BBA - Proteins and Proteomics 1866 (2018) 275–282

Contents lists available at ScienceDirect

BBA - Proteins and Proteomics

journal homepage: www.elsevier.com/locate/bbapap

The N-terminal domains of *Bacillus subtilis* CopA do not form a stable complex in the absence of their inter-domain linker

Liang Zhou, Kristine L. Kay[1], Oliver Hecht, Geoffrey R. Moore, Nick E. Le Brun*

Centre for Molecular and Structural Biochemistry, School of Chemistry, University of East Anglia, Norwich, NR4 7TJ, UK

ARTICLE INFO

Keywords:
Copper trafficking
Metallochaperones
Homeostasis
Metal transport

ABSTRACT

Copper-transporting P-type ATPases, which play important roles in trafficking Cu(I) across membranes for the biogenesis of copper proteins or for copper detoxification, contain a variable number of soluble metal-binding domains at their N-termini. It is increasingly apparent that these play an important role in regulating copper transport in a Cu(I)-responsive manner, but how they do this is unknown. CopA, a Cu(I)-transporter from *Bacillus subtilis*, contains two N-terminal soluble domains that are closely packed, with inter-domain interactions at two principal regions. Here, we sought to determine the extent to which the domains interact in the absence of their inter-domain covalent linker, and how their Cu(I)-binding properties are affected. Studies of a 1:1 mixture of separate CopAa and CopAb domains showed that the domains do not form a stable complex, with only indirect evidence of a weak interaction between them. Their Cu(I)-binding behaviour was distinct from that of the two domain protein and consistent with a lack of interaction between the domains. Cu(I)-mediated protein association was observed, but this occurred only between domains of the same type. Thus, the inter-domain covalent link between CopAa and CopAb is essential for inter-domain interactions and for Cu(I)-binding behaviour.

1. Introduction

Copper-transporting P-type ATPases, which belong to the P_{IB-1} subgroup of the P-type ATPase superfamily, have been identified in a wide variety of organisms, in which they function in moving Cu(I) across membranes, either for detoxification or for the biogenesis of a copper-containing protein [1,2]. Like all P-type ATPase transporters, they are integral membrane proteins with soluble phosphorylation, actuator and nucleotide-binding domains. The membrane-spanning region of these transporters, which consists of six to eight transmembrane helices, includes a CPX (cysteine-proline-cysteine/histidine/serine) motif that plays a key role in determining the specificity for Cu(I).

Also unique to the P_{IB-1} subgroup is a variable number of usually N-terminal metal-binding domains. In most cases, these have a ferredoxin-like βαββαβ fold and contain a conserved MXCXXC copper-binding motif [3]. Bacterial transporters feature either one or two metal-binding domains, while in eukaryotic transporters they number between two (e.g., in yeast) and six (e.g., in human) [4,5]. Furthermore, the nature of linking sequences between the domains is variable. In bacteria, transporters with two domains normally have a very short linker (a few residues), while in eukaryotes the linker is short between the 5th and 6th domains, but between other domains it varies between 10 and 90 residues [4].

The functions of the domains have been the subject of considerable debate. While some reports indicated that they are involved directly in the mechanism of Cu(I) transport [6], others revealed that they are not essential for Cu(I) transport [7–9]. Consistent with this, the X-ray structure of the Cu(I) transporter from *Legionella pneumophila* [10] and a cryo-EM based model of CopA from *Archaeoglobus fulgidus* [11] showed that the soluble domains are too far away from the proposed Cu(I)-entry site to be directly involved in transport. However, proximity to the actuator and nucleotide-binding domains, along with protein-protein interaction studies, led to the proposal that the metal-binding domain(s) fulfill a regulatory function through interactions with these domains [8,10–12].

