

Site-directed Mutagenesis of Human Ceruloplasmin

PRODUCTION OF A PROTEOLYTICALLY STABLE PROTEIN AND STRUCTURE-ACTIVITY RELATIONSHIPS OF TYPE 1 SITES*

Received for publication, August 8, 2000, and in revised form, October 18, 2000
Published, JBC Papers in Press, October 19, 2000, DOI 10.1074/jbc.M007176200

Pamela Bielli^{‡§**}, Gian Carlo Bellenchi^{‡¶}, and Lilia Calabrese^{‡||}

From the [‡]Department of Biology, University Roma Tre, Viale Marconi 446, 00146 Rome, Italy and ^{||}Consiglio Nazionale delle Ricerche Center of Molecular Biology, University La Sapienza, Piazzale Aldo Moro 5, 00185 Rome, Italy

A fully active recombinant human ceruloplasmin was obtained, and it was mutated to produce a ceruloplasmin stable to proteolysis. The stable ceruloplasmin was further mutated to perturb the environment of copper at the type 1 copper sites in two different domains. The wild type and the mutated ceruloplasmin were produced in the yeast *Pichia pastoris* and characterized. The mutations R481A, R701A, and K887A were at the proteolytic sites, did not alter the enzymatic activity, and were all necessary to protect ceruloplasmin from degradation. The mutation L329M was at the tricoordinate type 1 site of the domain 2 and was ineffective to induce modifications of the spectroscopic and catalytic properties of ceruloplasmin, supporting the hypothesis that this site is reduced and locked in a rigid frame. In contrast the mutation C1021S at the type 1 site of domain 6 substantially altered the molecular properties of the protein, leaving a small fraction endowed with oxidase activity. This result, while indicating the importance of this site in stabilizing the overall protein structure, suggests that another type 1 site is competent for dioxygen reduction. During the expression of ceruloplasmin, the yeast maintained a high level of Fet3 that was released from membranes of yeast not harboring the ceruloplasmin gene. This indicates that expression of ceruloplasmin induces a state of iron deficiency in yeast because the ferric iron produced in the medium by its ferroxidase activity is not available for the uptake.

Ceruloplasmin (Cp)¹ is a 130-kDa multicopper protein widely distributed in vertebrates. It occurs mainly in the plasma and plays an important role in iron homeostasis (1, 2). Other roles include its participation in the antioxidant defense (3–6) or in oxidative damage mechanisms (7, 8) and its involvement in a number of metabolic processes related to the metabolism of copper (9), biogenic amines (10), and nitric oxide (11).

* This work was supported by Consiglio Nazionale delle Ricerche “Progetto Finalizzato Biotecnologie.” The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Inst. of Microbiology and Genetics, Vienna Biocenter, University of Vienna, Dr. Bohrgasse 9/4, A-1030 Vienna, Austria. Tel.: 43-1-427754614; Fax: 43-1-42779546; E-mail: pamela@gem.univie.ac.at.

¶ Present address: Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France.

** This work was in partial fulfillment of a Ph.D.

¹ The abbreviations used are: Cp, ceruloplasmin; HCp, human ceruloplasmin; rHCp, recombinant human ceruloplasmin; T1, T2, and T3, type 1, 2, and 3 copper, respectively; PAGE, polyacrylamide gel electrophoresis; pPD, *p*-phenylenediamine; ET, electron transfer.

Ceruloplasmin supports such diverse functionalities by different catalytic activities. It has long been known that Cp is an oxidase and belongs to the class of multicopper “blue” oxidases (12). These enzymes couple the four-electron reduction of dioxygen to water to the four sequential one-electron oxidations of a variety of substrates. Other enzymes of the same group are ascorbate oxidase, laccase, and Fet3, a plasma membrane protein of yeasts, recently discovered (13). Among these enzymes, only Cp (14, 15) and Fet3 (16) are able to oxidize an inorganic substrate (*i.e.* Fe(II) ions) in addition to organic substrates such as diamines. While the precise physiologic function of Cp associated to its amine oxidase activity has not yet been defined, the ferroxidase activity is now considered the main function of ceruloplasmin. The complete absence of Cp, because of inherited mutations (17) or targeted disruption of the Cp gene (18), leads to a long term accumulation of parenchyma iron due to an impaired iron efflux from cells. The oxidation of the Fe(II) released from cells and its subsequent incorporation into apo-transferrin would be the mechanism whereby Cp is involved in mediating iron release from cellular stores (19, 20). Analogously, the ferroxidase reaction performed by Fet3 is also an essential reaction for the high affinity iron uptake in yeasts (13, 21).

Multicopper oxidases contain three distinct spectroscopic types of copper sites: at least one type 1 (T1), or “blue,” site in addition to one type 2 (T2), or “normal,” copper site and to one type 3 (T3), or binuclear, copper site lacking an EPR signal (12). The three copper atoms of these two last sites comprise a trinuclear cluster, observed in the x-ray structure of ascorbate oxidase (22), Cp (23), and laccase (24), which serves as the oxygen binding and reducing site during the catalysis. The T1 is the site of entry of electrons from substrate. The T1 sites of multicopper oxidases exhibit different geometry and different redox potential, due to differences in their coordination sphere (25). With the exception of Cp, there is generally one T1 site per trinuclear cluster in these enzymes. A conserved structural motif of the polypeptide chain, His-Cys His, where the Cys is a ligand of the T1 site and the two His residues are ligands of two copper atoms of the cluster, facilitates the intramolecular electron transfer (ET) between the two sites, ~13 Å apart (26, 27).

Ceruloplasmin contains multiple T1 sites. This is a long recognized peculiarity of Cp that has been outlined by numerous spectroscopic and catalytic studies and it has been confirmed by the x-ray structural studies on the human protein (23). Human ceruloplasmin (HCp) is a single chain of 1046 amino acids (28), with a carbohydrate content of 7–8% and a copper content of six integral copper atoms. The x-ray structure has shown that the molecule of HCp is composed of six compact domains, with large loop insertions, and that the six integral copper atoms are distributed in one trinuclear cluster, located

at the border between domains 1 and 6 and possessing ligands from each domain and in three mononuclear T1 sites. Those located in domains 4 and 6 are typical T1 sites such as that of ascorbate oxidase (22) and have a set of four ligands, two histidines, one cysteine, and one methionine, arranged in a distorted tetrahedral geometry. The one located in domain 2 is a tricoordinate T1 site in that it lacks the axial Met ligand, which is replaced in the amino acid sequence by Leu, as the single blue site of some laccases (26), and Fet3 (29). These latter sites have redox potential values higher than those of typical T1 sites. Whether and how all three T1 sites take part in the turnover reaction of Cp is presently a major question (27). Only the T1 site of domain 6 is connected by the cysteine-histidine linkages to the trinuclear cluster (23). Binding sites for iron (15) and amine substrates (10) have been identified in domains 4 and 6 by x-ray studies, which did not evidence equivalent structures on domain 2. This latter finding has been related to the peculiar redox properties of the T1 site of domain 2. A redox potential value of at least 1 V has been calculated for this site, which is apparently the highest one among T1 sites of blue proteins (30). On this basis, it has been postulated that the copper at this center stays permanently reduced, thus spectroscopically silent and not able to take part in the catalytic cycle of the oxidase.

