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Mass spectrometric studies of Cu(I)-binding to the N-terminal domains of *B. subtilis* CopA and influence of bacillithiol

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ABSTRACT

CopA is a Cu(I)-exporting transmembrane P_{1B}-type ATPase from *Bacillus subtilis*. It contains two N-terminal cytoplasmic domains, CopAab, which bind Cu(I) with high affinity and to form higher-order complexes with multiple Cu(I) ions. To determine the precise nature of these species, electrospray ionisation mass spectrometry (ESI-MS) under non-denaturing conditions was employed. Up to 1 Cu per CopAab resulted in Cu coordination to one or both CopAab domains. At > 1 Cu/CopAab, two distinct dimeric charge state envelopes were observed, corresponding to distinct conformations, each with $Cu_6(CopAab)_2$ as its major form. The influence of the physiologically relevant low molecular weight thiol bacillithiol (BSH) on Cu(I)-binding to CopAab was assessed. Dimeric CopAab persisted in the presence of BSH, with previously undetected $Cu_7(CopAab)_2$ and $Cu_6(CopAab)_2(BSH)$ forms apparent.

1. Introduction

Copper is an essential metal transported by dedicated copper chaperone proteins that deliver it to copper-requiring enzymes and detoxify it when in excess [1]. The latter process, which involves copper export across the cytoplasmic membrane, is essential to cell survival due to the potentially toxic nature of elevated cellular copper levels. Translocation of Cu(I) across the *Bacillus subtilis* cell membrane takes place *via* the integral membrane P-type ATPase, CopA [2,3].

P-type ATPase protein family members typically contain a central core of transmembrane helices, an actuator domain (A-domain) and an ATP-binding domain (ATP-BD), comprising two distinct sub-domains involved in nucleotide-binding (N) and phosphorylation (P) [4]. The P-type ATPases are further divided into subclasses based upon several structural features [5]. The P_{1B}-type ATPases contain an additional two helices in the transmembrane core, and are also distinguished by possession of a Cys-Pro-Cys (CPC) transmembrane metal-binding motif located in helix 4, and one or more soluble cytoplasmic domains (MBDs) at the N- and, sometimes, C-termini. The two N-terminal cytoplasmic MBDs in *B. sutbilis* CopA, CopAab, possess the same βαββαβ fold as that of the *B. subtilis* Atx1 (antioxidant protein 1, a eukaryotic copper chaperone)-like metallochaperone CopZ. Each individual domain, CopAa and CopAb, contains a conserved MXCXXC copper-binding motif with two solvent-exposed cysteines located between the end of the first loop and the beginning of the first α-helix [6].

The exact role of MBDs in ATPase function is unclear. It has been proposed that, in some cases, they are required for copper transfer activity [7,8], but in others it has been shown that they are not [9], and available structural data indicate that they are too remote from the transmembrane transport site to be directly involved. However, protein-protein interaction and protein phosphorylation studies, together with their proximity to the actuator and nucleotide-binding domains, have led to a model in which the MBDs perform a regulatory role through interactions with these domains that modulates not only Cu(I) transport activity but (in eukaryotes) also trafficking between sub-cellular locations [10–13].

Cu(I) binding to the MBDs of CopA, both as isolated domains (CopAa and CopAb) [14,15] and as a two domain protein, CopAab [16], has been characterised previously using spectroscopic and bioanalytical techniques. Initial binding of Cu(I) to CopAab occurs with high affinity ($K \approx 4 \times 10^{17}\,M^{-1}$) [17], and CopAab was shown to undergo Cu(I)-mediated dimerization above 1 Cu/protein. Data from AUC and SEC suggested that a small portion of dimer (≈5%) was present at 1 Cu/protein and by 2 Cu/protein the association state was fully dimeric [16]. Dimeric CopAab was also shown to be capable of accommodating multiple Cu(I) ions. Copper binding by CopAab at a ratio of 2 Cu/protein resulted in a highly luminescent species, predicted to be due to

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a $Cu_4(CopAab)_2$ form [17]. However, luminescence quenching beyond a ratio of 2:1 suggested that dimeric CopAab may be able to bind additional Cu(I) ions [17]. Recent studies of a 1:1 mixture of separate CopAa and CopAb showed that the domains do not form a stable complex in the absence of a covalent linker, though there was evidence of a weak interaction between them [18].

