Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1768 (2007) 3127–3134
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbamem

NMR and mutagenesis of human copper transporter 1 (hCtr1) show that Cys-189 is required for correct folding and dimerization

Sangwon Lee, Stephen B. Howell, Stanley J. Opella *

Department of Chemistry and Biochemistry and Department of Medicine, University of California, San Diego, La Jolla, CA, USA

Received 11 July 2007; received in revised form 30 August 2007; accepted 31 August 2007
Available online 21 September 2007

Abstract

The human high-affinity copper transporter (hCtr1) is a membrane protein that is predicted to have three transmembrane helices and two methionine-rich metal binding motifs. As an oligomeric polytopic membrane protein, hCtr1 is a challenging system for experimental structure determination. The results of an initial application of solution-state NMR methods to a truncated construct containing residues 45–190 in micelles and site-directed mutagenesis of the two cysteine residues demonstrate that Cys-189 but not Cys-161 is essential for both dimer formation and proper folding of the protein.

Keywords: Oligomerization; Copper transport; hCtr1; NMR; Disulfide bond

1. Introduction

Copper is an essential trace element in biology. Largely because of its roles in redox chemistry, it is involved in a wide variety of cellular functions including photosynthesis and respiration, free radical eradication, neurological development, and iron homeostasis [1,2]. As is the case for many essential trace metals, excess copper is toxic, and intracellular copper levels are tightly regulated so that sufficient copper is absorbed and excess copper is secreted. Menkes syndrome (copper deficiency) and Wilson disease (copper toxicosis) demonstrate the metabolic requirement for and toxicity of copper in humans; these diseases are caused by mutations in the P-type ATPases, ATP7A and ATP7B, respectively. Progress has been made toward understanding the mechanism of the intracellular copper transport through the metallochaperones, in large part due to the structure determinations of the key proteins [3–5]. In contrast, the mechanism of copper translocation across cellular membranes is largely unknown.

Copper transporters (CTR) are a family of proteins responsible for cellular copper uptake. Genetic studies in the yeast *Saccharomyces cerevisiae* provided the first identification of proteins involved in high-affinity copper uptake at the plasma membrane of eukaryotic cells, yCTR1 and yCTR3 [6,7]. The human high-affinity copper transporter (hCtr1) was subsequently identified by functional complementation studies of an *yCTR1* deletion mutant [8]. The 190-residue hCtr1 protein is predicted to have three transmembrane helical segments and two methionine-rich metal binding motifs. Several different models of the oligomerization state of the protein have been proposed, including a dimer [9], a trimer [10], and a dimer of trimers [11]. A connection between the resistance to extracellular copper and the resistance to cis-diamminedichloroplatinum(II) (cisplatin), a platinum-based chemo-therapeutic agent, has been observed [12–14]. The involvement of CTR1 in cisplatin transport has also been demonstrated by showing the effect of *ctr1* deletion on cisplatin resistance as well as competitive uptake between copper and cisplatin [15].

As an oligomeric polytopic membrane protein, hCtr1 is a challenging system for experimental structure determination. In order to simplify the problem somewhat and to focus on the membrane-associated domain of the protein, we prepared a truncated construct, $hCtr1_{TM}$, which includes residues 45–190, as indicated in Fig. 1. In this article we describe an initial application of solution-state NMR methods to $hCtr1_{TM}$ in

* Corresponding author.
E-mail address: sopella@ucsd.edu (S.J. Opella).

0005-2736/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2007.08.037

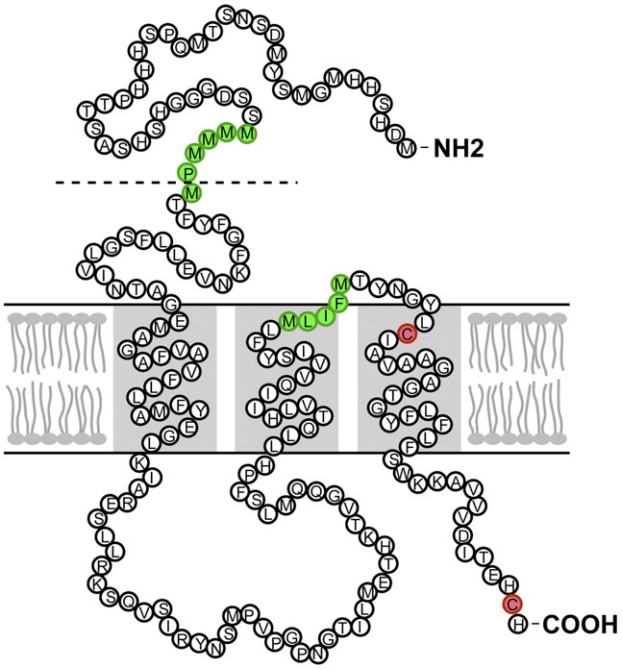

Fig. 1. The amino acid sequence of hCTR1 arranged to suggest a plausible structural arrangement. The two Cys residues mutated in this study are shown in red. The two copper binding motifs are shown in green. The N-terminal region was truncated at the position marked with the dashed line, leaving the sequence for the construct $hCTR1_{TM}$.

micelles. In the course of sample optimization and the preparation for the attachment of spin-labels for PRE (paramagnetic relaxation enhancement) measurements [16–18] we found that the protein was highly sensitive to the presence or absence of the cysteine residue at position 189 but not the one at position 161 as monitored by two-dimensional HSQC (heteronuclear single quantum correlation) NMR spectra and behavior on SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). These results demonstrate that not only is Cys-189 involved in the formation of stable dimers, but also is essential for the correct folding of the protein.

