

‡Determined from titrating BCS into Cu(I)-protein only.

§Determined at a Cu(I) concentration of 15 μM.

in coordinating Cu(I) in this form. The Cu(I) affinities of WT and His61Tyr Atx1, $PacS_N$ and $CtaA_N$ have also been measured at pH 7.5 (Table 1) to allow a comparison with estimated K_b values for the two transmembrane metal-binding sites of PacS and CtaA. Published Cu(I) affinities for the transmembrane sites in the ATPase CopA from *Archaeoglobus fulgidus* are 1.1×10^{15} and 1.3×10^{15} M^{-1} at pH 7.5 (45). Using the β value for $[Cu(BCA)_2]^{3-}$ [Cu(I) complex of bicinchoninic acid (BCA)] that we have determined (*SI Text*), Cu(I) affinities for these sites in CopA of 1.3×10^{17} and 1.5×10^{17} M^{-1} can be recalculated. The transmembrane domains of PacS and CtaA show high sequence identity (~40%) to that of CopA, and all of the ligands are conserved. Similar Cu(I) affinities are therefore anticipated for the transmembrane metal-binding sites of PacS and CtaA. Cu(I) affinities of WT and His61Tyr Atx1 and $CtaA_N$ have also been measured at pH 8.0 (Table 1), as Cu(I) transfer experiments with $CtaA_N$ were performed at this pH. The Tyr65His mutation has no effect on the Cu(I) affinity of $PacS_N$ (Table 1, Fig. S2 *D* and *E*). Plastocyanin has a Cu(I) affinity of $(2.6 \pm 0.5) \times 10^{17}$ M^{-1} while that of Cu_A-CCO (Cu^I_2-form) is one order of magnitude lower (Table 1, Fig. 3*A*, and Fig. S2 *F* and *G*). Atx1 possesses a weaker Cu(I) site when it binds more than one Cu(I) equivalent (35) with an affinity ~5 orders of magnitude lower than that of the tight site (*SI Text*, Fig. S2 *H* and *I*).

Effect of WT Atx1 Dimerization on Cu(I) Affinity. For WT Atx1 K_b values ranging from 5.6×10^{17} to 1.4×10^{18} M^{-1} are obtained from titrations of apo-protein against $[Cu(BCS)_2]^{3-}$ and from 5.0×10^{17} to 1.1×10^{18} M^{-1} for experiments in which BCS was added to the Cu(I)-protein. The observed Cu(I) affinity depends on the Cu(I)-protein concentration used in the titration due to WT Atx1 dimerization in the presence of Cu(I). A detailed analysis of the competition assays with BCS at multiple Cu(I) concentrations (Fig. 2*F*, Fig. S2*J*, and *SI Text*) provides a Cu(I) affinity for the monomeric WT protein of $(2.4–4.7) \times 10^{17}$ M^{-1} (Table 1) and a dimerization constant (K_{dim}) of $(2.4–4.1) \times 10^5$ M^{-1} for the Cu^I_1-form, the latter in good agreement with a value of

