Identification of the “missing domain” of the rat copper-transporting ATPase, atp7b: insight into the structural and metal binding characteristics of its N-terminal copper-binding domain

Mike J. Tsay, Negah Fatemi, Suree Narindrasorasak, John R. Forbes, Bibudhendra Sarkar

Abstract

Wilson disease is an autosomal disorder of copper transport caused by mutations in the ATP7B gene encoding a copper-transporting P-type ATPase. The Long Evans Cinnamon (LEC) rat is an established animal model for Wilson disease. We have used structural homology modelling of the N-terminal copper-binding region of the rat atp7b protein (rCBD) to reveal the presence of a domain, the fourth domain (rD4), which was previously thought to be missing from rCBD. Although the CXXC motif is absent from rD4, the overall fold is preserved. Using a wide range of techniques, rCBD is shown to undergo metal-induced secondary and tertiary structural changes similar to WCBD. Competition $^{65}$Zn(II)-blot experiments with rCBD demonstrate a binding cooperativity unique to Cu(I). Far-UV circular dichroism (CD) spectra suggest significant secondary structural transformation occurring when 2–3 molar equivalents of Cu(I) is added. Near-UV CD spectra, which indicate tertiary structural transformations, show a proportional decrease in rCBD disulfide bonds upon the incremental addition of Cu(I), and a maximum 5:1 Cu(I) to protein ratio. The similarity of these results to those obtained for the Wilson disease N-terminal copper-binding region (WCBD), which has six copper-binding domains, suggests that the metal-dependent conformational changes observed in both proteins may be largely determined by the protein–protein interactions taking place between the heavy metal-associated (HMA) domains, and remain largely unaffected by the absence of one of the six CXXC copper-binding sites.

Keywords: Copper; Wilson disease; ATP7B; LEC rat; HMA domain; Copper transport; Copper binding

1. Introduction

Copper is an essential trace element necessary for the proper functioning of many biological processes [1]. However, an excess or deficiency of copper in the body can lead to a diseased state, thus necessitating careful regulation, transport and storage of this metal [2]. Wilson disease is an autosomal recessive disorder in which copper homeostasis is severely affected and is characterized by the inability of hepatocytes to incorporate copper into ceruloplasmin or to effectively efflux copper from the liver [3]. These features of Wilson disease are caused by mutations in ATP7B, a gene encoding a transmembrane P-type ATPase (ATP7B) found in the liver [4,5]. Menkes disease, a disorder of copper absorption, is caused by mutations in ATP7A, a gene encoding a P-type ATPase (ATP7A) highly similar to
ATP7B protein but found in tissues other than the liver [6–8]. Although these two diseases have near-opposite manifestations, the proteins involved have a high amino acid sequence similarity to each other, and shown to have related functions [9]. In Wilson disease, dysfunctional ATP7B compromises biliary copper clearance, causing copper to accumulate in the liver. Eventually, an overload of metal leads to hepatic damage and the release of non-ceruloplasmin bound copper, which ultimately accumulates in peripheral organs such as the brain, cornea and kidney [2].

Many biochemical similarities of Wilson disease have been shown to exist in the Long Evans Cinnamon (LEC) rat [10,11]. The rat orthologue (atp7b) of ATP7B shows an amino acid sequence that is 80% similar to its human counterpart [10]. Furthermore, elevated serum copper concentrations and decreased amounts of ceruloplasmin establish the LEC rat as a suitable model for the study of Wilson disease. The disease causing mutation is a deletion of 900 bp in the 3’ terminus of atp7b gene; the gene is otherwise identical to the wild-type rat gene, even in the region encoding the N-terminus.

Over recent years, the N-termini of ATP7B and ATP7A have received considerable attention, with many groups hypothesizing that the function of this domain is vital for copper transport as well as a key component of translocation processes [12–15]. It is useful to compare characteristics of atp7b, ATP7B, and ATP7A because of their close similarities, yet having a difference involving the fourth domain of their N-terminal copper-binding region. Within this extended family of ATPases, studies on the N-terminal copper-binding region have focused on metal binding properties and stoichiometry [16–18], copper-binding states to heavy metal-associated (HMA) domains [19–22], cooperativity [17,23], and mutational studies [12–14,24,25]. Despite this extensive literature, work on comparative metal-induced secondary and tertiary structural changes of the N-terminus of these transporters is still incomplete.

