HAH1 Is a Copper-binding Protein with Distinct Amino Acid Residues Mediating Copper Homeostasis and Antioxidant Defense*

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HAH1 is a 68-amino acid protein originally identified as a human homologue of Atx1p, a multi-copy suppressor of oxidative injury in sod1Δ yeast. Molecular modeling of HAH1 predicts a protein structure of two α-helices overlaying a four-stranded antiparallel β-sheet with a potential metal binding site involving two conserved cysteine residues. Consistent with this model, in vitro studies with recombinant HAH1 directly demonstrated binding of Cu(I), and site-directed mutagenesis identified these cysteine residues as copper ligands. Expression of wild type and mutant HAH1 in atxΔΔ yeast revealed the essential role of these cysteine residues in copper trafficking to the secretory compartment in vivo, as expression of a Cys-12/Cys-15 double mutant abrogated copper incorporation into the multiciper oxydase Fet3p. In contrast, mutation of the highly conserved lysine residues in the carboxyl terminus of HAH1 had no effect on copper trafficking to the secretory pathway but eliminated the antioxidant function of HAH1 in sod1ΔΔ yeast. Taken together, these data support the concept of a unique copper coordination environment in HAH1 that permits this protein to function as an intracellular copper chaperone mediating distinct biological processes in eucaryotic cells.

Copper is an essential trace element in all living organisms. As a cofactor in enzymatic catalysis, this metal plays a key role in the biochemistry of cellular respiration, antioxidant defense, and iron metabolism in eucaryotes (1). Excess or free intracelullar copper is highly toxic; thus, specialized systems have evolved for the transport of this metal inside the cell. Insight into the mechanisms of intracellular copper trafficking has come from characterization of the genes involved in the inherited copper disorders, Wilson and Menkes disease (2–7). The genes for these diseases encode homologous P-type ATPases that reside in the trans-Golgi network of the cell and transport copper to the secretory pathway for incorporation into secretory proteins and cellular export (8–12). Studies on a homologous ATPase, Ccc2p, in Saccharomyces cerevisiae have revealed a remarkable evolutionary conservation of the mechanisms of cellular copper metabolism (13).

Although less is known about the mechanisms of cytoplasmic copper trafficking, recent studies have identified a soluble factor, Atx1p as well as an atxΔΔ homologue, HAH1, that functions in antioxidant defense and the delivery of copper to the transport ATPases (14–16). Together with studies identifying specific proteins responsible for the delivery of copper to cytochrome c oxidase in the mitochondria (17) and copper/zinc superoxide diamutase in the cytoplasm (18), these data suggest that the delivery of copper to specific intracellular enzymes is mediated by distinct copper transport proteins in the cell. The amino terminus of HAH1 contains a putative copper binding motif, MXXCXXC, that is repeated 6-fold in the Menkes and Wilson ATPases. This finding suggests that HAH1 is a copper-binding protein, and the current study was undertaken to directly examine this possibility and to determine the amino acid residues responsible for metal binding, copper trafficking to the secretory pathway, and antioxidant defense.

EXPERIMENTAL PROCEDURES

Molecular Modeling—Protein sequence data banks were searched using the Basic Local Alignment Search Tool (BLAST) network server at the National Center for Biotechnology Information with default parameterization (19). Hydrophobic cluster analysis was used for pairwise and multiple alignments as well as for secondary structure prediction (20). Manual editing of multiple alignments and profile data base screenings were performed utilizing the software package GCG 7.0 (Genetics Computer Group, Madison, WI). To measure the compatibility of the aligned sequences with a known three-dimensional structure, manual refinement of multiple sequence alignments was optimized as described (21). Structural models were then built using the known coordinates of the related folds and the refined coordinates of the NMR structure of MerP (22). Three-dimensional visualization was performed on a UNIX workstation using the Xnmol program (23).