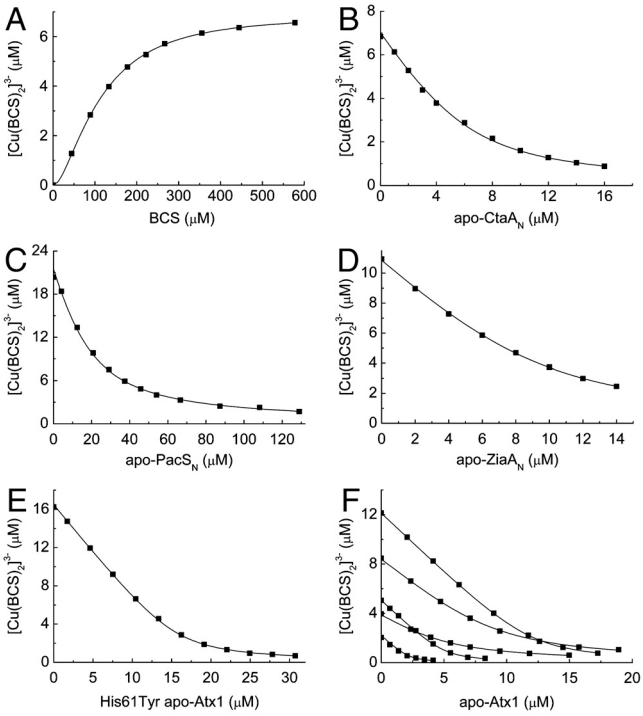

Fig. 2. Cu(I) affinities of the trafficking proteins. The titration of Cu^{I}_{1}-$CtaA_N$ (7 μM) in the presence of an excess of apo-$CtaA_N$ (5 μM) with BCS (*A*) and the titration of $[Cu(BCS)_2]^{3-}$ (7 μM) in the presence of an excess of BCS (26 μM) against apo-$CtaA_N$ (*B*). The lines show fits of these data to *SI Text: Eqs. S1 and S2* giving K_b values of $(7.1 \pm 0.1) \times 10^{16}$ and $(5.9 \pm 0.1) \times 10^{16}$ M^{-1}, respectively. Titrations of $[Cu(BCS)_2]^{3-}$ (20, 11, and 16 μM respectively) in the presence of an excess (78, 28, and 18 μM respectively) of BCS against fully reduced apo-$PacS_N$ (*C*), apo-$ZiaA_N$ (*D*) and His61Tyr apo-Atx1 (*E*). The lines show fits of these data to *SI Text: Eq. S2* giving K_b values of $(8.2 \pm 0.7) \times 10^{16}$, $(7.1 \pm 0.1) \times 10^{16}$, and $(2.1 \pm 0.2) \times 10^{17}$ M^{-1} respectively. (*F*) The titration of $[Cu(BCS)_2]^{3-}$ (2.0–12.0 μM) in the presence of an excess of BCS (46–166 μM) with WT apo-Atx1 and the simultaneous fit of the data to *SI Text: Eq. S3a* which gives a K_b of $(4.7 \pm 0.7) \times 10^{17}$ M^{-1} and a K_{dim} of $(2.4 \pm 1.0) \times 10^5$ M^{-1}. All experiments were performed in 20 mM Hepes pH 7.0 plus 200 mM NaCl.

$(5 \pm 2) \times 10^5$ M^{-1} obtained previously from gel filtration studies (35).

Copper(II) Affinity of Plastocyanin and a Calculated Reduction Potential. From titrations of L-histidine (His) into Cu^{II}-plastocyanin, and also of apo-plastocyanin into $Cu(His)_2$, Cu(II) affinities of $(6.6 \pm 0.2) \times 10^{14}$ and $(2.9 \pm 0.2) \times 10^{14}$ M^{-1} respectively at pH 7.0 (Table 1, Fig. 3*B*, and Fig. S2*K*) are obtained. From these affinities, that for Cu(I), and the reduction potential of the Cu^{II}/Cu^{I} aqua couple [164 mV vs. the normal hydrogen electrode (46)] a reduction potential of 328 ± 15 mV can be calculated for plastocyanin, in good agreement with the reported value of 345 mV (pH 7.0) (47).

Zinc(II) Affinities of Atx1, $PacS_N$, $CtaA_N$, and $ZiaA_N$. The binding of Zn(II) to the CXXC-containing motifs monitored (48) by UV-Vis (*SI Text*, Fig. S3 *A–C*) shows a linear decrease in absorbance at 240 nm up to 1 Zn(II) equivalent for $ZiaA_N$, whereas for Atx1, $PacS_N$ and $CtaA_N$ there is an inflection point after titrating ~0.5 equivalents of Zn(II). These titrations indicate the formation of a single Zn(II)-loaded species for $ZiaA_N$ [as previously seen by NMR (38)] and two Zn(II)-loaded forms (ZnP_2 and ZnP, where P is the apo-protein) of Atx1, $PacS_N$ and $CtaA_N$. Data at multiple concentrations of the Zn(II) chelator RhodZin-3 (49) have been simultaneously fit (*SI Text*) to determine the affinities (Table 2) of the apo-proteins for Zn(II) (K_{b1}), and also the affinities of

