ACCELERATED PUBLICATION

A novel copper site in a cyanobacterial metallochaperone

Gilles P. M. BORRELLY^{*1}, Claudia A. BLINDAUER^{†1}, Ralf SCHMID[‡], Clive S. BUTLER^{*}, Chris E. COOPER[§], Ian HARVEY[¶], Peter J. SADLER[†] and Nigel J. ROBINSON^{*2}

^{*}Cell and Molecular Biosciences, Medical School, University of Newcastle, Newcastle upon Tyne NE2 4HH, U.K., [†]School of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, U.K., [‡]Institute of Cell and Animal Population Biology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, U.K., [§]Department of Biological Sciences, University of Essex, Colchester, Essex CO4 3SQ, U.K., and [¶]CCLRC (Council for the Central Laboratory of the Research Councils) Daresbury Laboratory, Warrington, Cheshire WA4 4AD, U.K.

The thylakoid lumen of the cyanobacterium *Synechocystis* PCC 6803 is supplied with copper via two copper-transporting ATPases and a metallochaperone intermediary. We show that the copper site of this metallochaperone is unusual and consists of two cysteine residues and a histidine imidazole located on structurally dynamic loops. Substitution of this histidine residue enhances bacterial

two-hybrid interaction with the cytosolic copper exporter, but not the copper importer, suggesting that the interacting surfaces are distinct, with implications for metal transfer.

Key words: Atx1, copper metallochaperone, *Synechocystis* PCC6803, thylakoid.

INTRODUCTION

Cyanobacteria and plants contain internal thylakoid compartments where copper proteins that are vital for respiratory and/or photosynthetic electron transport, such as plastocyanin, reside (Figure 1A). Plastocyanin is imported into the thylakoid via a secretory system that carries unfolded proteins, thereby demanding a separate pathway for delivery of copper into this compartment in order to form the holoprotein. In cyanobacteria, such as *Synechocystis* PCC 6803, the thylakoid also contains other copper-requiring proteins, such as cytochrome *c* oxidase. We established previously (Figure 1A) that copper is supplied to thylakoid proteins in *Synechocystis* PCC 6803 via the metallochaperone Atx1 (related to *Enterococcus hirae*, *Bacillus subtilis* CopZ, *Saccharomyces cerevisiae* ATX1 and human Atox1 [1–4]), along with two P₁-type ATPases, CtaA and PacS [5,6]. In *Synechocystis* PCC 6803, in common with *Chlamydomonas reinhardtii* [7], plastocyanin is replaced by cytochrome *c*₆ for photosynthetic electron transport under copper-deficiency conditions. However, mutants in CtaA, PacS and Atx1 show impaired switching to plastocyanin for photosynthetic electron transport in copper-replete medium, accumulate negligible amounts of plastocyanin transcripts and have low cytochrome *c* oxidase activity in isolated membranes, all phenotypes attributable to impaired inward supply of copper to the thylakoid [5,6]. PacS has been localized to thylakoid membranes in *Synechococcus* PCC 7942 [8,9]. PacS mutants are copper-sensitive, but are silver-resistant, a phenotype also consistent with a transporter that delivers copper, or its analogue, silver, to silver-sensitive copper proteins, and CtaA mutants accumulate less total copper [5]. A chloroplast-targeted ATPase, functionally analogous to CtaA, has recently been identified in *Arabidopsis thaliana* [10]. It has been shown to be required for chloroplast copper accumulation and for plastocyanin maturation, and also for Cu/Zn superoxide dismutase activity within the stroma of this organelle.

Metal-transporting P₁-type ATPases typically contain a cytosolic N-terminal domain that forms a ferredoxin-like fold,

sometimes repeated [11,12]. Structurally similar metallochaperones, such as Atx1, interact with this domain, and hypothetical mechanisms of copper transfer from metallochaperones have been proposed in which Cu⁺ passes between the binding sites of the two proteins via ligand-substitution reactions [13]. *Synechocystis* PCC 6803 is currently the only organism known to contain a metallochaperone that interacts with both cytosolic copper-importing and -exporting P₁-type ATPases. Efficient delivery of copper to the thylakoid requires specific recognition between copper chaperone and ATPases, and efficient transfer of the copper from the donor to the acceptor. In the present study, we investigate the structure of the copper site of *Synechocystis* PCC 6803 Atx1 and the recognition of the two different ATPases.