Human copper-transporting P-type ATPases ATP7A (Menkes protein) and ATP7B (Wilson protein) each possess six N-terminal soluble domains [13]. The function of these domains is more complex than in their bacterial or yeast counterparts because they play an important

Abbreviations: CD, Circular dichroism; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSQC, heteronuclear single quantum correlation; ICP-AE, inductively coupled plasma-atomic emission; IPTG, isopropyl-β-D-thiogalactoside; LB, Luria-Bertani; LMCT, ligand to metal charge transfer; Mops, 3-morpholinopropanesulfonate; TOF, time of flight

* Corresponding author.
E-mail address: n.le-brun@uea.ac.uk (N.E. Le Brun).
[1] Present address: Plants for Human Health Institute, Food Bioprocessing & Nutrition Sciences, North Carolina State University, 600 Laureate Way, Kannapolis, NC 28081, USA.

https://doi.org/10.1016/j.bbapap.2017.11.008
Received 15 September 2017; Received in revised form 2 November 2017; Accepted 12 November 2017
Available online 14 November 2017
1570-9639/

role in trafficking the transporter to different cellular compartments in response to variance in copper levels [14]. While each soluble domain of ATP7A can bind Cu(I) [3,15], a protein containing all six domains preferentially converted to a more stable form with 4:1 stoichiometry [16], suggesting that Cu(I)-induced changes in tertiary structure result from cooperative interactions of the six N-terminal domains of ATP7A. On the other hand, NMR studies showed that the first and fourth domain formed a metal-mediated adduct with Hah1, the cognate copper chaperone, with Cu(I) then transferring to domains 5 or 6 [15,17]. In vivo studies showed that either the fifth or sixth domain alone was sufficient for trafficking to the plasma membrane/copper transport [18]. A similar picture has emerged for ATP7B, in which all domains are capable of binding Cu(I) [19] but only domains 5 and 6 are important for Cu(I)-dependent cellular localisation [20], and domains 1, 2 and 4 appear to interact preferentially with Hah1 [21].

The Cu(I)-transporting P-type ATPase from *Bacillus subtilis*, CopA, contains two soluble N-terminal domains with a two amino acid residue linker [22,23]. Both domains are able to bind Cu(I) and to interact with the chaperone CopZ [24]. The structure of the two domain protein CopAab revealed that it is organised in two closely packed ferredoxin-like domains, linked through only two residues, Val72 and Thr73, and spatially oriented such that the two Cu(I)-binding sites are far apart, see Fig. 1 [22,23]. There are two interacting regions between the two domains, one involving residues 11–12 and 72 with residues 119–122 and the other involving residues 9–10, 57, and 61 with residues 103–104. The protein undergoes Cu(I)-mediated dimerisation above 1 Cu(I) per protein, leading to a luminescent Cu(I) cluster [23,25]. Each soluble domain has also been studied in isolation, revealing different stabilities and, although both bind Cu(I), they do so in different ways [26,27], suggesting that they are functionally distinct.

In order to determine the importance of the covalent linkage between the two domains of CopAab, we employed spectroscopic and bioanalytical methods to study 1:1 mixtures of the separate CopAa and CopAb domains. The data reveal that, in the absence of the covalent link, the domains do not form a stable complex, with only indirect evidence of a weak interaction. Although Cu(I)-mediated domain association was observed, this occurred only between domains of the same type, consistent with a lack of inter-domain interaction. The physiological consequences of this are discussed.