Construction of the HAH1 cDNA, Mutagenesis, and Purification of Recombinant Protein—The coding region of the HAH1 gene was amplified by polymerase chain reaction utilizing oligonucleotides designed to introduce a 5′ NdeI and a 3′ EcoRI restriction site. The amplified product was ligated into the pET 28a(+) expression vector (Novagen) and used to transform Escherichia coli strain BL21 (DE3) (24). Site-directed mutagenesis of HAH1 cDNA was performed using KlenTaq polymerase (CLONTECH) and the ExSite mutagenesis kit (Stratagene) as described previously (11). In all cases, the fidelity of the cDNA sequence as well as the presence of the intended mutations was confirmed by dyeoxy nucleotide sequencing (25).

Transformed bacteria were grown to an optical density of 0.6 at 600 nm and induced with isopropl-1-thio-β-D-galactopopyranoside (Sigma). Cells were harvested by centrifugation and resuspended in 5 mM imidazole, 0.5 mM NaCl, 20 mM Tris-HCl, pH 7.9. After the addition of lysozyme, the bacterial cell suspension was disrupted by sonication at 4 °C, and DNase I was added. The suspension was then centrifuged at 100,000 × g in a Beckman Ti70 rotor at 4 °C for 1 h, and the supernatant was loaded onto a previously charged and equilibrated Ni-nitrilotriacetic acid column (Novagen). Wild type or mutant HAH1 proteins were cleaved from the column with thrombin in 20 mM Tris-HCl, pH 8.4.
used in this study were constructed as described (15). As described (28, 29).

Nuclear Magnetic Resonance and Mass Spectroscopy—Oxidized wild type apoprotein was prepared for NMR spectroscopy by dissolving in 90% 20 mM potassium phosphate, pH 8.0, 10% D2O (v/v). Nuclear Overhauser effect dipolar-correlated two-dimensional spectra (NOESY) and total correlation spectroscopy spectra were obtained at 298 K using a Varian 500 spectrometer. For mass analysis, wild type HAH1 was dialyzed in 20 mM potassium phosphate, pH 8.0, at 4 °C. Dithiothreitol was added to the buffer during dialysis to obtain the reduced form of HAH1. Oxidized and reduced apoprotein samples were subsequently lyophilized, and mass analysis was performed using a Finnigan (Thermoquest Corp., San Jose, CA) LCQ ion-trap mass spectrometer equipped with a standard electrospray source. Before spraying into the mass spectrometer, all protein samples were desalted on a Michron (Varian Corp.) LCQ ion-trap mass spectrometer equipped with a standard electrospray source. The eluent was infused directly into the LCQ source.

Metal Binding and Sulfhydryl Analysis—To examine cobalt binding, the recombinant HAH1 proteins were reduced in 0.3 mM β-mercaptoethanol, 100 mM Tris-HCl, pH 9.0. CoCl2 was added dropwise to a final concentration of 0.2 mM. The anaerobic reconstitution of HAH1 with cobalt was performed under argon after elimination of the reducing agent by a 10-fold dilution in the Tris buffer. After cobalt addition, an equal volume of 100 mM Tris-HCl pH 9.0 was added to the reconstituted protein solution, which was then centrifuged for 2 h in a Centricon-3 filter (Amicon). The proteins were then analyzed by ultraviolet-visible spectrophotometric wavelength scanning utilizing the eluent as a blank.

To examine copper binding, recombinant HAH1 proteins were reduced with dithiothreitol for 2 h before the addition of 62.5 pmol of 64Cu (35 mCi/μg of Cu) at 4 °C. After a 2-h incubation, the protein solutions were chromatographed by size exclusion on Sephadex G-25 (Pharmacia Biotech Inc.) columns previously equilibrated with 20 mM potassium phosphate, pH 8.0. Individual fractions were collected, and the radioactivity was analyzed in a 3-inch NaI crystal using a Packard γ counter. Aliquots were taken for the determination of protein content by the Bradford method using bovine serum albumin as standard (26).