MATERIALS AND METHODS

Bacterial strains and DNA manipulations

Escherichia coli strains JM101, SURE, BL21(DE3), were grown in Luria–Bertani (LB) medium. DNA manipulations were performed as described in [6]. The sequences of all PCR and QuikChange (Stratagene) products were validated by sequence determination (ABI Prism 377 DNA sequence analyser and Applied Biosystems 800 Molecular Biology Workstation for sample preparation). All buffers were prepared using Milli-Q deionized water. Hepes and Tris buffer salts were obtained from Melford Laboratories (Chelsworth, Ipswich, U.K.) and Boehringer Mannheim. All chromatography materials were purchased from Amersham Biosciences. Other reagents were obtained from Sigma.

Production and quantification of recombinant Atx1

pETATX1 was generated as described in [6] and recombinant protein produced in *E. coli* [BL21(DE3)] exposed to copper (0.5 mM) in LB medium. Lysates (2.5 ml) were applied to a Sephadex G-75 column (2.5 cm × 50 cm), and fractions (5 ml),

Abbreviations used: 2D, two-dimensional; 3D, three-dimensional; HSQC, heteronuclear single-quantum coherence; LB, Luria–Bertani; NOE, nuclear Overhauser effect.

¹ These authors made an equal contribution to this work.

² To whom correspondence should be addressed (e-mail n.j.robinson@ncl.ac.uk).

Figure 1 Copper trafficking in *Synechocystis* PCC 6803 and Atx1 structural elements

(A) Atx1 interacts with cytosolic N-terminal domains of the copper importer CtaA and the thylakoid importer PacS, delivering copper to plastocyanin and cytochrome *c* oxidase. Plastocyanin shuttles electrons between the cytochrome *b₆f* complex and Photosystem I. (B) $\text{CH}(\alpha)$ chemical-shift deviations from random coil ($\Delta\delta$ ppm, top) and selected NOE connectivities (bottom) indicative of a ferredoxin-like fold. (C) Experimental EXAFS (black) and single-scattering theoretical model (grey) for 2.2 (± 0.5) thiols at 2.24 (± 0.01) $\text{\AA}$, and 0.8 (± 0.3) nitrogen atoms at 1.93 (± 0.02) $\text{\AA}$. k , wavenumber; R , distance.

eluted in 25 mM Tris/HCl, pH 7.0, were analysed for total protein and for copper by atomic absorption spectroscopy. Pooled copper-peak fractions were applied to a Q-Sepharose column and were sequentially eluted with 25 mM Tris/HCl, pH 7.0, followed by 0.7 M NaCl and 25 mM Tris/HCl, pH 7.0. Fractions were again analysed for copper and protein, and copper-containing fractions were desalted on a Sephadex G-25 column in 25 mM Tris/HCl, pH 7.0. A single prominent band of the anticipated size was detected by PAGE, and the N-terminal ten residues of the sequence (Beckman LF 3000 protein sequencer) confirmed the identity of the purified protein. A further aliquot was hydrolysed and analysed by Alta Bioscience (University of Birmingham, Birmingham, U.K.) for amino acid composition to allow calibration of colorimetric estimations of Atx1 using Coomassie Blue R250 (correction factor 2.46 relative to BSA). ^{15}N -labelled Atx1 was prepared from cells grown in minimal medium containing M9 salts supplemented with $^{15}\text{NH}_4\text{Cl}$ and exposed to copper (0.02 mM).