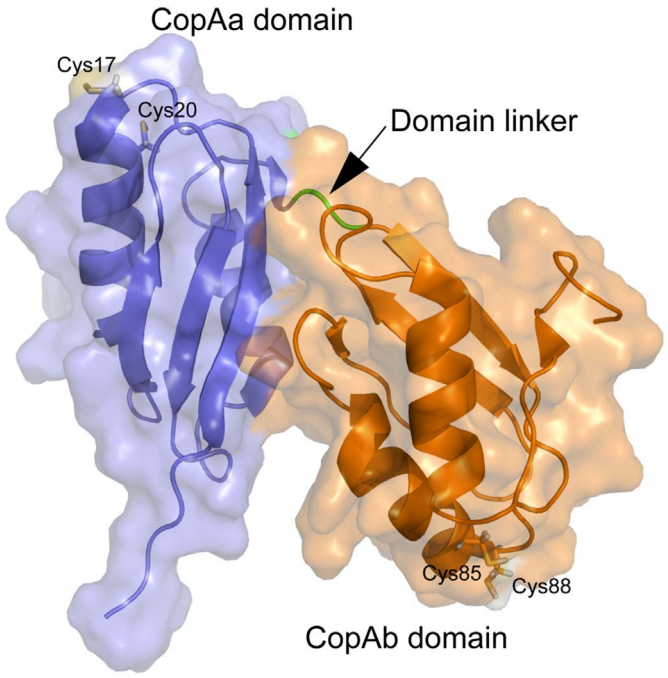

Fig. 1. The N-terminal metal binding domains of CopA. The structural image was generated using PyMol (www.pymol.org) [28] with PDB file:2RML [23]. The two domain protein is called CopAab, while the individual domains are CopAa and CopAb, as indicated in blue and orange, respectively. The two residue linker connecting the domains is shown in green. Cys residues at the Cu(I)-binding motifs of each domain are shown.

2. Material and methods

2.1. Purification of CopAa and CopAb and additions of Cu(I) ions

CopAa and CopAb containing, respectively, residues 1–72 and 73–147 of *B. subtilis* CopA were purified as previously described [26,27] using an Äkta Prime Plus (GE Healthcare). CopAa is known to be less stable than CopAb [26,27,29]. Unfolding appears to be concentration dependent because there was no evidence for it occurring prior to the dilution of CopAa from concentrated stock solution to generate working solutions for experiments. During experiments, precipitation did not occur (as evidenced by the lack of scattering measured by UV–visible absorbance spectroscopy). Prior to the addition of metal ions, the protein solutions were reduced using 5 mM DTT and excess DTT was subsequently removed using a desalting column (PD-10, GE Healthcare). The oxidation state of the cysteines following removal of DTT was assessed by reaction with Ellman's Reagent (5′5-dithio-bis (2-nitrobenzoic acid), DTNB), which confirmed the presence of ~2 reactive thiols per protein molecule. Anaerobic additions of Cu(I) were made using a microsyringe (Hamilton) as previously described [23,26,27]. Titration experiments were conducted using a single sample cuvette; after each metal ion addition, samples were incubated for 2 min before spectra were recorded. Data were corrected for dilution effects.

2.2. Absorbance and CD spectroscopies

UV–visible absorbance spectra were recorded on a Perkin-Elmer λ35 or JASCO V550 spectrophotometer. CD spectra in the far-UV range (190–250 nm) were recorded using a Jasco J-810 spectropolarimeter interfaced to a PC, with a slit width of 2 nm and scan speed of 100 nm/min. CD intensity is expressed as molar ellipticity ([θ]) in units of deg cm^2 dmol^{-1}. Spectra were corrected for intensity due to the buffer. Fluorescence emission spectra were recorded using a Perkin-Elmer LS55 spectrophotometer at 25 °C with excitation at 276 nm and excitation and emission slit widths of 9 nm. For the measurement of emission in the region 500–700 nm, slit widths were set to 10 nm (with excitation at 295 nm) and a 390 nm cut-off band pass filter was employed. Scan speed was 200 nm/min for all fluorescence measurements.

2.3. NMR spectroscopy

NMR spectra were acquired using a Bruker Avance III 800 MHZ spectrometer equipped with a triple resonance, pulsed field gradient probe, operating at frequencies of 800.23 MHz (^{1}H) and 81.09 MHz (^{15}N), using pulse sequences incorporated into the Bruker Topspin 2.1 software. Reduced apo-^{15}N-CopAa and apo-CopAb (300 μM of each in 100 mM phosphate, pH 7.0, 10% D_2O) were loaded into an NMR tube and 2D ^{1}H^{15}N HSQC spectra were recorded at 298 K and processed using NMRPipe [30]. The ^{1}H carrier frequency was positioned at the resonance of the water during the experiments, and the ^{15}N carrier frequency was at 115 ppm. Prior to Fourier transformation, a cosine-bell window function was applied to each dimension for apodization. The indirect dimensions were first linear-predicted to double the number of data points, and then zero-filled to round up the number of data points to the nearest power of 2.

2.4. Analytical gel filtration

Samples were prepared under anaerobic conditions containing 100 μM of each CopAa and CopAb in 100 mM MOPS, 100 mM NaCl, pH 7.5, at 0.5, 1.0 and 2.0 Cu(I) per domain. Each 500 μl sample was