Mechanisms of Copper Incorporation into Human Ceruloplasmin

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Abstract

Ceruloplasmin is a multicopper oxidase essential for normal iron homeostasis. To elucidate the mechanisms of copper incorporation into this protein, holoceruloplasmin biosynthesis was examined by immunoblot analysis and $^{64}$Cu metabolic labeling of Chinese hamster ovary cells transfected with cDNAs encoding wild-type or mutant ceruloplasmin. This analysis reveals that the incorporation of copper into newly-synthesized apoceruloplasmin in vivo results in a detectable conformational change in the protein. Strikingly, despite the unique functional role of each copper site within ceruloplasmin, metabolic studies indicate that achieving this final conformation driven state requires the occupation of all six copper-binding sites, with no apparent hierarchy for copper incorporation at any given site. Consistent with these findings, a missense mutation (G631R) resulting in aceruloplasminemia and predicted to alter the interactions at a single type I copper-binding site, results in the synthesis and secretion only of apoceruloplasmin. Analysis of copper incorporation into apoceruloplasmin in vitro reveals that this process is cooperative and that the failure of copper incorporation into copper-binding site mutants observed in vivo is intrinsic to the mutant proteins. These findings reveal a precise and sensitive mechanism for the formation of holoceruloplasmin under the limiting conditions of copper availability within the cell that may be generally applicable to the biosynthesis of cuproproteins within the secretory pathway.
Introduction

Copper is an essential trace element that plays a critical role in the biochemistry of all aerobic organisms (1). This metal permits the facile transfer of electrons by specific cuproenzymes in critical metabolic pathways. The chemical reactivity of copper also accounts for the toxicity of this metal when cellular homeostasis is impaired and therefore specific mechanisms have evolved which regulate copper trafficking and compartmentalization within the cell. Under physiological circumstances intracellular copper availability is extraordinarily restricted and as a result the delivery of copper to specific pathways and target proteins is determined by metallochaperones which protect this metal from intracellular scavenging (2,3). Biochemical and structural analysis of these copper chaperones indicates that copper transfer to specific targets within the cytoplasm is mediated by an intricate series of events involving direct protein-protein interaction (4). The majority of known cuproenzymes are synthesized within the secretory pathway and copper gains access to this compartment via the trans-Golgi resident copper transporting ATPases present in all cells (1). Despite the essential role of these cuproenzymes, there is currently little or no understanding of the mechanisms of copper incorporation into these proteins within the secretory pathway.

Ceruloplasmin is a copper-containing plasma ferroxidase that plays an essential role in mammalian iron homeostasis (5). This protein is a member of the multicopper oxidase family of enzymes, utilizing the electron chemistry of bound copper ions to couple iron oxidation with the four electron reduction of dioxygen. Inherited loss of function of ceruloplasmin in humans results in a progressive neurodegenerative disease due to excessive iron accumulation within the basal ganglia (6,7). Ceruloplasmin is synthesized in hepatocytes and secreted into the plasma following the incorporation of six atoms of copper in the secretory pathway (8,9). Copper does
not affect the rate of ceruloplasmin synthesis or secretion; however, failure to incorporate this metal during synthesis results in secretion of an unstable apoprotein that is devoid of oxidase activity and rapidly degraded in the plasma (10,11). In Wilson disease the absence or impaired function of a copper-transporting ATPase disrupts copper movement into the secretory pathway resulting in the decreased serum ceruloplasmin observed in affected patients (1).

