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A novel copper site in a cyanobacterial metallochaperone

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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 reinhardii [7], plastocyanin is replaced by cytochrome c6 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),
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 Sephadex G-25 and eluted with the appropriate buffer (see below). 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 15N-NMR were prepared under anaerobic conditions in a N2 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% 2H2O; 15N-NMR samples (0.2 mM) were prepared in 50 mM sodium phosphate, pH 7.0, and 10% 2H2O.

NMR spectroscopy

All NMR data for sequential assignment and shift-mapping were acquired at 298 K on a Bruker AVANCE™ 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 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 k x 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,15N] heteronuclear single-quantum coherence (HSQC) and three-dimensional (3D) [1H,1H,15N] TOCSY-HSQC (60 ms mixing time) and NOESY–HSQC (100 ms mixing time) spectra were acquired on uniformly 15N-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 k x 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 k x 256 x 64 data points. 1H-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 Gly46 to Val50, were integrated. Apart from the residues mentioned, the protons involved in these cross-peaks were from residues Glu17 to Thr20, Leu16, and Ile46 to Ala50. This procedure ( restraint statistics are available in supplementary Table I at http://www.BiochemJ.org/bj/378/bj3780293add.htm). The picked peaks were integrated, and upper distance limits of 3, 4 and 5 Å (1 Å = 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 Å (1 Å = 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 (Cys\(^{12}\), Cys\(^{15}\) and His\(^{61}\)) and of Ile\(^{68}\) and Ile\(^{69}\) 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 Cys\(^{12}\) and Cys\(^{15}\). 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–Ne 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-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 pacSN, ctaAN, atx1, atx1H61R, copAN and copZ

pTRGCTAAN\(_R\), pTRGPACSN\(_R\) and pBTAX1 were generated as described in [6]. Primers 5’-GGCTCGGCCGGGTCGCGAAGTT- PACSN 3’ and 5’-GAGTG-3’ and 5’-CACCTAATTTGCACCGCCGAAGC-3’ were used with the pBTAX1 template to convert codon 61 from histidine into arginine via QuikChange site-directed mutagenesis, according to the manufacturer’s protocols with introduction of an NruI site. The resulting plasmid was designated pBTAX1H61R. pTRGCOPAN\(_R\) 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 \(1^H/\)N-NMR studies of apo- and Cu\(^{+}\)-loaded Atx1 from *Synechocystis* PCC 6803. Sequential assignment, chemical shift indexing of CH\((\alpha)\) \(^1\)H shifts and backbone NOEs reveal a \(\beta\beta\beta\alpha\) fold, including connectivities from strand 3 to strands 1 and 2, characteristic of the expected ferredoxin fold, but with truncation of \(\beta\)-strand 4 (Figure 1B). Most importantly, His\(^{61}\) is located in loop 5, raising the possibility that this residue is proximal to the Cu\(^{+}\) site.

The copper site includes loop-5 His\(^{61}\) 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/b3780293add.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 (\(2\sigma\)) of 0.009 (± 0.003) and 0.023 (± 0.002) Å\(^2\) 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 Å\(^2\)), and the R-factor was decreased slightly, to 22.9 %. Modelling a 4 Å peak (using a Debye–Waller factor of 0.020 Å\(^2\)) 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
furnished exclusively models with His61 oriented towards the two cysteine residues at distances compatible with a trinodal 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,¹⁵N 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 [¹H,¹⁵N] 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. hiraee* [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 H₅₂ 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 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, PacS₅. 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* CopA₅, 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 CtaA₅, but exclusively enhanced interaction with PacS₅ (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 CtaA₅ will promote Cu⁺ acquisition by the metallochaperone, while displacement upon
interaction with PacS0 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.

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REFERENCES


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