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Characterization and Copper Binding Properties of Human COMMD1 (MURR1)†

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ABSTRACT: COMMD1 (copper metabolism gene MURR1 (mouse U2af1-rs1 region1) domain) belongs to a family of multifunctional proteins that inhibit nuclear factor NF-κB. COMMD1 was implicated as a regulator of copper metabolism by the discovery that a deletion of exon 2 of COMMD1 causes copper toxicosis in Bedlington terriers. Here, we report the detailed characterization and specific copper binding properties of purified recombinant human COMMD1 as well as that of the exon 2 product, COMMD-(61−154). By using various techniques including native-PAGE, EPR, UV−visible electronic absorption, intrinsic fluorescence spectroscopies as well as DEPC modification of histidines, we demonstrate that COMMD1 specifically binds copper as Cu(II) in 1:1 stoichiometry and does not bind other divalent metals. Moreover, the exon 2 product, COMMD(61−154), alone was able to bind Cu(II) as well as the wild type protein, with a stoichiometry of 1 mol of Cu(II) per protein monomer. The protection of DEPC modification of COMMD1 by Cu(II) implied that Cu(II) binding involves His residues. Further investigation by DEPC modification of COMMD(61−154) and subsequent MALDI MS mapping and MS/MS sequencing identified the protection of His101 and His134 residues in the presence of Cu(II). Fluorescence studies of single point mutants of the full-length protein revealed the involvement of M110 in addition to H134 in direct Cu(II) binding. Taken together, the data provide insight into the function of COMMD1 and especially COMMD(61−154), a product of exon 2 that is deleted in terriers affected by copper toxicosis, as a regulator of copper homeostasis.

Copper is an essential trace element that plays an important role in mammalian cellular metabolism (*1*). Although trace amounts of copper are needed to sustain life, excess copper is extremely toxic. Mutations of genes involved in copper homeostasis resulted in the disorder of copper metabolism in humans and animals. Cloning of the genes responsible for the two closely related major genetic disorders of copper metabolism in humans, Menkes disease (*ATP7A*) and Wilson disease (*ATP7B*) (*2*−*6*), was a major breakthrough in our understanding of intracellular copper transport. Both genes encode copper-transporting P-type ATPases. A crucial feature of the copper-transporting ATPases is the presence of a large N-terminal segment, which contains six copper-binding domains, each capable of binding one copper atom in the +1 oxidation state. The missense mutations found within metal-binding domains 1, 5, and 6 are known to give rise to Wilson disease, stressing their importance in function (*5*).

In Bedlington terriers suffering from an autosomal recessive disorder, copper toxicosis (BT/CT[1]), there is massive copper accumulation in the liver, seen as dense granules in lysosomes, which results in chronic hepatitis and cirrhosis (*7*). Recently, the *MURR1* gene has been identified, and a genomic deletion mutation of exon 2 of this gene, resulting in the complete absence of this protein probably due to protein instability, was detected in Bedlington terriers affected by this disease (*8*). This implies that MURR1 may play a role in copper metabolism (*8*). Furthermore, a new family of proteins in diverse species, including flies and yeast, that have structural and functional homology to MURR1 has been discovered (*9*). Thus, a new nomenclature for this family of proteins has been assigned as COMM (copper metabolism gene MURR1) domain or COMMD, which at present consists of 10 subgroups (COMMD1−COMMD10), where MURR1 is now known as COMMD1 (*9*). These proteins are defined by the presence of a conserved and unique motif that is leucine-rich, 70−85 amino acid long, and near the carboxyl terminus, which functions as an interface for protein−protein interactions.

COMMD1 is ubiquitously expressed in tissues and cell types (*10*). The protein is mainly located in cytoplasm but has also been found in cytoplasmic vesicular compartments of carcinoma cell lines (*10*) as well as in the perinuclear compartment (*11*). The actual function of COMMD1 is not

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[1] Abbreviations: MURR1, mouse U2af1-rs1 region 1; COMMD, copper metabolism gene MURR1 domain; BT/CT, Bedlington terriers with copper toxicosis; NF-κB, nuclear factor κB; XIAP, X-linked inhibitor of apoptosis; Trx, thioredoxin; GST, glutathione *S*-transferase; βME, β-mercaptoethanol; Sulfo-EGS, ethylene glycol bis (sulfosuccinimidylsuccinate); DEPC, diethylpyrocarbonate; BCA, bicinconinic acid; EDTA, ethylenediaminetetraacetic acid; TCEP, tris (2-carboxyethyl) phosphine; EPR, electron paramagnetic resonance; MS, mass spectrometry; MALDI, matrix-assisted laser desorption ionization.

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yet clear, but recently COMMD1 was found to interact specifically with ATP7B, a P-type ATPase involved in the transport of copper in the liver (*12*), but not with other copper-binding proteins, such as ATP7A, ATOX1, SOD, or CCS. COMMD1 was also found to interact with the human epithelial sodium channel and to inhibit its activity (*13*). COMMD1 has been reported to inhibit the degradation of IκBα, thus maintaining the transcription factor NF-κB in an inactive form, which is key in making the resting CD4 T-cells nonpermissive for HIV infection (*14*). It was further demonstrated that COMMD1 regulates the nuclear function of NF-κB by affecting the association of NF-κB with chromatin (*9*). These findings implicate a broader role of COMMD1 in the control of cellular functions.

A report of studies on human embryonic kidney 293 cells showed inverse correlation between the COMMD1 protein and the intracellular level of copper (*11*). Transfection of short interfering RNA (siRNA) targeting COMMD1 into 293 cells resulted in increased intracellular copper levels. Furthermore, a negative regulator of COMMD1 was identified as a protein called XIAP (X-linked inhibitor of apoptosis), which is a potent suppressor of apoptosis (*11*). Ectopic expression of XIAP resulted in increased copper levels, whereas transformed fibroblasts derived from Xiap-deficient mice had reduced cellular copper level in conjunction with increased COMMD1 level. Likewise, the copper level in the liver tissue of Xiap-deficient mice was lower than that in control animals. XIAP was found to decrease the level of COMMD1 by promoting the ubiquitination and degradation of COMMD1 (*11*). Most recently, XIAP has also been reported to bind copper, which resulted in the reversible change in conformation as well as in enhancing the degradation of this protein (*15*). However, in the same report, XIAP levels were markedly reduced under the conditions of copper overload, such as those in Wilson disease or other copper toxicosis disorders and in cell cultures under high copper conditions.

In spite of various reports that suggested the role of COMMD1 in copper metabolism, there has been no evidence of its ability to bind copper. Here, we report the expression, purification, and characterization of recombinant human COMMD1 and demonstrate that COMMD1 specifically binds copper in the Cu(II) form with a stoichiometry of 1 mol Cu(II) per mol of protein. The Cu(II)-binding site is located in the exon 2 product, which is deleted in Bedlington terriers affected by copper toxicosis disease.

EXPERIMENTAL PROCEDURES

Cloning of Recombinant Human COMMD1 and Its Truncated Mutants. The expression vector for human COMMD1 was constructed from cDNA, kindly provided by Dr. Diane Cox (Department of Genetics, University of Alberta, Canada). First, the pET32(a) vector (Novagen), which expresses Trx and His_6-tag fusion protein, was modified by replacing the enterokinase cleavage site with the human rhinovirus 3C protease ("Prescission" protease(Amersham)) cleavage site containing amino acid sequence LEVLFQGP. Briefly, the following nucleotides 5′GATCATCTGGAAGTTCTGTTCCAGGGGCC 3′ and 5′ CATGGGCCCCTGGAACAGAACTTCCAGAT 3′ that encode LEVLFQGP were inserted into pET32(a) precut with BglII and NcoI restriction enzymes. The resulting vector is called pET32-(p).

The coding sequence of full length COMMD1 and its truncated mutants, namely, COMMD(61−154), which is the product of exon 2 and COMMDΔ (61−154), the product of exon1 and exon 3, were cloned into the pET32(p) plasmid between NcoI and Not1 sites. The coding sequences of full length and COMMD(61−154) were amplified by PCR using appropriate primers containing NcoI at the 5′-end and NotI at the 3′-end. The PCR products were digested with NcoI and NotI, purified, and ligated to the precut pET32(p). For COMMDΔ(61−154), two fragments of DNA were amplified. One contains the sequence encoding amino acid residues 1−60, between the NcoI and HinDIII sites, and the other contains the sequence encoding residues 155−190, between the HinDIII and NotI sites. Each fragment was digested with its appropriate restriction enzymes, combined, and ligated to pET32(p) precut with NcoI and NotI.

Site-directed mutagenesis was performed using the QuickChange mutagenesis kit (Stratagen) with appropriate primers, according to the manufacturer's instructions. The DNA sequence of constructs and mutants was verified by automated DNA sequencing (DNA Sequencing Facility, Center for Applied Genomics, Hospital for Sick Children, Toronto, Canada).