2. Methods and materials

2.1. Cloning of wt-hCTR1$_{TM}$, C161S-hCTR1$_{TM}$, C189S-hCTR1$_{TM}$, and C161S/C189S-hCTR1$_{TM}$

cDNA corresponding to amino acid residues 45–190 of hCtr1, $hCtr1_{TM}$, was PCR amplified from the DNA encoding full-length hCtr1 using the oligonucleotides 5′-GGAATTCCATATGACCTTCTACTTTGG and 5′-CCGCTCGAGATGGCAATGCTCTGTG. The PCR products were digested with the restriction enzymes *Nde*I and *Xho*I (New England Biolabs, Ipswich, MA), and purified by agarose gel electrophoresis using the protocol supplied with a Gel Extraction Kit (Qiagen, Valencia, CA). The DNA fragment was ligated into the vector pET-31b(+) (EMD Biosciences, La Jolla, CA) which was cut by the same restriction enzymes and purified. The ligation product was transformed into DH5α competent cells (Invitrogen Corp., Carlsbad, CA) and the DNA sequence was verified. A PCR test with the same oligonucleotide primers was performed to confirm if the ligation product contains the target PCR product. Further confirmation came from digesting with the restriction enzyme *Eco*RV to ensure the restriction digest yields the fragments of correct sizes. The final recombinant plasmid, $pEThCTR1_{TM}$, codes for amino acid residues 45–190 of hCTR1 with the amino acids LEHHHHHH attached at the C-terminus.

A single rare codon for *Escherichia coli* was found within the gene for $hCtr1_{TM}$ at the position encoding Arg-90 (CGA) that was optimized by changing it to CGC using the standard site-directed mutagenesis protocol (QuikChange, Stratagene, La Jolla, CA) with the primers listed in Table 1. Two cysteine residues in the $hCTR1_{TM}$ polypeptide, Cys-161 and Cys-189, were changed to serine residues following the standard site-directed mutagenesis protocol with the primers listed in Table 1.

Four different $hCTR1_{TM}$ constructs were generated using the primers listed in Table 1: wt-$hCtr1_{TM}$, C161S-$hCtr1_{TM}$, C189S-$hCtr1_{TM}$, and C161S/C189S-$hCtr1_{TM}$. All mutations were performed using the *Pfu* polymerase-mediated reaction on the full-length plasmids. The sequences of the constructs were verified by sequencing the plasmid using T7 promoter as a primer.

2.2. Expression and Western blot analysis of hCTR1$_{TM}$ constructs

The recombinant plasmids were transformed into *E. coli* competent cells C43(DE3) (Imaxio, France) and grown on LB agar plates containing 100 μg/mL carbenicillin. A single colony was picked and inoculated in 5 mL LB media with 100 μg/mL carbenicillin at 37 °C, 260 rpm for 2 h. 500 μL of the culture was transferred into 100 mL M9 minimal media containing 3% LB media and incubated at 37 °C overnight, then 30 mL of the culture was transferred to 1 L M9 minimal media containing 3% LB media. The target protein expression was induced using 0.4 μM isopropyl-β-D-thiogalactoside (IPTG) when the OD_{600} reached a value of ~0.6, followed by incubation for an additional 4 h at 37 °C. The cells were harvested by centrifugation at 12,000×*g* for 45 min at 4 °C using a Beckman Avanti J-20 XP centrifuge with a JLA-8.1 rotor. Isotope labeling was achieved by utilizing ^{15}N-ammonium sulfate (Cambridge Isotope Laboratories, Inc., Andover, MA) as the sole nitrogen source in the growth media.

Western blot analysis was performed on whole cells using an antibody directed against C-terminal amino acid residues of hCTR1 (Novus biologicals, Inc., Littleton, CO) with the standard immunoblotting protocol. The samples were loaded onto a 12% SDS-PAGE gel and electroblotted onto a polyvinylidene difluoride (PVDF) transfer membrane by applying 30 V for 1 h. The blotted membrane was blocked using TBS-Tween20 buffer (25 mM Tris–Cl, pH 7.4, 150 mM NaCl, 0.05% Tween-20) containing 5% milk for 1 h and incubated overnight with TBS-Tween20 buffer containing anti-C-terminal antibody diluted to 1:1000. The membrane was washed with TBS buffer three times for 10 min and incubated for 1 h with secondary ECL horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) diluted to 1:1000. The bands were visualized using SIGMAfast 3,3-diaminobenzidine (DAB) tablets (Sigma-Aldrich, St. Louis, MO). InVision His-tag In-gel Stain (Invitrogen Corp., Carlsbad, CA) was used to detect the proteins with hexa-Histidine tag following the standard protocol.

2.3. Purification of hCtr1$_{TM}$

Cells containing the expressed target proteins were resuspended in 20 mL of lysis buffer (50 mM sodium phosphate, 1 M NaCl, 5 mM EDTA, pH 7.6) per

Table 1
Oligonucleotides used in the site-directed mutagenesis for $hCTR1_{TM}$

R90CGC forward	GGACTCAAGATAGCC**CGC**GAGAGCCTGCTGCG
R90CGC reverse	CGCAGCAGGCTCTC**GCG**GGCTATCTTGAGTCC
C161S forward	CCTACAACGGGTACCTC**AGC**ATTGCAGTAGCAGCAGG
C161S reverse	CCTGCTGCTACTGCAAT**GCT**GAGGTACCCGTTGTAGG
C189S forward	GGATATCACAGAGCAT**AGC**CATCTCGAGCACCACC
C189S reverse	GGTGGTGCTCGAGATG**GCT**ATGCTCTGTGATATCC

Targeted mutation points are shown in bold. Primers are shown in the 5′ to 3′ direction.

gram of wet cells with protease inhibitor cocktail (cOmplete, Roche Applied Science, Indianapolis, IN). The resuspended cells were lysed by sonicating on ice for 4 min (5 s on, 5 s off) three times. Unlysed cells and inclusion body fractions were removed by centrifugation at 6000×*g* for 30 min at 4 °C using a Beckman Avanti J-20 XP centrifuge with a JA-25.50 rotor. The membrane fraction containing the target protein was separated from the fraction containing soluble proteins by centrifuging the supernatant at 90,000×*g* for 3 h at 4 °C using a Beckman Optima L-100 XP Ultracentrifuge with a 45Ti rotor. The isolated membrane fraction was then dissolved in 1% w/v dodecylphosphocholine (DPC, Fos-Choline-12, Anatrace, Inc., Maumee, OH) in 20 mL of buffer A (50 mM sodium phosphate, 1 M NaCl, pH 7.6) per gram wet cells and incubated overnight at 4 °C with gentle agitation. The extracted solution was centrifuged at 90,000×*g* for 20 min at 4 °C to separate undissolved materials left in the membrane fractions.

