Biochemical and Biophysical Research Communications 470 (2016) 663–669

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Copper binding triggers compaction in N-terminal tail of human copper pump ATP7B

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ARTICLE INFO

Article history:
Received 10 January 2016
Accepted 14 January 2016
Available online 18 January 2016

Keywords:
Protein–protein interactions
Metalloenzymes
Copper transport
NMR
Circular dichroism
Conformational changes

ABSTRACT

Protein conformational changes are fundamental to biological reactions. For copper ion transport, the multi-domain protein ATP7B in the Golgi network receives copper from the cytoplasmic copper chaperone Atox1 and, with energy from ATP hydrolysis, moves the metal to the lumen for loading of copper-dependent enzymes. Although anticipated, conformational changes involved in ATP7B's functional cycle remain elusive. Using spectroscopic methods we here demonstrate that the four most N-terminal metal-binding domains in ATP7B, upon stoichiometric copper addition, adopt a more compact arrangement which has a higher thermal stability than in the absence of copper. In contrast to previous reports, no stable complex was found in solution between the metal-binding domains and the nucleotide-binding domain of ATP7B. Metal-dependent movement of the first four metal-binding domains in ATP7B may be a trigger that initiates the overall catalytic cycle.

1. Introduction

Copper (Cu) is found in the active sites of essential proteins that participate in cellular reactions such as respiration, antioxidant defense, neurotransmitter biosynthesis, connective-tissue biosynthesis and pigment formation [1–3]. To avoid Cu toxicity, the intracellular concentration of Cu is regulated via dedicated proteins that facilitate uptake, efflux as well as distribution of Cu to target Cu-dependent proteins and enzymes [4–6]. In the human cytoplasm, after Cu has entered the cell via Ctr1 [7], the Cu chaperone Atox1 transports the metal to cytoplasmic metal-binding domains in ATP7A/B (Menkes and Wilson disease proteins, respectively), which are two homologous multi-domain P_{1B}-type ATPases located in the trans-Golgi network. Once transferred to ATP7A/B, the Cu ion is thought to be channeled between metal sites within the protein to the lumen of the Golgi and there loaded on target Cu-dependent proteins.

ATP7A/B are multi-domain proteins consisting of eight membrane-spanning helices, an actuator domain, as well as nucleotide- and phosphorylation-domains, with nucleotide-binding site and an invariant Asp (transiently phosphorylated during the catalytic cycle), respectively, protruding into the cytoplasm. In addition, ATP7A/B have six cytoplasmic metal-binding domains connected by peptide linkers of various lengths constituting the N-terminal tail [8]. There are many detailed studies reported on individual ATP7A/B domains and on yeast and bacterial homologs [9,10]. Each metal-binding domain in ATP7A/B, as well as Atox1, has a ferredoxin-like α/β fold and a surface-exposed invariant CXXC motif (X = any residue) in which a single Cu can bind via the cysteine sulfurs. In contrast to humans, bacterial and yeast P_{1B}-type ATPases have only one or two metal-binding domains. The reason for multiple metal-binding domains in ATP7A/B has been proposed to involve regulation of Cu transfer activity to the Golgi lumen and Cu-mediated protein trafficking between the Golgi and the plasma membrane [11–14].

During the catalytic cycle that requires ATP hydrolysis and ultimately results in Cu movement to the lumen side of the membrane, ATP7A/B are likely to undergo significant conformational changes and altered domain–domain interactions [8]. Available predictions for how the ATP7A/B proteins work come from analogy with the calcium pump SERCA, for which structures of different enzymatic stages have been resolved [15]. Since there is no high-resolution structural information on the arrangement of the six metal-binding domains within full length ATP7A/B, it is unclear how these domains participate in the catalytic cycle and are arranged relative to each other at different stages of the process.

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http://dx.doi.org/10.1016/j.bbrc.2016.01.085
0006-291X/

Nonetheless, because Atox1 can deliver Cu to the metal-binding domains [16], one may speculate that Cu-triggered conformational changes among these domains initiate the catalytic cycle.