Expression and Purification of COMMD1 and Mutants. The plasmids were transformed into BL21(DE3) cells, and the proteins were expressed according to standard protocols. COMMD1 and its mutants were expressed mainly in the inclusion bodies. Briefly, the proteins were purified as follows. After the induction of expression, cells were harvested and resuspended in 50 mM Tris-HCl buffer and 300 mM NaCl at pH 8.0 (buffer A), followed by the addition to the final concentration of 1 mM PMSF, 5 mM Benzamidine-HCl, 0.1 mg/mL lysozyme, and 0.5% Triton X-100. Cells were lysed by repeated freeze−thaw cycles. The cell lysate was incubated with 1 μg/mL Dnase1 at 37 °C for 30 min followed by centrifugation at 30,000 rpm for 30 min. The supernatant was discarded and the pellet was homogenized in 6 M Urea and 50 mM Tris-HCl at pH 8.0 and the solution was centrifuged at 45,000 rpm for 45 min. The supernatant was refolded by dialysis against 50 mM Tris-HCl and 15% glycerol at pH 8.0, with one change of buffer. The dialyzed protein solution was adjusted to contain 300 mM NaCl before binding to a Co^{2+}-IMAC column (Talon column (CloneTech)), pre-equilibrated with 50 mM Na/K-phosphate and 300 mM NaCl at pH 7.0 (buffer B). After washing extensively with buffer B, the fusion protein was eluted with buffer B and 150 mM imidazole. The fractions containing the fusion protein were concentrated by Amicon filtration to about one-third of the original volume, followed by the addition of Prescission protease (Amersham) at 0.3 units per mg of fusion protein. The mixture was dialyzed, at 4 °C versus buffer B and 1 mM βME to carry out digestion and the removal of imidazole at the same time. After the digestion was completed, the mixture was further dialyzed versus buffer B without βME before passing through a small GSH-Sepharose column to remove the protease, followed by application to another Talon column. The cleaved protein, collected in the flow-through and wash fractions from the Talon column, was concentrated and the buffer adjusted to contain 50 mM phosphate, 150 mM NaCl at pH 7.0 (PBS),

and 10% glycerol. The purity of the protein after this stage was more than 90%. The protein was stored at −80 °C in small aliquots. Typical yield was between 10 and 20 mg per liter of cell culture.

After protease cleavage, the final protein products contained some extra amino acids as follows: GP at the N-terminus of wild type and GPMG at the N-terminus of COMMD(61−154). For COMMDΔ(61−154), there was GP at the N-terminus and an L that links aa residue 60 to residue 155 of COMMD1.

Polyacrylamide Gel Electrophoresis. SDS−PAGE was performed according to Laemmli's method (*16*), and native-PAGE was carried out by omitting SDS from the separating gel and running buffer. For native-PAGE, preliminary results indicated that there was no difference whether the gel was pre-run before sample loading or not; therefore, all experiments were performed without pre-electrophoresis of the gels.

Chemical Cross-Linking. Cross-linking reactions with Sulfo-EGS (ethylene glycol bis(sulfosuccinimidylsuccinate) (Pierce), with a spacer arm of 16.1 Å) were carried out in the absence and presence of metal ions. Reactions were performed by incubating 10 μM protein with 10 mM cross-linking agent in PBS at room temperature for 30 min. The reaction was quenched with 0.1 M Tris-HCl at pH 8.0 for at least 15 min. The cross-linked products were analyzed on SDS−PAGE. Control reactions, without the cross-linker, were performed in parallel.

UV−Visible Spectra of Metal−Protein Complexes. All UV−visible spectra were recorded on a Hitachi U-3210 Spectrophotometer. For the UV spectra of Cu(II), protein complexes were generated by direct titration of aliquots of $CuCl_2$ solution with 10−20 μM protein solution in PBS. The spectra between wavelengths 240−360 nm were recorded, and Cu(II) complex formation was followed by measuring an increase in absorbance at 254 nm. The visible spectra between wavelengths 300−800 nm of the Cu(II)−protein complex were obtained from 250 μM Cu(II)−COMMD1 by using a concentration of apoprotein that was the same as that of reference.

Stoichiometry of Copper Binding to COMMD1. Aliquots of 10−50 μM protein solution were incubated with 5×−10× excess of Cu(II) solutions, followed by dialysis versus buffer B. The dialyzed protein solutions were determined for copper content and protein concentration as well as gel electrophoretic behaviors. The total copper content in copper bound proteins was determined by the BCA (bicinchoninic acid) reaction described by Brenner and Harris (*17*), after the metal was released by TCA treatment. Protein concentration was estimated by the method of Bradford (*18*) and by the BCA protein assay (*19*).

Chemical Modification of Histidines by DEPC. DEPC modification of COMMD1 was carried out as described in refs 20 and 21 (*20*, *21*). Briefly, small aliquots of 10−20 μM protein solution in PBS were incubated with DEPC (dissolved in absolute ethanol) at a final concentration of 1 mM, in the absence and presence of varying concentrations of Cu(II) or Zn(II) at 25 °C for 30 min. The number of histidine residues modified was determined from the difference in absorbance at 242 nm between protein solutions containing DEPC and those with the same amount of ethanol (control tubes), using the molar absorption coefficient, 3200 M^{-1} cm^{-1}.

Mapping the Copper-Binding Region of COMMD1 and COMMD(61−154) Using DEPC and MS Analysis. A 5-fold molar excess of DEPC was reacted with 33 μM COMMD1 and 49 μM COMMD(61−154) in PBS for 30 min at room temperature. In the case of the copper-bound protein, samples were incubated with 3-fold molar excess of $CuCl_2$ for 60 min on ice. The samples were dialyzed against 2 mM ammonium bicarbonate buffer overnight and subjected to MS analysis. Small portions of the samples were digested with trypsin (sequencing grade, Roche) at the ratio of 1:100 at 37 °C for 3 h. The digested samples were dried in vacuum and redissolved in 10 μL of 2 mM ammonium bicarbonate and subjected to MALDI-MS and tandem MS/MS analysis.

The tryptic digests were analyzed on the Appiled Biosystems/Sciex QStar XL mass spectrometer with an oMALDI 2 source, after mixing with the MALDI matrix (2,5-dihydroxybenzoic acid, 160 mg mL^{-1}). The *m*/*z* spectrum was calibrated externally with two standard peptides (dalargin and melittin) and was acquired using a UV nitrogen laser (337 nm). Both mass-mapping and tandem MS measurements (MS/MS) on the proteolytic peptides as well as the measurement of the overall protein masses were carried out on this instrument.

Fluorescence Spectroscopy and Titration with Metals. Steady-state fluorescence spectra were monitored at room temperature on a Photon Technology International Fluorometer, model C-60. Emission spectra were collected from 310 to 400 nm (with ϵ_{ex} = 280 nm, 1 s/nm and bandwidth = 1 nm), and fluorescence intensities from 310 to 380 nm were integrated. For Cu(II) binding experiments, 1 mL aliquots of 1 μM COMMD1 or COMMD(61−154) in PBS were mixed with $CuCl_2$ at various concentrations from 0.2−50 μM. The fluorescence intensity of each sample was quantified. The percentage of decrease in fluorescence intensity due to the addition of Cu(II), relative to the sample without metal, was calculated and used to represent the degree of bound copper. The data were fitted using the Sigma plot 8 program (Jandel) to obtain the binding constant (K_d) of Cu(II) to the proteins. To compare metal binding selectivity, 1 μM COMMD1 was mixed with 50 μM $CuCl_2$, $ZnCl_2$, $NiCl_2$, $CoCl_2$, or $CdCl_2$, and the effect on fluorescence intensities was compared.

EPR Spectroscopy. One milliliter aliquots of COMMD1 in PBS were incubated with 3−5× excess of Cu(II) solutions, followed by dialysis in PBS. The dialyzed protein solutions were determined for copper content and protein concentration. The samples of 0.8 mM COMMD1 were loaded into a 50 μL capillary tube for EPR measurement. The EPR spectra were recorded on a Bruker ECS 106 spectrometer operating at ν = 9.38 GHz using a microwave power of 10.0 mW, and a modulation amplitude of 9.698 G. All X-band spectra were acquired at 100 K. The sample was placed in the cavity where the temperatures were fixed by the addition of nitrogen liquid. Spectra were recorded from 2600 to 3800 G. 200 scans were performed and accumulated. Simulation of the EPR spectrum was performed by using Xsophe Computer Simulation Software Suite (version 1.1.2), developed by the Center for Magnetic Resonance and Department of Mathematics, the University of Queensland, Australia, and EPR parameters determined by Bruker Biospin, Rheinstetten, Germany. For each transition, the resonance field was calculated using perturbation theory and assuming that all

tensors are coaxial. The resulting stick spectrum was convoluted with Gaussian functions.