After the selective extraction, the supernatant containing the target protein dissolved in DPC was incubated with Ni-NTA resin (Qiagen, Valencia, CA) pre-equilibrated with buffer A, for 1 h at 4 °C with gentle agitation. The protein-DPC micelle mixture bound to Ni-NTA resin was loaded onto an empty column, and washed with >2 bed volumes of 0.2% w/v DPC solution containing 50 mM sodium phosphate, 1 M NaCl, and 5 mM imidazole at pH 7.6. Nonspecifically bound proteins were removed by washing with >2 bed volumes of buffer containing 0.2% w/v DPC, 50 mM sodium phosphate, 1 M NaCl, and 30 mM imidazole at pH 7.6. The target protein was eluted with buffer containing 0.2% w/v DPC, 50 mM sodium phosphate, 1 M NaCl, and 300 mM imidazole.

2.4. NMR sample preparation and experiments

5 mM EDTA was added to the sample purified with the nickel affinity chromatography, and the sample was dialyzed against water for ~2 days in a dialysis bag with a molecular weight cutoff of 1 kDa. The precipitated sample was collected by centrifugation followed by lyophilization. The NMR sample was made by directly adding a stock solution of 100 mM DPC to the lyophilized protein powder. 10% D_2O was added to the sample, and the pH was adjusted to 5.0.

Standard ^{15}N,^{1}H-HSQC experiments were performed on a Bruker DRX 600 spectrometer with a TXI-cryoprobe and on a Bruker AVANCE 800 spectrometer. The data were processed with NMRPipe [32], and analyzed with SPARKY (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco).

3. Results

3.1. Cloning, expression, and purification of $hCtr1_{TM}$

Since hCtr1 consists of 190 amino acids with three putative transmembrane domains and oligomerizes, the challenges for NMR spectroscopy start with sample preparation. The polypeptides need to be over-expressed in *E. coli* for uniform isotopic labeling, purified to a high degree, and reconstituted into lipid environments that are conducive not only to proper folding and oligomerization, but also for NMR experiments [19].

The plasmid pEThCtr1$_{TM}$ (Fig. 2A) was designed so that the hCtr1 constructs could be expressed in *E. coli* with a hexa-Histidine (His6) tag added at the C-terminus to facilitate purification with immobilized metal affinity chromatography. The target protein for the studies described in this article, hCtr1$_{TM}$, consists of residues 45–190 of the native full-length hCtr1. As shown in Fig. 1, the 146-residue hCtr1$_{TM}$ is expected to contain all three transmembrane domains and the metal-binding motif located between TM2 and TM3, but not the second metal-binding motif located in the N-terminal region. The His_6 tag was placed at the C-terminus to prevent non-specific interactions that might occur if the tag was at the N-terminus of the protein. The pET-31b(+) (EMD Biosciences, Inc., San Diego, CA) plasmid, originally developed to over-express the target protein with the ketosteroid isomerase (KSI) fusion protein, was used to design the plasmid pEThCtr1$_{TM}$, with the KSI-encoding region removed by the restriction enzymes *Nde*I and *Xho*I.

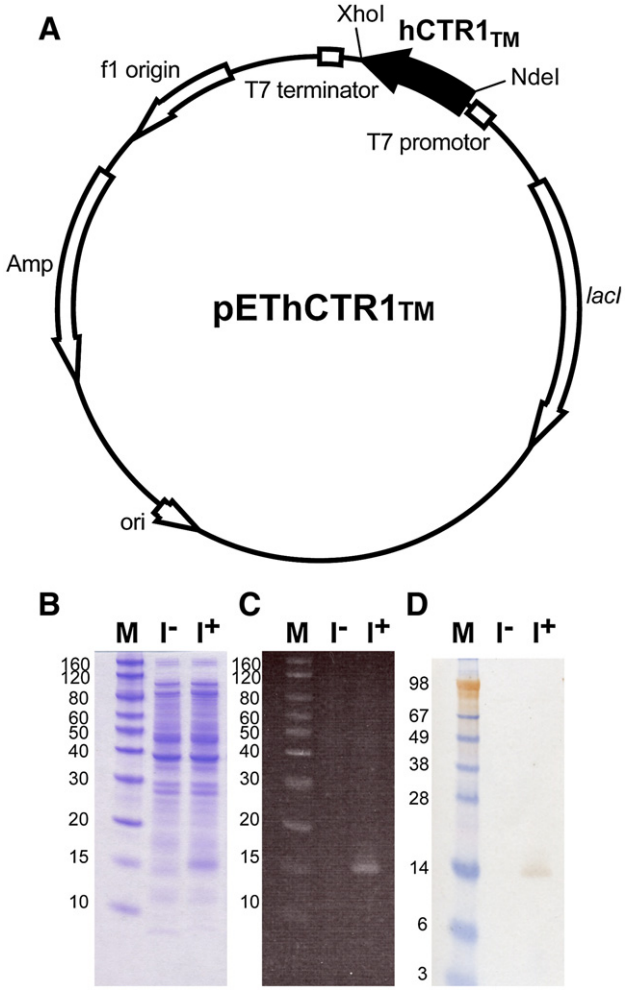

Fig. 2. (A) A schematic diagram of the plasmid pEThCTR1$_{TM}$. The expression of hCTR1$_{TM}$ was confirmed by (B) SDS-PAGE, (C) InVision His-tag In-gel Stain, and (D) Western blot detected with anti-C-terminal hCTR1. Lanes marked as "M" indicate the protein standards, with the standardized molecular weights in kDa indicated next to the lanes. Samples containing the whole cells before the induction were labeled "I^-", and 4 h after the induction, "I^+". The cells loaded onto each lane were 'normalized' according to OD_{600} values.