For sulfhydryl group analysis, HAH1 proteins were incubated with 5,5′-dithiothreitol (2-nitrobenzoic acid) in 20 mM potassium phosphate buffer, pH 8.0, and the production of 20% D2O (v/v). Nuclear Overhauser effect dipolar-correlated two-dimensional spectra (NOESY) and total correlation spectroscopy spectra were obtained at 298 K using a Varian 500 spectrometer. For mass analysis, wild type HAH1 was dialyzed in 20 mM potassium phosphate, pH 8.0. Individual fractions were collected, and the radioactivity was analyzed in a 3-inch NaI crystal using a Packard γ counter. Aliquots were taken for the determination of protein content by the Bradford method using bovine serum albumin as standard (26).

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RESULTS

To develop a molecular model of HAH1, the amino acid sequence of this protein was aligned with several previously identified homologous sequences. Secondary structure prediction of these sequences using hydrophobic cluster analysis suggested the presence of amphipathic β-strands and α-helices (Fig. 1). This prediction was used to search for three-dimensional folds comprising a βαββαβ domain in the protein data base. Three-dimensional structures satisfying these secondary structure constraints consisted of two α-helical segments lying on the same side of a four-stranded antiparallel β-sheet. This secondary structure corresponded to that of MerP which, as previously noted (14), shares 21% sequence identity with HAH1.

A molecular model was constructed using the known coordinates of the related folds and the refined coordinates of the NMR structure of MerP. This revealed the formation of a hydrophobic core at the interface of the two α-helices and one face of the anti-parallel β-sheet (Fig. 2). The loop connecting the first β-strand to the first α-helix contained residues potentially involved in a metal binding site. Among these amino acids, two cysteines, Cys-12 and Cys-15, were expected to contribute two sulfur ligands to the metal binding site. The methionine, Met-10, is also conserved (Fig. 1), but a direct role for this residue as a metal ligand is unclear. The location of a third cysteine residue, Cys-41, was revealed to be distant from the metal binding site as indicated (Fig. 2). A cluster of lysine residues at the carboxyl terminus represents a conserved motif in the human and yeast copper chaperones (Fig. 1), and two of these residues, Lys-57 and Lys-60, were predicted to be in the vicin-
ity of the metal binding site according to the model as shown. Two-dimensional $^1$H NMR of recombinant HAH1 confirmed the presence of the $\beta$-strands and $\alpha$-helices according to the chemical shifts observed in the peaks of the fingerprint region (data not shown).

The three-dimensional model of HAH1 suggested a potential approach to begin to characterize the structure and function of this protein. Therefore, recombinant HAH1 was overexpressed in bacteria, and the purified protein was analyzed by mass spectroscopy (Fig. 3). The recombinant protein contains three additional residues, GSH, at the amino terminus, and mass analysis of the purified protein identified a single polypeptide of the predicted molecular weight, suggesting that the wild type apoprotein is purified in the oxidized form (Fig. 3A). Reduction of HAH1 by the addition of dithiothreitol to the dialysis buffer during purification resulted in a molecular species with a mass difference of two daltons (Fig. 3B). These data indicated the formation of a disulfide bond in the oxidized protein sample. Spectroscopic analysis of wild type and cysteine mutants of oxidized HAH1 with 5,5'-dithiobis(2-nitrobenzoic acid) revealed the presence of one free sulfhydryl group in the wild type protein, one free sulfhydryl group in the C12G,C15G double mutant and two free sulfhydryl groups in the C12G single mutant (data not shown). Taken together, these data suggest that the disulfide bridge observed by mass spectroscopy most likely forms between residues Cys-12 and Cys-15 as predicted from the HAH1 model (Fig. 2).