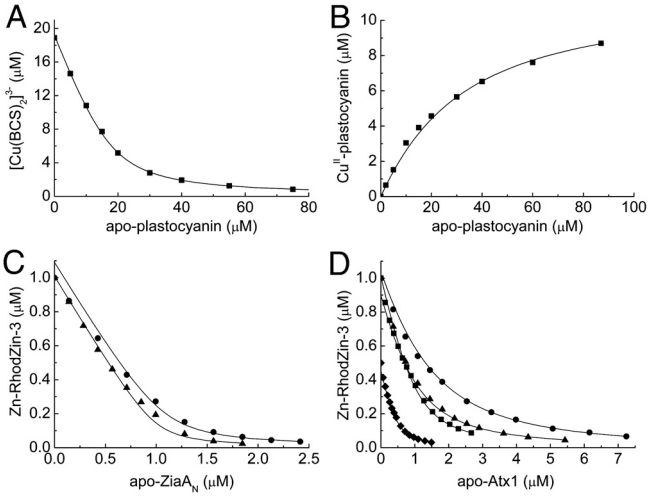

Fig. 3. Cu(I) and Cu(II) affinities of plastocyanin and the Zn(II) affinities of $ZiaA_N$ and Atx1. The titration of $[Cu(BCS)_2]^{3-}$ (19 μM) in the presence of an excess of BCS (82 μM) (*A*) and of $Cu(His)_2$ (11.4 μM) plus 10 mM His (*B*) with apo-plastocyanin in 20 mM Hepes pH 7.0 plus 200 mM NaCl. The lines show fits of these data to *SI Text: Eq. S2* giving K_b values of $(3.1 \pm 0.1) \times 10^{17}$ and $(2.9 \pm 0.2) \times 10^{14}$ M^{-1}, respectively. The titration of Zn-RhodZin-3 (1 μM) and excess RhodZin-3 [4 (triangles) and 9 (circles) μM] with apo-$ZiaA_N$ (*C*) and Zn-RhodZin-3 [1 μM, except (diamonds) 0.5 μM] and excess RhodZin-3 [0.75 (diamonds), 1.5 (squares), 4 (triangles), and 9 (circles) μM] with apo-Atx1 (*D*) in 25 mM Hepes pH 7.4 plus 100 mM NaCl. The simultaneous fit of the data for $ZiaA_N$ to *SI Text: Eq. S4* gives a K_{b1} of $(1.1 \pm 0.1) \times 10^{10}$ M^{-1}, and for Atx1 to *SI Text: Eq. S5* gives a K_{b1} of $(7.2 \pm 0.3) \times 10^8$ M^{-1} and K_{b2} of $(1.5 \pm 0.1) \times 10^5$ M^{-1}.

apo-Atx1, apo-$PacS_N$, and apo-$CtaA_N$ for the Zn(II)-proteins (K_{b2}) (Fig. 3 *C* and *D*, Fig. S3 *D* and *E*). The K_{b2} values for Atx1, $CtaA_N$ and $PacS_N$ are in the 10^4–10^5 M^{-1} range. The K_{b1} values [also determined using MagFura-2 for Atx1 (50), Table 2] are tighter and increase in the order $PacS_N$ < Atx1 ~ $CtaA_N$ < $ZiaA_N$, with the Zn(II) affinity for $ZiaA_N$ being more than two orders of magnitude greater than that of $PacS_N$ (Table 2).