Mutation studies have been used to clarify the influence of ligands and the contribution of other elements of the protein matrix to the properties of each type of copper (31–33). Although a recombinant human ceruloplasmin has been expressed in mammalian cells and it has been partially purified (34), the physicochemical properties of the recombinant protein were not assessed. In this paper, we report the expression of human ceruloplasmin in *Pichia pastoris* and the use of site-directed mutagenesis to explore its structure to function relationships. The residues responsible for the proteolytic susceptibility of the protein, Arg⁴⁸¹, Arg⁷⁰¹, and Lys⁸⁸⁷ had to be mutated in tandem ahead of targeted mutations at the copper sites. Mutation of Leu³²⁹ to Met and of Cys¹⁰²¹ to Ser in the coordination sphere of the T1 sites of domains 2 and 6, respectively, demonstrated a different role for these two sites. During the expression of the heterologous oxidase, the yeast maintained its endogenous oxidase Fet3, although a soluble, fully active, derivative of Fet3 was found in the medium.

EXPERIMENTAL PROCEDURES

Chemicals and Biologicals—Superdex 200, Sephacryl S-200, Sephadex G-75, and Sepharose 4B were from Amersham Pharmacia Biotech. DE52 was from Whatman. Enzymes for DNA manipulation were from New England Biolabs or Promega. Anti-HCp polyclonal antibodies were from Sigma. Peptide:N-glycosidase was from Roche Diagnostic GmbH. Media for yeast cultures were from Difco. The *Pichia* Expression Kit was from Invitrogen. All other reagents were purchased from Sigma unless otherwise noted and used without further purification.

Strains, Culture, and Media—The *P. pastoris* strain used for the heterologous expression of HCp was GS115 *his4* and was routinely grown in buffered glycerol complex medium; buffered methanol complex medium was used for the induction. The 10F' *Escherichia coli* was grown in Luria-Bertani medium.

Integration of HCp Expression Cassette in *P. pastoris* Genome and Site-directed Mutagenesis—The cDNA of HCp, encoding the precursor protein with a 19-amino acid signal peptide, was kindly provided by Professor J. D. Gitlin (Washington University, St. Louis, MO). Site-directed mutagenesis was performed on the cDNA cloned into the Bluescript II KS plasmid (pBS) with the following mutagenic primers: 5'-GAAAAAGGCTTGCATACCGGC-3' for the mutation L329M; 5'-CC-CAGAGCGCAAGTGTGCCTC-3' for the mutation R481A; 5'-CTCAGACTGCGCCCTGCATTG-3' for the mutation R701A; 5'-ACCTACTTG-GCAGTATCAATCC-3' for the mutation K887A; 5'-GTTACTCCACT-CCCATGTGACC-3' for the mutation C1021S. The megaprimer technique (35) was used to obtain the K887A mutant first, and then the mutations of R⁴⁸¹ and R⁷⁰¹ to Ala were introduced in a second round of mutagenesis according to Ref. 36 to obtain the triple mutant R481A/

R701A/K887A. The mutations L329M and C1021S were introduced on the triple mutant with the megaprimer technique. All mutations were verified by automated sequencing at the ENEA-BIOGEN sequencing facility.

The cDNA encoding each HCp (wild type or mutant) with its own signal peptide was subcloned from pBS into the expression vector pHIL-D2, which has a single *EcoRI* cloning site adjacent to the alcohol oxidase 1 gene promoter, and used to transform *E. coli* (TOP 10F') cells. Plasmid DNA was purified from selected colonies, linearized by digestion with *NotI*, and used to transform GS115 *his4* cells by spheroplasting according to the manufacturer's instructions. Histidine-independent transformants were selected and subsequently screened for their slow methanol utilization phenotypes. Positive clones were induced with methanol and screened for HCp production by Western blotting of the culture medium.

Yeast Cultivation and Ceruloplasmin Production—Yeast colonies that had undergone the appropriate recombination events to incorporate the Cp gene into the *P. pastoris* genome were used to inoculate the buffered glycerol complex medium (800 ml). The flasks (2 liters) were incubated at 30 °C in a shaking incubator (250 rpm) until the cells attained an A₆₀₀ of ~6. The cells were harvested by centrifugation at 3000 × g, and the cell pellet was washed extensively and resuspended in the buffered methanol complex medium containing 300 μM CuSO₄ and 30 μM FeSO₄ to an approximate A₆₀₀ of 100. These conditions were settled by monitoring the effect of various concentrations of these salts on the level of Cp secreted. The baffled flasks were shaken at 30 °C. The cultures were monitored for up to 7 days. The daily addition of methanol to a final concentration of 0.5% maintained the induction conditions of the alcohol oxidase 1 promoter.

Aliquots of cultures of induced *P. pastoris* cells were collected at various times during the induction period, and cells were removed by centrifugation. The supernatants were analyzed either by SDS-PAGE followed by Western blot analysis probed with anti-HCp polyclonal antibodies or by native PAGE followed by staining with *o*-dianisidine at pH 5, to monitor the oxidase activity. In this case, the samples (5 ml) were fractionated on small columns of Sephadex G-75, and the high molecular weight fraction was collected and concentrated to ~50 μl. Total membrane extracts from cell pellets were obtained according to Ref. 21 and were analyzed by non denaturing SDS-PAGE (37) followed by staining with *o*-dianisidine for oxidase activity.

Ceruloplasmin Purification and Analysis—The purification of rHCp was routinely performed after a 72-h induction. At variance with authentic HCp, which was isolated from plasma by a single step on a column of activated Sepharose 4B (38), the isolation of recombinant ceruloplasmin required multiple steps due to the abundance of brownish components in the induction medium. The amount of this material was somehow variable, and any effort to limit its presence in the medium was in vain. It was adsorbed by DE52 added in batch (60 g/500 ml) to the supernatant obtained from induced cultures. After 15 min, the resin was filtered off, and the medium was depleted of low molecular weight components and brought to low ionic strength by gel filtration on a column of Sephadex G-75 (10 × 60 cm) equilibrated at pH 7 with 50 mM phosphate buffer containing 0.5 mM EDTA and 5 mM amino caproic acid. The fractions containing ceruloplasmin were loaded on a bed of the activated Sepharose (2.5 × 20 cm). Extensive washings with 120 mM phosphate buffer were necessary to completely elute Fet3. Nearly 50% of the original content of rHCp was found in these fractions. The remaining rHCp was eluted at 200 mM phosphate buffer. This fraction was diluted and applied again to a small column of activated Sepharose to concentrate ceruloplasmin. The procedure was accomplished in 10 h, and the yield was nearly 1 mg of rHCp from 500 ml of induction medium, which represented ~20% of the recombinant protein secreted in the medium. Very often, however, the concentrated sample of the recombinant protein still contained yellow component(s) responsible for a huge absorption below 500 nm. These samples required treatment with DE52, added in batch, and gel filtration on either Superdex 200 by fast protein liquid chromatography or Sephacryl S-200, that, however, lowered the yield to ~0.6 mg.

Deglycosylation was performed on ceruloplasmin denatured at 90 °C in 50 mM Tris-Cl buffer, pH 8, containing 0.5% SDS, 0.1 M 2-mercaptoethanol, by the addition of 5 units of peptide:N-glycosidase F in the presence of 20 mM 1,10-phenanthroline, 2% Triton X-100. The mixture was incubated for 2 h at 30 °C.

The amine oxidase activity was measured at pH 6 and pH 7, 0.2 M phosphate buffer, by a coupled NADH/PPD assay (39). The ferroxidase activity was measured at pH 6 in 0.3 M acetate buffer by monitoring the appearance of ferric ions at 315 nm (40).