After transformation into several trial strains of bacteria and optimization of growth conditions, C43(DE3) was found to give the expression level suitable for preparation of the milligram quantities required for NMR studies. C43(DE3) and C41(DE3) are the mutant strains of *E. coli* strain BL21(DE3) known to survive when the expressed proteins are toxic to the host [20]. Nonetheless, we found it essential to test the specific host–plasmid combinations in order to obtain optimal expression levels. Using identical growth and expression protocols, BL21 (DE3) cells did not survive (the solution becomes clear and OD_{600} goes down) 2 h after the induction at 37 °C; in contrast, C43(DE3) survive 5 h after the induction.

Interestingly, bacterial culture in rich media, such as the Luria–Bertani (LB) broth, did not yield protein expression at a detectable level, but when the growth was switched to M9 minimal media, the expression level went up. This is a fortunate situation for NMR studies where minimal media is required for introducing stable isotopes, in this case ^{15}N into all nitrogen sites. Transformation of the plasmid into competent *E. coli* cells designed for overcoming the codon-bias problem, such as Rosetta 2 (DE3) (EMD Biosciences, Inc., San Diego, CA) yielded a small increase in the expression level, and a single rare codon was found at the position encoding Arg-90, which was changed to a more 'abundant' codon CGC by standard site-directed mutagenesis. Varying IPTG concentrations from 0.2 μM to 1 μM did not change the expression level significantly with the optimum IPTG concentration about 0.4 μM, but adding thiamine did help increase the expression level although the BL21 strain is not vitamin B1 deficient. Also, addition of 3%–5% LB media to the minimal media helped increase the expression level as well as the growth rate. Various induction times were tested to optimize the expression level, and a significant reduction in OD_{600} was observed after 6 h of induction at 37 °C. Lowering the incubation temperature following induction is known to reduce the toxicity caused by the over-production of membrane proteins and localize the target protein within the membrane, thus increasing the final yield of the protein. For $hCtr_{TM}$, inducing at 25 °C for 12–16 h resulted in significantly higher cell density than inducing at 37 °C for 5 h, however, it did not increase the amount of purified protein per liter of culture.

Following these optimization steps, the expression of $hCtr1_{TM}$ results in a clearly visible band on the SDS-PAGE at a position corresponding to a molecular weight of around 15 kDa (Fig. 2B). $hCtr1_{TM}$ has a calculated monomeric molecular weight of 17.4 kDa. The cell concentrations loaded in each of the lanes in Fig. 2 were normalized according to the OD_{600}, thus the SDS-PAGE shows the relative levels of polypeptides in the samples. Proteins with the exposed His_6 tag were detected using InVision His-tag In-gel Stain (Invitrogen, Corp., Carlsbad, CA). The InVision His-tag In-gel Stain consists of a fluorescent dye conjugated to a nickel–nitrilotriacetic acid (NTA) complex and allows the specific detection of proteins with a polyHis-tag when exposed to the ultraviolet light, as shown in Fig. 2C. An immunoblot using the antibody raised against the C-terminal region of hCtr1 shown in Fig. 2D indicates that the protein expressed after the induction contains the same amino acid sequence as the C-terminal region of hCtr1.

Harvested cells were resuspended in a standard lysis buffer for *E. coli* containing sodium phosphate buffer, ethylenediamine tetraacetate (EDTA), and NaCl. Added protease inhibitors were essential to keep the protein intact. The pellet containing inclusion bodies and any unlysed cells was separated from the solution containing the soluble proteins and the membrane proteins associated the lipid membranes by low speed centrifugation. A majority of the expressed $hCtr1_{TM}$ was found in the solution according to SDS-PAGE, which may explain some of the anomalous expression results. This is also in agreement with previous reports about the subcellular localization of hCtr1 [10,21]. We made many attempts to isolate the target protein from the soluble fraction by solubilizing the membrane proteins using various detergents such as Triton X-100 and Empigen BB followed by purification using the metal affinity chromatography, but the purity and the yield were not sufficient for NMR studies. The purity was increased when the total membrane fraction was separated from the solution using high-speed centrifugation, and dissolved in a high concentration of detergent. When a solution of the mild detergent n-dodecyl-β-D-maltoside (DDM) was used, extraction of $hCtr1_{TM}$ from the membrane occurred, while a solution of n-octyl-β-D-glucopyranoside (OG) was unable to solubilize the target protein.

Ni^{2+} affinity chromatography in the presence of 0.2% DDM enabled purification of $hCtr1_{TM}$ after optimizing conditions such as salt concentration, pH, detergent concentration, and imidazole concentration. However, when the final sample was collected and the $^{15}N/^{1}H$-HSQC spectrum was obtained, the presence of the detergent DDM had adverse effects on the spectrum, and the low critical micelle concentration (CMC) of DDM (~0.2 mM) prevented complete removal using dialysis. Dodecylphosphocholine (DPC) has been established as an excellent micelle system, and has been used in a number of NMR studies of membrane proteins, including Pf1 coat protein [22], diacylglycerol kinase [23], phospholamban [24], PagP [25] and OmpA [26]. When 1% DPC solution was added to the membrane fraction isolated by high speed ultracentrifugation and incubated overnight at 4 °C, a majority of the $hCtr1_{TM}$ was solubilized and ready to be loaded onto the Ni-NTA resin. Nickel affinity chromatography was carried out in the presence of 0.2% DPC and the binding of $hCTR1_{TM}$ to Ni-NTA (Qiagen, Valencia, CA) resin was very effective even without the presence of reducing agent.

SDS-PAGE of a protein sample from the final step of purification is shown in Fig. 3A. The apparent molecular weight of about 28 kDa suggests that it is a dimer. When the strong reducing agent DTT was added to the sample, the major band on SDS-PAGE was detected around 15 kDa corresponding to the size of $hCtr1_{TM}$ in the monomeric state. The weak band at around 28 kDa was also detected, and this is presumably a small amount of residual dimer. This agrees with previous reports that show that full-length hCtr1 migrates as a monomer and a dimer in non-reducing SDS-PAGE [9,10,27]. These data indicate that the oligomerization properties characteristic of the 190-residue hCtr1 are preserved in the 146-residue $hCtr1_{TM}$, missing the N-terminal 44 residues. The samples loaded onto the SDS gel were purified by nickel affinity chromatography with the protein dissolved in 0.2% DPC, 50 mM sodium phosphate pH 7.6, 1 M NaCl and 300 mM imidazole, before the lithium dodecyl sulfate (LDS) sample buffer (Invitrogen Corp., Carlsbad, CA) was added. This suggests that protein fold is not disrupted by the presence of DPC, which is a similar result to that reported for the pentameric phospholamban [24].

