

*CHAPTER I*

**GENERAL INTRODUCTION**

Mitochondria play a central role in cellular oxidative metabolism, converting the products from carbohydrate, fat, and protein catabolism into chemical energy stored in ATP. In these metabolic pathways, synthesis of ATP is taken place in the respiratory chain (electron transport system) that locates on the inner membrane of mitochondria. Cytochrome c oxidase (CCO) is the terminal enzyme of the respiratory chain, catalyzing the transfer of electrons from reduced cytochrome c to molecular oxygen. The deficiency of the enzymatic activity of CCO causes a wide range of encephalomyopathic disorders (Shoubridge *et al.*, 2001).

The mammalian CCO is composed of thirteen polypeptides, three of which are encoded by mitochondrial DNA (COX I, II and III) and the other ten of which are encoded by nuclear genes. It has been reported that CCO requires a total of three copper ions to be inserted into two subunits, COX I and II: COX I has a mononuclear copper-binding site which buried within the inner membrane, COX II has a binuclear copper-binding site which sticks out into the mitochondrial intermembrane space (Yoshikawa *et al.*, 1995). Despite the considerable progress in analyzing the structural organization of CCO, little is known about when or how copper is transported from cytosol and inserted into the enzyme.

Besides CCO, there are several copper-depending enzymes (cuproenzymes) such as superoxide dismutase (SOD, which is involved in superoxide dismutation), ceruloplasmin, catechol oxidase (which is involved in ferroxidation, synthesis of melanin), dopamine- $\beta$ -monooxygenase (which is involved in norepinephrine biosynthesis), and peptidylglycine  $\alpha$ -amidating monooxygenase (which is involved in  $\alpha$ -amidation of peptide hormone/neuropeptides). Thus, copper is an essential component of many enzymes and important for cells. On the other hand, an excess accumulation of copper is highly toxic due to its proclivity to engage in redox reactions that result in the

formation of hydroxyl radical, a reactive species that causes extensive damage to nucleic acids, proteins, and lipids. Therefore, cells have a variety of mechanisms to deal with this essential, yet toxic trace element (Ricardo *et al.*, 1998). The mechanisms of detoxification of extra amount of metal ions have been almost cleared by the studies about metallothionein. However, the mechanisms of copper transport to cuproenzymes had been unknown for a long time.

Recently, a new class of cytosolic copper-binding peptides (copper chaperones) have been identified in yeast (Fig. I-1) (O'Halloran and Culotta, 2000; Valentine and Gralla, 1997). And it has been believed that these peptides deliver copper to their respective cuproenzymes. To date, three copper chaperones, Atx1, Lys7 and Cox17p, have been identified in yeast (Fig. I-1): Atx1 directs copper to a post-Golgi compartment, by way of a P-type adenosine triphosphatase Ccc2, a transmembrane copper transporter; Lys7 delivers copper to the Cu/Zn-SOD which is a primary antioxidant enzyme in the cytosol; Cox17p guides copper to CCO in the mitochondria (O'Halloran and Culotta, 2000; Himelblau and Amasino, 2000). The mammalian or plant homologues of the yeast copper chaperones have already been cloned or purified (Culotta *et al.*, 1997; Glerum *et al.*, 1996; Lin *et al.*, 1995; Valentine and Gralla, 1997). And this fact suggests the possibility that most eucaryotic cells utilize these peptides for delivering copper to the donor enzymes.

Cox17p was first isolated from the respiratory-deficient mutant of yeast that failed to assemble the functional CCO by Tzagoloff and his colleagues in 1996 (Glerum *et al.*, 1996). The yeast Cox17p localizes to both cytosol and intermembrane space of the mitochondria (Glerum *et al.*, 1996), forms oligomer (homomeric dimer or tetramer), and binds three atoms of copper per molecule (Heaton *et al.*, 2000). These indicate the possibility that this peptide is involved in copper transport from cytosol to mitochondria. Fig. I-2 shows the

current model of the copper transport to mitochondria. At first, copper is reduced by one of the plasma membrane reductases. And then, the reduced copper (Cu (I)) is incorporated across the plasma membrane by the high affinity copper transporter, Ctr1. Cox17p guides the copper from cytosol to the mitochondrial intermembrane space (Beers *et al.*, 1997; Glerum *et al.*, 1996; Srinivasan *et al.*, 1998), and Sco1 and 2 incorporate the copper into CCO (COX II).

In mammalian cells, by contrast, little is known about the mitochondrial copper transport, although several mammalian homologues of the yeast Cox17p have already been cloned or purified (Amaravadi *et al.*, 1997; Chen *et al.*, 1997; Nishihara *et al.*, 1998; Takenouchi *et al.*, 1999; Kako *et al.*, 2000). Fig I-3 shows a comparison of amino-acid sequence of yeast, mouse, rat, porcine, and human Cox17p (Kako *et al.*, 2000). The murine Cox17p shares more than 90% identity with other mammalian Cox17p, and 46% identity with the yeast homologue (Kako *et al.*, 2000). Furthermore, the putative copper-binding motif (KPCXC) is highly conserved among all the species (Kako *et al.*, 2000). The intracellular localization of mammalian Cox17p is also both cytosol and mitochondria (Kako *et al.*, 2000). Analysis of expression profile revealed that Cox17p mRNAs express ubiquitously and especially, high levels of Cox17p messages were detected in tissues such as heart, brain, kidney and skeletal muscle where the metabolic activities are relatively high (Fig. I-4) (Kako *et al.*, 2000). Taken together, it is suggested that the mammalian Cox17p may be also involved in cellular respiration, such as the yeast system.