Characterization of Fet3—The limited proteolysis experiments and

the purification of the soluble derivative of Fet3 were carried out as already reported (41). To test the susceptibility of the purified soluble Fet3 to proteases, the protein, in 0.1 M phosphate buffer, pH 7.4, was incubated with 2 μ M trypsin and 2 μ M chymotrypsin, for 10 h. Oxidase activity measurements and SDS-PAGE analyses were performed on aliquots from the mixture at various times.

To isolate full-length Fet3 from transformed *P. pastoris*, the cells were collected 48 h after the induction with methanol. The same purification procedure (41) applied to the membranes of untransformed GS115 cells, grown in the presence of bathophenanthroline disulfonate, was used. Nondenaturing SDS-PAGE (37) was used to monitor the electrophoretic heterogeneity of Fet3 during the purification; the fractions of Fet3 at the various steps reproduced the pattern of membranes, although a decrease of the slower component was noticed along the entire procedure. For the N-terminal sequence determination, the gels were soaked in the SDS buffer, containing 2.5% mercaptoethanol, after the electrophoretic run and incubated for 10 min at 90 °C prior to the transfer to the polyvinylidene difluoride membranes.

Analytical Methods—Protein concentration was determined by the copper/bicinchoninic acid assay (Pierce). Copper was determined by flameless atomic absorption spectrophotometry on a PerkinElmer Life Sciences model 3030 instrument equipped with graphite furnace. The N-terminal sequence analyses of electrophoretic bands transferred on polyvinylidene difluoride membranes were performed on an Applied Biosystems model 475A sequencer. EPR spectra were obtained using a Bruker ESP300 spectrometer equipped with a variable temperature controller. Samples were run at 100 K, 9.43-GHz microwave frequency, 10-milliwatt power, and 10-Gauss modulation amplitude. Paramagnetic copper content was estimated by double integration of the sample signal by using a copper-EDTA standard. Absorbance spectra were recorded on a PerkinElmer Life Sciences λ 14 model instrument. Figures representing views of the crystal structure of HCp and of the copper site were created by using crystallographic coordinates taken from the Protein Data Bank (Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ) with the entry code 1KCW. The two loop regions and the C-terminal portion of the polypeptide chain not clearly defined in the electron density map were modeled as described previously (42).

RESULTS

Expression of Human Ceruloplasmin in *P. pastoris*—Expression and recovery of recombinant human ceruloplasmin was accomplished by subjecting *P. pastoris* cells to an induction time of 3 days in complete medium containing iron and copper salts. The behavior of Fet3, the endogenous multicopper oxidase of yeast, was carefully monitored over this time. As shown in Fig. 1A, the electrophoretic analyses of the culture medium, performed under nondenaturing conditions to detect the oxidase activity, revealed the presence of two bands, both exhibiting a different mobility with respect to the authentic human serum Cp. The two bands exhibited an opposite behavior. The one with higher electrophoretic mobility was very intense at 24 h from the induction, and then it gradually decreased at longer times. Although it was not recognized by the anti-Cp antibodies, this oxidase mimicked the behavior of authentic HCp during purification (38). It was retained by activated Sepharose, and it was found in the high ionic strength fractions when the isolation of recombinant HCp was afforded at 24 h postinduction. The SDS-PAGE analysis (Fig. 1D) showed that the principal component of these fractions had an apparent molecular mass of \sim 100 kDa. Its N-terminal sequence, ETHHTWNFTTGFV, turned out to be identical to that determined for the soluble derivative of Fet3 obtained by limited proteolysis of membrane suspensions of *P. pastoris* (41).

Since Fet3 was not found in the media of cultures of uninduced cells, this suggested that proteolytic attack(s) at the extracellular portion of membrane-bound Fet3 could release its catalytic domain, upon exposing yeasts to the conditions of the induction. The susceptibility of this domain to massive proteolytic attacks once in the medium can explain the time-dependent decrease of the higher mobility band shown in Fig. 1A. This hypothesis was supported by the observation that the soluble

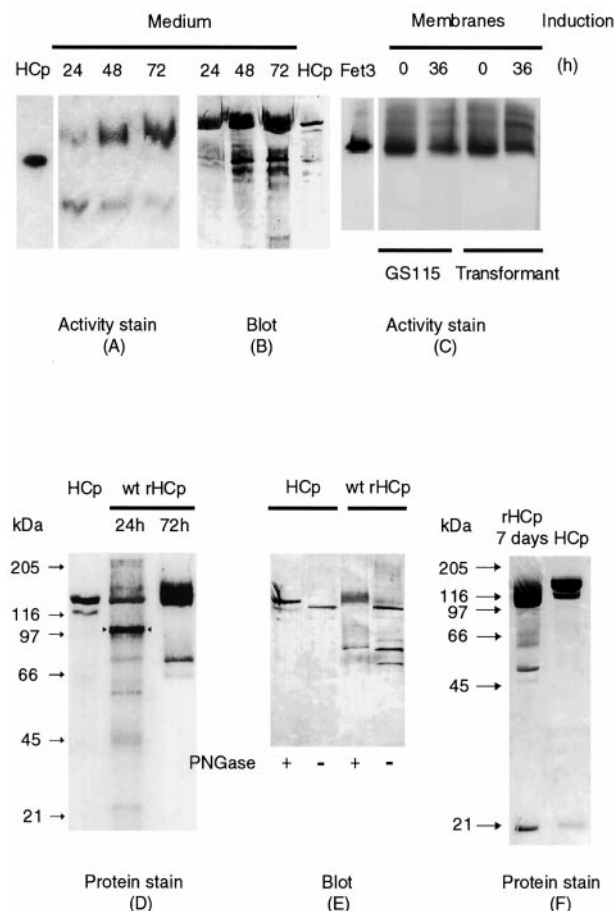


FIG. 1. Expression of human ceruloplasmin in *P. pastoris*. *Top*, analysis of proteins secreted following methanol induction. Aliquots of culture media of *P. pastoris* were removed at the indicated times postinduction and centrifuged. The supernatants were fractionated on Sephadex G-75 and concentrated 100 \times before loading on gels for PAGE and in-gel assay activity by using *o*-dianisidine (A) and loaded as such on gels for denaturing SDS-PAGE and Western blot analysis by using anti-HCp (B). C, membrane extracts from cell pellets from either uninduced cells grown in buffered glycerol complex medium (0), and from cells collected 36 h postinduction were analyzed by nondenaturing SDS-PAGE. The behavior of untransformed GS115 is compared with the transformed yeast. *Bottom*, SDS-PAGE analyses of preparations of wild type (*wt*) recombinant human ceruloplasmin. D, the 200 mM fraction from activated Sepharose of a preparation at 24 h postinduction is compared with recombinant HCp purified at 72 h postinduction; the arrows indicate the band corresponding to the sequence of Fet3. E, the effect of peptide:N-glycosidase treatment on the sample at 72 h postinduction and on authentic Cp. Samples were denatured at 90 °C and deglycosylated with 5 units of peptide:N-glycosidase at 30 °C for 24 h. F, electrophoretic pattern of samples purified 7 days postinduction showing the proteolytic fragments. HCp and Fet3 in both A and B refer to the purified proteins run in each case as control.

derivative of Fet3, purified from *P. pastoris* cells subjected to limited proteolysis as already described (41), was more than 90% inactivated after 10 h of incubation at room temperature with the same proteases. After this time, the electrophoretic analyses showed that not only the oxidase-active band but also the protein band had nearly vanished (results not shown).