Copper(I) Transfer. The transfer of Cu(I) between trafficking proteins, including the influence of mutating the loop 5 residue, has been investigated (Table S4). In all cases equilibrium is reached in less than 5 min and the exchange equilibrium constants (K_{ex} values) are in agreement with those calculated from K_b values (Table S4). Cu(I) transfer from Atx1 to $PacS_N$ and $CtaA_N$ gives K_{ex} values of 0.03 and 0.05 at pH 7.0 and 8.0, respectively (Table S4). This limited transfer is partly due to the dimerization of Cu^{I}_{1}-Atx1, and the recalculated experimental K_{ex} values for Cu(I) transfer from monomeric Atx1 to $PacS_N$ and $CtaA_N$ are 0.13–0.16 and 0.16–0.20, respectively (Table S4, *SI Text*). K_{ex} values of 0.3–0.4 are obtained for His61Tyr (monomeric) Atx1

Table 2. Zn(II) affinity constants (K_{b1} values) for Atx1, $PacS_N$, $CtaA_N$, and $ZiaA_N$, and the affinity constants of apo-protein for Zn(II)-protein (K_{b2} values) for Atx1, $PacS_N$, and $CtaA_N$*†

Protein	K_{b1} (M^{-1})	K_{b2} (M^{-1})
WT Atx1	$(7.2 \pm 0.3) \times 10^8$ $(5.1 \pm 0.6) \times 10^{8,\dagger}$	$(1.5 \pm 0.1) \times 10^5$
$PacS_N$	$(4.4 \pm 0.4) \times 10^7$	$(2.6 \pm 0.3) \times 10^5$
$CtaA_N$	$(1.1 \pm 0.1) \times 10^9$	$(2.9 \pm 1.4) \times 10^4$
$ZiaA_N$	$(1.1 \pm 0.1) \times 10^{10}$	

*Determined using RhodZin-3 in 25 mM Hepes pH 7.4 plus 100 mM NaCl.

†Determined from competition with MagFura-2 (in the fit K_{b2} was kept constant at 1.5×10^5 M^{-1}, as a single MagFura-2 concentration was used).

with $PacS_N$ and $CtaA_N$ (Table S4). Most notably, Cu(I) transfer occurs between Atx1 and $ZiaA_N$, with K_{ex} values for transfer from monomeric Atx1 of 0.07–0.16 (Table S4). Atx1 and $PacS_N$ can also exchange two electrons when the metal-binding Cys residues form an intramolecular disulfide in one of the proteins (*SI Text*). Cu(I) transfer to oxidized $PacS_N$ is ~6–10 times more thermodynamically favorable than to reduced $PacS_N$, but is significantly slower (Table S4 and *SI Text*).

Discussion

Copper trafficking to the thylakoid compartments is essential for photosynthesis and respiration in cyanobacteria. The Cu(I) affinities of the soluble copper-binding proteins and domains in the cytoplasm of *Synechocystis* PCC 6803 have been determined (Fig. 4*A*). For the trafficking proteins, Zn(II) affinities are compared to that for a protein involved in zinc homeostasis (Fig. 4*B*). These studies demonstrate the factors important for copper and zinc allocation in a photosynthetic prokaryote, and allow comparisons to be made to other copper-trafficking systems. The excellent agreement between the reduction potential of plastocyanin calculated from Cu(I) and Cu(II) affinities and that determined directly (47) demonstrates that the affinities reported are absolute values. Further evidence for absolute affinities is the similarity of the value of Atx1 for Zn(II) measured using two different chelators. A thorough comparison is possible between the affinities of proteins for a particular metal, and also of the same protein for potentially competing metals, circumventing many of the well documented problems associated with metal affinity determinations (23, 51).

The absolute Cu(I) affinities of the cytosolic copper-trafficking proteins in *Synechocystis* range from 5×10^{16} to 5×10^{17} M^{-1} at pH 7.0, which covers values obtained previously for copper metallochaperones and MBDs from both prokaryotes and eukaryotes (18, 20–23, 51–53), if a unified method of determination is applied (23, 51). Extremely tight binding of Cu(I) appears to be a conserved feature of cellular copper trafficking. Monomeric WT Atx1 from *Synechocystis* has a Cu(I) affinity that is 3–7-fold higher than those of $PacS_N$ and $CtaA_N$, and K_{ex} values of approximately 0.2 for transfer to the MBDs, similar to those observed for His61Tyr (monomeric) Atx1 (Table S4), are found. In all previous studies, mainly in eukaryotes, shallow, but favorable, thermodynamic gradients exist for Cu(I) transfer from the metallochaperone to the MBDs of ATPases (15, 17–23), although K_{ex} values close to 1 have been found (17). The *Synechocystis* system provides the first example where the thermodynamics of copper transfer to the MBDs is unfavorable, which could indicate that the Cu(I) affinity of Atx1 may not be a determining factor for copper trafficking in this organism. Alternatively, copper trafficking in cyanobacteria may require tighter control. Dimerization of WT Cu(I)-Atx1 (35, 44) at low micromolar concentrations (35) results in an increased affinity for Cu(I). In *Saccharomyces cerevisiae*, Atx1 is present at low micromolar concentrations (54) and if similar levels occur in *Synechocystis* dimerization will restrict Cu(I) transfer to the MBDs when Cu(I) concentrations increase (Table S4), which may have a regulatory role in copper trafficking.