RESULTS AND DISCUSSION

COMMD1 Exists as a Mixture of Monomeric and Dimeric Proteins. When purified COMMD1 was separated on SDS−PAGE in the absence of a reducing agent, there were two protein bands. The major one has a MW of about 21 and a minor band with a MW of about 40 (Figure 1A, lane 1). However, in the presence of a reducing agent, there was only one major protein band of MW about 21 (Figure 1A, lane 2). This indicates that COMMD1 exists as a mixture of monomers and dimers (which resulted from disulfide bond formation). When the protein was chemically cross-linked with Sulfo-EGS, the major product appeared as a dimer plus some higher aggregates with only a small percentage of the monomeric band (Figure 1A, lane 3). The proportion of dimeric product, relative to the monomer, from cross-linking experiments did not correlate well with the proportion of these two forms seen in Figure 1A, lane 1. This raises the question as to whether the disulfide bond formation is a prerequisite for dimer formation. To address this issue, a mutant, COMMD(C160A), with Cys residue 160 (there is only one Cys in COMMD1) replaced by Ala was generated, purified, and characterized. As shown in Figure 1A, lanes 5, 6, and 7, there is only a major band of monomer both in the presence and absence of the reducing agent, yet the cross-linked product still shows the presence of the dimer, similar to what was observed with the wild type protein. These results indicate that COMMD1 dimerization does not require disulfide bond formation. However, a disulfide bond is probably formed as a result of protein dimerization because of the close proximity of the Cys residue in each monomer to the dimerization interface. Furthermore, the protein may dimerize weakly without the disulfide bond; the presence of the disulfide bond further strengthens the protein dimer formation.

The COMM domain, which comprises amino acid residues 116−185 of COMMD1, was reported to function as an interface for protein−protein interaction between itself and other proteins in the COMMD family (*9*). To further pinpoint which part of the COMM domain contributes to this function, two truncated peptides, COMMD(61−154), a peptide product of exon 2, and COMMDΔ(61−154), the peptide product of exons 1 and 3, were expressed and purified. Both truncated peptides were expressed adequately and were in inclusion bodies similar to the full-length protein. The purified peptides were compared for their abilities to dimerize. As shown in Figure 1B (lanes 3 and 7), both peptides were able to form dimers as well as some higher oligomers. However, the ratio of dimer/monomer was higher in COMMDΔ(61−154) than in COMMD(61−154) and was almost similar to that detected in the full-length protein. Some of the COMMDΔ(61−154) dimer also contains the disulfide bond (Figure 1B, lane 5), which suggests that the stretch of the dimerization interface includes the Cys 160 residue. Because each of the two peptides contains an almost equal half of the COMM domain and each is capable of forming a dimer, it is likely that the dimerization interface lies at the junction between exons 2 and 3. Burstein et al. (*9*) have found that the exon 1 and 3 product had a significant impairment in COMMD−COMMD interaction by using a protein analogous to the purified protein COMMDΔ(61−154). However, in contrast, we found that the carboxy terminus part of COMM domain was able to form a dimer (and oligomers) almost as strongly as the full-length protein. This suggests a major role for this acidic residue-rich region of the COMM domain in protein interaction. Indeed, the most recent studies by de Bie et al. (*22*) on the interaction between COMMD1 and COMMD6 also

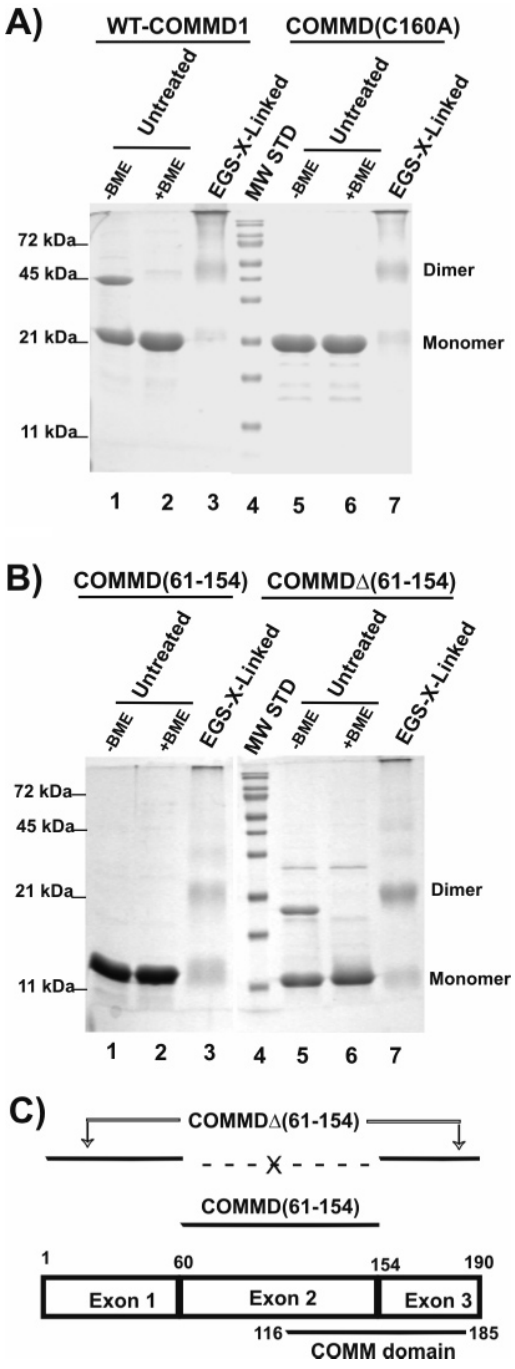


FIGURE 1: (A and B) SDS−PAGE of wild type COMMD1, lanes 1−2A; COMMD(C160A), lanes 5−6A; COMM(61−154), lanes 1−2B; and COMMDΔ(61−154), lanes 5−6B in the absence and presence of β-mercaptoethanol. Their cross-linked products (lanes 3A, 7A, 3B, and 7B) were analyzed in the presence of only β-mercaptoethanol. An aliquot of 10 μg protein samples was applied per lane. Protein bands were stained with the Commassie blue dye. (C) Schematic representation of COMMD1 and the amino acid residues that are the boundaries of each exon as well as the size of truncated mutants, COMMD(61−154) and COMMDΔ(61−154).

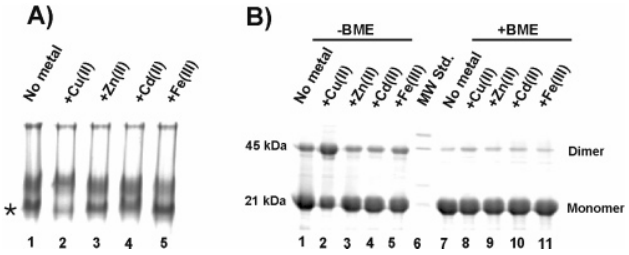


FIGURE 2: Effect of divalent metals on the gel electrophoretic pattern of COMMD1. (A) Native-PAGE of COMMD1 in the presence and absence of divalent metals. Ten microgram aliquots of COMMD1 were incubated without and with 0.5 mM $CuCl_2$, $ZnCl_2$, $CdCl_2$, or $FeCl_2$ before being applied to a 10% native-PAGE. (B) SDS−PAGE of COMMD1 in the presence of metals. The same amount of protein in the absence and presence of divalent metals were applied to 16% SDS−PAGE without (lanes 1B−5B) and with β-mercaptoethanol (lanes 7B−11B). The protein bands were stained with Commassie blue dye.

indicate that COMMD1 from dog exon 1 + 3 (same as our COMMDΔ(61−154)) was capable of interacting with COMMD6 as well as full-length COMMD1.

COMMD1 Specifically Binds with Cu(II) but Not with other Divalent Metals. When a purified COMMD1 was separated on native-PAGE there appeared to be two protein bands with equal intensity on the gel (lane 1A), suggesting that there are two populations of the protein in the sample. This probably represents the monomer and dimer as indicated by the cross-linking result shown in Figure 1. When COMMD1 was incubated with $CuCl_2$ and separated on native-PAGE (Figure 2A), the intensity of the fast migrating band (*) decreased significantly (lane 2A), whereas other divalent metals did not have such pronounced effect. SDS−PAGE (Figure 2B) without the reducing agent also showed that the presence of Cu(II) increased dimer formation compared to that of other metals (lane 2B). We speculate that Cu(II) binding may facilitate dimerization because of ligand sharing between the two monomers; however, further investigation is necessary to support this hypothesis. This is the first evidence that COMMD1 can specifically interact with Cu(II) and not with other metals tested.