Preparation of apo- and copper-bound Atx1 for physical analyses

Atx1 was incubated with reductant (10 mM dithiothreitol), transferred to a N_2 atmosphere chamber, and fractionated on a Sephadex G-25 column equilibrated in, and eluted with HCl, pH 2.0, with no added reductant. If metallated protein was required, recovered material was exposed to an equivalent molar concentration of CuCl before the addition of 1 M Hepes, pH 7.0

(to return to pH 7.0). The sample was further concentrated on a Q-Sepharose column and sequentially eluted with 25 mM Hepes, pH 7.0, followed by 0.7 M NaCl/25 mM Hepes, pH 7.0. Fractions were again analysed for protein, and selected fractions were desalted on Sephadex G-25 and eluted with the appropriate buffer (see below). If the apoprotein was required, the same procedure was used, but without the addition of copper to the protein. All samples for EPR, EXAFS, ^1H -NMR and ^{15}N -NMR were prepared under anaerobic conditions in a N_2 chamber and sealed before subsequent analyses. EPR samples (<0.3 mM) were prepared in 100 mM Hepes, pH 7.0. EXAFS samples (0.6 mM) were prepared in 100 mM sodium phosphate, pH 7.0; ^1H -NMR samples (0.6 mM) were prepared in 100 mM sodium phosphate, pH 7.0, and 10% $^2\text{H}_2\text{O}$; ^{15}N -NMR samples (0.2 mM) were prepared in 50 mM sodium phosphate, pH 7.0, and 10% $^2\text{H}_2\text{O}$.

NMR spectroscopy

All NMR data for sequential assignment and shift-mapping were acquired at 298 K on a Bruker AVANCETM 600 spectrometer, operating at 599.87 MHz for protons, equipped with a triple-resonance-triple axis (TXI) probe head with Z gradients. ^1H resonances were referenced to the residual HDO peak. The XWINNMR software package on SGI Workstations was used for data processing. Resonance assignment was carried out within the program SPARKY [14].

Two-dimensional (2D) homonuclear [^1H , ^1H] double-quantum-filtered 2D correlation spectra, total correlation spectra (65 ms spin-lock time) and nuclear Overhauser effect (NOE) spectra (100 ms mixing time) were acquired into $2\text{ k} \times 512$ increments [phase-sensitive hypercomplex time-proportional phase incrementation (States-TPPI) acquisition]. Water suppression was effected by excitation sculpting with gradients. All data for the 2D homonuclear spectra were zero-filled in F1 to 2 k, and apodized with squared sine-bell functions before Fourier transformation. Sensitivity-enhanced 2D [^1H , ^{15}N] heteronuclear single-quantum coherence (HSQC) and three-dimensional (3D) [^1H , ^1H , ^{15}N] TOCSY-HSQC (60 ms mixing time) and NOESY-HSQC (100 ms mixing time) spectra were acquired on uniformly ^{15}N -labelled samples. For the 2D shiftmap, 2 k data points were acquired into 96 increments (phase-sensitive acquisition using Echo/Antiecho-TPPI gradient selection), and processed into $2\text{ k} \times 512$ data points. The 3D data sets were acquired with 2 k data points into 160 increments in F3 and 40 increments in F1. The data were processed into $1\text{ k} \times 256 \times 64$ data points. ^{15}N -decoupling was achieved using the GARP (Gly-Ala-Arg-Pro) sequence, and the water signal was suppressed by moderate presaturation.

Comparative modelling

All NOE cross-peaks pertaining to protons from Ile¹⁰ to Ala¹⁶, and from Gly⁶⁰ to Val⁶³, were integrated. Apart from the residues mentioned, the protons involved in these cross-peaks were from residues Glu¹⁷ to Thr²⁰, Leu³⁶, and Ile⁵⁶ to Ala⁵⁹. This procedure yielded a total of 108 NOE-derived restraints; redundant distance restraints (e.g. vicinal intraresidue distances) were not incorporated (restraint statistics are available in supplementary Table 1 at <http://www.BiochemJ.org/bj/378/bj3780293add.htm>). The picked peaks were integrated, and upper distance limits of 3, 4 and 5 $\text{\AA}$ (1 $\text{\AA}$ = 0.1 nm) were assigned according to the peak volumes. When NMR-degenerate methyl protons were involved, the restraint was defined in relation to the respective C atom, adding 1 $\text{\AA}$ (1 $\text{\AA}$ = 0.1 nm). For the diastereomeric methylene protons of Cys¹² and Cys¹⁵, both assignment possibilities were investigated

in separate runs, and the combination yielding models with the lower target functions was used in the final run.

