## Translation 3

**Role of External Loops of Human Ceruloplasmin in Copper Loading by ATP7B and Ccc2p**

Ceruloplasmin is a multicopper oxidase required for correct iron homeostasis. Previously, we have identified a ceruloplasmin mutant associated with the iron overload disease aceruloplasminemia, which was unable to acquire copper from the mammalian pump ATP7B but could be produced in an enzymatically active form in yeast. Here, we report the expression of recombinant ceruloplasmin in the yeast *Pichia pastoris* and the study of the role of five surface-exposed loops in copper incorporation by comparing the efficiencies of mammalian ATP7B and yeast Ccc2p. The possibility to "mix and match" mammalian and yeast multicopper oxidases and copper ATPases can provide clues on the molecular features underlying the process of copper loading in multicopper oxidases.

Ceruloplasmin (Cp)3 is a complex protein present in vertebrates, which belongs to the family of the multicopper oxidases. These enzymes are able to couple the single electron oxidation of substrates to complete reduction of dioxygen to water. Multicopper oxidases possess multiple copper-binding sites with different structural and functional properties; type 1 blue copper is the primary electron acceptor from the substrate, and a trinuclear cluster formed by type 2 and binuclear type 3 copper constitutes the oxygen binding and reduction site (1). Cp is a multidomain protein made up of six plastocyanin- like domains, the interface between domains 6 and 1 hosting the catalytically essential trinuclear copper cluster and domains 2, 4, and 6 harboring a type 1 copper site each. Cp is predominantly secreted by hepatocytes, where the P-type ATPase ATP7B incorporates copper into apo-Cp during transit in the trans-Golgi network (2). A GPI-anchored form of Cp (Cp-GPI) has also been identified, mainly in the brain, where it resides on the plasma membrane of astrocytes (3). Synthesis of this isoform is via alternative splicing, causing the replacement of the five C-terminal amino acids of the secreted protein by 30 alterative residues that lead to the addition of the GPI anchor (4). The ferroxidase activity of Cp is required for proper iron homeostasis; lack of oxidase-active Cp leads to internalization and degradation of ferroportin (Fpn), the only known mammalian iron exporter (5). Furthermore, genetic defects of the Cp gene cause aceruloplasminemia, a rare autosomal iron overload disease with clinical manifestations, including retinal degeneration, diabetes mellitus, and neurological symptoms, which include ataxia, involuntary movements, and dementia (6). Missense Cp mutants associated with aceruloplasminemia are beginning to be characterized and can be broadly classified in different groups according to their ability to stabilize Fpn on the plasma membrane of cells silenced for endogenous Cp-GPI. Nonfunctional mutants are inactive due to retention in the endoplasmic reticulum or secretion as apo-Cp lacking copper, and partially or fully functional mutants are enzymatically active. Mutant Cp R701W is atypical in that it is found in an unusually young patient in heterozygous form (7).

We have previously reported that Cp R701W is unable to be loaded with copper by ATP7B, but it can acquire the prosthetic metal from the yeast copper ATPase Ccc2p. Moreover, this mutant is dominant over wild type Cp and induces fragmentation of the Golgi complex with relocalization of ATP7B (8). Arg701 is located in a large solvent-exposed loop connecting domains 4 and 5, and corresponding loops connect the other domains of Cp. Despite a low degree of sequence homology, all these loops start with a CX(R/K) motif, with the cysteine residue stabilizing the loop by forming a disulfide bridge (Fig. 1 and supplemental Fig. S1). We have recently shown that mutation of Cp basic residues Lys340 or Arg883 into tryptophan on two of these loops causes rapid degradation of Fpn. However, K340W and R883W mutants were found to be not dominant over wild type Cp, at variance with the homologous R701W mutant. On the other hand, all three mutants (R701W, K340W, and R883W) are enzymatically active when produced in yeast (8). These findings suggest that Cp loops could play a critical role in copper incorporation and that the process of copper loading in yeast versus mammalian cells is less structurally demanding.

Heterologous expression of multicopper oxidases is generally quite challenging; a survey of the literature shows that bacteria are useful only for expression of prokaryotic multicopper oxidase. Eukaryotic enzymes have been expressed with varying luck in yeast, mammalian, or insect cells. Human Cp has been produced in the methylotrophic yeast *Pichia pastoris* under control of the inducible *AOX1* promoter (9). However, not only yields were well below 1 mg/liter but the whole procedure was quite complex and took several days to be completed. In this work, we have set up a different system based on an engineered *P. pastoris* strain harboring an inactivated gene for the endog- enous ferroxidase Fet3p, and we have used the strong constitutive glyceraldehyde-3-phosphate dehydrogenase promoter to drive expression of recombinant Cp. The rationale for using a fet3A strain was that of avoiding contamination of recombinant Cp by the endogenous yeast ferroxidase.

Expression of Cp mutants in this system has allowed us to expand our investigation on a peculiar dominant negative Cp mutant associated with aceruloplasminemia, i.e. R701W. Moreover, the role of all surface-exposed loops in the process of copper incorporation has been analyzed by comparing yeast ATPase Ccc2p with the mammalian homologous ATP7B for their ability to deliver copper to Cp. The possibility of “mixing and matching” yeast and mammalian ferroxidases and copper ATPases can provide clues on the molecular mechanism of copper incorporation into complex proteins such as the multicopper oxidases.