Ceruloplasmin is characterized by the presence of three types of spectroscopically distinct copper sites. Three type I copper sites result in strong absorption at 600 nm conferring an intense blue color to this protein. A single type II copper is coordinated by four imidazole nitrogens and is in close proximity to two antiferromagnetically coupled type III copper ions which absorb at 330 nm. The type II and type III coppers have been shown to form a trinuclear copper cluster which is the site of oxygen binding during the catalytic cycle (12). Resolution of the structure of human ceruloplasmin by X-ray crystallography has confirmed the presence of this trinuclear cluster as well as the identity of each of the amino acid copper ligands (13). In the current study, this molecular information, taken together with a well-defined expression system that reflects the physiological mechanisms of ceruloplasmin synthesis and secretion, provided the opportunity to directly examine the mechanisms of copper incorporation into this protein within the secretory pathway.
Experimental Procedures

Cell Culture and Metabolic Labeling. HepG2 and Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection and maintained in media with 10% fetal bovine serum as previously described (14). CHO cells were transfected with Lipofectamine (Invitrogen) according to the manufacturer’s instructions and forty-eight hours later incubated for 20 minutes with $[^{35}\text{S}]$methionine and $[^{35}\text{S}]$cysteine, chased for the indicated times in serum-free media, followed by collection of lysate and media for immunoprecipitation and 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) (14). For copper labeling, CHO cells were incubated for the indicated times with 300 $\mu$Ci/ml $^{64}\text{Cu}$, chased with serum-free media containing 100-fold excess copper and media was collected, concentrated using Centricon-30 filters (Millipore) and subjected to SDS-PAGE on 4-20% Tris-HCl gradient gels under non-reducing conditions as described previously (8). Following quantitative analysis of $^{64}\text{Cu}$ by PhosphorImager (Molecular Dynamics), proteins were transferred to nitrocellulose membranes, cross linked by ultraviolet radiation and denatured in 25 mM Tris, pH 8, 2% SDS. Immunoblot analysis was performed using an anti-ceruloplasmin antisera as previously described (14). In some experiments, cells were pretreated for 48 hours with 50 $\mu$M of the copper chelator tetraethylenepentamine (TEPA), followed by extensive washing in phosphate-buffered saline (PBS) prior to the addition of $^{64}\text{Cu}$. Treatment of cells with this concentration of TEPA reduces the intracellular copper content by 50% without any effect on cell viability (15). $^{64}\text{Cu}$ (750 Ci/mmol) was prepared by fast neutron bombardment of a natural zinc target as described previously (16). Molecular modeling of the G637R mutation was accomplished using the software program InSight (Accelrys) and the published x-ray crystallographic structure of human ceruloplasmin (13).
Ceruloplasmin Gene Sequencing and Site-Directed Mutagenesis. DNA was isolated from peripheral blood leukocytes, followed by automated nucleotide sequencing of each of the exons of the ceruloplasmin gene as described (7). Site-directed mutagenesis was performed in pcDNA3 (Invitrogen) containing the entire human ceruloplasmin open-reading frame, using Klentaq polymerase (CLONTECH), 5’ sense and 3’antisense oligonucleotides and primer pairs corresponding to the mutations noted in Table I (14). The presence of specific mutations and fidelity of the entire cDNA sequence were verified by automated fluorescent sequencing (Perkin-Elmer). Research involving human subjects was approved by the institutional review boards of Washington University and Erasmus University and all clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

Urea Denaturation and Limited Proteolysis. 5 µg of purified human ceruloplasmin (Vital Products) was resuspended in PBS with increasing concentrations of urea for 30 min at 37°C. Samples were then subjected to SDS-PAGE followed by detection of protein with Coomassie blue dye. For limited trypsin proteolysis, media was harvested from CHO cells following transfection with a cDNA encoding wild-type human ceruloplasmin and concentrated on a Centricon-30 column. 5 µl aliquots of concentrated media were then incubated in 25µl of 20 mM Hepes pH 7.5, 100 mM KCl, 10% (v/v) glycerol containing 2.5-50 ng/µl bovine pancreatic trypsin (Sigma) for 30 min at 20°C (17). Proteolysis was quenched by addition of Laemmli sample buffer followed by SDS-PAGE, transfer to nitrocellulose and detection of ceruloplasmin by immunoblotting.