The structural model and spectroscopic studies of recombinant HAH1 support the concept of metal binding involving the MXCXXC motif of this protein. To directly examine this possibility, cobalt binding was studied using ultraviolet-visible wavelength scanning spectroscopy. When cobalt was added to reduced recombinant wild type HAH1 under anaerobic conditions in the absence of excess reducing agent, a strong signal in the near ultraviolet range was observed that was absent for the C12G,C15G double mutant (Fig. 4A, shoulders at 280 and 308 nm). This absorbance was attributed to charge transfer, suggesting that the cobalt was bound to sulfur atoms. Reconstitution of HAH1 with cobalt in the presence of excess $\beta$-mercaptoethanol resulted in the appearance of two additional signals at 398 and 478 nm attributed to coordination of the metal by additional sulfur atoms provided by the reducing agent (Fig. 4B). A similar, but much weaker, pattern of absorption was observed with cobalt and $\beta$-mercaptoethanol alone. These spectral properties of HAH1 in the presence of reducing agents suggest a different metal binding site geometry possibly due to the coordination of cobalt by external ligands, supporting the concept from the structural model that the metal binding site is solvent-accessible (Fig. 2).

In the reducing environment of the cell, copper binding would be anticipated to occur as Cu(I) and in support of this, ultraviolet-visible wavelength scanning spectroscopy of recombinant HAH1 reconstituted with Cu(II) revealed no absorption peak in the visible region (data not shown). To directly examine

**FIG. 3.** Electrospray mass spectra of purified recombinant HAH1. The oxidized (A) and reduced (B) forms of wild type HAH1 were prepared as described under "Experimental Procedures." Rel. Int., relative intensity.
copper binding to HAH1, wild type or C12G,C15G double-mutant proteins were incubated with $^{64}$Cu as CuCl$_2$ in the presence of excess reducing agent. In these studies, ubiquitin, a similarly sized, non-copper-binding protein served as a control for nonspecific binding. As can be seen in Fig. 5, under these conditions, recombinant HAH1 specifically bound Cu(I), and this binding was abrogated by mutation of the cysteine residues in the putative metal binding motif. These findings were supported by studies of HAH1 copper binding using bicinechonic acid chelation, which also indicated that copper was bound as Cu(I) (data not shown).

The metal binding data indicated that HAH1 bound copper as Cu(I) in vitro and that the Cys-12 and Cys-15 residues serve as ligands in this binding. To directly examine the functional role of these residues, recombinant HAH1 specifically bound Cu(I), and this binding was abrogated by mutation of the cysteine residues in the putative metal binding motif. These findings were supported by studies of HAH1 copper binding using bicinechonic acid chelation, which also indicated that copper was bound as Cu(I) (data not shown).

The function of HAH1 in these transformants was examined by analyzing copper incorporation into newly synthesized Fet3p. After metabolic labeling with $^{64}$Cu, equivalent amounts of crude plasma membrane fractions from $atu\Delta$ transformants were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, incubated with HAH1 antibody, and analyzed by chemiluminescence. The arrow indicates the HAH1 protein in the total cell lysates. kD, kilodalton.

containing wild type (Fig. 6, lane 1) or mutant HAH1 cDNA (Fig. 6, lanes 3–7). As can be seen in this analysis, a single 7.5-kDa protein was observed in each of the transformants that was equivalent in size to that observed for HAH1 in human tissues and cell lines.