Interactions among the six metal-binding domains of ATP7A/B (perhaps resembling stacks of logs) have been proposed to allow for long-range signal transmission among the domains [11,17]. In some support of this, we discovered that in a construct of domains 5 and 6 of ATP7B (WD5-6), minute variations in salt and pH conditions perturbed domain–domain relative fluctuations such that the efficiency of Atox1-mediated Cu delivery to these domains was modulated [18]. Moreover, based on earlier proteolysis and circular dichroism (CD) data, local interactions among WD domains have been postulated to depend on Cu loading and phosphorylation [11,12,19]. From pull-down studies using cell lysate, an interaction between a construct of all six ATP7B metal-binding domains (WD1-6) and the ATP-binding domain (abbreviated N-domain) was detected [12,20]. This interaction appeared to block ATP binding to the N-domain but was eradicated by Cu loading of WD1-6 [12]. This suggested the interaction between the N-domain and WD1-6 to be inhibitory with respect to Cu transfer activity in the full length protein.

Here, we used spectroscopic methods to investigate the overall dimensions of the first four metal-binding domains (WD1-4) of ATP7B as a function of Cu loading. Although lacking domains 5 and 6, WD1-4 can be considered a biologically relevant unit because the linker to WD5-6 is 57 amino acids (and thus WD1-4 may act as an individual segment) and Atox1 prefers to deliver Cu to WD2 and WD4 [16] making WD1-4 the likely place for initial Cu loading. We also searched for evidence of the reported interaction between the N-domain and the metal-binding domains (in the absence of Cu) using purified protein domains in solution. Our results demonstrate conformational changes in WD1-4 as a function of Cu loading (specifically, compaction in parallel with domain–domain interactions) whereas we find no stable interaction between the N-domain and the metal-binding domains in solution (implying K_D > ~5 mM).