The regulation of cellular Cu(I) levels could be influenced by cytoplasmic low molecular weight thiol-containing species. For example, glutathione may serve as a copper-binding intermediate between the eukaryotic importer Ctr1 (high affinity copper transporter 1) and cytoplasmic chaperones [19]. A role in metal trafficking has also been suggested for bacillithiol (BSH), the cytoplasmic low molecular weight thiol of *B. subtilis*, and related species [20–22]. BSH has been shown to detoxify xenobiotic compounds and antibiotics and serve a protective effect against cysteine oxidation through protein S-bacillithiolation [20,21]. Cu(I)-induced *copZA* induction was elevated in BSH-null mutants of *B. subtilis*, suggesting the absence of BSH led to elevated labile Cu(I) levels and the presence of BSH plays a role in Cu(I) buffering. Recent studies of Cu(I)-binding to *B. subtilis* CopZ using electrospray ionisation mass spectrometry (ESI-MS) under non-denaturing conditions, in the absence and presence of BSH, revealed bacillithiolation and a range of BSH adducts of CopZ in which the thiol most likely serves as an exogenous Cu(I) ligand [22]. Low molecular weight thiols have previously been shown to influence CopAab association state behaviour [17], but the effects of BSH have not been investigated.

Despite the previous characterisation of complex Cu(I) binding to CopAab, the precise nature of species and mixtures of species which form as Cu(I) levels vary is unknown. The recent investigation of Cu(I)-binding to CopZ using ESI-MS revealed precise information about the nature of Cu(I)-CopZ species, suggesting that similar high resolution information about CopAab could be available. Thus, non-denaturing ESI-MS studies of Cu(I)-binding to CopAab, in the absence and presence of BSH, were carried out. The data are consistent with solution spectroscopic and bioanalytical studies, demonstrating Cu(I)-mediated dimerization of CopAab, and the formation of a $Cu_6(CopAab)_2$ form at high copper loadings. While the non-physiological thiol dithiothreitol (DTT) severely inhibited dimerization of CopAab, BSH, at ratios to CopAab of 1–10, was found to promote dimerization and to influence the distribution of Cu(I)-bound species. In contrast to CopZ [22], bacillithiolation and BSH-adduct formation were not readily detected.

2. Materials and methods

2.1. Purification of CopAab

Overexpression and purification of CopAab was carried out as previously described [16], except for the inclusion of an additional step to remove contaminating nucleic acids following ion exchange chromatography. $(NH_4)_2SO_4$ was added to fractions containing CopAab (determined by SDS-PAGE analysis), to a final concentration of 3 M. The protein solution was then loaded onto a 15 mL phenyl-sepharose hydrophobic interaction chromatography (HIC) column (GE Healthcare), previously equilibrated with 5 CV 100 mM HEPES, 100 mM NaCl, 3 M $(NH_4)_2SO_4$, 15 mM DTT, pH 7.0. The column was washed with 20 CV binding buffer before applying a 100 mL gradient of 3–0 M $(NH_4)_2SO_4$. Fractions containing CopAab (as determined by SDS-PAGE analysis) were buffer exchanged into 100 mM HEPES, 100 mM NaCl, pH 7.0 and concentrated to < 2.5 mL, using a centrifugal ultrafiltration unit (Vivaspin; Millipore) at 8000 ×*g* and 4 °C. The protein solution was passed through a 0.45 μm filter (Sartorius) and DTT added to a final concentration of 15 mM before applying to the Sephacryl S-100 gel filtration column. Subsequent purification steps were carried out as previously described [16].