To verify that a decrease of the membrane-bound Fet3 was occurring, concomitant to the appearance of Fet3 in the medium, the amine oxidase activity of membrane extracts was assayed by using *p*PD as substrate (41). Transformed cells showed only a modest decrease of the specific oxidase activity, which accounted for \sim 90% that of uninduced cells at 24 h postinduction and remained at these levels over the induction period. In contrast, the parent GS115 strain, subjected to same conditions, showed a variable batch-to-batch, but important

(50–80%), decline of its oxidase activity, checked at different times postinduction. The electrophoretic analyses of the membrane fraction from cell pellets harvested before and after the induction to monitor the membrane-bound Fet3 confirmed these results (Fig. 1C), thus suggesting that coding for a secreted heterologous oxidase was responsible for the behavior of the transformed yeast. It should be noted that the electrophoretic pattern of transformed cells at 36 h postinduction showed a more pronounced heterogeneity due to an increased intensity of the faster migrating of the bands with slower mobility with respect to the principal band of Fet3. This behavior appears to be typical of *P. pastoris* subjected to low iron conditions in that this band (and occasionally other components) is well resolved also in the electrophoretic pattern of the membranes isolated from cells grown in the presence of 80 μM bathophenanthroline disulfonate (41). Fet3, purified from transformed cells induced with methanol, retained this component, which gave the same N-terminal sequence (ETHTWNFT-TGFVNANPDG) as the principal band of Fet3.

This analysis was also performed on the oxidase-active bands of the fractions obtained at early steps of the purification and revealed that Fet3 was the major component of the slower band. The other sequences found varied batch-to-batch and did not match any known sequence. Since the anomalous electrophoretic mobility of Fet3, as well as that of Cp (37), in non-denaturing gels strongly correlates with the overall protein conformation, a plausible explanation is that the slower band represents a fraction of Fet3 locked in a different conformation or, more simply, an aggregated form of the protein.

The oxidase-active band, much broader and slower than authentic HCp on native gels (Fig. 1A), was recognized by anti-HCp antibodies (Fig. 1B), and the parallel enhancement of these bands at longer times of induction indicated the gradual accumulation of an active rHCp in the medium. The presence of multiple bands on Western blot (Fig. 1B), however, indicated that rHCp might be suffering proteolytic attack(s), probably from the same proteases responsible for the decline of the soluble Fet3 in the medium. Thus, the induction was usually stopped after 72 h to avoid excessive degradation of recombinant Cp.

Molecular Properties of Recombinant Ceruloplasmin—As described under “Experimental Procedures,” the isolation of rHCp from the culture medium was quite laborious with respect to the single-step method employed to isolate HCp from plasma (38). The electrophoretic analyses, performed on the purified protein, confirmed the results obtained on the medium. rHCp as isolated appeared as a broad band on native gels and dissociated under denaturing conditions in two principal and heterogeneous components (Fig. 1C). The N-terminal sequence determinations gave the following results. The cluster of bands at 130–170 kDa gave only one sequence, that of the mature HCp, KEKHYYIGIETT. The sequence SVPP-SASHVA was obtained from the major component of the cluster at around 80 kDa, and this result indicated that a proteolytic attack at Arg⁴⁸¹ had cut the polypeptide chain of rHCp nearly in two halves. The sequence of the other fragment, *i.e.* the N-terminal sequence of HCp, was found in the multiple bands scattered in the region above and below 80 kDa. The heterogeneity of these components was undoubtedly due to the carbohydrate moiety, since treatment of denatured samples of rHCp with peptide:N-glycosidase F induced a mobility shift of these bands consistent with a decrease in the molecular weight. In particular, the high molecular weight bands were reduced to a more homogeneous species that migrated with an apparent mass of ~ 120 kDa (Fig. 1E), corresponding to that obtained from authentic HCp.

Different preparations of rHCp were analyzed for copper content taking into account a molecular mass value of 120 kDa. A value of 5.9 ± 0.2 g atoms of copper/120 kDa was obtained, which indicated the full occupancy of the integral copper sites. The specific oxidase activity of rHCp as isolated was, at pH 6, 0.11 ± 0.03 $\mu\text{mol}/\text{min}/\text{mg}$ of protein with *p*PD as substrate, a value consistent with that of authentic HCp run as control, 0.14 ± 0.02 $\mu\text{mol}/\text{min}/\text{mg}$ of protein. At pH 7, the activity decreased nearly 1 order of magnitude. Same results were obtained in assays of the ferroxidase activity (see below).

Storage induced a decrease of the enzymatic activity, $\sim 50\%$ decrease after two months, which was more pronounced with respect to that of HCp and that was paralleled by changes in the electrophoretic pattern. The recombinant protein gradually shifted from the pattern in Fig. 1D to that shown in Fig. 1F, which was typically obtained from samples of rHCp purified at the longer induction times and/or higher cellular density in an attempt to raise the yield of the recombinant protein. The fragmentation pattern now reproduced that usually found in HCp (28), with fragments at ~ 116 , 19, and 50 kDa, produced by the cleavage of the polypeptide chain at Lys⁸⁸⁷.

Catalytic and Spectroscopic Properties of Recombinant Ceruloplasmin—Optical and EPR spectroscopies were used to analyze the state of copper sites (Fig. 2). The EPR spectrum of rHCp was superimposable to that of HCp isolated by the single-step method from serum, reproducing the peculiar features of this form of protein (Fig. 2B), namely a low content of T2 copper, as detected by the low field hyperfine line, and a low content of paramagnetic copper, which accounted for $\sim 40\%$ of the total copper content. According to these findings, a low absorption intensity of the blue sites was also found (Fig. 2A); the molar extinction coefficient at 610 nm was determined to be ~ 7000 $\text{M}^{-1} \text{cm}^{-1}$. For reference, this value in other multicopper oxidases, having a single T1 site, is ~ 5000 $\text{M}^{-1} \text{cm}^{-1}$ (26, 29). Below 450 nm, the optical spectra of purified rHCp samples invariably exhibited a slightly higher intensity with respect to HCp. The presence of extra bands in the region of the 330-nm chromophore (12) of rHCp could, however, be excluded on the basis of differential spectra, obtained by subtracting the absorption of authentic Cp from rHCp spectra. Transitions occurring at lower wavelengths, due to traces of the strongly absorbing pigment(s) of the induction medium, are the most reasonable explanation for this difference.

The peculiar spectroscopic properties of HCp are due to the tendency of a fraction of its T1 sites to stay reduced in the resting protein (30, 43). Binding of Cl^- to this protein activates intramolecular ET to the cluster with a resulting increase of the absorbance at 610 nm (44). The addition of 100 mM Cl^- to purified rHCp samples to probe the oxidation level of their T1 sites did not affect the spectroscopic properties. In contrast, a considerable increase of the optical absorption at 610 nm (results not shown) and of the intensity of the EPR signal (Fig. 2C) was noticed when, still impure, the recombinant protein was tested immediately after elution from the activated Sepharose. The extent of the spectroscopic changes varies considerably among samples of HCp, ranging between 10 and 60%. The reasons of these differences have never been investigated. Thus, to understand the behavior of the recombinant protein, the reactivity of authentic Cp was carefully investigated, and it was found that samples of serum Cp that exhibited a 40% increase of the visible absorption, immediately after the isolation, became nearly unresponsive to chloride when tested after they were subjected to the same chromatographic steps employed to obtain rHCp samples shown in Fig. 2, A and B.