Size exclusion chromatography. Forty-eight hours following transfection, CHO cells were pulsed for 1.5 hours in 300 µCi/ml $^{64}$Cu followed by a 2 hr chase period in Ham’s F12 media. Media was then concentrated on Centricon-30 columns and chromatographed on Sephacryl S-100
(Amersham Pharmacia Biotech) equilibrated in 50mM Hepes, 150mM NaCl, pH 7.4. 500 µl fractions were collected and analyzed directly for radioactivity in a 3 inch NaI crystal Packard γ Counter. Window settings were established empirically and counting efficiency determined using aliquots of isotope and correcting for half life ($^{64}$Cu $t_{1/2} = 12.8$ hr). All samples were measured in triplicate. For detection of ceruloplasmin in column fractions, 30 µl of each fraction was analyzed by immunoblotting.

**In Vitro Copper Incorporation.** Media was isolated from CHO cells expressing wild-type or mutant ceruloplasmin, concentrated with Centricon-30 columns and dialyzed twice against 20 X volume of universal buffer containing 14.3 mM boric acid, 14.3 mM KP0₄, 14.3 mM citric acid, 14.3 mM barbital and 1 mM sodium ascorbate pH 7.4 (18). 50 µl aliquots were then incubated with 500 µl of 50 mM sodium acetate pH 5.6, 1mM sodium ascorbate and specified concentrations of CuCl₂, CuSO₄ or Cu(CH₃COO)₂ for 1 hr at 20ºC. Samples were then concentrated using Microcon-30 filters, subjected to SDS-PAGE and immunoblotted for ceruloplasmin. For quantitative copper incorporation studies, the amount of copper present in media samples was determined by atomic absorption spectroscopy (Perkin-Elmer).

Autoradiographs were quantitated using UnScanIt imaging software (Silk Scientific) and data fitted to the curve using Sigma Plot (SPSS Science). For experiments with Fet3, wild-type or mutant proteins containing a FLAG epitope were isolated and purified as described previously (19). 5 µg of these purified proteins were resuspended in either PBS or 50 mM sodium acetate pH 5.6, 1mM sodium ascorbate followed by addition of 500 µCi/ml $^{64}$Cu. Samples were incubated for 1 hr at 20ºC followed by concentration with Centricon-30 filters, SDS-PAGE and analysis for copper incorporation as indicated above and described previously for Fet3 (20).
Results

As can be seen in Fig. 1A, immunoblot analysis of ceruloplasmin in the media of HepG2 cells following SDS/PAGE under non-reducing conditions demonstrates the presence of two bands of 135 and 85 kDa. We have previously shown that these two bands represent apo and holoceruloplasmin that have distinct electrophoretic mobility under these conditions and that the 85 kDa species represents oxidase active holoceruloplasmin containing all six coppers (8). In support of this concept, the 85 kDa holoceruloplasmin band is undetectable following prior treatment of cells with the copper chelator TEPA but is increased in intensity if these same cells are previously grown in media containing excess CuCl2 (Fig. 1A, lanes 2,4). Similar analysis of the media of HepG2 cells following metabolic labeling with $^{64}$Cu also detects the 85 kDa holoceruloplasmin band by direct autoradiography (Fig. 1A, lane 5) as demonstrated previously (8). Analysis of the media of CHO cells transfected with a cDNA encoding human ceruloplasmin reveals identical findings to those observed for HepG2 cells indicating the validity of this approach for dissecting the mechanisms of copper incorporation into ceruloplasmin (Fig. 1A lanes 6-8).

The electrophoretic analysis suggested the possibility of unique conformational differences between the apo and holo forms of ceruloplasmin. This concept was examined further by subjecting purified human ceruloplasmin to denaturation in increasing concentrations of urea, followed by non-reducing SDS-PAGE and detection with Coomassie blue staining. As can be seen in Fig. 1B, this treatment results in the conversion of the 85 kDa holoceruloplasmin to the 135 kDa apoprotein with no intermediate forms detectable. To detect these conformational differences in vivo, media from transfected CHO cells was subjected to limited trypsin proteolysis followed by non-reducing SDS-PAGE and immunoblot detection of ceruloplasmin.
As illustrated in Fig. 1C, this treatment results in distinct cleavage patterns for apo and holoceruloplasmin. Furthermore, apoceruloplasmin was more sensitive to trypsin digestion with complete cleavage taking place at 10 ng/µl (Fig. 1C). Taken together with the findings from the urea denaturation these data support the concept of conformational differences between apo and holoceruloplasmin.