Copper Interferes with the Intrinsic Fluorescent of COMMD1. To further confirm that there is a specific interaction between COMMD1 and Cu(II), the effect of metals on the intrinsic fluorescence of COMMD1 was investigated. There are three Trp and two Tyr residues present in COMMD1. When excited at 280 nm, the emission spectrum of COMMD1 had a maximum wavelength at 328 nm. Addition of Cu(II) to the protein solution resulted in a decrease in fluorescence intensity in a concentration-dependent manner, as demonstrated in Figure 3A. The shape of the emission spectrum of COMMD1 remained unchanged upon binding, thus suggesting a lack of large scale conformational change by the Cu(II)−protein complex. The binding curve shown in Figure 3B, depicts saturation binding between COMMD1 and Cu(II). The average K_d of Cu(II) was between 3 and 5 μM. Figure 3B also shows that the presence of Zn(II) or other divalent metals had no effect on the fluorescence of COMMD1. These results strongly suggest that COMMD1 specifically binds Cu(II). Interestingly, the presence of TCEP, a strong reducing agent, abolished the effect of Cu(II) (Figure 3B), implying that COMMD1 has affinity exclusively for Cu(II) not for Cu(I). The perturbation effect on intrinsic

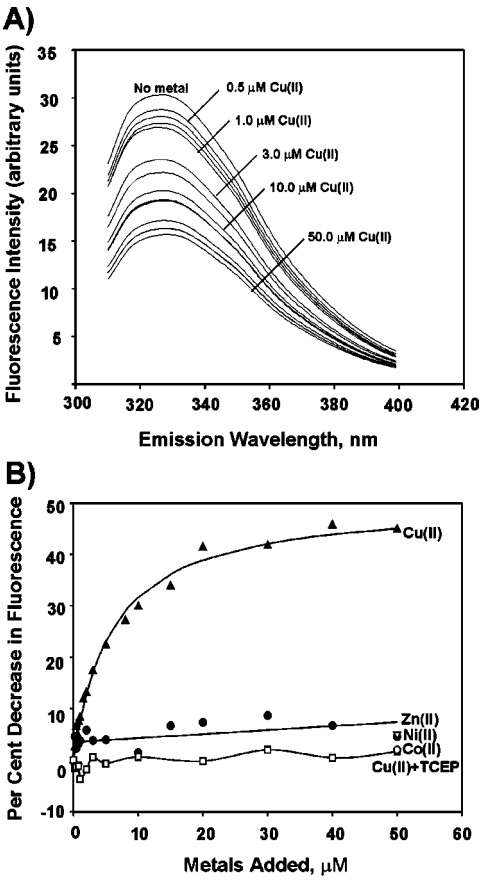


FIGURE 3: (A) Fluorescent spectra of the titration between COMMD1 and Cu(II). One milliliter aliquots of a 1 μM solution of COMMD1 in PBS were incubated with various concentrations of $CuCl_2$. The fluorescent spectra were obtained as described in the Experimental Procedures section. (B) Plots of the percent decrease in fluorescence caused by the presence of metals vs the concentration of metals added. For Zn(II), Cu(II), and Cu(II) + TCEP, the metals were used through the range from 0 to 50 μM. For Ni(II) and Co(II), the metals were used only at 50 μM.

fluorescence of COMMD1 by Cu(II) could be reversed by the presence of EDTA (data not shown).

Next, the stoichiometry of Cu(II) bound to COMMD1 was determined. When COMMD1 was incubated with excess Cu(II) followed by extensive dialysis to remove unbound Cu(II), it was found that Cu(II) bound to COMMD1 at a metal/protein ratio of 1 mol (0.982 ± 0.214) of Cu(II) per mol of COMMD1 monomer.

UV−Visible Spectral Characteristics of the COMMD1−Cu(II) Complex. When COMMD1 was titrated with increasing amounts of Cu(II), there was a small increase in absorbance around 254 nm (Figure 4A), indicating the presence of a Cu−thiolate bond, which may be contributed by Cys or Met residues. This effect was abolished when Cu(II) titration was performed in the presence of TCEP (Figure 4B), which again confirms the similar observation (Figure 3B) that COMMD1 binds the oxidized form of copper. For titration and the determination of protein spectra in the visible region, when a high concentration of protein was used (due to the low sensitivity of the absorption), a greenish blue color was observed when Cu(II) was added to protein solution. There was no color formed in a control tube containing PBS and the same amount of Cu(II). In the visible region, there was an increase in the absorbance peak at about 360 nm

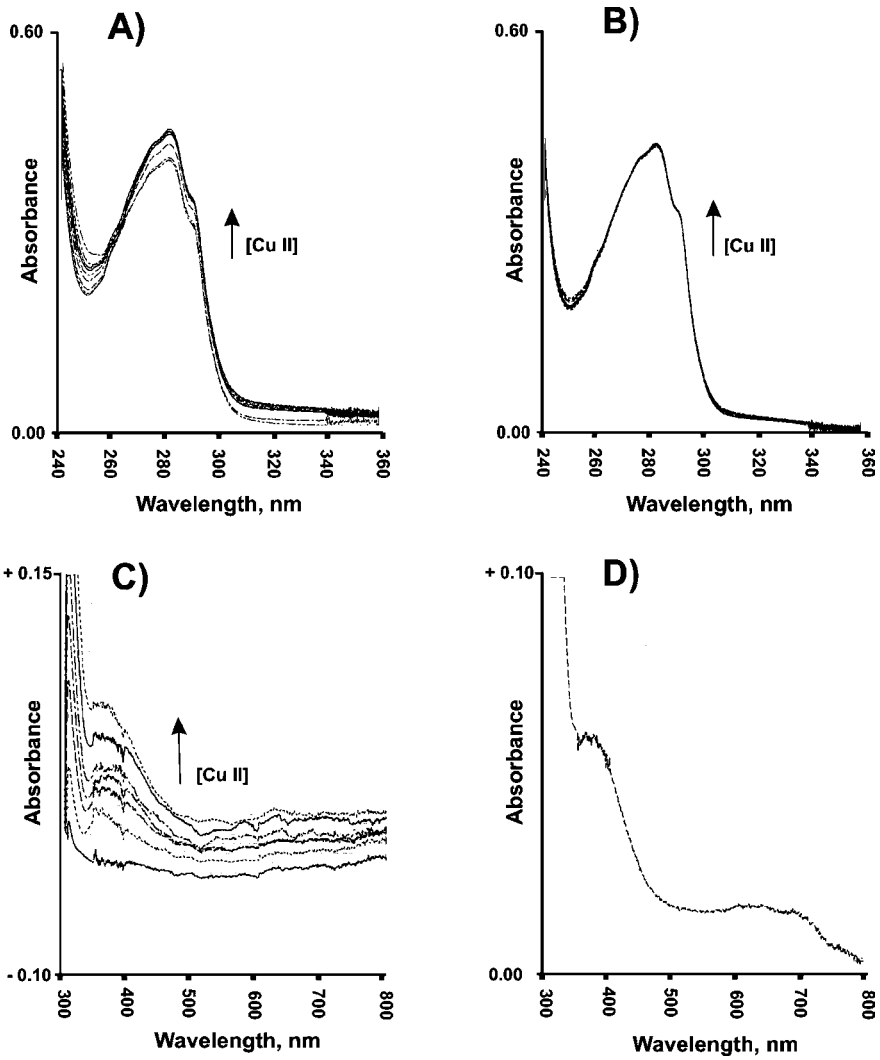


FIGURE 4: UV−visible spectra of COMMD1 in the presence of Cu(II). (A) COMMD1 titration with Cu(II). A 1 mL aliquot of 20 μM COMMD1 in PBS was titrated with Cu(II) at a molar ratio of Cu(II)/protein from 0.2:1 to 2:1 against PBS. (B) COMMD1 titration with Cu(II) in the presence of 1 mM TCEP at conditions similar to those in A. (C) COMMD1 titration with Cu(II). A 1 mL aliquot of 253 μM was titrated with Cu(II) at a [Cu(II)]/protein ratio from 0.2:1 to 2:1 against the same concentrations of apo-COMMD1. (D) Cu(II)-bound COMMD1 against the same concentrations of apoprotein. Cu(II)-bound COMMD1 was prepared by incubating the apo protein with Cu(II) at 3-fold molar excess. The mixture was extensively dialyzed to remove free Cu(II).

and a broad peak between 600 and 700 nm after each addition of Cu(II) (Figure 4C). Figure 4D also shows the similar spectra of actual Cu(II)-bound COMMD1.

EPR Spectroscopy. The detection of the EPR signal clearly demonstrates the presence of Cu(II) bound to the protein (Figure 5). Three of four hyperfine lines in the low field parallel region of the spectrum are detectable, and the fourth is masked by a large signal in the perpendicular region (Note: The four lines in this region arise from hyperfine coupling to the $I = 3/2$ Cu nucleus.) The EPR parameters for the Cu(II)−COMMD1 sample were obtained by simulation with tensors of axial symmetry: $g_{\|} = 2.246$, $g_{\perp} = 2.031$, $A_{Cu\|} = 170 \times 10^{-4}$ cm^{-1}, $A_{Cu\perp} = 10 \times 10^{-4}$ cm^{-1}. The A values for Cu(II) are the mean values corresponding to the two ^{63}Cu and ^{65}Cu isotopes with their natural abundances. The EPR parameters differ significantly from those of a frozen aqueous solution of hydrated copper (*23*), confirming the binding of Cu(II) to COMMD1. The principal values of the g tensor ($g_{\|} > g_{\perp}$) indicate that the Cu(II)−COMMD1 complex has an elongated axial geometry.