Comparative modelling was carried out with the program MODELLER [15], using the solution structure of copper-loaded ATX1 from yeast as a template {Protein Data Bank (PDB) code 1FD8; [16]}. This template was chosen because it refers to a monomeric copper chaperone, as opposed to the only available Cu^+ -containing X-ray structure [17], which is for a dimer. It also has the advantage that, in the pairwise alignment, only one gap, situated in loop 2 (after helix 1), is present. The other monomeric, Cu^+ -containing NMR structure, CopZ from *Bacillus subtilis* (PDB code 1K0V; [2]) is available only as an ensemble of 20 NMR structures. Although the sequential similarity of this protein to our target sequence is slightly higher than that of yeast ATX1, we did not attempt to generate an average structure or to use a representative conformer.

Of the 108 NMR restraints, between none and three were violated by more than 0.5 Å in each of the 100 final models. The models were inspected manually to select representative best models based on criteria such as the MODELLER 'objective function', violation of NMR-derived restraints and geometric features. The selected models were submitted to the program SCWRL [18], which optimizes side-chain conformations. Alterations of the metal ligand residues (Cys¹², Cys¹⁵ and His⁶¹) and of Ile¹⁰ and Ile⁵⁶ were excluded in the SCWRL run. Inspection of the various modelling stages revealed that the side-chain conformations of all other residues involved in NMR-derived restraints were not changed significantly in the SCWRL run, and therefore were accepted as such.

Finally, the Cu^+ ion was incorporated into the models, initially bound only to the thiol groups of Cys¹² and Cys¹⁵. The structure was energy-minimized employing the TRIPOS force field, after specific bond and angle parameters for Cu^+ had been implemented. Cu-ligand distances were taken from the EXAFS data (2.24 Å), and the ideal S-Cu-S bond angle was initially set to 180°, using a weak (0.042 kJ/mol) angle bend constant. The minimization protocol applied 100 steps of the Powell algorithm to the whole molecule, to remove clashes and bad geometries; the NMR restraints were also taken into account. The final model was generated by introducing a Cu–N ϵ bond, constrained to 1.93 Å, and a further 100 steps of energy minimization. In this model, no NMR restraint is violated by more than 0.5 Å. Throughout the modelling procedure, models were manually inspected as well as checked by the 'biotech validation suite for protein structures' (<http://biotech.embl-ebi.ac.uk:8400/>) which incorporates WHATIF [19] and PROCHECK [20].

X-ray absorption spectroscopy

X-ray spectra were recorded at the Cu *k*-edge on EXAFS station 9.3 of the Synchrotron Radiation Source [CCLRC (Council for the Central Laboratory of the Research Councils) Daresbury Laboratory] operating at 2 GeV. A silicon (111) double crystal monochromator and vertically focusing mirror (for harmonic rejection) were used. A total of 28 scans of 30 min were collected at 12 K (helium cryostat) in fluorescence mode using a 13-element solid-state germanium detector, and in *k* space using a *k*³-weighted regime for counting time. Data reduction and analysis were performed using standard procedures [21] and EXCURV98 [22].

Generation of bacterial two-hybrid constructs containing *pacS_N*, *ctaA_N*, *atx1*, *atx1H₆₁R*, *copA_N* and *copZ*

pTRGCTAA_N, pTRGPACS_N and pBTATX1 were generated as described in [6]. Primers 5'-GCCTCGGCGGGTCGCGAAGTT-

GAGTG-3' and 5'-CACTCAACTTCGCGACCCGCCGAGGC-3' were used with the pBTATX1 template to convert codon 61 from histidine into arginine via QuikChange site-directed mutagenesis, according to the manufacturer's protocols with introduction of an *Nru*I site. The resulting plasmid was designated pBTATX1H₆₁R. pTRGCOPA_N and pBTCOPZ were obtained as described in [23].