The further examine the conformational changes that result following copper incorporation into apoceruloplasmin, missense mutations were generated by site-directed mutagenesis at each of the six integral copper-binding sites in ceruloplasmin. To abrogate copper incorporation at each of these sites, histidine to glutamine substitutions were made at each type I Cu-binding site (H276Q, H637Q, and H1026Q), the type II Cu-binding site (H101Q), and each type III Cu-binding site (H103Q and H163Q) (Table I). To ensure that the results were not due to the replacement of histidine residues, a type I Cu-binding site mutant in which cysteine was converted to serine (C680S) was also generated. Pulse-chase analysis following transfection of each mutant into CHO cells demonstrated kinetics of synthesis and secretion identical to that of wild-type ceruloplasmin, indicating that none of these copper-binding site mutations results in grossly impaired protein folding or trafficking (Fig. 2). Despite this finding, metabolic labeling with $^{64}$Cu revealed the striking observation that each mutation results in a complete abrogation of copper incorporation into ceruloplasmin (Fig. 3A). The inability of these copper-binding site mutants to form holoceruloplasmin was verified by immunoblot analysis of the media which revealed the presence only of apoceruloplasmin (Fig. 3B). In contrast to findings with wild-type ceruloplasmin (Fig. 1A), incubation of cells transfected with mutant ceruloplasmin in media containing excess CuCl$_2$ did not result in any detectable holoceruloplasmin (data not shown). These findings were not the result of the electrophoretic methodology used to detect apo and
holoceruloplasmin, as size exclusion chromatography of the media of transfected cells following

$^{64}$Cu labeling also revealed copper incorporation only in the wild-type protein (Fig. 4A).

Interestingly, under these conditions, apo and holoceruloplasmin separate in identical fractions supporting the concept that the size differences observed in SDS-PAGE reflect conformational differences accentuated with this technique (Fig. 4B). Failure to detect $^{64}$Cu holoceruloplasmin with this method was not due to loss of the transfected proteins as immunoblot analysis of chromatographic fractions revealed abundant apoceruloplasmin in the media from cells transfected with the copper-binding site mutants (Fig. 4B).

The observation that interference with copper incorporation at any one of the six copper-binding sites abrogates copper incorporation into apoceruloplasmin without disrupting apoceruloplasmin synthesis or secretion suggests a precise mechanism of holoceruloplasmin biosynthesis in the secretory pathway. To directly examine the physiological relevance of these findings, a similar analysis was undertaken using a missense mutation in the ceruloplasmin gene from a patient with aceruloplasminemia. The proband in this family had undetectable levels of circulating serum ceruloplasmin and was found to be homozygous for the mutation G631R (data not shown). As observed previously for the copper-binding site mutants, pulse-chase analysis of the G631R mutation revealed that this protein is synthesized and secreted with kinetics identical to that of wild-type ceruloplasmin (Fig. 5A). Nevertheless, both $^{64}$Cu metabolic labeling and immunoblot analysis reveals that the G631R mutant is synthesized and secreted only as the apoprotein (Fig. 5B,C). As noted with the copper-binding mutants, size exclusion chromatography of media following $^{64}$Cu metabolic labeling also failed to reveal any copper incorporation into the G631R mutant ceruloplasmin (data not shown). Molecular modeling of the targeted G631 residue reveals that it is in close proximity to and interacts with H637, one of the
critical ligands of a type I copper-binding site (Fig. 6). This finding suggests the possibility that the substituted arginine in the G631R mutation may interfere with copper binding at this type I site either through disruption of hydrogen bonding or through more distant conformational effects. As predicted from data in Fig. 3, such an effects would then prevent copper incorporation at all other sites. The predicted outcome of this mutation, consistent with the clinical findings, is the secretion of an apoceruloplasmin that is devoid of ferroxidase activity and rapidly degraded in the plasma.