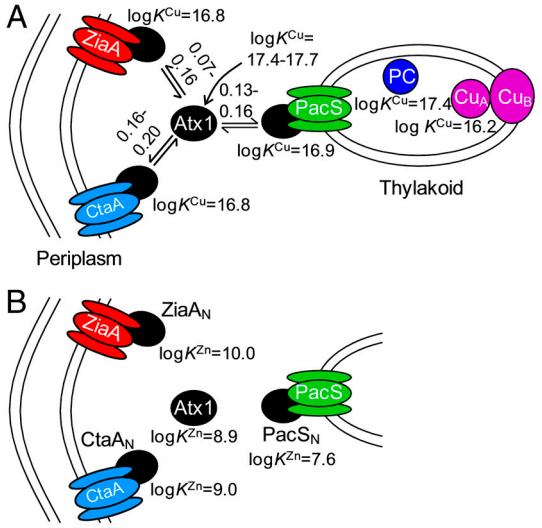

Fig. 4. The Cu(I) and Zn(II) affinities determined in this study. (*A*) The Cu(I) affinities (log K_b values labeled as log K^{Cu}) at pH 7.0 for the cytosolic copper proteins of *Synechocystis* along with the K_{ex} range for Cu(I) exchange (pH 8.0 for the Atx1-$CtaA_N$ transfer) involving monomeric Atx1 and the MBDs shown between the trafficking proteins. The Zn(II) affinities (log K_{b1} values labeled as log K^{Zn}) at pH 7.4 of the trafficking proteins are shown in (*B*). All of the protein abbreviations have been defined in the legend for Fig. 1.

There is current uncertainty about whether the MBDs of P-type ATPases are directly involved in copper transfer or whether copper loading regulates their enzymatic activity (37, 53, 55–57). If copper loading of the MBDs by Atx1 in *Synechocystis* has a regulatory role, then it will proceed under equilibrium conditions, and the high Cu(I) affinity of Atx1 will result in very tight control of copper-transporting activity. In *A. fulgidus* it has been shown that the copper metallochaperone CopZ can deliver Cu(I) directly to the transmembrane copper-binding sites of CopA (53). The Cu(I) affinities of the transmembrane sites of CopA are 2-fold tighter than that of CopZ (45, 53). The homology of the transmembrane domains of PacS and CtaA to that of CopA, including conservation of all of the proposed copper ligands (45), allows the Cu(I) affinities of their transmembrane metal-binding sites to be estimated as approximately 1×10^{17} M^{-1} at pH 7.5. These values are very similar to the Cu(I) affinities of $PacS_N$ and $CtaA_N$ at this pH (Table 1). Copper transfer from Atx1 to either the MBDs or the transmembrane sites of PacS and CtaA would appear to be equally unfavorable in terms of thermodynamics, which may be overcome by ATP-driven metal translocation (2, 5).