The function of HAH1 in these transformants was examined by analyzing copper incorporation into newly synthesized Fet3p. After metabolic labeling with $^{64}$Cu, equivalent amounts of crude plasma membrane fractions from $atu\Delta$ transformants were separated by SDS-polyacrylamide gel electrophoresis and analyzed by autoradiography. As can be seen in Fig. 7A, a single 80-kDa radioactive band corresponding to holoFet3p was observed under these conditions in membrane fractions from...
the atx1Δ mutant transformed with wild type HAH1 (Fig. 7A, lane 1), directly demonstrating restoration of copper incorporation into Fet3p. As anticipated, no Fet3p was detected under these same conditions in membrane extracts from atx1Δ mutants transformed with vector alone (Fig. 7A, lane 2) or in the fet3Δ strain (Fig. 7A, lane 8). Mutation of Cys-12 decreased the amount of copper incorporated into Fet3p (Fig. 7A, lane 4), and mutation of both Cys-12 and Cys-15 eliminated holoFet3p biosynthesis (Fig. 7A, lane 6), confirming the essential role of these cysteine residues in copper binding. In contrast, mutation of Cys-15 or Met-10 alone had no effect on copper delivery to Fet3p (Fig. 7A, lanes 3 and 5). HoloFet3p synthesis was likewise unaffected by mutation of the conserved lysine residues in the carboxyl terminus (Fig. 7A, lane 7). These findings were not attributable to alterations in the amount of Fet3p among the transformants, as revealed by immunoblot analysis of Fet3p using equivalent amounts of membrane protein (Fig. 7B). As observed previously, when analyzed under these conditions, the protein that is devoid of copper migrated more slowly than the holoprotein and is detected as a doublet due to differences in glycosylation (11).

Yeast strains lacking copper/zinc superoxide dismutase (SOD1) are sensitive to dioxygen and auxotrophic for lysine when grown aerobically. The ATX1 gene was originally isolated by virtue of its ability to reverse the lysine auxotrophy of sod1Δ yeast in a copper-dependent fashion, and previous studies demonstrated that expression of HAH1 in these strains restores growth on lysine-deficient media (16). As the antioxidant function of Atx1p is copper-dependent (14), the role of HAH1 metal binding residues in this antioxidant function was examined. Wild type and mutant HAH1 cDNAs were introduced into sod1Δ yeast, and these transformed yeast were then analyzed for their ability to grow aerobically on plates lacking lysine (Fig. 8). Although all transformants were able to grow in oxygen in complete medium containing lysine (Fig. 8, SD), only the wild type HAH1 and the M10I mutant were able to restore growth in lysine-deficient medium, indicating that both the Cys-12 and Cys-15 residues are essential for this function of HAH1. Unexpectedly, mutation of the conserved lysines in the carboxyl terminus also prevented HAH1 from restoring the lysine auxotrophy in the sod1Δ yeast (Fig. 8, K57G,K60G) revealing a direct role for these residues in the antioxidant function of HAH1. These findings were not due to differences in the amount of HAH1 expressed in the sod1Δ transformants as revealed by immunoblot analysis of HAH1 (data not shown). Furthermore, identical findings were obtained when the point mutations shown were made with serine instead of glycine as the substituting amino acid (data not shown).

**DISCUSSION**

The results of this study demonstrate that HAH1 binds Cu(I) and that this binding is dependent upon the cysteine residues in the conserved MXXCXC motif in the amino terminus of the protein. The data also indicate that these cysteine residues are essential for HAH1 to function in copper trafficking to the secretory pathway and in antioxidant defense in vivo. In the wild type protein, the methionine in this motif would not appear to be essential for either of these functions. However, holoFet3p biosynthesis was preserved in atx1Δ strains transformed with HAH1 lacking only one of these cysteines, suggesting that under certain circumstances this methionine residue may contribute as a copper ligand. The quantitative difference in Fet3p copper incorporation between the Cys-12 and Cys-15 mutants (Fig. 7F) may reflect steric differences in the ability of this methionine to form a copper coordination with these two residues. Within the reducing environment of the cell, an exogenous thiol may also coordinate as a weak copper ligand as revealed by the cobalt binding studies, which demonstrate a spectral change in the presence of excess reducing agent suggestive of solvent accessibility of the bound metal (Fig. 4B).

The results of the site-directed mutagenesis studies reported here are supported by structural and functional studies of the bacterial Hg(II) transport protein MerP, which demonstrate that the bound mercury is bicoordinate with the cysteine ligands in the conserved MXXCXC motif (22, 32). Although an analogous copper binding structure has not been previously described, recent spectroscopic studies from O'Halloran and co-workers (33) on the yeast HAH1 homologue Atx1p suggest that this protein can adopt a two- or three-coordinate copper ligand site involving the conserved cysteines and either the proximate methionine or an exogenous thiol. Although analysis of these HAH1 mutants is consistent with this data, the precise nature of this ligand environment must await x-ray crystallographic analysis.