Here we have combined a wide range of techniques to probe the structure of the rat copper-transporting ATPase N-terminal copper-binding region (rCBD) and to compare it to the Wilson disease N-terminal copper-binding region (WCBD). We use homology modelling to reveal the presence of a domain in rCBD, which was previously thought to be absent from atp7b [10]. We show that this domain, the fourth domain (rD4) of rCBD, has retained the typical fold associated with HMA domains, even though it is missing the CXXC (where X is any amino acid) motif of copper-binding. We establish that rCBD and WCBD both contain six domains, but unlike the six HMA domains found in WCBD, only five of the domains in rCBD are HMA domains containing the amino acid sequence CXXC. Our 65Zn(II)-competition blotting and circular dichroism (CD) spectroscopic techniques reveal extensive similarities between the metal-binding properties and metal-induced conformation changes observed in rCBD and WCBD. We propose that the conformational changes observed in the N-terminal copper-binding region are triggered initially by cooperative metal binding to the domains, and then driven to completion by favourable protein–protein contacts established among some of the domains, and depend to a lesser extent on metal binding.

2. Materials and methods

2.1. Construction of atp7b copper-binding region cDNA

The cDNA fragment was generated by the polymerase chain reaction (PCR) directly from a clonal phage lysate (clone 7; [10]), containing the entire atp7b coding region cDNA. PCR was carried out in a 50-µl volume using 1 unit of pfu polymerase (Stratagene), 2 mM each of dATP, dCTP, dGTP, and dTTP, 1.5 mM magnesium chloride, 50 ng of each oligonucleotide primer and template cDNA in the buffer supplied by the manufacturer. The reaction conditions were 5 min at 95 °C for initial denaturation of template cDNA followed by 20 cycles of 30 s 95 °C denaturation, 30 s 58 °C annealing, and 3 min 72 °C extension. The primers used were as follows 5’-ACTGGATCCATGCCTGAA-CAGGAGAAAG-3’ and 5’-ACTGGTCGACT-CACTGTATTATTCGCTTCGTTGTG-3’. These primers incorporated a 5’ BamHI and a 3’ SalI restriction endonuclease site to enable cloning of the amplified cDNA fragment. Following PCR amplification, the cDNA fragment was purified by agarose gel electrophoresis, and recovered from the excised gel slice using a QiaQuick Gel purification kit (Qiagen) according to the manufacturer’s protocol. The purified cDNA fragment was double digested with BamHI and SalI restriction endonucleases (New England Biolabs) then subjected to a further round of gel purification as described above. This cDNA fragment was ligated into pUC19 vector (Pharmacia) double-digested with BamHI and SalI restriction endonucleases (New England Biolabs) and gel-purified as described above. Ligations were carried out at 4 °C overnight using T4 DNA ligase (New England Biolabs) in buffer supplied by the manufacturer. Ligated DNA was transformed into XL-1Blue E. coli (Stratagene) by electroporation (BioRad). Plasmid DNA was prepared from carbenicillin selected E. coli clones using Qiagen Miniprep Spin columns according to the manufacturer’s protocol. rCBD cDNA was excised out of the pUC19 vector and cloned into a glutathione-S-transferase (GST) fusion expression vector, pGEX-6P-2 (Amersham Pharmacia Biotech Inc.) to form the construct pGEX-rCBD.