Two-dimensional $^{15}N/^{1}H$ HSQC NMR spectra were obtained from samples of uniformly ^{15}N labeled $hCtr1_{TM}$ in DPC micelles. The optimal sample conditions (Fig. 4A) were ~1 mM protein concentration and 100 mM DPC at pH 5.0. Following nickel affinity chromatography the polypeptide was dialyzed for 2 days against water, and lyophilized to powder. The NMR

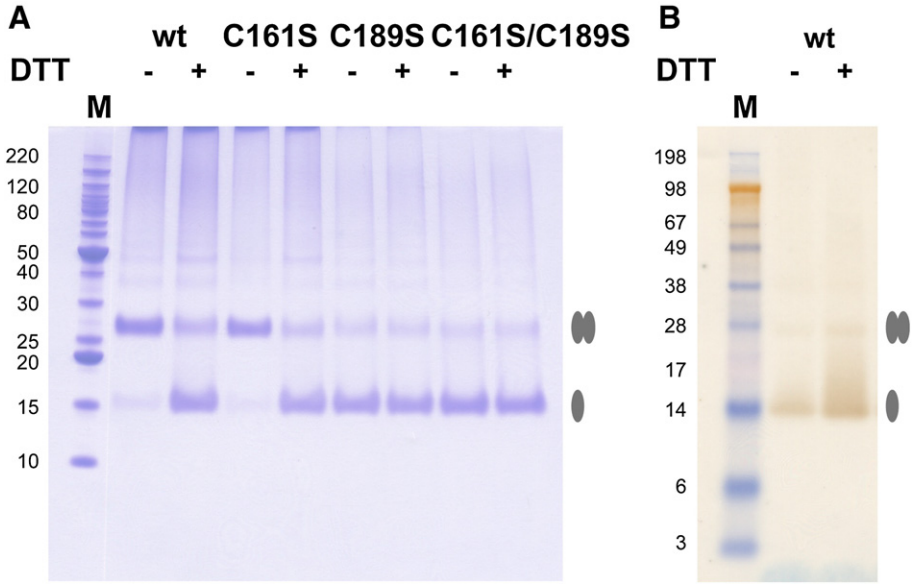

Fig. 3. (A) Benchmark protein standard (Invitrogen Corp., Carlsbad, CA), labeled "M", was used as a molecular weight standard with the standardized molecular weight values in kDa indicated next to the lane. Various $hCTR1_{TM}$ cysteine mutants were analyzed by (A) SDS-PAGE and (B) Western blot detected using C-terminal hCTR1 antibody. The presence or absence of DTT was indicated as + or − on top of the figures. The grey ovals displayed to the right indicate the locations of the plausible oligomeric states of $hCTR1_{TM}$.

samples were prepared by adding the DPC-containing aqueous solution directly to the powder. When a strong reducing agent, such as DTT or tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), was added to an NMR sample, the resolution of the spectrum degraded significantly. Although highly reproducible, this is exactly the opposite of the usual result where the addition of DTT improves the spectral resolution by reducing the cysteine sidechains and eliminating both disulfide linked oligomers and any incorrectly cross-linked polypeptides.

Many different detergents were surveyed in order to find the conditions where $hCtr1_{TM}$ would yield reproducibly well-resolved spectra in the presence of a reducing agent. The lipids examined included 1-myristoyl-2-hydroxy-*sn*-glycero-3-[phospho-RAC-(1-glycerol)] (LMPG), 1,2-dicaproyl-1-*sn*-glycero-3-phosphocholine (DHPC), n-octyl-β-D-glucopyranoside (OG), sodium dodecyl sulfate (SDS), and DDM (n-dodecyl-β-D-maltoside). Most of the tested detergents were not compatible with $hCtr1_{TM}$. Due to low solubility of the protein in LMPG, DHPC, and OG, there were no detectable signals in the $^{15}N/^{1}H$-HSQC NMR spectra. DDM and SDS did solubilize the protein, but did not yield spectra as well resolved as those obtained in DPC. In the spectra in Fig. 4A and B, the number of distinct resonances is approximately the same as the number of residues in the protein. This kind of variation of spectral quality among detergents is typical of many membrane proteins [28]. It is the response to reducing agents that makes $hCtr1_{TM}$ atypical.

3.2. Mutation of cysteine residues and the effect on the oligomerization

The preparation of mutant $hCtr1_{TM}$ polypeptides containing only a single cysteine residue allowed us to examine the influence of the individual sulfhydryl groups on the properties of the protein in the presence of SDS (on gels) and DPC (in NMR samples). The two cysteines in $hCtr1_{TM}$, Cys-161 and Cys-189, were mutated into serines. Since Cys-161 is located in the middle of the third transmembrane helix, labeling this residue with nitroxide spin label could potentially provide sufficient distance information for obtaining the backbone structure of the protein. Three mutants were generated to examine the effect of cysteine substitutions: two single amino acid substitution mutants, C161S and C189S, and a double substitution mutant, C161S/C189S. All the mutants were generated by following the simple site-directed mutagenesis protocol (www.stratagene.com/manuals/200518.pdf), and were transformed into the same C43(DE3) cell line. The same expression, purification, and sample preparation protocols were applied to all of the protein species. All of the proteins were expressed at high levels within the membrane fractions. The SDS-PAGE of all four $hCtr1_{TM}$ proteins in the presence and absence of reducing agents are compared in Fig. 3.

Clearly, the presence of the reducing agent DTT affects the oligomerization state of wild type and C161S. The intensities of the bands corresponding to the monomeric state of $hCtr1_{TM}$, as indicated as single grey oval in the figure, increase when DTT was added to the wild type or C161S proteins. However, the intensities of the bands for C189S and C161S/C189S are not affected by the presence of DTT. This result indicates that the dimeric state of $hCtr1_{TM}$ is stabilized by Cys-189, which is located at the end of C-terminus. Using an antibody directed against the C-terminal residues of hCtr1, the bands corresponding to the monomeric state of $hCtr1_{TM}$ were identified in Fig. 3B. This antibody failed to detect the dimer bands in the absence of DTT, which could be due to the presence of a disulfide linkage preventing the access of the antibody or other factors.