Although the pulse-chase experiments suggest no difference in the rate of synthesis or secretion between the wild-type and mutant forms of ceruloplasmin, it remains possible that the mutant proteins are trafficked abnormally in the late secretory pathway preventing targeting to the compartment where copper is incorporated. To directly examine if the mutant proteins are capable of incorporating copper, media from transfected CHO cells was subjected to *in vitro* copper exchange with increasing concentrations of CuCl₂. This treatment resulted in an increase in holoceruloplasmin in media from cells transfected with wild-type ceruloplasmin, while neither the G631R mutation nor the copper-binding site mutants were capable of incorporating copper under these same conditions (Fig. 7A). These *in vitro* findings suggest that the conformation of apoceruloplasmin is the critical determinant for copper incorporation and that each copper binding site plays a key role in this process. The copper concentrations indicated in Fig. 7A reflect the amount added to the media. To precisely quantitate the copper-dependent conversion to holoceruloplasmin under these conditions, experiments were performed in media where the total amount of copper was determined by atomic absorption spectrophotometry. This quantitative analysis of copper incorporation into apoceruloplasmin *in vitro* revealed a sigmoidal curve, diagnostic of cooperative binding (Fig. 7B). This same cooperative binding curve was
observed when these experiments were repeated with purified apoceruloplasmin suggesting that
this finding is not the result of competition with copper-binding factors in the media (data not
shown).

Spectroscopic studies of the homologous multicopper ferroxidase Fet3 in *Saccharomyces
cerevisiae* have shown that mutations at one copper-binding site do not abrogate copper
incorporation into the other copper-binding sites of this protein (19). To directly compare these
results to our findings with ceruloplasmin, a similar *in vitro* analysis was performed using wild-
type Fet3 or Fet3 containing a missense mutation at the type I (C484S) or type II (H81Q) copper
binding sites. These experiments reveal copper incorporation into all three Fet3 proteins,
confirming the previous spectroscopic findings (Fig. 8A). Identical findings were observed when
these proteins were copper-labeled utilizing the same tissue culture media as employed for the
ceruloplasmin studies (data not shown). The ability to detect partially labeled Fet3 (type I or type
II mutants) also suggests that the failure to detect copper-labeled ceruloplasmin under these same
conditions is not the result of loss of copper during the electrophoresis. As observed previously
in yeast, analysis of copper incorporation into Fet3 under these conditions results in a detectable
signal between 75 and 100 kDa due to the glycosylation of this protein (20). Detection of copper
incorporation into mutant Fet3 indicates that our inability to observe copper incorporation into
mutant ceruloplasmin (Fig. 3A, 5B) is not the result of the methodological approach utilized and
likely reflects unique differences between these two proteins. In support of this concept, while
recent studies reveal that chloride is a critical allosteric effector for copper loading into Fet3 (18),
copper incorporation into ceruloplasmin was found to be equivalent using either CuCl₂,
Cu(CH₃COO)₂, or CuSO₄ as the copper source (Fig. 8B).
Discussion

These data, together with previous biochemical studies (8,14), indicate that ceruloplasmin is an allosteric protein which enters the late secretory pathway in a copper-competent conformation, where copper serves as a homotropic modulator facilitating the transition to a functional holoprotein. This concept is supported by physical and spectroscopic studies of ceruloplasmin indicating that copper removal results in an apoprotein with altered sedimentation velocity and electrophoretic mobility but retained secondary structure, albeit with weaker tertiary interactions than observed in the holoprotein (21,22). We propose that this folding intermediate may be similar to that described for proteins such as apomyoglobin and staphylococcal nuclease A where secondary structure is preserved but flexibility permits the interval motions required for cofactor insertion (23,24). In the case of ceruloplasmin this copper-competent state would allow for folding and exit from the endoplasmic reticulum and serve to protect the protein from unfolding in the acidic milieu of the late secretory pathway while still permitting the critical small scale fluctuations required to accommodate copper incorporation.