β-Galactosidase assays

These were performed via a microtitre-plate-based procedure [24]. Cells were used when the *D*₅₉₅ was 0.6, following 20 h of growth at 30 °C. Data shown in each Figure relate to the results of a replicated experiment performed on a single day. Equivalent experiments were repeated on separate days with separate transformants generated from independent transformations, giving equivalent trends.

RESULTS AND DISCUSSION

Synechocystis Atx1 adopts a ferredoxin-fold

Atx1 from *Synechocystis* PCC 6803 shares 20–28% sequence identity with known copper chaperones. We carried out 2D and 3D ¹H/¹⁵N-NMR studies of apo- and Cu^+ -loaded Atx1 from *Synechocystis* PCC 6803. Sequential assignment, chemical shift indexing of CH(α) ¹H shifts and backbone NOEs reveal a βαβα fold, including connectivities from strand 3 to strands 1 and 2, characteristic of the expected ferredoxin fold, but with truncation of β-strand 4 (Figure 1B). Most importantly, His⁶¹ is located in loop 5, raising the possibility that this residue is proximal to the Cu^+ site.

The copper site includes loop-5 His⁶¹ in addition to loop-1 cysteine residues

We probed the copper co-ordination sphere using EXAFS, and the spatial arrangements of the residues in loops 1 and 5 in Cu-loaded Atx1 were investigated by 2D NOESY NMR spectroscopy.

Synechocystis PCC 6803 Atx1 binds Cu^+ (see Supplementary Figure 1 at <http://www.BiochemJ.org/bj/378/bj3780293add.htm>). A sample concentration of 0.6 mM was used for EXAFS, since the protein forms oligomers at higher concentrations. As this relatively low concentration limited the achievable data range and signal/noise ratio, only first-shell, single-scattering analysis was possible. A single-scattering theoretical model (shown in Figure 1C) gives copper co-ordinated to 0.8 (± 0.3) nitrogen atoms at 1.93 (± 0.02) Å, and 2.2 (± 0.5) sulphur atoms at 2.24 (± 0.01) Å with Debye–Waller factors (2σ²) of 0.009 (± 0.003) and 0.023 (± 0.002) Å² respectively. The Fit Index was 0.548, and the *R*-factor was 23.5%. With histidine ligation, peaks were usually observed in the Fourier transform at approx. 3 and 4 Å, corresponding to the other atoms within the imidazole ring. The experimental Fourier transform showed a 3 Å peak, but no 4 Å peak. Using multiple scattering and an ideal imidazole, the 3 Å peak was simulated well (Debye–Waller factor 0.013 Å²), and the *R*-factor was decreased slightly, to 22.9%. Modelling a 4 Å peak (using a Debye–Waller factor of 0.020 Å²) showed that the limited signal/noise ratio can explain the absence of this feature. A Cu–S (thiolate) distance of 2.24 Å is characteristic of trigonal Cu^+ , and thus the presence of a third ligand is confirmed.

Structural models of *Synechocystis* PCC 6803 Atx1 were generated by comparative modelling using yeast Cu^+ -Atx1 as a template. The incorporation of 108 NOE-derived distance restraints for loops 1 and 5 (Figure 2A) in the modelling procedure

Figure 2 NMR-refined structural model of *Synechocystis* PCC 6803 Atx1

(A) NMR-derived distance restraints (magenta) used to define the region of the Cu^+ site. Note the numerous connectivities for His⁶¹ (H82). (B) Ribbon diagram of *Synechocystis* PCC 6803 Cu^+ -Atx1, generated using MOLMOL [26], showing the co-ordinated copper atom (green) and ligands from loops 1 (Cys¹² and Cys¹⁵, yellow) and 5 (His⁶¹ imidazole- N_ϵ , dark blue).

furnished exclusively models with His⁶¹ oriented towards the two cysteine residues at distances compatible with a trigonal Cu^+ site. Crucially, this is not the case in structures modelled without NMR restraints (see Supplementary Figure 2 at <http://www.BiochemJ.org/bj/378/bj3780293add.htm>). The final energy-minimized model which incorporates Cu^+ bound to His⁶¹ (Figure 2B) is consistent with the observed NOEs.