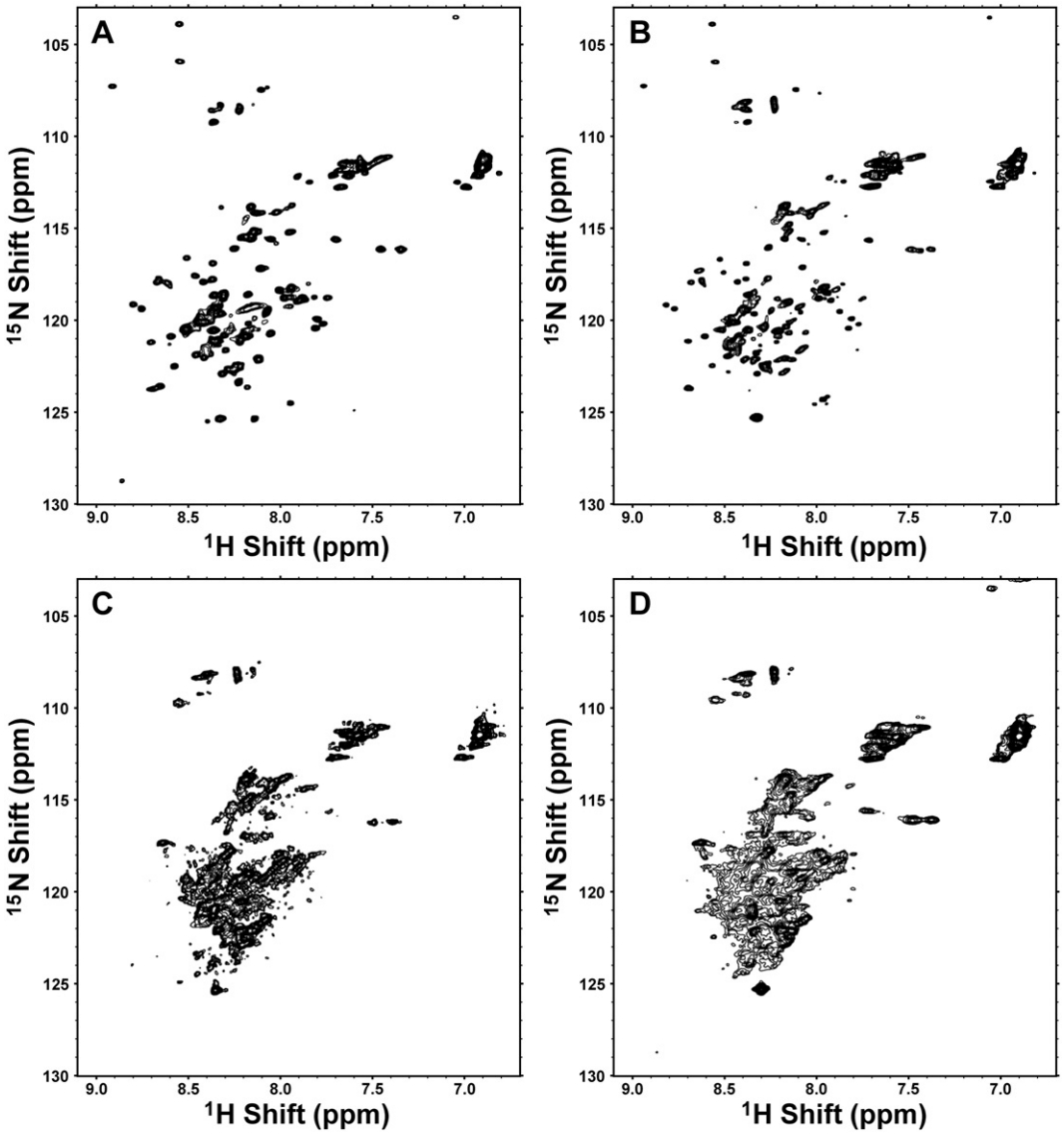

Fig. 4. ^{15}N,^{1}H-HSQC spectra of hCTR1$_{TM}$ cysteine mutants, (A) wild type, (B) C161S, (C) C189S, and (D) C161S/C189S. The proteins were solubilized in 100 mM DPC, and the pH was adjusted to 5.0. All the spectra were acquired at 323 K.

The intriguing, yet somewhat contradictory nature of these findings was reiterated in the NMR spectra of the proteins, which are shown in Fig. 4. The spectra of proteins containing Cys-189 are reasonably well resolved, at least for a polytopic membrane protein in micelles, and those of proteins without Cys-189 are of poor quality. Remarkably, the resolution of the ^{15}N/^{1}H-HSQC spectra is inversely correlated with what would be expected from the oligomerization state of hCtr1$_{TM}$ under the conditions of the SDS-PAGE. The reduced monomers give poorly resolved spectra, and the larger dimers give well-resolved spectra. The ^{15}N/^{1}H-HSQC spectra of the proteins missing Cys-189, C189S and the double mutant C161S/C189S, are indistinguishable from those obtained from samples of wild type hCtr1$_{TM}$ in the presence of DTT, and exhibit the characteristics typical of those from partially or completely unfolded proteins, e.g. intense, highly overlapped signals with very limited ^{1}H chemical shift dispersion. This suggests that replacement of the cysteine residue at position 189 by serine interferes with the oligomerization and folding of hCtr1$_{TM}$. The intermolecular disulfide bond formation was not reversible in this case: once DTT was added to the wild type hCtr1$_{TM}$, raising pH, adding hydrogen peroxide, adding a thiol-specific oxidizing agent *N*,*N*,*N*,*N*-Tetramethylazodicarboxamide (diamide) and/or re-purifying in the absence of DTT did not recover the spectral quality.