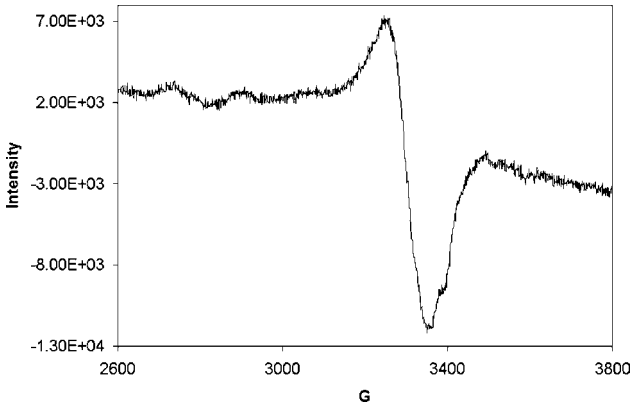


FIGURE 5: Electronic paramagnetic resonance (EPR) spectrum of Cu(II)−COMMD1 at low temperature (100 K) showing the characteristic signal of copper binding to COMMD1 in the Cu(II) oxidation state. Divalent copper is a d^9 transition metal ion and is readily observable by EPR

DEPC Modification of His Residues of COMMD1. Because histidine is a common amino acid residue involved in

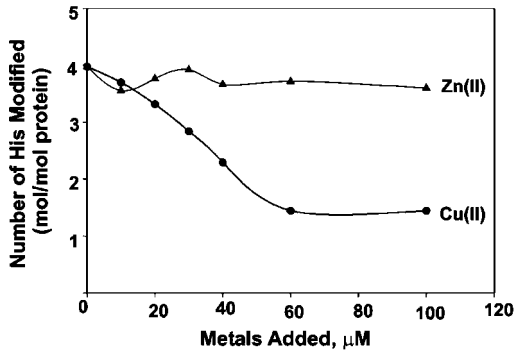


FIGURE 6: Protection of DEPC modification of His residues by Cu(II). Aliquots of 20 μM COMMD1 in PBS were preincubated with various concentrations of Cu(II) or Zn(II) prior to reacting with DEPC as described in Experimental Procedures.

copper binding in the majority of copper-binding peptides and proteins, particularly those binding Cu(II) (*24*), we were interested in determining whether any of the His residues in COMMD1 are involved in Cu(II) binding. When COMMD1 was interacted with the histidine-modifying reagent DEPC, there were four His residues being modified. The addition of Cu(II) protected this chemical modification in a concentration-dependent manner, whereas the addition of Zn(II) did not provide any protection (Figure 6). The maximum number of His residues protected in the presence of Cu(II) was about 2.5 mol His per mol protein.

There are four His residues per mol of COMMD1, as shown in Scheme 1. The finding that all of the His residues could be modified by DEPC suggests that all are located at the surface or easily accessible to the reagent. The finding that Cu(II) could protect between two and three His residues per mol protein indicates that a majority of the His residues may be essential for ligating Cu(II). Alternatively, the protection afforded by Cu(II) could be due to the drastic change in protein conformation that resulted in the internalization of His residues from the surface. More importantly, three His residues reside in the region missing in COMMD1 of dogs with copper toxicosis. It is possible that this region may be the Cu(II) binding domain. To probe this possibility, the binding of Cu(II) to COMMD(61−154) was investigated by a fluorescence titration technique. The fluorescence spectrum of COMMD(61−154) is somewhat different from that of wild type protein (Figure 7A). When excited at 280 nm, the maximum emission wavelength was at 343 nm compared to 328 nm for the wild type protein. This is likely due to the different conformations of the two proteins. The maximum intensity of fluorescence of COMMD(61−154) was less than that of the wild type, in spite of the fact that all three Trp residues are present, thus implying that some of the Trp residues in the truncated protein may be buried. Additionally, two tyrosine residues present in the wild type protein and not present in COMMD(61−154) may also contribute significantly to the overall fluorescence in COMMD1. Their absence red-shifts the maxima because now the fluorescence is only due to tryptophan residues, which generally have emission maxima at a higher wavelength. The addition of Cu(II) to COMMD(61−154) resulted in a decrease of fluorescence intensity in a concentration dependent fashion (Figure 7B), with the K_d value for Cu(II) binding being similar to that of wild type COMMD1. This result shows that the region between residues 61−154 is sufficient to bind Cu(II). The stoichiometry of Cu(II) was also found to be at 1 mol per mol protein. However, at protein concentrations higher than 10 μM, COMMD(61−154) aggregated in the presence of Cu(II); thus, the UV−visible absorption and other physical characterizations of this peptide could not be carried out. Nevertheless, the aggregation of this peptide in the presence of Cu(II) could be reversed by the chelation of Cu(II) by EDTA.

Site-Directed Mutagenesis of His and Met Residues. The fact that Cu(II) could protect two to three His residues from being modified by DEPC and that COMMD(61−154), which has three His residues within its sequence, could bind Cu(II) suggests that some of these residues are essential for the Cu(II) binding properties of COMMD1. Also, the results from the UV spectra of the Cu(II)−COMMD1 complex suggest the involvement of sulfur-containing amino acid in binding. To study the role of these residues, three His mutants, namely, COMMD(H101A), COMMD(H134A), and COMMD(H139A), as well as two Met mutants, COMMD(M55A) and COMMD(M111A), were generated. These mutants could be expressed and purified with yields in quantities similar to those of the wild type protein. The mutants were investigated for their ability to bind Cu(II) using the fluorometric technique. The fluorescence spectra of all of the mutants had the same characteristics and intensity as those of the wild type protein (data not shown), indicating that a single point mutation did not have any effect on over all protein structure. When titrated with Cu(II), the mutants COMMD(M110A) and COMMD(H134A) were less responsive to Cu(II) addition. The maximum change of fluorescence was only 20−25% compared to that of the wild type, which had a maximum change of about 50% (Figure 8). The results suggest that these two residues, M110 and H134, may play an important role in Cu(II) binding. However, other mutants, COMMD(H101A), COMMD(H139A), and COMMD(M55A) as well as C160A, had only a small change in the degree of perturbation of fluorescence from that of the wild type (Figure 8). Therefore, these residues may not be involved in Cu(II) binding, or they may contribute as a minor coordinating partner so that in their absence, individually, the protein will still function normally. Indeed, there are many reports of Cu(II) binding proteins that have one of the Cu(II) binding residues mutated and still retain characteristics of the wild type proteins with only minor changes (*25*−*28*).

Although all of the mutants were apparently soluble in the presence of Cu(II) under the conditions used (1 μM) for fluorescence study, it was observed that at higher concentrations (20 μM and up), one particular mutant, COMMD(M55A), was found to aggregate heavily in the presence of Cu(II). This aggregation was reversible upon the addition of EDTA. This observation implies that M55 may play a role in maintaining the structure and stability of COMMD1. In its absence, Cu(II) may bind to the protein in a constrained fashion, which results in the collapsing of protein structure, leading to the aggregation of the protein. Interestingly, as described earlier, the truncated peptide, COMMD(61−154) also had the tendency to reversibly aggregate in the presence of Cu(II). This peptide lacks one M55 residue as well.

DEPC Mapping of Wild Type COMMD1 and Truncated Peptide COMMD(61−154). DEPC mapping experiment has been successfully used previously to demonstrate Cu(II)

Scheme 1: Alignment of Amino Acid Sequences of COMMD1 in Humans, Dogs, and Bedlington Terriers That Were Affected by Copper Toxicosis (BT/CT)[a]

```
                1                                                  50                                                 100
COMMD1 Human MAAGELEGGK PLSGLLNALA QDTFHGYPGI TEELLRSQLY PEVPPEEFRP FLAKMRGILK SIASADMDFN QLEAFLTAQT KKQGGITSDQ AAVISKFWKS
COMMD1 Dog   MAA.ELEGSK ALGGLLSGLA QEAFHGHHGI TEELLRSQLY PEVSLEEFRP FLAKMRGILK SIASADMDFN QLEAFLTAQT KKQGGITSDQ AAVISKFWKN
COMMD1 BT/CT MAA.ELEGSK ALGGLLSGLA QEAFHGHHGI TEELLRSQLY PEVSLEEFRP FLAKMRGILK ------.--- ---------- --------.- ----------
               101                                                150                                                190
COMMD1 Human HKTKIRESLM NQSRWNSGLR GLSWRVDGKS QSRHSAQIHT PVAIIELEIG KYGQESEFLC LEFDEVKVNQ ILKTLSEVEE SISTLISQPN
COMMD1 Dog   HKTKIRESLM NQSRWDSGLR GLSWRVDGKS QSRHSAQIHT PVAIMELEIG KSGQESEFLC LEFDEVKVSQ LLKKLSEVEE SLSTLM.QPA
COMMD1 BT/CT -.------.- ---------- ---------- ---------- ---------- ---ESEFLC LEFDEVKVSQ LLKKLSEVEE SLSTLM.QPA
```

[a] Amino acid residues that are not identical are in bold. The conserved amino acid residues that are possible candidates for ligating Cu(II) are colored.