Altogether, these results confirmed the identity of recombinant ceruloplasmin with authentic ceruloplasmin. They also

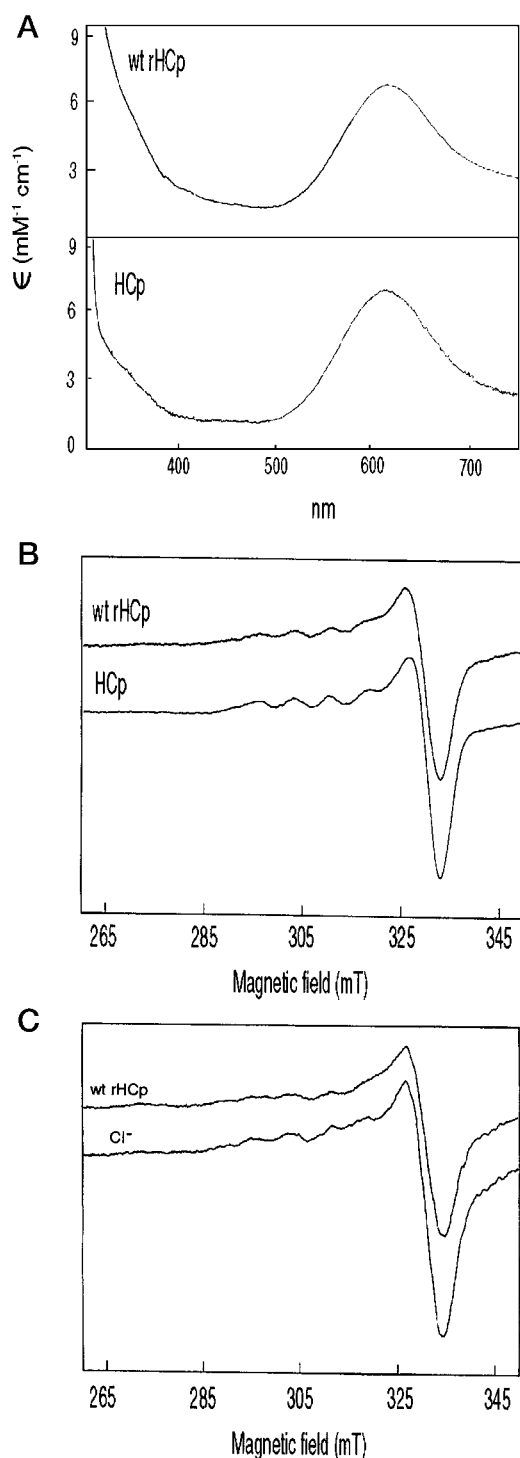


FIG. 2. Spectroscopic analyses of wild type (*wt*) recombinant HCp. The visible spectrum (A) and the EPR spectrum (B) of the recombinant protein purified at 72 h postinduction are compared with those of authentic human ceruloplasmin. The EPR spectrum of a concentrated sample of the 200 mM fraction from activated Sepharose (C) is shown before and after the addition of 100 mM Cl^- . EPR spectra were recorded at 100 K.

indicated that the varied carbohydrate moiety was not affecting the functional properties of the protein.

Site-directed Mutagenesis at the Proteolytic Sites of Ceruloplasmin—As shown in Fig. 3A, the three residues Arg⁴⁸¹, Arg⁷⁰¹, and Lys⁸⁸⁷, responsible for the lability of HCp to proteases, appear to be excluded from the compact six-domain core of the molecule harboring the copper sites. On this basis, site-

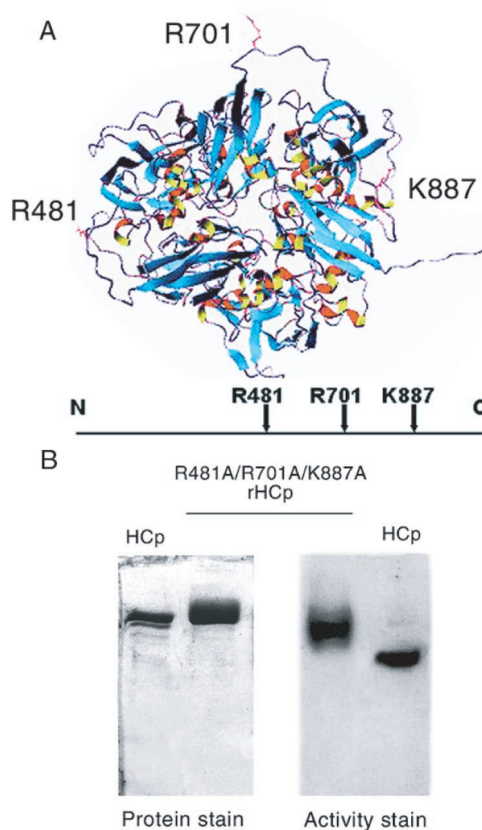


FIG. 3. Site-directed mutagenesis at the proteolytic sites of ceruloplasmin. A, view of the HCp molecule (*top*) and scheme of the polypeptide chain (*bottom*) showing the location of the proteolytic sites. The molecule is oriented to present its flat surface to the observer. B, electrophoretic analyses of the R481A/R701A/K887A triple mutant by SDS-PAGE (*left*) and by native PAGE (*right*). In both cases purified HCp was run as a control.

directed mutagenesis of these residues was attempted to protect the recombinant protein against degradation. A mutant was then produced where each residue of the three sites was replaced by alanine. The triple mutant, R481A/R701A/K887A rHCp (R,R,K/A rHCp), turned out to be stable to proteolysis. It was free of the low molecular weight bands in the SDS-PAGE analysis under denaturing conditions (Fig. 3B). Storage did not induce the appearance of fragments. Its spectroscopic and catalytic properties matched exactly those of wild type rHCp already described. This mutant was then used to afford the mutagenesis at the copper sites.

Site-directed Mutagenesis at the Copper Sites of Ceruloplasmin—Two different mutations, L329M and C1021S, were performed to modify the coordination sphere of the copper of the sites of domains 2 and 6.

The T1 site of domain 2 is the most critical metal binding site of HCp. This site is considered unable to engage redox reaction with known ceruloplasmin substrates, due to its high redox potential value (30). It is a tricoordinate site, and it is characterized by the lack of the axial Met ligand, which is replaced in the amino acid sequence by a leucine residue unable to coordinate copper (Fig. 4A). It is not clear how this difference in geometry, with respect to the typical T1 sites of domains 4 and 6, and/or the nature of surrounding protein matrix are responsible for its properties. As shown in Fig. 4A, a methionine residue can replace the leucine 329, without destroying the site, and the mutated site assumes a geometry more similar to that of a typical T1 site (25).