4. Discussion

In the course of developing and optimizing samples for NMR structural studies of the membrane protein hCtr1, we discovered the crucial roles of Cys-189 in both oligomerization and folding of the protein. This finding is in partial agreement with previous studies that showed cysteine residues are responsible for the stable oligomerization of hCtr1, as detected from

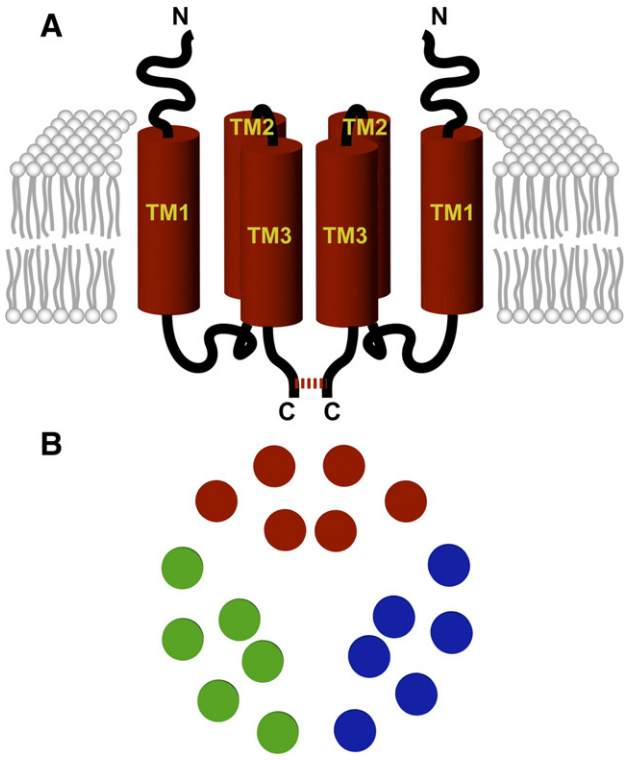

Fig. 5. A model of hCTR1 oligomerization consistent with the experimental results in Figs. 3 and 4. (A) The possible dimeric arrangement of hCTR1, with the dotted line representing the intermolecular disulfide bond between Cys-189 residues that seems to be necessary for the correct folding. The third transmembrane domain helix was arranged to be located inside of the core as suggested by previous report [11]. (B) Top view of hypothetical 'trimer of dimer' arrangement of hCTR1.

the DDM-solubilized membrane fraction [27] or from the total membrane fraction [29]. However, the studies of Eisses and Kaplan suggest that both of the cysteine residues are responsible for the oligomerization and they showed a synergistic effect on the copper transport. In contrast, the result reported here suggests that Cys-189 is the only cysteine responsible for formation of stable dimers. Other studies suggested that the oligomerization of hCtr1 is stabilized by the Gly-X-X-X-Gly (GG4) motif in the third transmembrane domain through helix packing [30], and later, the same group reported a 6-Å resolution projection structure of hCtr1 using electron microscopy, supporting the importance of the third transmembrane domain for multimerization and helix packing and the trimeric arrangement [11] that was initially suggested by the Thiele group using crosslinking experiments [10]. However, the trimeric arrangement cannot be explained by the formation of intermolecular disulfide bonds if, as we find, only one of the two cysteine residues, is involved in oligomerization. The Unger group also noticed the disulfide-dependent profile during the purification using gel filtration chromatography.

We suggest a model in Fig. 5 that reconciles many of the new and earlier findings. This model has the individual polypeptides linked as covalent dimers through their Cys-189 residues. Unger and coworkers suggest that the GG4 motif within the third transmembrane domain stabilizes helix packing [30] and that the third transmembrane domain may be located on the inside of the 'pore' based on the projection density map from the electron microscopy [31]. The locations of the first and second transmembrane domains are currently unknown, although the N-terminal extracellular domain has been reported to interact with itself [9]. This self-association between N-terminal extracellular domains suggests the hypothetical model shown in Fig. 5B, where the top view of the 'trimer of dimer' model is depicted based on the previously reported projection density map [11]. The circles with the same color represent the transmembrane helices within the dimer assembled through the disulfide bond as shown in Fig. 5A, and different colors represent different subunits of the dimers. The model shown here assumes that the third transmembrane domains are located on the inside of the 'pore', and the trimeric arrangement comes from the interactions between the N-terminal extracellular domains, which is excluded in the current study. Note that this model is based on the assumption that the dimerization occurs first through the intermolecular disulfide bond between the Cys-189 residues. It is possible that the other residues, such as the GG4 motif, contribute to the oligomerization process by assisting the helix packing. However, the current study shows that residue Cys-189 plays a critical role in the oligomerization and folding process, and it is impossible to explain the oligomerization through the intermolecular disulfide bonds with the trimer model that has been suggested previously.

In summary, this article describes the cloning, expression and purification of the transmembrane domain of hCtr1, $hCtr1_{TM}$, presents initial NMR spectra of $hCtr1_{TM}$, and identifies residue Cys-189 as crucial for oligomerization and folding of $hCtr1_{TM}$ through the formation of intermolecular disulfide bonds. By integrating these results with previous findings of other groups we develop a plausible model for the organization of hCtr1 in membrane environments that can be used as the basis for planning future structural and mechanistic studies.

Acknowledgements

This research was supported by grants RO1GM075877 and RO1CA095298 from the National Institutes of Health, and it utilized the Resource for NMR Molecular Imaging of Proteins supported by grant P41EB002031.