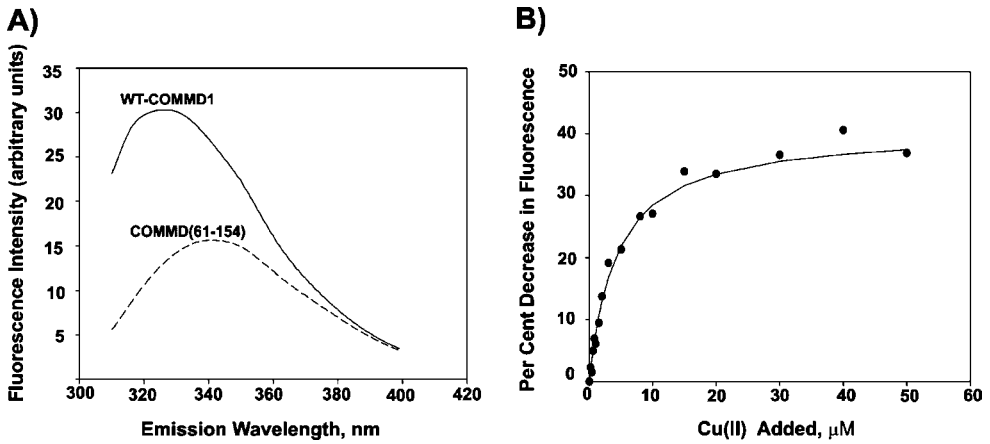


FIGURE 7: (A) Intrinsic fluorescence of COMMD(61−154) compared to that of the wild type. (B) Fluorescence titration between COMMD(61−154) and Cu(II). One milliliter aliquots of 1 μM COMMD(61−154) in PBS were incubated with various concentrations of $CuCl_2$. The percent decrease in fluorescence caused by the presence of Cu(II) was plotted vs Cu(II) concentration.

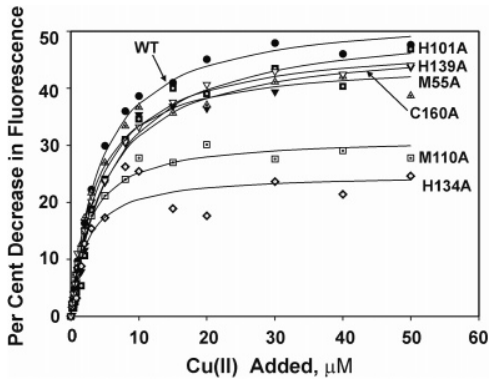
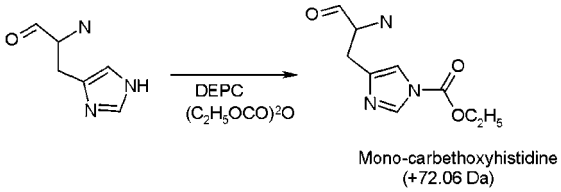


FIGURE 8: Comparison of the fluorescence titration between Cu(II) and COMMD1 or its mutants. The experimental procedure is the same as that described in Figure 6.

binding to His residues in prion protein (*29*). We employed similar strategies to identify the involvement of His residues in Cu(II) binding to COMMD1. When COMMD1 was subjected to DEPC modification followed by molecular mass determination of the intact protein as well as peptide mapping of its tryptic fragments using mass spectrometry, the analysis shows characteristic increase in the molecular mass of the protein by 72.06 Da per DEPC adduct because of the monocarbethoxylation of the His residue. (Scheme 2).

The copper binding properties of both COMMD1 as well as COMMD(61−154) were found to be similar in the spectroscopic analysis. Our preliminary MS experiments on COMMD1 indicated that it did not give 100% sequence coverage with either trypsin or chymotrypsin proteolysis such that peptides in the region of residues 61−154 were not detected easily under experimental conditions. However, we found that Cu(II) binding did not protect H25 from DEPC modification (data not shown). This suggested that the Cu(II) binding site could be located in the region between 61 and 154 residues, which contains three histidine residues. Also, COMMD(61−154) gave almost 100% sequence coverage by trypsin proteolysis as described below. On the basis of these facts, we have carried out a detailed MS analysis only on COMMD(61−154).

Scheme 2: Carbethoxylation Reaction of Histidine Residues[a]



[a] The reaction results in the increase in molecular mass by 72.06 Da per modification.

The DEPC modification of COMMD(61−154) yielded 5 to 6 adducts as shown in Figure 9A. COMMD (61−154) has three His residues at the 101, 134, and 139 positions. It is known that in addition to His residues, DEPC also shows reactivity toward tyrosine, lysine, and serine residues and the N-terminal NH_2 group (*30*). Therefore, the number of adducts is always greater than the number of His residues. This effect on selective histidine modification can be minimized using optimal conditions and an appropriate Cu(II) concentration. In the presence of Cu(II), the number of DEPC adducts is decreased by at least two (Figure 9B), indicating the protection of two residues. This is in agreement with the spectrophotometric determination.

To identify the actual His residues involved in Cu(II) binding, the DEPC-modified and unmodified proteins were subjected to tryptic digestion followed by MALDI-MS and tandem MS/MS analysis. From the MALDI-MS analysis, two peptide fragments at *m*/*z* 1013.57 and 2304.23 were identified

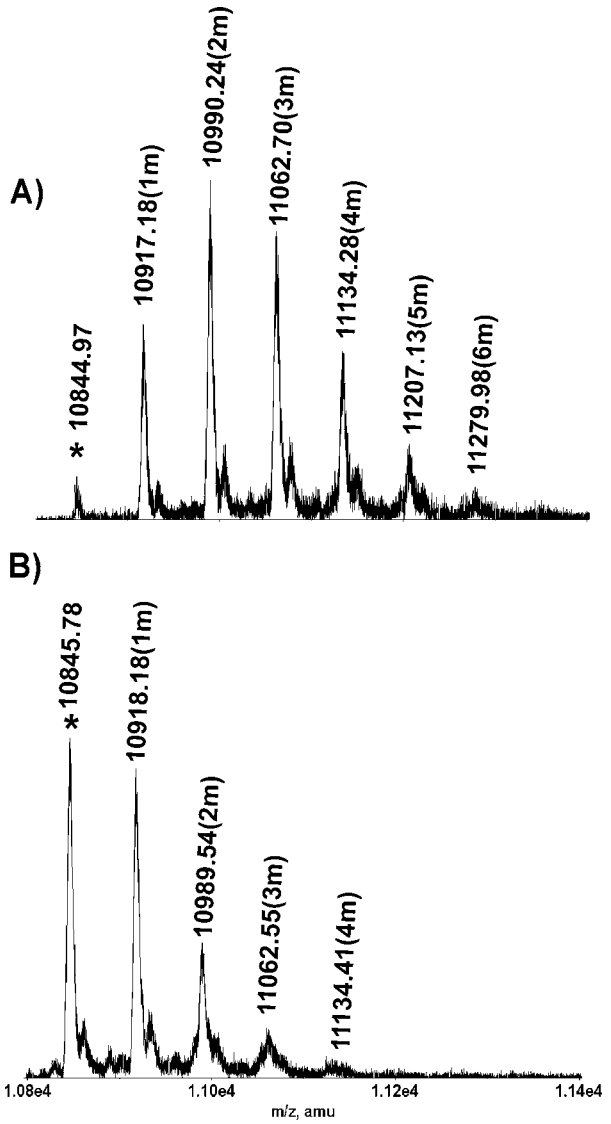


FIGURE 9: DEPC modification of COMMD(61−154) in the absence and presence of Cu(II). (A) MALDI-MS spectrum of COMMD-(61−154) in the absence of Cu(II) showing peaks corresponding to the unmodified protein at *m*/*z* 10844.97 (*) and successive DEPC adducts at *m*/*z* 10917.18 (1 m), 10990.24 (2 m), 11062.70 (3 m), 11134.28 (4 m), 11207.13 (5 m), and 11279.98 (6 m) respectively. (B) In the presence of Cu(II), a large peak of unmodified protein at *m*/*z* 10845.78 (*) with successive DEPC adducts at *m*/*z* 10918.18 (1 m), 10989.54 (2 m), 11062.55 (3 m), and 11134.41 (4 m) are observed.

as potential target peptides on the basis of an observation of corresponding modified peaks with an increment of approximately 72 Da. The peptide at *m*/*z* 1013.57 had a significant adduct peak at *m*/*z* 1085.59 in Figure 10A. The MS/MS analysis demonstrated that this fragment at *m*/*z* 1013.57 corresponds to residues SHKTKIR (residues 100−106, molecular mass 869 Da) with two modifications at K102 and K104 (+144 Da). MS/MS sequencing of the peptide at *m*/*z* 1085.57 reveals an extra modification at H101 based on the MS/MS fragment at *m*/*z* 297.12, corresponding to b_2 + 72 Da (SH + 72 Da) in the absence of Cu(II). Similarly, the peptide at *m*/*z* 2304.23 shows two low abundance adduct peaks at *m*/*z* 2376.26 and *m*/*z* 2448.28 upon DEPC treatment (Figure 10B).