The MXXCXC motif constituting the HAH1 copper binding site detected in this study is also present in the amino terminus of all known copper-transporting P-type ATPases (34). This motif is repeated 6-fold in the human Wilson and Menkes ATPases, and recent studies indicate that Cu(I) is bound by the conserved cysteine residues in the corresponding regions of these two proteins (29). As Atx1p and HAH1 have been shown to be directly involved in the pathway of copper transfer to the ATPases, these findings suggest the possibility of direct copper...
transfer from HAH1 to the Wilson and Menkes proteins via these homologous motifs. Indeed, recent studies reveal a copper-dependent, protein-protein interaction between Atx1p and Ccc2p involving these homologous copper binding motifs (33). This observation has led to the proposal that the copper binding motifs of the ATPases may contribute cysteine ligands to the solvent-accessible copper in Atx1p, thereby providing a facile mechanism for the rapid transfer of copper between these proteins (33). The MXCXXXC motif may thus be ideally suited as a functional protein domain permitting the binding and sequestration of copper and yet allowing for the rapid exchange of this metal as needed to specific proteins at diverse cellular sites. Such a concept is supported by the identification of a homologous motif in the recently characterized yeast and human copper chaperones for superoxide dismutase (18).

The data in this study also reveal a specific role for the conserved lysines in the carboxyl terminus of HAH1, as mutation of these residues prevented rescue of lysine auxotrophy in sod1Δ yeast (Fig. 8). These lysine residues are conserved among the ATX1 homologues but are not present in the related mercury transport proteins (Fig. 1). Studies by Culotta and Lin (14) originally identified ATX1 as a multi-copy suppressor of oxidative damage in yeast strains lacking superoxide dismutase. This antioxidant function of ATX1 is copper-dependent, and this is consistent with the site-directed mutagenesis studies reported here (Fig. 8). Although the mechanism of the antioxidant function of HAH1 is unknown, Atx1p has no antioxidant activity in vitro (15), suggesting that the antioxidant function is mediated by transfer of copper to a target downstream of HAH1. Interestingly, unlike the findings with holoFet3p biosynthesis, the single cysteine HAH1 mutants did not retain the ability to suppress lysine auxotrophy, perhaps indicating that different ligand structures may be utilized for copper transfer in this antioxidant pathway. Alternatively, these findings may reflect differing sensitivities of the assays used in this study. The mutagenesis studies indicate that this pathway is independent of copper transport to the secretory compartment, and this is supported by studies demonstrating that the antioxidant activity of Atx1p is maintained in sod1Δ/ccc2Δ yeast (15). The dependence of HAH1 antioxidant function upon the conserved lysine residues in the carboxyl terminus suggests that, analogous to the ATPase interaction discussed above, this region may function as a recognition motif for protein-protein interaction between HAH1 and a novel copper-dependent protein.

Taken together the results of these studies support the concept that HAH1 is a member of the newly proposed class of intracellular proteins termed copper chaperones (18, 33). These chaperones are proposed to bind and transport copper to the appropriate protein in the cell and, in some cases, to facilitate formation of the active site of the target protein. This emerging picture of copper chaperones may have important biomedical implications. For example, recent studies reveal that gain-of-function mutations in copper/zinc superoxide dismutase result in amyotrophic lateral sclerosis due to altered copper-dependent functions of this enzyme (35–36). Recent findings that copper chelation may ameliorate the course of neurodegeneration in a transgenic model of this disease (37) provide a compelling example that copper chaperones may be ideal pharmacotherapeutic targets to manipulate intracellular copper homeostasis. Further studies on the mechanism of metal binding and transfer by such proteins as well as a careful analysis of their genetic and metabolic regulation in mammalian cells should therefore be useful.

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