Cu binding strongly influences His⁶¹

^1H , ^{15}N shift-mapping (Figures 3A and 3B) reveals substantial differences between apo- and Cu^+ -Atx1 in loops 1 and 5, crucially with dramatic Cu^+ -induced changes for His⁶¹. In the $[\text{H}^1, \text{N}^{15}]$ HSQC spectrum of Cu^+ -Atx1, resonances are sharp and well-resolved, whereas several peaks in the apo-Atx1 spectrum are broadened, doubled or weak. NH resonances for residues Thr⁹-Glu¹³ were absent from the spectra of apo-Atx1, preventing a complete sequential assignment and indicating that the copper site is not pre-organized, similar to spectral observations for CopZ from *B. subtilis*, but converse to *Ent. hirae* [25]. A comparison of 2D NOESY spectra of Cu^+ - and apo-Atx1 (Figure 3C) reveals that all long-range NOE cross-peaks identified for the H82 proton of His⁶¹ in Cu^+ -Atx1 are absent in the apo-Atx1 NOESY spectrum. His⁶¹ cross-peaks are much weaker in the apo-Atx1 spectra, and at least three conformers can be distinguished. These

Figure 3 Structural dynamics of Atx1

(A) Superposition of 2D $[\text{H}^1, \text{N}^{15}]$ HSQC spectra of Cu^+ -Atx1 (blue) and apo-Atx1 (red). Black labels, residues not influenced by copper binding; green boxes, residues undergoing significant changes in chemical shift; blue boxes, residues observed only for the Cu^+ -bound form (Thr⁹-Glu¹³). (B) ^1H and ^{15}N amide chemical-shift differences, calculated as shown, between Cu^+ - $[\text{N}^{15}]$ Atx1 and apo- $[\text{N}^{15}]$ Atx1 with observed elements of secondary structure below. Cross-peaks for loop 1 were absent in apo-Atx1 spectra. (C) 2D ^1H , ^1H -NMR strips for His⁶¹ H82 proton. H82 shows many more intra- and inter-residue NOE cross-peaks for Cu^+ -Atx1 (left) than apo-Atx1 (right), plus peak splitting in apo-Atx1.

observations indicate that His⁶¹ is well-ordered in Cu^+ -Atx1, but conformationally flexible in apo-Atx1.

Substitution of His⁶¹ enhances interaction with PacS, but not CtaA

We used bacterial two-hybrid assays to investigate the contribution of His⁶¹ to the magnitude of interactions with the two different ATPases. Previous assays [6] detected interaction between Atx1 and the N-terminal domain of the copper-accepting ATPase, PacS_N. The present experiments (Figure 4A) show that although the electrostatic properties of *Synechocystis* Atx1 are expected to be similar to *B. subtilis* CopZ, the interaction of the chaperone with its ATPase is highly specific, with no detectable cross-interactions between Atx1 and *B. subtilis* CopA_N, or between CopZ and PacS.

Mutation of His⁶¹ in Atx1 to a non-co-ordinating residue, arginine, had no effect upon bacterial two-hybrid interaction with CtaA_N, but exclusively enhanced interaction with PacS_N (Figure 4B). The experiment formally demonstrates that the residue at position 61 can interact differently with the complementary surfaces of the two different copper-ATPases.

In conclusion, we have shown that the Atx1 copper metallochaperone from *Synechocystis* PCC 6803 has an atypical Cu^+ site in comparison with related proteins from other species (Figure 2B). We propose that this is a special adaptation to allow both removal of Cu^+ from one P₁-type ATPase in addition to release of Cu^+ to another such protein in the cyanobacterium. Entry of His⁶¹ into a shared site with CtaA_N will promote Cu^+ acquisition by the metallochaperone, while displacement upon