2.2. Expression and purification

Expression and purification were performed as previously described with modifications [17]. Briefly, pGEX-rCBD was amplified using E. coli DH5α cells to retain plasmid integrity. The vector sequence was confirmed through Sanger dideoxy method (DNA Sequencing Facility, The
Centre for Applied Genomics, The Hospital for Sick Children). The construct was then isolated by Miniprep (Qiagen) from DH5α cells and transformed into E. coli strain BL21(DE3) cells for increased expression. Cells were grown in normal LB media and induced at mid-log phase for 3.5 h with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Cells were collected, centrifuged for 15 min at 5000 rpm (Beckman Jm-10) and submitted to one to two freeze thaw cycles as necessary in lysis buffer (25 mM Tris–HCl, pH 8.0, 130 mM NaCl, 1% Triton X-100, 1 mM ethylene diamine tetra-acetic acid (EDTA), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.15 mg/ml lysozyme). The cells were ultracentrifuged (Beckman L-90K) at 118,000 × g for 45 min. The supernatant liquid was applied to a GST-affinity column (Amersham Pharmacia), the pellet was homogenized in solubilization buffer (25 mM Tris–HCl, pH 8.0, 6 M urea, 130 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT)), and reultracentrifuged as before. Solubilization was repeated as necessary. The solubilization supernatant from all centrifugations was dialysed extensively against 25 mM Tris–HCl, pH 8.0, 10% glycerol, 1 mM EDTA, 1 mM DTT, 1% Triton X-100 (BioRad) and then applied to a GST-affinity column. The GST-fusion protein was eluted using buffer containing 25 mM Tris–HCl pH 8.0, 6 M urea, 80 mM NaCl, 1 mM EDTA, 1 mM DTT. The quality of fractions was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Further purifications were performed on Sepharose Fast-Q anion exchange column and eluted using the same buffer but with a NaCl gradient from 80 to 200 mM. Purified fusion protein was then dialysed into refolding buffer (elution buffer without urea) and cleaved using 0.3 units/ml PreScission ™ protease (Amersham Pharmacia Biotech). rCBD was purified from uncleaved fusion protein and GST by GST affinity column. Protein quantitation was performed by BCA assay (Pierce). Protein was stripped of metal when necessary using a method previously described for the preparation of WCBD [26,27].

Fig. 1. Purification of rCBD. Standard Marker (A). GST-rCBD expression in BL21(DE3) cells without IPTG (B) and following induction with IPTG (C). Initial purification by GST affinity chromatography (D) and then by ion exchange chromatography (E). Purified GST-rCBD was cleaved with PreScission protease (P). rCBD was purified from GST and GST-rCBD on a GST affinity column (G).

Fig. 2. Thermal denaturation of rCBD. The CD spectrum of thermal denaturation of rCBD (1 mg/ml) at 222 nM shows a sigmoidal curve, typical of a folded protein.

Fig. 3. Competition of rCBD with 65Zn(II) and transition metals. rCBD (10 μg/lane) was electrophoresed on 10% SDS-PAGE and transferred to nitrocellulose. Nitrocellulose strips of rCBD were incubated with 65Zn(II) and one of a variety of transition metals as described in Section 2. Zn intensity refers to the digital quantitation of autoradiograph bands and should not be considered equivalent to binding affinity. Autoradiographs were overexposed until binding patterns appeared; comparisons of various metal binding should be made relative to other metals (not to the baseline) and only qualitatively. Digital quantitation of autoradiographs revealed the relation in (A) between 65Zn(II) and various metals and (B) in more detail between 65Zn(II) and Cu(I).
2.3. Thermal denaturation analysis

In order to confirm proper folding after various steps of purification, samples were examined by thermal denaturation analysis using CD spectroscopic analysis carried out at 222 nm from 25 to 95 °C in 25 mM Tris–Acetate pH 8.0. All spectra were corrected against buffer and noise reduced. Spectra were collected and analyzed using an AVIV 62A DS spectrometer with a 0.1-cm rectangular CD cell. Data points were collected every 2 °C with intermediary equilibration times of 1 min.

2.4. 65Zn(II)-competition blotting

Purified rCBD was electrophoresed on a 10% SDS-PAGE gel, transferred to nitrocellulose membrane (BioRad) in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) (Sigma), pH 11.0 at 300 mA for 1 h 45 min. The membrane was then cut into strips and soaked in a reducing binding buffer (100 mM Tris–Cl, pH 7.2, 50 mM NaCl, 1 mM DTT) for 2 h. Individual strips were placed in incubation tray wells, briefly washed in binding buffer without DTT. Strips were incubated with 65Zn(II) to a final concentration of ~4.2 μM in 3 ml of binding buffer with various concentrations of competitor metal for 30 min. When copper was the competitor, DTT was included to reduce Cu(II) to Cu(I). Strips were washed twice for 15 min in the same buffer and then exposed to Kodak Biomax MR film for 16–24 h at −70 °C. Autoradiographs of nitrocellulose blots were over-exposed to reveal the lower limit of binding and binding trends. Therefore, competition results are reported according to Zn(II) intensity, and due to the design of the experiment, are only interpreted qualitatively.