The mutation of Leu³²⁹ to Met was introduced into the triple mutant; the resulting quadruple mutant was produced to the

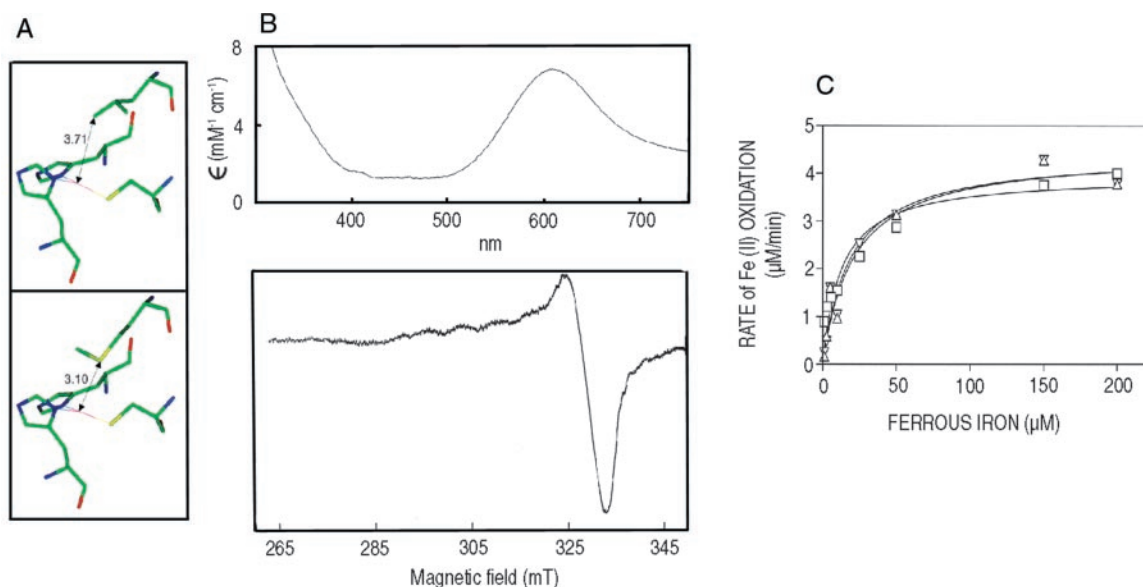


FIG. 4. Site-directed mutagenesis at the tricoordinate copper site of domain 2 of human ceruloplasmin. A, view of the site showing in addition to the three copper ligands (two histidines and one cysteine) the leucine 329 (*top*), replaced by methionine (*bottom*). The distances (Å) of these two latter residues from copper are indicated. Spectroscopic (B) and catalytic (C) properties of (R,R,K/A)L329M rHCp are shown. The kinetics of the ferroxidase activity of the L329M mutant (∇) as a function of iron concentration are shown in comparison with authentic HCp (\square) and wild type rHCp (\triangle). All proteins were $0.05 \mu\text{M}$ in the assay. *mT*, millitesla.

same extent as the wild type rHCp and the triple mutant. The spectroscopic properties of (R,R,K/A)L329M rHCp were carefully analyzed; however, no qualitative and quantitative differences of either the optical absorption or of the EPR signal were found (Fig. 4B). The measurements of the enzymatic activities, both the ferroxidase activity (Fig. 4C) and the amine oxidase activity ($0.10 \pm 0.03 \mu\text{mol}/\text{min}/\text{mg}$ versus *pPD*), indicated that the presence of methionine was also ineffective in producing significant modifications of the catalytic properties. Upon the addition of Cl^- to a partially purified sample of this mutant, *i.e.* to the 200 mM fraction from activated Sepharose, the behavior of wild type rHCp shown in Fig. 2C was again observed in that the anion elicited the spectroscopic properties of this mutant, but this did not entail the appearance of new species (results not shown).

Cys¹⁰²¹ is a ligand of the typical T1 site of domain 6, together with His⁹⁷⁵, His¹⁰²⁶, and Met¹⁰³¹ (23). Its flanking His residues are ligands for two of the copper atoms of the trinuclear cluster so to furnish a bifurcate pathway to the electron donated to the copper center by a substrate. The mutation of Cys¹⁰²¹ to Ser was afforded to destroy the catalytic activity pertinent to this site (10, 15) to ascertain whether the other T1 site(s) could sustain the enzymatic activity of the mutated protein. The mutant C1021S rHCp proved to be very difficult with respect to the other recombinant ceruloplasmins in that it was produced at lower levels. Furthermore, it exhibited an anomalous behavior during purification, since only part of it bound to activated Sepharose and the fraction retained by the resin eluted at low ionic strength, with the 100 mM buffer. During the purification, small fragments of ~ 40 kDa, were noticed in all fractions. The overall result was that only a minute amount of this mutant, a few hundred μg , could be obtained.

The electrophoretic analyses revealed the extreme heterogeneity of this mutant (Fig. 5). The copper content of these samples was exceedingly low, ~ 0.1 g atom of copper/120 kDa, with respect to the expected stoichiometric value of 5/6; therefore, the spectroscopic characterization was not attempted, nor were careful activity measurements done. However the electrophoretic analyses performed under nonreducing conditions showed a quite interesting finding, since they revealed the

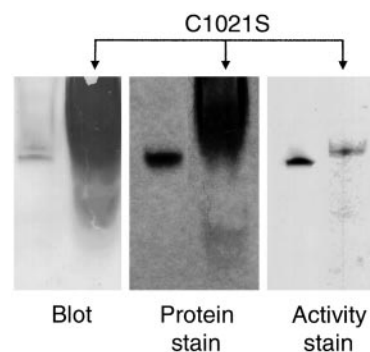


FIG. 5. Properties of recombinant ceruloplasmin mutated at the copper site of domain 6. The native PAGE analyses of C1021S mutant were performed on $2 \mu\text{g}$ of protein for Western blot (*left*) and $10 \mu\text{g}$ of protein for both protein staining (*middle*) and activity staining with *o*-dianisidine (*right*). In each case, HCp was run as control in the following amounts (from left to right): 250 ng, 1 μg , and 0.5 μg .

presence of an oxidase-active band (Fig. 5, *right*) among the multiple inactive bands, which are likely to be various forms of the recombinant protein, completely or partially depleted of copper. The active band is clearly the only form of the recombinant protein retaining a full complement of copper at the trinuclear cluster accompanied by the occupancy of at least one of the two other T1 copper sites. As to the amount of this active form, it should be noted that the gel had to be overloaded with $10 \mu\text{g}$ of protein to observe the active band.

DISCUSSION

We succeeded in obtaining a recombinant human ceruloplasmin displaying all of the properties of the authentic protein as purified from plasma. Unfortunately, the recombinant protein retained also the susceptibility of HCp to proteolysis, a property that has hampered all studies on this protein (45). The stability of the triple mutant that we obtained by substituting Arg⁴⁸¹, Arg⁷⁰¹, and Lys⁸⁸⁷ with alanine demonstrates that only these residues are responsible for the fragmentation pattern that affects all ceruloplasmin preparations.

It should be recalled that the lability of human ceruloplasmin is such that it is found fragmented even in the plasma (38).

The fragments initially do not dissociate and mimic the intact molecule, but the protein suffers changes of its redox properties that lead to a redistribution of electrons within its copper sites (46). In fact, the antioxidant or the prooxidant activities of Cp are specific to the cleaved and to the intact molecule, respectively (8). Thus, it was of utmost importance to have a stable Cp to work with before attempting mutagenesis targeted to the copper sites.

The mutagenesis has been focused on the two T1 copper sites more critical to the function of ceruloplasmin (*i.e.* those on domains 2 and 6). The aim of these experiments was not only studying the role of the ligands on the properties of the sites, as it is the case of current studies on multicopper oxidases containing a single T1 site. In the case of ceruloplasmin, the major problem concerns the reasons for having multiple T1 sites with different disposition with respect to a single trinuclear cluster (23). This is a peculiarity of ceruloplasmin that cannot be simply related to the necessity of increased potentiality of electron capture from substrates. Fet3 has only one T1 site (29) and is as able as Cp to oxidize both Fe(II) ions and amines. Structural and catalytic studies are trying to find plausible electron transfer pathways in the molecule to disclose the functional relations of the three sites, if any (27), and their interaction with the cluster during catalysis (15).