2. Materials and methods

2.1. Protein expression and purification

Constructs of the first four (WD1-4), and all six (WD1-6) metal-binding domains of ATP7B, as well as the N-domain of ATP7B (Val1036 to Asp1196 of the full length protein) [21,22] were ordered from GenScript (NJ, USA) and cloned into a pET-3a vector carrying an N-terminal His-tag. Plasmids for WD1-4 and WD1-6 were transformed into *E. coli* BL21 (DE3) competent cells and were grown in 1xLB (100 mg/L carbenicillin) at 37 °C until OD_{600} of ~0.6. Protein expression was induced with 1 mM IPTG and further grown over night. Cells were harvested by centrifugation (6000 rpm, 4 °C, 30 min) and pellets were re-suspended in 20 mM Tris, 50 mM NaCl, 10 mM imidazole, 2 mM DTT, 5% glycerol, pH 7.5. For purification, DNase I was added to a final concentration of 0.01 units/μL together with one tablet protease inhibitor (Roche Applied Science) per 60 mL of cell suspension. The solution was sonicated and centrifuged (20,000 rpm, 4 °C, 30 min) and the pelleted cells were lysed by re-suspension in 20 mM Tris, 50 mM NaCl, 7 M guanidinium chloride, pH 7.5 and centrifuged (20,000 rpm, 4 °C, 30 min). The supernatant was dialyzed in buffer A (20 mM Tris, 50 mM NaCl, 10 mM imidazole, 2 mM DTT, pH 7.5); filtered through a 0.20 μm filter, and loaded to a 20 mL Ni-sepharose column (GE healthcare). The column was washed with buffer A followed by elution of His-tagged WD1-4 and WD1-6 proteins using imidazole buffer (20 mM Tris, 50 mM NaCl, 250 mM imidazole, 2 mM DTT, pH 7.5). To cleave the His-tag, 20 mM β-mercaptoethanol was added, followed by addition of Caspase-7 (1:100 w:w), incubated overnight at 4 °C. The samples were loaded on HiTrap Q Sepharose FF anion exchange column and washed with buffer B (20 mM Tris, 2 mM DTT, 1 mM EDTA, pH 7.5). WD1-4 and WD1-6 were eluted with buffer B containing 1 M NaCl. The elution fractions containing WD1-4 or WD1-6 were concentrated using Ultra-15 centrifugal filter devices (Amicon). The concentrate was loaded on a HiLoad 16/60 Superdex 75, (GE Healthcare) column equilibrated with buffer C (20 mM Tris, 50 mM NaCl, 2 mM DTT, pH 7.5). For purification of the N-domain, harvested cells were re-suspended in buffer A followed by sonication. The solution was centrifuged (20,000 rpm, 4 °C, 30 min), and the supernatant filtered through a 0.20 μm filter, and loaded to a 20 mL Ni-sepharose column (Ni Sepharose 6 Fast Flow, GE healthcare). The column was washed with buffer A and His-tagged N-domain was eluted with imidazole buffer (20 mM Tris, 50 mM NaCl, 250 mM imidazole, 2 mM DTT, pH 7.5). 20 mM β-mercaptoethanol was added, followed by Caspase-7 (1:100 w:w) to cleave the His-tag (incubation overnight at 4 °C). The sample was loaded on a HiTrap Q Sepharose FF anion exchange column (GE Healthcare) and washed with buffer B. The N-domain was eluted with buffer B including 1 M NaCl. The protein-containing fractions were concentrated and loaded on a HiLoad 16/60 Superdex 75, (GE Healthcare) column equilibrated with buffer C. ^{15}N-labeled WD1-4 and N-domain proteins were grown in M9 media using the same procedures as mentioned for the unlabeled proteins. Expression and purification of Atox1 followed the procedure reported in Ref. [23]. All purification steps were performed in presence of 2 mM DTT. For all purified proteins, sample purity was confirmed by a single band on SDS-PAGE and a single elution peak in size exclusion chromatography. The concentrations of WD1-4, WD1-6, N-domain and Atox1 were determined using the absorption at ε_{280} of 19,940 M^{-1} cm^{-1}, 24,410 M^{-1} cm^{-1}, 8480 M^{-1} cm^{-1}, and 2980 M^{-1} cm^{-1}, respectively (values based on amino acid composition). To load Cu(I) into WD1-4, the apo protein was mixed with stoichiometric amounts of $Cu(II)Cl_2$ in the presence of five-fold excess of DTT to assure reduction of Cu(II) to Cu(I) prior to protein binding.

2.2. Far-UV circular dichroism (CD)

CD was measured in a Jasco-720 spectropolarimeter (200–300 nm; 1 mm quartz cuvette; 4 averaged scans) for 20 μM WD1-4 in the absence and the presence of increasing amounts of copper (buffer: 20 mM Tris, 50 mM NaCl, pH 7.5, 200 μM DTT). Background spectra were always subtracted prior to analysis. Thermally-induced unfolding of 20 μM WD1-4 were probed at 220 nm (1 °C/min; 20–90 °C) in absence and in presence of 80 μM Cu and, in the absence of Cu, comparing low (50 mM NaCl) and high salt (350 mM NaCl) concentrations. The 10-fold molar excess of DTT over protein in all experiments was included to assure reduction of Cu prior to protein binding. Unfolding reversibility and scan-rate dependence were tested. The thermal curves were fitted to an equation for a two-state transition to identify the T_m (midpoint temperature of the unfolding transition).

2.3. NMR spectroscopy

All NMR experiments were performed using a Bruker Avance III HD 850 MHz spectrometer equipped with a z-gradient cryoprobe and protein samples containing 5% D_2O (v/v) in 50 mM MOPS, 50 mM NaCl, pH 7.4, 5 mM DTT at 25 °C unless stated otherwise. The temperature was calibrated prior to the experiments by inserting a temperature probe into the sample compartment of the spectrometer. For WD1-4, TROSY-based ^{1}H–^{15}N correlation experiments were applied to increase the spectral resolution and