2.5. Structural analysis by CD spectroscopy

Separate aliquots of apo-rCBD (29.4 μM) were combined with various concentrations of copper (0–300 μM) in the form of CuSO4 with 1 mM DTT. The solutions were equilibrated for 30 min, by which time it was assumed that all metals were reduced and bound to available sites. Excess metal and DTT were dialysed out in argon-purged buffer (50 mM Tris–Acetate pH 8.0). DTT was included to reduce protein and Cu(II) to Cu(I) but necessarily dialysed out because of interference experienced in the near-UV range. Buffer was purged with argon to maintain reduced conditions. CD spectra of Cu(I) samples were collected and analyzed using a Jasco J-720 spectropolarimeter. Far-UV CD spectroscopic analyses were employed to qualitatively monitor the change in structure as increasing amounts of metal are bound. The effect of metal binding on tertiary structure was observed by near-UV spectroscopic analyses that measure the specific aromatic residues and disulfide content of the protein, factors which are likely to be dependent on the intrinsic properties of rCBD. Secondary
structure (far-UV, 300–190 nm) analysis was performed in a 0.1-cm path length cylindrical CD cell. Tertiary structure (near-UV, 400–250 nm) changes were recorded using a 2-cm path length cylindrical CD cell.

2.6. Homology modelling of the N-terminal domains

The rCBD was subjected to homology modelling based on the solved structures of other HMA domains. SWISS-Model was used to model the HMA domains of the rCBD and SWISS-PDB-viewer was used to generate a pictorial representation of the domains [28–30].

3. Results

3.1. Cloning, expression and purification

We have cloned the DNA segment coding for the rat N-terminal copper-binding region into a GST pGEX-6P-2 vector. Subsequent expression of this plasmid and cleavage of the fusion protein with PreScission™ protease resulted in free rCBD. rCBD was purified from uncleaved fusion protein and GST protein by GST affinity and anion exchange columns. A typical purification process is shown in Fig. 1. The purity of the final product, rCBD protein, was determined to be ~95% pure by 10% SDS-PAGE. Because at least part of the purification process involved denaturation by urea, thermal denaturation was employed to confirm refolding. Thermal denaturation curve of purified rCBD observed at 222 nm displays a sigmoid shape (Fig. 2), indicating that the protein has a compact conformation typical of folded structures and native proteins. This was also supported by earlier investigation of WCBD showing that the CD profile of protein that had undergone denaturation and refolding during purification was similar to protein purified under non-denaturing conditions [26].

3.2. 65Zn(II)-competition blotting

The binding of certain transition metals was investigated by the use of a 65Zn(II)-blot assay; a summary of results is given in Fig. 3. Zinc(II) has previously been shown to bind to the metal-binding domain of ATP7B [17]. Fig. 3A shows that various transition metals were able to successfully bind to the protein and displace 65Zn(II) while others did not. rCBD shows an affinity for Cu(I)>Ag(I)>Hg(II)>Au(III)>Cd(II)>Cr(III)>Fe(III). As the concentration of Cu(I), Ag(I), Hg(II) and Au(III) was increased, 65Zn(II) was displaced in a linear fashion (Fig. 3A). Co(II), Mg(II), and Ca(II) did not appear to be able to compete with 65Zn(II) at all, while Cd(II), Cr(III) and Fe(III) competed to a lesser extent. A more detailed analysis of copper as the competitor to 65Zn(II) reveals a sigmoidal pattern, characteristic of a cooperative interaction of metal with the protein (Fig. 3B).