Biochemical analysis of the G631R patient mutation underscores the essential nature of this copper competent state in the formation of an active, functional ferroxidase (Fig. 5). In transfected cells this mutation did not affect the critical stages of protein folding required for exit of ceruloplasmin from the endoplasmic reticulum or secretion from the cell but clearly altered the intrinsic protein structure in such a way as to abrogate copper incorporation as revealed with in vitro copper incorporation studies (Fig. 7). Although greater than 90% of serum ceruloplasmin is in the form of the holoprotein, the marked differences in the serum half-lives of apo and holoceruloplasmin predicts an equivalent rate of hepatic synthesis under steady state conditions
(25). This is precisely what is observed in CHO cells transfected with wild-type ceruloplasmin (Fig. 1A), confirming the validity of this approach for analysis of the G631R mutation.

One of the most critical features of the data in this current study is the observation that copper incorporation into ceruloplasmin requires competency of all six ligand binding sites with no apparent hierarchy for incorporation at any one site. Obviously, at some point during this process, intermediates must form and it remains possible that these represent copper binding to low affinity sites such that only the final step of copper incorporation into a high affinity site is required to bring the protein into the trypsin resistant, stable holo conformation. Under such circumstances the binding at these low affinity sites may still occur in an ordered or hierarchical fashion. While we are currently evaluating the possibility of such a model with fluorescence spectroscopic studies of copper binding to recombinant, purified apoprotein, the existence of such low affinity intermediates seems unlikely given the inability to detect these forms even when excess copper is added to the cell cultures and electrophoresis conditions (data not shown) or in the in vitro copper loading experiments (Fig. 7).

In contrast to our findings with ceruloplasmin, partial copper intermediates have been observed for the multicopper oxidases Fet3 and bilirubin oxidase (19,26) and in the case of Fet3 these were readily detected with the methods used in this current study (Fig. 8). Ceruloplasmin is unique amongst these three multicopper oxidases in containing three type I copper centers and it is possible that this may result in structural or biochemical differences that account for the all-or-none process of copper incorporation. Consistent with this possibility, it has been proposed that the type I copper in domain II of ceruloplasmin may serve in a structural role and not be involved in electron transfer as the high reduction potential at this site prevents oxidation at physiologic pH (27). As the data in this current study clearly indicate an essential role for this type I copper
site in holoprotein formation (Fig. 3 H276Q) this does raise the intriguing possibility that this copper, as Cu^{1+}, may serve a structural role in protein folding. Regardless of such a possibility, further work will be needed to elucidate the structural mechanisms of the copper incorporation process observed in this current study. Although the precise role of copper in protein folding is not well understood, further study of these mechanisms in ceruloplasmin may have a more broad relevance given recent observations that copper binding to the cellular prion protein induces thermodynamic changes favoring pathologic β-sheet formation (28).

Ceruloplasmin is a single subunit protein with multiple binding sites for copper (Table I). This suggested to us the hypothesis that the allosteric effects of copper binding observed in Fig. 1 might result in an increase in the affinity for subsequent copper binding. This concept is supported by the binding studies in Fig. 7B which clearly illustrate the cooperative nature of this process. The allosteric effects giving rise to such cooperativity are presumably mediated by conformational changes transmitted within the protein upon copper binding. As cooperativity permits a more sensitive response to ligand concentrations, taken together these data reveal a precise and sensitive mechanism to permit the rapid synthesis of holoceruloplasmin under the normal physiological circumstances of restricted copper availability in the cell. This proposed mechanism is consistent with the considerable evidence indicating that copper is incorporated into ceruloplasmin at or beyond the trans-Golgi network (5). Under such circumstances, the partially folded, copper competent apoceruloplasmin would then be sequestered with copper in an acidic vesicular environment where the cooperative mechanisms of protein folding permits holoceruloplasmin synthesis. Although such a model is distinct from that utilized to ensure copper delivery in the cytoplasm, it seems plausible given the enormous diversity of cell specific expression as well as structure and function of the cuproenzymes synthesized in the secretory
pathway (1). While further studies will be needed to directly test this hypothesis, support is found in recent experiments examining copper incorporation into tyrosinase demonstrating specific mutations that abrogate copper incorporation but do not affect trafficking of the apoprotein to the melanosome (29).
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Figure Legends