A regulatory role of the MBDs may not rely solely on Cu(I) loading but on the interactions with Atx1 (58, 59), which have been studied in detail (2, 16, 32, 34, 56, 58–61). These heterocomplexes are usually stabilized by electrostatic interactions and the presence of Cu(I) in both prokaryotes and eukaryotes (16, 34, 60). In humans certain MBDs of the Menkes and Wilson ATPases can form complexes with HAH1 that are detectable by NMR (58, 59), with these interactions thought to regulate activity (56, 58, 59). Other MBDs do not form detectable complexes with HAH1 but are still able to acquire Cu(I) from the metallochaperone (58, 59), proposed to be due to less stable intermediates (59, 61). In *Synechocystis* both $PacS_N$ and $CtaA_N$ form detectable complexes with Atx1 (32, 34), which is not the case for $ZiaA_N$ (33, 38). We show that Cu(I) transfer occurs between Atx1 and $ZiaA_N$, presumably via a more transient complex (38). Given that the Cu(I) affinities of $PacS_N$, $CtaA_N$, and $ZiaA_N$ are almost identical, the stability of the complex with Atx1 appears to be a key factor which allows the metallochaperone to distinguish between the MBDs of the copper and zinc transporters.

The Zn(II) affinity of $ZiaA_N$ is 1–2 orders of magnitude higher than those of Atx1, $CtaA_N$, and $PacS_N$, and zinc selectivity may be controlled by a trafficking protein's affinity for this metal. Tighter binding is presumably due to Asp18, adjacent to the CXXC motif, acting as an additional Zn(II) ligand in $ZiaA_N$ [the C-terminal His-rich region of $ZiaA_N$ may also influence the affinity of this Zn(II) site]. The Zn(II) affinities are much lower (≥ seven orders of magnitude) than the Cu(I) affinities, consistent with calculations on a bis-Cys model site (62), and values for the MBDs of zinc and copper transporters in *Arabidopsis thaliana* (52). The activity of ZiaA may be regulated by zinc loading of $ZiaA_N$, as suggested for the MBDs of other Zn(II) and Cd(II) ATPases (63, 64), in which case the high Zn(II) affinity of $ZiaA_N$ will ensure maximal zinc export is achieved at very low levels of this

metal [less than ~ one free zinc atom per cyanobacterial cell (24, 65)]. If Atx1 does bind zinc in vivo (39), the difference in Zn(II) affinities will drive metal transfer from Atx1 to $ZiaA_N$ and not to $PacS_N$ or $CtaA_N$, resulting in zinc export from the cytoplasm.

A cyanobacterium provides a system in which two copper-requiring target proteins, plastocyanin and cytochrome *c* oxidase, are located in the same compartment (the thylakoids). Limited information about copper acquisition within cellular compartments is currently available, but appears to involve complex processes in both prokaryotes and eukaryotes (36, 66). The presence and nature of available copper pools in the thylakoid are unknown, but tight binding of Cu(I) by the transmembrane sites of the copper transporters would make Cu(I) release to solution unlikely. Copper-plastocyanin forms only when a sufficient amount of the metal is available, and is replaced in photosynthetic electron transfer by the iron-containing cytochrome c_6 under copper depletion (29). The Cu_A-site is essential for respiration at the thylakoid membrane (67), and copper depletion will result in cytochrome *c* oxidase being preferentially copper loaded. The observation that the Cu(I) affinity of the Cu_A site is ~ 15-fold lower than that of plastocyanin indicates that under limiting conditions, the delivery of copper to these targets cannot be dictated by thermodynamics. The Sco proteins, which are essential for Cu_A assembly in mitochondrial cytochrome *c* oxidase (7), and also in most prokaryotes, are absent in cyanobacteria (68). Mechanisms to ensure copper delivery to cytochrome *c* oxidase (for respiration) and plastocyanin (for photosynthesis), possibly involving currently unidentified metallochaperones, appear to be required.

The cyanobacterial periplasm is believed to contain more labile pools of copper (and zinc) than the cytoplasm (36), providing a potential location for proteins to safely acquire this metal (11). CucA (a quercetin dioxygenase), the most abundant soluble periplasmic copper protein in *Synechocystis* is exported unfolded and metal-free to this compartment preventing the need for more labile cytosolic copper pools which could result in the mis-metallation of metalloproteins (11). However, CucA does not directly acquire copper from these periplasmic pools in vivo (36), and CtaA, $PacS_N$, and Atx1 have all been shown to be involved in copper loading CucA, although the mechanism of this process is unknown. The lack of a copper affinity gradient in the cytoplasm of *Synechocystis* (Fig. 4*A*) could therefore be important for copper redistribution, which has been highlighted as an important attribute of copper homeostasis in photosynthetic organisms (69). When this essential trace element becomes limiting, relocation between different targets or compartments can be achieved with minimal energetic expense.