In the presence of Cu(II), there is a peak at *m*/*z* 1013.57, but the intensity of the peak at *m*/*z* 1085.59 is markedly reduced (Figure 11A) because of the protection of the H101 residue against DEPC modification, indicating its role in Cu(II) binding. Similarly, the two adduct peaks show marked changes in the presence of Cu(II) (Figure 11B). The peak around *m*/*z* 2448 is completely abolished, whereas the peak at *m*/*z* 2376.25 shows marked decrease in intensity. This region contains H134 and H139. Interestingly, it is observed that the presence of Cu(II) protects only one residue against DEPC modification because the intensity of the peak at *m*/*z* 2376.25 is still significant (Figure 11B).

The MS/MS analysis indicates that the peak at *m*/*z* 2304.25 corresponds to the tryptic peptide fragment 134−153 (HSAQIHTPVAIIELELGKYG) (Figure 12). However, MS/MS analysis of the peak at *m*/*z* 2376.29 shows that this peptide was modified at K151 alone. These data are validated by the presence of the b_{18} + 72 Da ion at *m*/*z* 2011.12, corresponding to the peptide fragment (134−151) + 72 Da from the MS/MS data (Figure 13 A). This is an interesting result because the region 134−151 contains two histidine residues, and this finding implies that only one of the two histidine residues is involved in Cu(II) coordination.

The MS/MS analysis of the fragment at *m*/*z* 2448.30 shows that the peptide is modified at K151 and H134 and is confirmed by the presence of the b_1 + 72 Da ion at *m*/*z* 210.09, corresponding to H134 + 72 Da and b_{18} + (2 × 72 Da) at *m*/*z* 2082.11, corresponding to the peptide (134−151) + (2 × 72 Da) (Figure 13B). Subsequently, the fragment corresponding to b_1 + 72 at *m*/*z* 210 Da was found to be absent in the MS/MS spectrum of 2376.29 Da. This observation reveals two interesting features about this region: (a) Cu(II) binding involves H134 alone, and (b) His139 does not bind Cu(II) and is resistant or inaccessible to DEPC modification even in the absence of Cu(II).

In summary, DEPC modification and MS analysis clearly points out that the copper binding site is located in the region encompassing 61−154 residues, and His101 and His134 are protected on Cu(II) addition. His139 is not accessible to DEPC modification under the experimental conditions used. The involvement of H134 in direct Cu(II) binding was also seen in our fluorescence experiments (Figure 8). However, we did not find the involvement of H101 in Cu(II) binding in our fluorescence experiments, which suggests that protection against DEPC modification might result from Cu(II) induced conformational changes rather than direct binding.

Possible Significance of Cu(II) Binding to COMMD1. We have presented compelling evidence that COMMD1 is a Cu(II) binding protein and that Cu(II) binding involves His and Met residues. Most importantly, the finding that the Cu(II) binding site (or a major part of it) is located in the region that is deleted in dogs affected by the copper toxicosis disease implies that there is a correlation between the copper binding of COMMD1 and the control of copper homeostasis. Other than its inhibitory effect on NF-κB, the overall biological function of COMMD1 is not yet known. Recent reports suggest that COMMD1 can translocate to various cell compartments (*10*) and can interact with diverse proteins (*9*, *11*−*14*). Copper(II) binding to COMMD1 may modulate these interactions. The biological function of COMMD1 may also be controlled in part by signal transduction pathways involving protein phosphorylation, and Cu(II) binding may

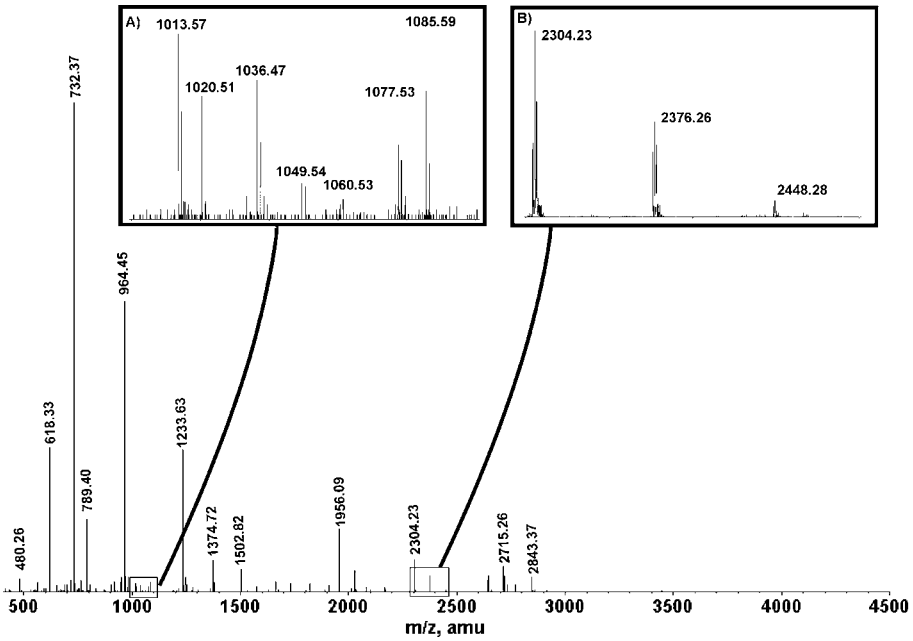


FIGURE 10: MALDI-MS of a tryptic digest of COMDD(61−154) upon DEPC modification in the absence of Cu(II). (A) In the absence of Cu(II), a strong peak at *m*/*z* 1085.59 due to His101 modification is observed in addition to the peak at *m*/*z* 1013.57 corresponding to peptide 100−106 (SHKTKIR) and (B) two peaks at *m*/*z* 2376.26 and *m*/*z* 2448.28 due to DEPC modifications of the peptide at *m*/*z* 2304.23 corresponding to residues 134−153 (HSAQIHTPVAIIELELGKYG).

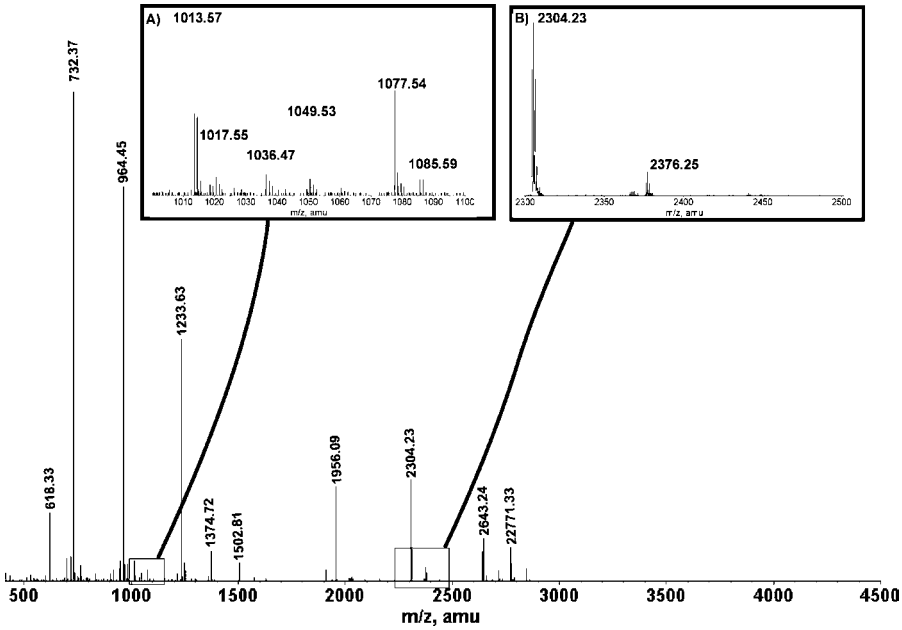
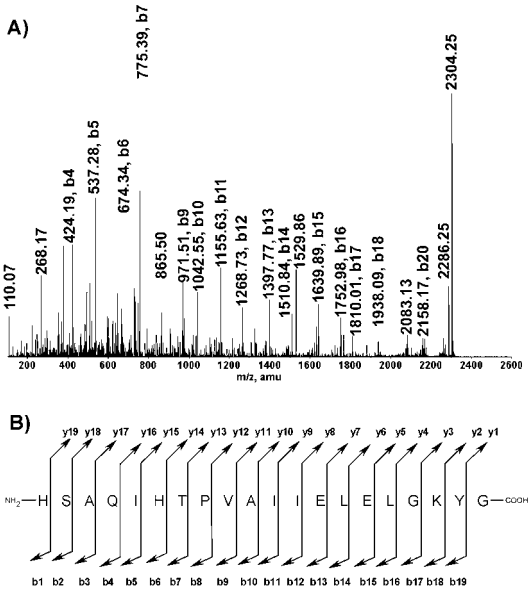