The mutation of Cys¹⁰²¹ to Ser was performed for this purpose. Since it has been ascertained that mutation of Cys at the T1 site is fatal to multicopper oxidases having a single T1 site, this mutation in the T1 site of domain 6 of Cp should clarify whether this site is the entrance to the bifurcate ET pathway to the cluster also for electrons (possibly) released to the other T1 sites. The same mutation in Fet3 succeeded in producing an inactive T1-depleted protein that conserved unaltered the structure of the T2 and T3 sites (33). An analogous mutation in bilirubin oxidase produced an inactive protein that did not, however, conserve the trinuclear cluster intact (31). Our results on the C1021S mutant, its anomalous behavior during purification, and its much less than stoichiometric copper content indicate that the occupancy of the T1 site of domain 6 contributes to stabilizing the other copper binding sites and/or the protein structure. The observation of a species of this mutant retaining the enzymatic activity indicates that another T1 site is able to engage a redox reaction with the cluster. This site is most likely that of domain 4, since x-ray experiments locate on it the binding site for amine substrates (10).

Since the substitution of Cys abolishes the character of the T1 site, the mutation of Cys¹⁰²¹ is also important to shed some light on the spectroscopic properties of HCp. Our results indicate that future studies aimed at producing rHCp mutated at domain 6 that is suitable for spectroscopic studies have to be carefully planned. In fact, the mutation C1021D failed to produce transformants with meaningful expression.²

HCp typically occurs in a partially reduced state (30, 44). The protein treated with oxidants still contains reduced copper, and its molar absorptivity does not exceed the value of 9000 M⁻¹ cm⁻¹, which is consistent with only two T1 sites bearing Cu(II) ions (26). The EPR spectra of the resting, as isolated, and of the oxidized forms of HCp can, however, be simulated on the basis of two (30) as well as of three (43, 56) nonequivalent T1 components. This problem is related to the state and the function of the T1 site of domain 2 which has been the target of the other mutation, L329M.

Methionine is not an indispensable residue to produce the peculiar nature of the T1 site, which seems rather to be related to the ligation of Cys within the trigonal core ligation His-Cys-

His (25). Its more likely role is to tune the redox potential and the spectroscopic properties of the site, as indicated by extensive site-directed mutagenesis of blue proteins (47–50) and also by results on multicopper oxidases (31). That the mutation of leucine 329 to methionine in the polypeptide chain of human ceruloplasmin is silent under all aspects is an intriguing result that can, however, shed some light on the properties of tricoordinate T1 sites of multicopper oxidase.

Assuming that Met³²⁹ is able to bind the copper atom of the site of domain 2, a conclusion would be that Met³²⁹ binds copper without any effect on the structure of the site. The recent study on *Trametes villosa* laccase shows that all properties of the enzyme are affected by the mutation of Phe⁴⁶³ to Met (32). The changes of the spectroscopic properties observed in this enzyme, a 13-nm shift in the maximal absorption wavelength and the variation of magnetic parameters of the EPR spectrum, are such that they had to be detected even when masked by the contribution of other T1 sites, as in rHCp. A decrease of 0.1 V of the redox potential value accompanies the variation of the spectroscopic properties in mutated laccase, resulting in an EPR signal intermediate between that of the wild type laccase and of the typical T1 site of plastocyanin. As to the variation of the redox potential expected for mutated T1 site of HCp, it has to be considered that the value calculated for this site in authentic HCp exceeds 1 V (30) so that even when lowered by hundreds of mV, according to the results on laccase and those obtained in model systems (51), it would remain an exceedingly high value, the highest among T1 sites. Assuming that it is in the reduced state in the wild type protein, it will probably remain in this state also in the L329M mutant, explaining why the mutation fails to affect spectroscopic and catalytic properties of HCp. The fact that the introduction of Met is unable to render this site more similar to the other two T1 sites confirms that other elements of the protein matrix play an important role beyond ligands in tuning the structure of T1 sites (32, 52, 53). In other words, it is locked in a definite frame, and this should confer a specialized function to it. It might either play an active role in the reactions of the oxidase, under the specific control of effectors affecting its redox properties, or be responsible to other functions, specific to the human protein. This consideration comes from the observation that the three T1 sites of other mammalian ceruloplasmin (54) do not differ between each other to the extent that characterizes human ceruloplasmin.

An additional finding of this work was that secreted ceruloplasmin can affect the iron metabolism of the yeast. The fact that the transformed yeast cells tend to maintain a constant level of Fet3 on their membranes balancing the leakage of the protein indicates that they are in a condition of iron deficiency (13, 21). The only way to explain this result is to consider that recombinant ceruloplasmin is under turnover conditions as a ferroxidase in the medium of induced cells, as demonstrated by its partially reduced state upon isolation. This would shift the redox state of iron to an oxidized ferric form not available to cells, confirming that uptake of iron by yeast requires Fe(III) ions produced by a member (Fet3) of its transport system (55). The ferroxidase reaction of ceruloplasmin, which decreases the level of free Fe(II) in the plasma, favoring release of iron from cells, results in a condition of iron deficiency for yeast with consequent induction of Fet3.

REFERENCES

1. Frieden, E., and Hsieh, H. S. (1976) *Adv. Enzymol.* **44**, 187–236
2. Gitlin, J. D. (1998) *Pediatr. Res.* **44**, 271–276
3. Gutteridge, J. M. C. (1983) *FEBS Lett.* **157**, 37–40
4. Miyajima, H., Takahashi, Y., Serizawa, M., Kaneko, E., and Gitlin, J. D. (1996) *Free Radical Biol. Med.* **20**, 757–760
5. Cha, M. K., and Kim, I. H. (1999) *Biochemistry* **38**, 12104–12110
6. Inoue, K., Akaike, T., Miyamoto, Y., Okamoto, T., Sawa, T., Otagiri, M.,

² P. Bielli, G. C. Bellenchi, and L. Calabrese, unpublished results.