3.3. Homology modelling of rCBD

The common belief has been that ATP7B contains five HMA domains while ATP7B contains six; this has been considered to be the key difference between these two copper transporting P-type ATPases. Our homology modelling of the rCBD has revealed an additional domain, which had not been immediately obvious from the primary sequence alone (Fig. 4A). Due to the absence of the CXXC sequence motif from this region, which corresponds to the fourth domain in WCBD, rCBD was considered to be missing this domain [10], and remained undetected until now. The sequence alignment of the domains within rCBD reveals that although only five contain the CXXC Cu(I)-binding sites (Fig. 4B), the six domains not only share significant sequence similarity and conservation of hydrophobic residues, but more importantly they all possess the fold typical of HMA domains (Fig. 4C).
3.4. Structural analysis

Molar ellipticity at 220 nm (Fig. 5A) shows that the secondary structure of rCBD increases in direct relation to the addition of Cu(I). Above a ratio of 2–3 mol of Cu(I) per mole of rCBD, no significant changes in the secondary structure are observed. Near-UV CD was employed to further characterize the metal binding characteristics of Cu(I) reconstituted rCBD. At 260 nm, the molar ellipticity increases proportionally to an incremental addition of Cu(I) until 2 mol of Cu(I) is added. Upon the addition of 2 and 3 mol of copper per mole of protein, there is a radical change in the direction of the spectra indicating a critical change in the environment surrounding aromatic residues (Fig. 5B). Extrapolation from this wavelength clearly demonstrates that major tertiary structural changes are occurring between 2 and 3 mol of Cu(I) per mole of rCBD (Fig. 6A). Although the aromatic residues clearly reflect the structural changes of rCBD, disulfide spectra may more clearly elucidate events at the individual binding sites. Molar ellipticities at 240–350 nm are responsible for a broad disulfide signal; however, because of interferences by tryptophan and tyrosine, disulfide signals are only measurable when they make a significant contribution to the signal and when measured at above 320 nm [31]. An analysis of CD spectra at an absorbance of 330 nm shows that the molar ellipticity/residue decreases in direct relation to the addition of Cu(I) to apo rCBD but does not change beyond 5 mol Cu(I) per mole of protein (Fig. 6B).

4. Discussion

This report describes the structural and metal-binding characteristics of the rCBD, in light of the discovery of the presence of the fourth HMA-like domain, which was previously thought to be missing in rCBD. The ability of different transition metals to compete with Zn(II) and to bind to rCBD confirms earlier reports of multi-metal binding to copper-transporting ATPases [17]. Indeed, our experiments show that metals other than copper are binding to rCBD; this is not surprising considering that the CXXC site in the metal-binding domains is ubiquitously found across species in different kinds of metal transporting proteins such as in the Escherichia coli Zn(II) transporter, ZntA [32] or the mercury-transporting protein MerP [33]. rCBD’s diverse metal affinity implies that in vivo atp7b is able to discriminate among these metals by a mechanism other than binding ability alone. Copper(I) and Ag(I) (data not shown) are the only metals among those capable of binding to rCBD that displayed cooperativity upon binding. Considering that atp7b is a known copper-transporting protein, and that Cu(I) [12,13,24,34–36] and Ag(I) [34] are the only two metals capable of inducing the kind of metal dependent trafficking that has become the hallmark of this class of proteins, it is reasonable that the interaction of Cu(I) and Ag(I) with rCBD would induce a response distinct from those of other transition metals. Previous studies have shown that the most likely configuration for Cu(I) bound to HMA domains is that of a bent 2-coordinate linear system [19,22]. It is interesting to point out that despite rCBD having one less CXXC copper-binding site than WCBD, they both display a similar pattern of cooperativity upon Cu(I) binding [17]. This indicates that the tertiary structure of the rCBD may be an important factor in the cooperative metal binding character displayed. It appears that the absence of the CXXC site from the fourth domain does not alter the cooperative binding of metal to the remaining domains, as long as the HMA fold is present, and the important tertiary protein–protein contacts between the HMA domains are maintained.

This raises an interesting question: if a conformational change is a necessary precursor for the translocation of the protein from the trans-Golgi network to the plasma membrane [35,37] as already hypothesised [22,26], is this cooperativity of copper binding related to the copper induced translocation?