Fig. 1 Copper incorporation into apoceruloplasmin. (A). Immunoblot analysis of ceruloplasmin in media from HepG2 cells (lanes 1-4) or CHO cells transfected with a cDNA encoding human ceruloplasmin (lanes 6-7). In some experiments cells were incubated in 50 µM TEPA (lanes 2, 7) or 50 µM CuCl₂ (lane 4) for 48 hr prior to analysis or 200 µCi/ml of ⁶⁴Cu for three hours (lanes 5, 8). Media was subjected to non-reducing SDS-PAGE on 4-20% Tris-HCl gradient gels followed by transfer to nitrocellulose and immunoblotting or direct autoradiography for detection of ⁶⁴Cu. (B) Purified human serum ceruloplasmin, a mixture of apo and holoceruloplasmin (arrows) was incubated in increasing concentrations of urea as indicated at 37°C, subject to non-reducing SDS-PAGE on 4-20% Tris-HCl gradient gels and visualized by Coomassie staining. (C) Media from transfected CHO cells grown in the presence of TEPA (apo) or following in vitro copper exchange to reconstitute holoceruloplasmin (holo) was concentrated and incubated in increasing concentrations of trypsin, followed by non-reducing SDS-PAGE, transfer to nitrocellulose and immunoblot analysis for ceruloplasmin. Ctrl lane is media from transfected CHO cells without treatment. Asterisks indicate unique bands.

Fig. 2 Biosynthesis and secretion of copper-binding site mutants. CHO cells transfected with either wild-type (wt) or the indicated Cu-binding site mutants were pulse labeled with [³⁵S] methionine and [³⁵S] cysteine for 20 minutes and chased for the indicated times in media containing excess methionine. Ceruloplasmin was immunoprecipitated from cell lysates (IC) and media (EC) and analyzed by 7.5% SDS-PAGE.

Fig. 3 Copper incorporation into copper-binding site mutants (A) HepG2 cells or CHO cells transfected with either wild-type (wt) ceruloplasmin or the indicated Cu-binding site mutants were incubated in serum-free media containing 200 µCi/ml ⁶⁴Cu for 1 hr followed by chase in
media containing 100-fold excess copper for 2 hr. Media was subjected to non-reducing SDS-PAGE on 4-20% Tris-HCl gradient gels and analysis of $^{64}$Cu following exposure of gels to PhosphorImager. (B) Immunoblot analysis of ceruloplasmin from media of HepG2 cells or CHO cells transfected with either wild-type (wt) ceruloplasmin or the indicated copper-binding site mutants.

**Fig. 4** Gel filtration analysis of copper incorporation into binding site mutants. (A) $^{64}$Cu elution profiles following Sephacryl S-100 chromatography of media from CHO cells transfected with either vector alone, wild-type ceruloplasmin (wt) or the indicated copper binding site mutants. (B) Immunoblot analysis of ceruloplasmin in chromatography fractions 4-12 from each of the experiments indicated above in (A). Apo (a) and holoceruloplasmin (h) are indicated.