Materials and Methods

Protein Purification, Preparation, Cu(I) Loading, and Quantification. WT and mutants of Atx1 and $PacS_N$, and also PacS95 (34), were purified as described previously (35). For Tyr65His $PacS_N$ induction was performed at 20 °C, and the protein was treated with 50 mM ethylenediaminetetraacetic acid to remove zinc before the final chromatographic step. $CtaA_N$ was purified from inclusion bodies (35). $ZiaA_N$ was purified using a modified version of a reported method (38), including an additional step on a metal affinity column (*SI Text*). Plastocyanin and Cu_A-CCO purifications were adapted from previously reported procedures (47, 70), as described in *SI Text*. All apo-proteins (except plastocyanin and Cu_A-CCO) were reduced with dithiotreitol, transferred to an anaerobic chamber (Belle Technology, $[O_2] \ll 2$ ppm), and exchanged into 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) pH 7.0 as described previously (22, 35). Oxidized apo-Atx1 and apo-$PacS_N$ were prepared as described in *SI Text*. Cu(I) loading of the CXXC-containing proteins was performed as described previously (35) and for plastocyanin and Cu_A-CCO the procedures are described in *SI Text*. The quantification of fully reduced apo-proteins was based on free thiol concentrations determined with 5,5′-dithiobis-(2-nitrobenzoic acid), as described previously (35). The concentration of Cu(I)-proteins and samples in which the copper-binding Cys residues were oxidized was determined as described previously (35). The concentration of Cu^{II}-plastocyanin was determined from the absorbance at 603 nm ($\epsilon_{603} = 4{,}600\ M^{-1}\ cm^{-1}$) (47).

Determination of Cu(I) and Cu(II) Affinities Using Competitive Chelators, and Cu(I) Transfers. Competition between protein and chelators was performed as described previously (22), with details provided in *SI Text*. Cu(I) affinity constants (K_b values) were determined in 20 mM Hepes pH 7.0 or 7.5 [in some cases also in 25 mM N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid pH 8.0] usually in the presence of 200 mM NaCl, using competition assays with BCS. BCA was used to determine the Cu(I) affinity of the weak site of Atx1 as described in *SI Text*. For WT Atx1, the affinity data at multiple Cu(I) concentrations were simultaneously fit to a model which includes the dimerization of Cu^{I}_{1}-Atx1 (*SI Text*). For reduced Cu_A-CCO, the Cu(I) affinity was measured assuming that the protein concentration is half the copper concentration [determined by atomic absorption spectroscopy or with BCA or BCS (*SI Text*)] and that the two copper ions have equal affinity for the protein [no Cu(I) binding cooperativity]. The Cu(II) affinity of plastocyanin was determined using His as the competing ligand (*SI Text*). Cu(I) transfer experiments were performed using a previously described approach (35) with details given in *SI Text*.

Determination of Zinc Affinities. Zinc affinity constants (K_{b1} and K_{b2} values) were determined in 25 mM Hepes pH 7.4 plus 100 mM NaCl by titrating apo-protein into Zn-RhodZin-3 and excess RhodZin-3 (49) as described in *SI Text*. Data at multiple RhodZin-3 concentrations were used to determine K_{b1} for $ZiaA_N$ and both K_{b1} and K_{b2} for Atx1, $CtaA_N$ and $PacS_N$ (*SI Text*). The Zn(II) affinity of Atx1 was also determined with MagFura-2 (50) using a similar approach (*SI Text*).

ACKNOWLEDGMENTS. We thank Christian Obinger for the genes for plastocyanin and the Cu_A domain of cytochrome *c* oxidase and Nigel Robinson for DNA encoding ZiaA. This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) grant BB/E016529.

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