FIGURE 11: MALDI-MS of a tryptic digest of COMMD1(61−154) upon DEPC modification in the presence of Cu(II). The intensity of the peak around *m*/*z* 1085 is markedly decreased, indicating the protection of the H101 residue against DEPC modification (inset A). The extent of modification is markedly decreased as indicated by the apparent reduction in intensity of the peak at *m*/*z* 2376.25, whereas the peak around *m*/*z* 2448 is almost gone (inset B), suggesting the protection of H134 in Cu(II) binding.

play a role in fine-tuning these processes. We have recently observed a similar phenomenon in our studies of the effect of copper on phosphorylation (*31*). Indeed, we found that COMMD1 can be phosphorylated *in vitro* by protein kinase A and protein kinase C and that the presence of Cu(II) inhibited the phosphorylation by both kinases (Narindrasorasak, S., and Sarkar, B., unpublished data). Alternatively, COMMD1 may act as a copper chaperone that delivers Cu(II) to other proteins and enzymes. In general, copper chaperones have a moderate affinitys with K_d values in the micromolar range (*32*−*34*). This would be energetically favorable for delivering copper to other target proteins that have higher affinity (*35*). The K_d of Cu(II) binding to COMMD1, as determined by a fluorescence titration experiment, is between 3 and 5 μM, which seems to fit this criterion. Finally, COMMD1 may act as a carrier for Cu(II) excretion. Two hypotheses can be advanced to explain the accumulation of copper in affected dogs. First, the process by which copper is excreted via the bile canalicular membrane requires a complex formed between COMMD1 and copper-bound ATP7B. Analagous to the interaction of COMMD1 and RelA, which form a stable complex and



Residue	Mass	b	y
H, His	137.06	138.07	2233.21
S, Ser	87.03	225.10	2096.16
A, Ala	71.04	296.14	2009.12
Q, Gln	128.06	424.19	1938.09
I, Ile	113.08	537.28	1810.03
H, His	137.06	674.34	1696.94
T, Thr	101.05	775.38	1559.89
P, Pro	97.05	872.44	1458.84
V, Val	99.07	971.51	1361.78
A, Ala	71.04	1042.54	1262.72
I, Ile	113.08	1155.63	1191.68
I, Ile	113.08	1268.71	1078.60
E, Glu	129.04	1397.75	965.51
L, Leu	113.08	1510.84	836.47
E, Glu	129.04	1639.88	723.38
L, Leu	113.08	1752.96	594.34
G, Gly	57.02	1809.99	481.26
K, Lys	128.10	1938.08	424.24
G, Gly	57.02	1995.10	296.14
Y, Tyr	163.06	2158.17	239.12
G, Gly	57.02	2215.19	76.06

FIGURE 12: (A) Tandem MS/MS spectrum of a peptide at *m*/*z* 2304.23. (B) Fragmentation scheme for peptide 134−153 (HSAQIHTPVAIIELELGKYG). The N-terminal fragments are indicated with the letter b, and C-terminal fragments are labeled with the letter y. The table below the Figure corresponds to the calculated masses for the respective b and y fragments

migrate to the nucleus (*9*), COMMD1 may interact with ATP7B and translocate together, as a protein complex, to the bile canalicular membrane for the excretion of copper. This process does not function in BT/CT dogs because of the lack of the protein−protein interaction domain. Second, biliary excretion of copper may require the Cu(II)-bound form, and COMMD1 may mediate copper availability for excretion. ATP7B is known to bind Cu(I) (*36*), and we have shown here that COMMD1 binds Cu(II). The intriguing question is how the oxidation state of copper comes into play in the physiological function of ATP7B, COMMD1, and other components. At present, very little is known, and

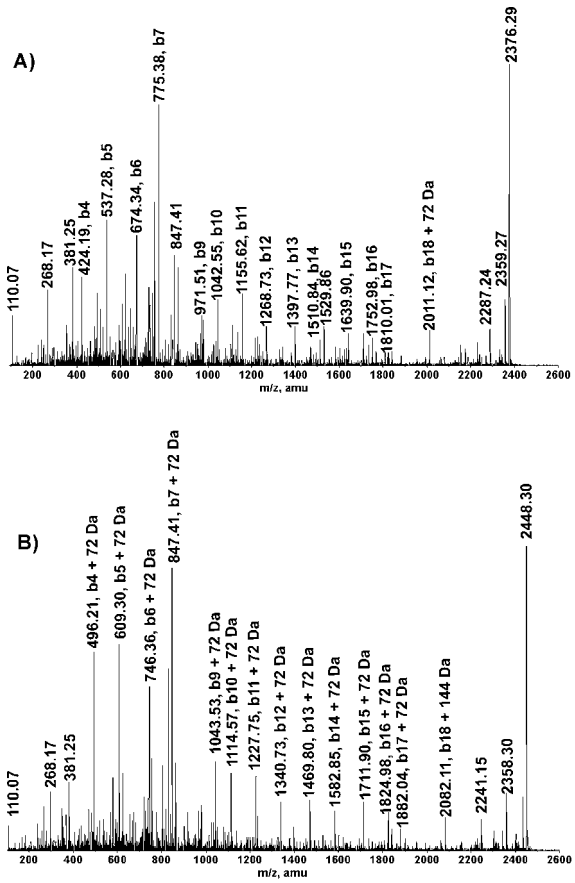


FIGURE 13: MS/MS spectra for the modified peptide fragment (134−153). In the presence of Cu(II) (A), the MS/MS of the *m*/*z* 2376.29 peptide shows a characteristic peak at *m*/*z* 2011.12 corresponding to peptide (134−151) + 72 Da, indicating the modification of only K151 but not the H134 residue. In the absence of Cu(II) (B), the MS/MS of the *m*/*z* 2448.30 peptide shows a peak at *m*/*z* 2082.11 corresponding to peptide (134−151) + (2 × 72 Da), indicating the modification of both H134 and K151 residues.

we can only speculate that some redox reaction occurs in the sequence of events in the process of copper excretion.

It is also interesting to point out that dog albumin lacks the ability to bind copper with high affinity because of the absence of a histidine residue at the third position (*37*, *38*), which is most likely the reason for a high content of copper in the dog liver even when there are no mutations in the MURR1 gene (*39*). In this situation, excess copper accumulation in BT/CT dogs puts them in a double burden of copper toxicity.

Unlike most copper proteins that bind copper in both Cu(II) and Cu(I) oxidation states or other divalent metals (*40*−*44*), COMMD1 specifically binds only Cu(II). This could reflect a specific function of COMMD1 for handling Cu(II) in the cell. If the role of COMMD1 is for the delivering/receiving of Cu(II) to a specific location, then distinguishing Cu(II) from other metals would ensure the efficiency of the process.

CONCLUSIONS

Purified recombinant COMMD1 exists as a mixture of monomers and dimers as well as some oligomers. The region involved in dimerization lies within the COMM domain, but the major contribution is in the carboxy terminus of the

molecule. COMMD1 specifically binds Cu(II) with a concomitant increase in protein dimerization. Copper(II) binding quenches the intrinsic fluorescence of COMMD1 in a concentration-dependent manner. This effect could be reversed in the presence of TCEP or EDTA. The K_d for Cu(II) binding is approximately 3−5 μM. UV−visible spectra and EPR results confirm the presence of Cu(II) in the copper−protein complex. The Cu(II)−COMMD1 complex had a light blue-green color with an absorption maximum at about 360 nm and a broad peak between 600 and 700 nm. Most importantly, the truncated protein COMMD(61−154), which is the product of exon 2, a region deleted in dogs affected by copper toxicosis, is able to bind Cu(II) in a manner similar to that used by the wild type protein. DEPC modification experiments reveal the involvement of histidine residues in Cu(II) binding. H134 was identified as the residue involved in direct Cu(II) binding as demonstrated by MS analysis. Furthermore, from the fluorescence studies of different mutants, M110 was identified as another potential binding site for Cu(II) along with H134. These residues, H134 and M110, reside in the exon 2 product and, therefore, emphasize the important role of this region in Cu(II) binding. It is possible that one or more additional residues that are involved in direct Cu(II) binding remain to be identified. This finding, reveals for the first time the specificity of this domain for Cu(II) binding and raises the possibility of its role in copper homeostasis. Further efforts to elucidate the role of Cu(II) binding on the overall function of COMMD1 are underway.

ACKNOWLEDGMENT

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