- Suzuki, S., Yoshimura, T., and Maeda, H. (1999) *J. Biol. Chem.* **274**, 27069–27075
7. Swain, J., and Gutteridge, J. M. C. (1995) *FEBS Lett.* **368**, 513–515
8. Mukhopadhyay, C. K., Mazumder, B., Lindley, P. F., and Fox, P. L. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 11546–11551
9. Harris, E. D. (1993) *Prog. Clin. Biol. Res.* **380**, 163–179
10. Zaitsev, V., Zaitseva, I., Papiz, M., and Lindley, P. F. (1999) *J. Biol. Inorg. Chem.* **4**, 579–587
11. Bianchini, A., Musci, G., and Calabrese, L. (1999) *J. Biol. Chem.* **274**, 20265–20270
12. Malmstrom, B. G. (1982) *Annu. Rev. Biochem.* **51**, 21–59
13. Askwith, C., Eide, D., Van Ho, A., Bernard, P. S., Li, L., Davis-Kaplan, S., Sipe, D. M., and Kaplan, J. (1994) *Cell* **76**, 403–410
14. Osaki, S. (1966) *J. Biol. Chem.* **241**, 5053–5059
15. Lindley, P., Card, G., Zaitseva, I., Zaitsev, V., Reinhammar, B., Selin-Lindgren, E., and Yoshida, K. (1997) *J. Biol. Inorg. Chem.* **2**, 454–463
16. de Silva, D. M., Davis-Kaplan, S., Fergestad, J., and Kaplan, J. (1997) *J. Biol. Chem.* **272**, 14208–14213
17. Harris, Z. L., Takahashi, Y., Miyajima, H., Serizawa, M., MacGillivray, R. T. A., and Gitlin, J. D. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 2539–2543
18. Harris, Z. L., Durely, A. P., Man, T. K., and Gitlin, J. D. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 10812–10817
19. Osaki, S., Johnson, D. A., and Frieden, E. (1966) *J. Biol. Chem.* **241**, 2746–2751
20. Chidambaram, M. V., Barnes, G., and Frieden, E. (1983) *FEBS Lett.* **159**, 137–140
21. Yuan, D. S., Stearman, R., Dancis, A., Dunn, T., Beeler, T., and Klausner, R. D. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 2632–2636
22. Messerschmidt, A., Ladenstein, R., Huber, R., Bolognesi, M., Avigliano, L., Petruzzelli, R., Rossi, A., and Finazzi Agrò, A. (1992) *J. Mol. Biol.* **224**, 179–205
23. Zaitseva, I., Zaitsev, V., Card, G., Moshkov, K., Bax, B., Ralph, A., and Lindley, P. (1996) *J. Biol. Inorg. Chem.* **1**, 15–23
24. Ducros, V., Brzozowski, A. M., Wilson, K. S., Brown, S. H., Ostergaard, P., Schneider, P., Yaver, D. S., Pedersen, A. H., and Davies, G. J. (1998) *Nat. Struct. Biol.* **5**, 310–316
25. Holm, R. H., Kennepohl, P., and Solomon, E. I. (1996) *Chem. Rev.* **96**, 2239–2314
26. Solomon, E. I., Sundaram, U. M., and Machonkin, T. E. (1996) *Chem. Rev.* **96**, 2563–2605
27. Farver, O., Bendahl, L., Skov, L. K., and Pecht, I. (1999) *J. Biol. Chem.* **274**, 36135–36140
28. Ortel, T. L., Takahashi, N., and Putnam, F. W. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 4761–4765
29. Hassett, R., Kosman, D. J., Yuan, D. S., and Kosman, D. J. (1998) *J. Biol. Chem.* **273**, 23274–23282
30. Machonkin, T. E., Zhang, H. H., Hedman, B., Hodgson, K. O., and Solomon, E. I. (1998) *Biochemistry* **37**, 9570–9578
31. Shimizu, A., Know, J. H., Sasaki, T., Satoh, T., Sakurai, N., Sakurai, T., Yamaguchi, S., and Samejima, T. (1999) *Biochemistry* **38**, 3034–3042
32. Xu, F., Palmer, A. E., Yaver, D. S., Berka, R. M., Gambetta, G. A., Brown, S. H., and Solomon, E. I. (1999) *J. Biol. Chem.* **274**, 12372–12375
33. Blackburn, N. J., Ralle, M., Hassett, R., and Kosman, D. J. (2000) *Biochemistry* **39**, 2316–2324
34. Rafiq, M., Suen, C. K. M., Choudhury, L., Joannou, C. L., White, K. N., and Evans, R. W. (1997) *FEBS Lett.* **407**, 132–136
35. Sarkar, G., and Sommer, S. S. (1990) *BioTechniques* **4**, 404–407
36. Aiyar, A., and Leis, J. (1993) *BioTechniques* **3**, 366–369
37. Sato, M., and Gitlin, J. D. (1991) *J. Biol. Chem.* **266**, 5128–5134
38. Musci, G., Bonaccorsi di Patti, M. C., Fagiolo, U., and Calabrese, L. (1993) *J. Biol. Chem.* **268**, 13388–13392
39. Musci, G., Carbonaro, M., Ariani, A., Lania, A., Galtieri, A., and Calabrese, L. (1990) *Arch. Biochem. Biophys.* **279**, 8–13
40. Bonomi, F., Kurtz, D. M., and Cui, X. (1996) *J. Biol. Inorg. Chem.* **1**, 67–72
41. Bonaccorsi di Patti, M. C., Belenchi, G. C., Bielli, P., and Calabrese, L. (1999) *Arch. Biochem. Biophys.* **372**, 295–299
42. Musci, G., Polticelli, F., and Calabrese, L. (1999) *Adv. Exp. Med. Biol.* **448**, 175–182
43. Musci, G., Bonaccorsi di Patti, M. C., and Calabrese, L. (1993) *Arch. Biochem. Biophys.* **306**, 111–118
44. Musci, G., Bonaccorsi di Patti, M. C., and Calabrese, L. (1995) *J. Protein Chem.* **14**, 611–619
45. Rydén, L. (1984) in *Copper Proteins and Copper Enzymes* (Lontie, R., ed) Vol. 3, pp. 37–100, CRC Press, Inc., Boca Raton, FL
46. Calabrese, L., and Musci, G. (1997) *Molecular Properties of Ceruloplasmin from Different Species in Multi-Copper Oxidase* (Messerschmidt, A., ed) pp. 307–354, World Scientific Publications, Singapore
47. Canters, G. W., and Gilardi, G. (1993) *FEBS Lett.* **325**, 39–48
48. Romero, A., Hoytink, C. W., Nar, H., Huber, R., Messerschmidt, A., and Canters, G. W. (1993) *J. Mol. Biol.* **229**, 1007–1021
49. Libeu, C. A. P., Kukimoto, M., Nishiyama, M., Horinouchi, S., and Adman, E. T. (1997) *Biochemistry* **36**, 13160–13179
50. Hall, J. F., Kanbi, L. D., Strange, R. W., and Hasnain S. S. (1999) *Biochemistry* **39**, 12675–12680
51. Olsson, M. H. M., and Ryde, U. (1999) *J. Biol. Inorg. Chem.* **4**, 654–663
52. Wittung-Stafhede, P., Hill, M. G., Gomez, E., Di Bilio, A. J., Karlon, B. G., Leckner, J., Winkler, J. R., Gray, H. B., and Malmstrom, B. G. (1998) *J. Biol. Inorg. Chem.* **3**, 367–370
53. Malmstrom, B. G., and Leckner, J. (1998) *Curr. Opin. Chem. Biol.* **2**, 286–292
54. Musci, G., Fraterrigo, T. Z. L., Calabrese, L., and McMillin, D. R. (1999) *J. Biol. Inorg. Chem.* **4**, 441–446
55. Askwith, C., and Kaplan, J. (1998) *Trends Biochem. Sci.* **23**, 135–138

**Site-directed Mutagenesis of Human Ceruloplasmin: PRODUCTION OF A
PROTEOLYTICALLY STABLE PROTEIN AND STRUCTURE-ACTIVITY
RELATIONSHIPS OF TYPE 1 SITES**

Pamela Bielli, Gian Carlo Bellenchi and Lilia Calabrese

J. Biol. Chem. 2001, 276:2678-2685.

doi: 10.1074/jbc.M007176200 originally published online October 19, 2000

Access the most updated version of this article at doi: [10.1074/jbc.M007176200](https://doi.org/10.1074/jbc.M007176200)

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 54 references, 14 of which can be accessed free at
<http://www.jbc.org/content/276/4/2678.full.html#ref-list-1>