References

[1] E.D. Harris, Cellular copper transport and metabolism, Annu. Rev. Nutr. 20 (2000) 291–310.
[2] R.M. Llanos, J.F. Mercer, The molecular basis of copper homeostasis copper-related disorders, DNA Cell Biol. 21 (2002) 259–270.
[3] D.L. Huffman, T.V. O'Halloran, Function, structure, and mechanism of intracellular copper trafficking proteins, Ann. Rev. Biochem. 70 (2001) 677–701.
[4] A.C. Rosenzweig, Metallochaperones: bind and deliver, Chem. Biol. 9 (2002) 673–677.
[5] S.J. Opella, T.M. DeSilva, G. Veglia, Structural biology of metal-binding sequences, Curr. Opin. Chem. Biol. 6 (2002) 217–223.
[6] A. Dancis, D.S. Yuan, D. Haile, C. Askwith, D. Eide, C. Moehle, J. Kaplan, R.D. Klausner, Molecular characterization of a copper transport

protein in *S. cerevisiae*: an unexpected role for copper in iron transport, Cell 76 (1994) 393–402.
[7] S.A. Knight, S. Labbe, L.F. Kwon, D.J. Kosman, D.J. Thiele, A widespread transposable element masks expression of a yeast copper transport gene, Genes Dev. 10 (1996) 1917–1929.
[8] B. Zhou, J. Gitschier, hCTR1: a human gene for copper uptake identified by complementation in yeast, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 7481–7486.
[9] A.E. Klomp, J.A. Juijn, L.T. van der Gun, I.E. van den Berg, R. Berger, L.W. Klomp, The N-terminus of the human copper transporter 1 (hCTR1) is localized extracellularly, and interacts with itself, Biochem. J. 370 (2003) 881–889.
[10] J. Lee, M.M. Pena, Y. Nose, D.J. Thiele, Biochemical characterization of the human copper transporter Ctr1, J. Biol. Chem. 277 (2002) 4380–4387.
[11] S.G. Aller, V.M. Unger, Projection structure of the human copper transporter CTR1 at 6-A resolution reveals a compact trimer with a novel channel-like architecture, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 3627–3632.
[12] R. Safaei, S.B. Howell, Copper transporters regulate the cellular pharmacology and sensitivity to Pt drugs, Crit. Rev. Oncol./Hematol. 53 (2005) 13–23.
[13] X. Lin, T. Okuda, A. Holzer, S.B. Howell, The copper transporter CTR1 regulates cisplatin uptake in *Saccharomyces cerevisiae*, Mol. Pharmacol. 62 (2002) 1154–1159.
[14] A.K. Holzer, G.H. Manorek, S.B. Howell, The contribution of the major copper influx transporter CTR1 to the cellular accumulation of cisplatin, carboplatin and oxaliplatin, Mol. Pharmacol. 70 (2006) 1390–1394.
[15] S. Ishida, J. Lee, D.J. Thiele, I. Herskowitz, Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 14298–14302.
[16] W.L. Hubbell, A. Gross, R. Langen, M.A. Lietzow, Recent advances in site-directed spin labeling of proteins, Curr. Opin. Struck. Biol. 8 (1998) 649–656.
[17] J.L. Battiste, G. Wagner, Utilization of site-directed spin labeling and high-resolution heteronuclear nuclear magnetic resonance for global fold determination of large proteins with limited nuclear Overhauser effect data, Biochemistry 39 (2000) 5355–5365.
[18] B. Liang, J.H. Bushweller, L.K. Tamm, Site-directed parallel spin-labeling and paramagnetic relaxation enhancement in structure determination of membrane proteins by solution NMR spectroscopy, J. Am. Chem. Soc. 128 (2006) 4389–4397.
[19] S.J. Opella, F.M. Marassi, Structure determination of membrane proteins by NMR spectroscopy, Chem. Rev. 104 (2004) 3587–3606.
[20] B. Miroux, J.E. Walker, Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels, J. Mol. Biol. 260 (1996) 289–298.
[21] J.F. Eisses, Y. Chi, J.H. Kaplan, Stable plasma membrane levels of hCTR1 mediate cellular copper uptake, J. Biol. Chem. 280 (2005) 9635–9639.
[22] K.J. Shon, Y. Kim, L.A. Colnago, S.J. Opella, NMR studies of the structure and dynamics of membrane-bound bacteriophage Pf1 coat protein, Science 252 (1991) 1303–1305.
[23] K. Oxenoid, H.J. Kim, J. Jacob, F.D. Sonnichsen, C.R. Sanders, NMR assignments for a helical 40 kDa membrane protein, J. Am. Chem. Soc. 126 (2004) 5048–5049.
[24] K. Oxenoid, J.J. Chou, The structure of phospholamban pentamer reveals a channel-like architecture in membranes, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 10870–10875.
[25] P.M. Hwang, W.Y. Choy, E.I. Lo, L. Chen, J.D. Forman-Kay, C.R. Raetz, G.G. Prive, R.E. Bishop, L.E. Kay, Solution structure and dynamics of the outer membrane enzyme PagP by NMR, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 13560–13565.
[26] A. Arora, F. Abildgaard, J.H. Bushweller, L.K. Tamm, Structure of outer membrane protein A transmembrane domain by NMR spectroscopy, Nat. Struct. Biol. 8 (2001) 334–338.
[27] J.F. Eisses, J.H. Kaplan, Molecular characterization of hCTR1, the human copper uptake protein, J. Biol. Chem. 277 (2002) 29162–29171.
[28] R.C. Page, J.D. Moore, H.B. Nguyen, M. Sharma, R. Chase, F.P. Gao, C.K. Mobley, C.R. Sanders, L. Ma, S.D. Sonnichsen, S. Lee, S.C. Howell, S.J. Opella, T.A. Cross, Comprehensive evaluation of solution nuclear magnetic resonance spectroscopy sample preparation for helical integral membrane proteins, J. Struct. Funct. Genomics 7 (2006) 51–64.
[29] J.F. Eisses, J.H. Kaplan, The mechanism of copper uptake mediated by human CTR1: a mutational analysis, J. Biol. Chem. 280 (2005) 37159–37168.
[30] S.G. Aller, E.T. Eng, C.J. De Feo, V.M. Unger, Eukaryotic CTR copper uptake transporters require two faces of the third transmembrane domain for helix packing, oligomerization, and function, J. Biol. Chem. 279 (2004) 53435–53441.
[31] C.J. De Feo, S.G. Aller, V.M. Unger, A structural perspective on copper uptake in eukaryotes, Biometals 20 (2007) 705–716.
[32] F. Delaglio, S. Grzesiek, G.W. Vuister, G. Zhu, J. Pfeifer, A. Bax, NMRPipe: a multidimensional spectral processing system based on UNIX pipes, J. Biomol. NMR 6 (1995) 277–293.