CD spectroscopy was used to monitor the conformational changes in the rCBD upon the binding of Cu(I). The changes observed in the far-UV CD spectra are indicative of changes in secondary structure, and the changes observed in the near-UV spectra are indicative of changes in the
environment of aromatic residues and of tertiary structure in general. When apo rCBD is slowly titrated with Cu(I), significant changes occur in the secondary structure and aromatic regions of the CD spectra, most distinctive is the transition from the apo to the 1:1 state. In vivo this can translate to a transition from low Cu(I) to elevated Cu(I) conditions, inducing a conformational change in the rCBD and influencing the function of atp7b. These results may point to the conformational change in the N-terminal region as a possible trigger of the translocation event to the plasma membrane; this is also suspected to be the case from the results of the mutational studies on ATP7B [14] and ATP7A [12,13].

Current literature tends to support two hypotheses on the function of the metal-binding region of the copper-transporting ATPases. It is commonly accepted that although not necessary for copper-transport across the membrane, the metal-binding region plays a central role in the copper-dependent trafficking and regulation of copper-transporting ATPases. In general, Cu(I) is believed to bind to the HMA domains and then transport across the membrane, likely by a channel formed by the eight transmembrane helices present in the ATPases. The metal-binding region may also serve as a second function, that of a metal “sensor” [6,17,18,34]. The gradual change in secondary structure, as observed by far-UV CD spectra, supports a model where Cu(I) binds to sensor HMA domains and induces conformational change.

The results in the near-UV/tertiary structure region support results from both far-UV experiments and 65Zn(II) competition assays. The changes in molar ellipticity/residue in response to the addition of copper to rCBD, at 260, 290 and 330 nm are similar in pattern to those observed for WCBD [26]. The molar ellipticity/residue at 330 nm decreases linearly both in the case of rCBD and WCBD but does not change beyond 5 and 6 mol of copper for each, respectively. Considering that only five of the six domains of the rCBD contain the CXXC copper-binding site, and are likely to have similar metal-binding affinities due to sequence and structural similarities, this result is in agreement with the results obtained from structural homology modelling and provides further evidence that copper binds at the CXXC motif and that there is only one copper binding per CXXC containing HMA domain. Also, these results suggest that the ratio of fully saturated rCBD is 5 mol of copper bound per mole of rCBD.

It should be noted that although rCBD contains five metal-binding motifs and the WCBD contains six, the breakpoint for structural change in rCBD (Fig. 3) is similar to that found in WCBD [26] despite the missing CXXC Cu(I)-binding site. This observation is supported by the homology modelling of the rCBD, which indicates the presence of six domains having the HMA domain fold (Fig. 4). Since rD4 lacks the CXXC metal-binding motif (Fig. 4A), the fourth CXXC does not appear to be crucial for cooperative binding copper binding to rCBD or structural/conformational changes as long as the overall HMA domain fold is present. In addition, the lack of this site does not appear to affect the multi-metal binding affinity of rCBD observed in competition studies (Fig. 3), similar to studies with WCBD [17]. Another possibility may be that the rD4 may not serve a vital function in copper transport or that the loss of the CXXC sequence motif is an evolutionary adaptation that enables copper to bind or transport more efficiently. The similarity in the pattern of binding as observed in the WCBD [26] and in rCBD tends to imply that the structural response to metal binding will also be similar. The fact that both the WCBD and the rCBD have an equal number of structurally homologous HMA domains is in support of this argument. However, since there are no studies confirming the order in which copper binds to ATP7B or related proteins, it is difficult to make definitive conclusions about the specific role that HMA domain 4 in ATP7B and atp7b may play.

It has been known for some time that not all of the copper-binding domains of the N-terminal region are required for the copper-transport function of the ATPase [13,14], and that the conformational changes in the N-terminal copper-binding region in response to metal binding may have functional consequences for the ATPase [12,13,15]. Our studies propose that the metal-dependent conformational changes observed in the N-terminal region of these ATPases may not require the presence of a CXXC motif on every domain, as long as the domain has the proper shape, which is an HMA-domain-like fold, and as long as some of them contain the CXXC metal binding sites. The breakpoint in the binding trend, observed at around 2–3 mol of copper in the near-UV CD spectra, may well correspond to the breakpoint determined by 65Zn(II)-blots. This suggests that at a certain point during the cooperative binding of Cu(I), protein–protein interactions among the HMA domains begin to dominate the conformational changes in rCBD, while metal binding to the HMA domains makes a smaller contribution.

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References