2.2. Mass spectrometry

ESI-MS samples of CopAab were prepared by first adding 15 mM DTT (Formedium) and removing excess reductant by passage down a G25 Sephadex column (PD10, GE Healthcare) in an anaerobic glovebox (Faircrest Engineering, O_2 concentration < 2 ppm) using 20 mM ammonium acetate (Sigma), pH 7.4, as the elution buffer. Protein concentrations were calculated using an extinction coefficient, $\varepsilon_{276\ nm}$, of 5800 $M^{-1}\,cm^{-1}$ [17], before anaerobic addition of Cu(I) using a microsyringe (Hamilton). UV–visible absorbance spectra were recorded on a Jasco V-550 spectrophotometer. To prepare Cu(I)-bound CopAab samples, a deoxygenated solution of Cu(I)Cl prepared in 100 mM HCl (Sigma), 1 M NaCl was added to anaerobic, reduced CopAab using a microsyringe (Hamilton) in an anaerobic glovebox. Unbound Cu(I) was removed by passage of the sample down a G25 Sephadex column (PD10, GE Healthcare) equilibrated with 20 mM ammonium acetate, pH 7.4. The protein sample was diluted with 20 mM ammonium acetate to a working sample concentration of 5 μM. Thiol experiments were carried out using dithiothreitol (DTT) (Formedium) or BSH (synthesised as described previously [23]) and prepared anaerobically using deoxygenated LC-MS grade water (HiPerSolv, VWR). Solutions of CopAab were prepared at 4.0 Cu(I)/protein (as described above), where thiol solution was added to yield ratios of 1, 5, 10, or 25 thiol per protein. Mass spectra were acquired using a Bruker micrOTOF-QIII electrospray ionisation (ESI) time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Coventry, UK), in positive ion mode. The ESI-TOF was calibrated using ESI-L Low Concentration Tuning Mix (Agilent Technologies, San Diego, CA). Native protein samples were introduced to the ESI source at 4 °C *via* a syringe pump (Cole-Parmer) at 5 mL/min, and data acquired for 2 min, with ions scanned between 500 and 3000 *m/z*. MS acquisition was controlled using Bruker oTOF Control software, with parameters as follows: dry gas flow 5 L/min, nebuliser gas pressure 0.8 Bar, dry gas 180 °C, capillary voltage 4500 V, offset 500 V, isCID (in-source collision induced dissociation) energy 0 eV, quadrupole radio frequency (RF) stepping set at 2000 Vpp (peak to peak volts; 25%) and 3200 Vpp (75%). Processing and analysis of MS experimental data were carried out using Compass DataAnalysis version 4.1 (Bruker Daltonik, Bremen, Germany). The spectra were deconvoluted using the ESI Compass version 1.3 Maximum Entropy deconvolution algorithm over a mass range of 15,000–35,000 Da. Exact masses were determined from peak centroids, with 3-point Gaussian smoothing applied only to spectra acquired in the presence of BSH. Predicted masses are given as the isotope average of the neutral protein or protein complex, in which Cu(I)-binding is expected to be charge compensated For ions due to CopAab-Cu species, *m/z* values corresponded to $[M + x(Cu) + (n - x)H]^{n+}/n$, where M is the molecular mass of the protein and x the number of coordinated Cu^+ ions, which offsets the number of protons required to achieve the observed charge state (n+) [22,24].

3. Results and discussion

3.1. ESI-MS studies reveal Cu(I)-mediated dimerisation of CopAab and formation of $Cu_6(CopAab)_2$

ESI-MS under non-denaturing conditions preserves protein structure and non-covalent protein-metal and protein-protein interactions, and can provide high resolution information about the species present in complex mixtures [24,25]. The *m/z* mass spectra for CopAab containing 0.5, 1.0, 2.0 and 4.0 Cu/protein are presented in Fig. 1. Three charge state envelopes were observed, corresponding to monomeric (+8, +9) and dimeric (+11 – +14; and +16 – +18) forms of CopAab. At 0.5 and 1.0 Cu/CopAab (Fig. 1A and B, respectively), the monomer charge envelope (8+, 9+) was predominant, with no significant intensity due to dimeric charge states. The dimer charge states increased in intensity at higher levels of copper, such that they matched or exceeded the monomeric charge state envelope in intensity at a level of 4.0 Cu/CopAab (Fig. 1C and D).