**Fig. 5** Analysis of mutation in aceruloplasminemia. (A). CHO cells transfected with either wild-type (wt) or G631R mutant were pulse labeled with $[^{35}$S] methionine and $[^{35}$S] cysteine for 20 minutes and chased for the indicated times in media containing excess methionine. Ceruloplasmin was immunoprecipitated from cell lysates (IC) and media (EC) and analyzed following 7.5% SDS-PAGE. (B) CHO cells transfected with either vector alone, wt, or G631R ceruloplasmin were incubated in serum-free media containing $200 \mu$Ci/ml $^{64}$Cu for 1 hr followed by chase in media containing excess copper for 2 hr. Media was subjected to non-reducing SDS-PAGE on 4-20% Tris-HCl gradient gels and analysis of $^{64}$Cu following exposure of gels to PhosphorImager. (C) Immunoblot analysis of ceruloplasmin in media from CHO cells transfected with either vector alone, wt, or G631R ceruloplasmin. The presence of apo (a) and holoceruloplasmin (h) is indicated.
**Fig. 6 Structural model illustrating G631.** Structural model of ceruloplasmin showing G631 and the adjacent copper-binding H637. The α-carbon on G631 is separated from the ε-carbon on H637 by a distance of 7.10 Å.

**Fig. 7 In vitro copper incorporation into ceruloplasmin.** (A) CHO cells were transfected with either wild-type (wt) ceruloplasmin or the indicated mutants, incubated for 48 hr in 50 µM TEPA and the media collected, concentrated and incubated in 50 mM sodium acetate pH 5.6, 1mM sodium ascorbate and indicated amounts of CuCl₂. Ceruloplasmin was detected by immunoblot analysis following non-reducing SDS-PAGE. (B) Quantitative analysis of copper incorporation (µM) into apoceruloplasmin using the media from CHO cells transfected with wild-type ceruloplasmin as in (A). Copper content was determined by atomic absorption spectrophotometry and apo and holoceruloplasmin detected by immunoblot analysis and quantitated by scanning of the autoradiograph.

**Fig. 8 Copper incorporation into Fet3 and ceruloplasmin.** (A) Purified wild-type or mutant Fet3 or media from CHO cells transfected with wild-type ceruloplasmin were incubated in PBS (-) or 50 mM sodium acetate pH 5.6, 1mM sodium ascorbate (+) with 500 µCi/ml ⁶⁴Cu followed by non-reducing SDS-PAGE and analysis of ⁶⁴Cu by PhosphorImager. The gel was subsequently transferred to nitrocellulose and immunoblot analysis performed using antisera to the FLAG epitope or ceruloplasmin. (B) Media from CHO cells incubated without or with 50 µM TEPA and transfected with wild-type ceruloplasmin was dialyzed twice against 20 X volume of universal buffer, incubated in 50 mM sodium acetate pH 5.6, 1mM sodium ascorbate and 0.5 µM CuCl₂, CuSO₄ or Cu(CH₃COO)₂ as indicated followed by non-denaturing SDS-PAGE and immunoblot analysis of ceruloplasmin.
### Table A

|          | wt Fet3 | C484S Fet3 | H81Q Fet3 | wt Cp |
|----------|---------|------------|-----------|-------|
| Cu exchange: | - +     | - +        | - +       | - +   |

### Image A

- **64Cu-Fet3**
- **FLAG**
- **64Cu Cp**
- **apo Cp**
- **holo Cp**

### Table B

|          | CuCl₂ | Cu-acetate | CuSO₄ |
|----------|-------|------------|-------|
| 0.5µM Cu: | -     | -          | -     |
| TEPA:    | - +   | - +        | - +   |

### Image B

- **apo**
- **holo**
| Cu atom       | Amino Acids | Mutations |
|--------------|-------------|-----------|
| Type I (domain II) | H276, C319, H324, L329 | H276Q |
| Type II (domain IV) | H637, C680, H685, M690 | H637Q, C680S |
| Type III (domain VI) | H975, C1021, H1026, M1031 | H1026Q |
| Type II      | H101, H978  | H101Q     |
| Type III     | H163, H980, H1020 | H163Q     |
| Type III     | H103, H161, H1022 | H103Q     |
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