Figure 4 Bacterial two-hybrid assays

(A) pBT (bait) or pTRG (target) (BacterioMatch™, Stratagene) alone, or with one or both plasmids containing indicated translational fusions. Elevated reporter activity is detected with complementary partners, CopZ–CopA_N from *B. subtilis* or Atx1–PacS_N from *Synechocystis* PCC 6803. No cross-species interactions were detected. (B) Conversion of Atx1–His⁶¹ into arginine exclusively enhances interaction with PacS_N (note scale); controls (from left) pBT–pTRG, Atx1–pTRG, Atx1–H₆₁R–pTRG, pBT–PacS_N, pBT–CtaA_N. All assays were performed in triplicate on a single occasion ($\pm$ S.E.M.) and repeated further on two different occasions using three independent transformations to obtain similar trends (results not shown). Day-to-day variation impairs direct comparison of absolute values between data sets (note the magnitude of the controls are twice those in A). (C) Copper-transfer model. Black ovals represent Atx1 with cysteine (CXXC) associated with loop 1 and His⁶¹ (H₆₁) within a dynamic C-terminal region. The latter becomes less dynamic when bound to copper. Light grey ovals represent the ferredoxin-fold subdomain of CtaA, unfilled ovals the ferredoxin-fold subdomain of PacS. In this model, an imidazole nitrogen atom of His⁶¹ enters a shared copper site in Atx1–CtaA complexes, favouring metal-retention by Atx1 at dissociation. In an Atx1–PacS complex it is proposed that the C-terminal region of Atx1, and hence His⁶¹, is displaced by the contact surface of PacS, encouraging copper release from Atx1. This could encourage reversal of the vectors for Atx1 copper-transfer at the cytosolic importer and exporter.

interaction with PacS_N will promote release (Figure 4C). This model is consistent with the proposal [16] that outward movement of loop 5 is a conserved mechanism that triggers Cu⁺ release from related proteins such as human Atox1 and other similar metallochaperones that have a digonal site.

This work was supported by the BBSRC (Biotechnology and Biological Sciences Research Council) Plant and Microbial Sciences Committee (grants to N. J. R. and G. P. M. B.) and Wellcome Trust (Edinburgh Protein Interaction Centre and Essex EPR facility). We thank Dr Dimitri Svistunenko (University of Essex) for assistance with the EPR spectroscopy.

REFERENCES

- Odermatt, A. and Solioz, M. (1995) Two *trans*-acting metalloregulatory proteins controlling expression of the copper-ATPases of *Enterococcus hirae*. *J. Biol. Chem.* **270**, 4349–4354
- Banci, L., Bertini, I., Del Conte, R., Markey, J. and Ruiz-Duenas, F. J. (2001) Copper trafficking: the solution structure of *Bacillus subtilis* CopZ. *Biochemistry* **40**, 15660–15668

- Lin, S. and Culotta, V. (1995) The ATX1 gene of *Saccharomyces cerevisiae* encodes a small metal homeostasis factor that protects cells against reactive oxygen toxicity. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3784–3788
- Klomp, L. W. J., Lin, S.-J., Yuan, D. S., Klausner, R. D., Culotta, V. C. and Gitlin, J. D. (1997) Identification and functional expression of HAH1, a novel human gene involved in copper homeostasis. *J. Biol. Chem.* **272**, 9221–9226
- Totter, S., Rich, P. R., Rondet, S. A. M. and Robinson, N. J. (2001) Two Menkes-type ATPases supply copper for photosynthesis in *Synechocystis* PCC 6803. *J. Biol. Chem.* **276**, 19999–20004
- Totter, S., Rondet, S. A. M., Borrelly, G. P. M., Robinson, P. J., Rich, P. R. and Robinson, N. J. (2002) A copper metallochaperone for photosynthesis and respiration reveals metal-specific targets, interaction with an importer, and alternative sites for copper acquisition. *J. Biol. Chem.* **277**, 5490–5497
- Merchant, S. and Bogorad, L. (1987) Metal ion regulated gene expression: use of a plastocyanin-less mutant of *Chlamydomonas reinhardtii* to study the Cu(II)-dependent expression of cytochrome *c*-552. *EMBO J.* **6**, 2531–2535
- Phung, L., Ajilani, G. and Haselkorn, R. (1994) P-Type ATPase from the cyanobacterium *Synechococcus* 7942 related to the human Menkes and Wilson disease gene products. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9651–9654
- Kanamaru, K., Kashiwagi, S. and Mizuno, T. (1994) A copper-transporting P-type ATPase found in the thylakoid membrane of the cyanobacterium *Synechococcus* species PCC 7942. *Mol. Microbiol.* **13**, 369–377
- Shikanai, T., Müller-Moulé, P., Munkage, Y., Niyogi Krishna, K. and Pilon, M. (2003) PAA1, a P-type ATPase of *Arabidopsis*, functions in copper transport in chloroplasts. *Plant Cell* **15**, 1333–1346
- Soliz, M. and Vulpe, C. (1996) CPx-type ATPases: a class of P-type ATPases that pump heavy metals. *Trends Biochem. Sci.* **21**, 237–241
- Lutsenko, S. and Kaplan, J. H. (1996) P-type ATPases. *Trends Biochem. Sci.* **21**, 467
- Pufahl, R. A., Singer, C. P., Peariso, K. L., Lin, S. J., Schmidt, P. J., Fahrni, C. J., Culotta, V. C., Penner-Hahn, J. E. and O'Halloran, T. V. (1997) Metal ion chaperone function of the soluble Cu(I) receptor Atx1. *Science* **278**, 853–856
- Goddard, T. D. and Kneller, D. G. (2000) SPARKY3, University of California, San Francisco
- Marti-Renom, M. A., Stuart, A. C., Fiser, A., Sanchez, R., Melo, F. and Sali, A. (2000) Comparative protein structure modeling of genes and genomes. *Annu. Rev. Biophys. Biomol. Struct.* **29**, 291–325
- Arnesano, F., Banci, L., Bertini, I., Huffman, D. L. and O'Halloran, T. V. (2001) Solution structure of the Cu(I) and Apo forms of the yeast metallochaperone, Atx1. *Biochemistry* **40**, 1528–1539
- Wernimont, A. K., Huffman, D. L., Lamb, A. L., O'Halloran, T. V. and Rosenzweig, A. C. (2000) Structural basis for copper transfer by the metallochaperone for the Menkes/Wilson disease proteins. *Nat. Struct. Biol.* **7**, 766–771
- Bower, M. J., Cohen, F. E. and Dunbrack, Jr, R. L. (1997) Prediction of protein side-chain rotamers from a backbone-dependent rotamer library: a new homology modeling tool. *J. Mol. Biol.* **267**, 1268–1282
- Vriend, G. (1990) WHAT IF: a molecular modeling and drug design program. *J. Mol. Graphics* **8**, 52–56
- Laskowski, R. A., MacArthur, M. W., Moss, D. S. and Thornton, J. M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* **26**, 283–291
- Guo, M., Harvey, I., Yang, W., Coghill, L., Campopiano, D. J., Parkinson, J. A., MacGillivray, R. T. A., Harris, W. R. and Sadler, P. J. (2003) Synergistic anion and metal binding to the ferric ion-binding protein from *Neisseria gonorrhoeae*. *J. Biol. Chem.* **278**, 2490–2502
- Binstead, N. (1998) EXCURV98: Daresbury Laboratory Computer Program, Daresbury Laboratory, Warrington
- Radford, D. S., Kihlken, M. A., Borrelly, G. P. M., Harwood, C. R., Le Brun, N. E. and Cavet, J. S. (2003) CopZ from *Bacillus subtilis* interacts *in vivo* with a copper exporting CPx-type ATPase CopA. *FEMS Microbiol. Lett.* **220**, 105–112
- Rutherford, J. C., Cavet, J. S. and Robinson, N. J. (1999) Cobalt-dependent transcriptional switching by a dual-effector MerR-like protein regulates a cobalt-exporting variant CPx-type ATPase. *J. Biol. Chem.* **274**, 25827–25832
- Wimmer, R., Herrmann, T., Solioz, M. and Wüthrich, K. (1999) NMR structure and metal interactions of the CopZ copper chaperone. *J. Biol. Chem.* **274**, 22597–22603
- Koradi, R., Billeter, M. and Wüthrich, K. (1996) MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graphics* **14**, 51–55, 29–32

Received 31 October 2003/22 December 2003; accepted 7 January 2003

Published as BJ Immediate Publication 7 January 2003, DOI 10.1042/BJ20031669