Then, does Cox17p really function as a mitochondrial copper chaperone in mammalian system? The purpose of this study is to determine the physiological role of copper chaperone Cox17p in mammalian system. At first, I cloned and analyzed mouse *COX17* genomic DNA to suppose the physiological function of Cox17p by genetic approach (CHAPTER II). Mouse

*COX17* was a single copy gene that composed of three exons interrupted by two introns, and located on the chromosome 16 on mouse genome. Next, I cloned the 5'-up stream fragment of this gene, and characterized its promoter region (CHAPTER III). The transcriptional regulation of *COX17* gene was characteristic for the regulation of other respiratory genes such as cytochrome c, COX4, 5b, 6c and 7a. And finally, I generated and analyzed mice with targeted disruption of the *COX17* gene (CHAPTER IV). In the CHAPTER IV, I demonstrated that mammalian Cox17p plays an important role not only for the activation of CCO but also for the embryonic development. I also elucidated a possibility that the enzymatic activity of CCO is not essential for the early stage of embryonic development (discussed in CHAPTER IV).

Most recently, the gene disruption of *CTR1* and the other copper chaperones, *ATOX1* and *CCS* (mammalian homologue of yeast Atx1 and Lys7, respectively) were reported (Kuo *et al.*, 2001; Lee *et al.*, 2001; Hamaza *et al.*, 2001; Wong *et al.*, 2000). In CHAPTER V, I summarize and generally discuss about my study (CHAPTER II to IV) including recent movement of the studies of copper chaperones and future plan of my study.

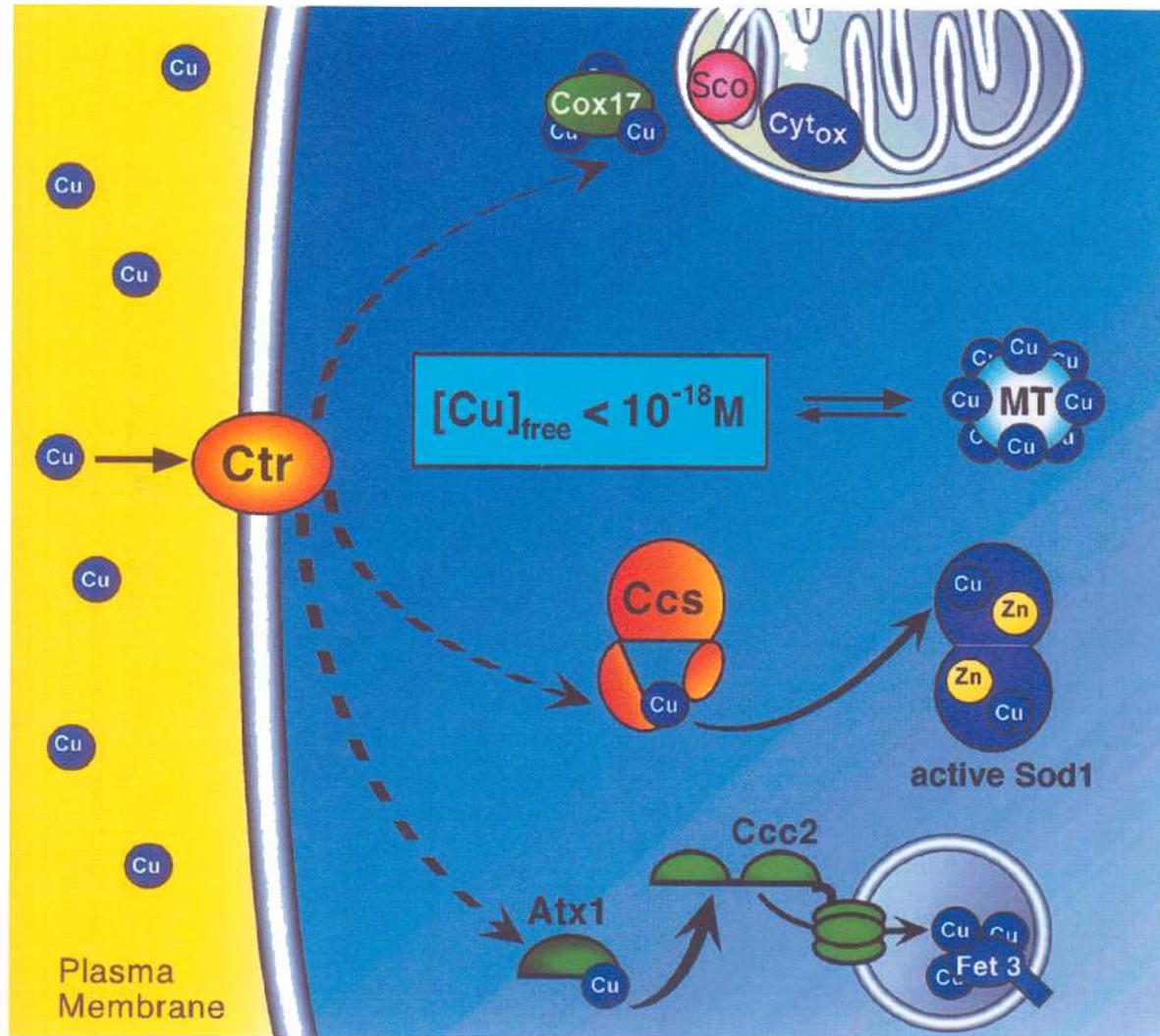
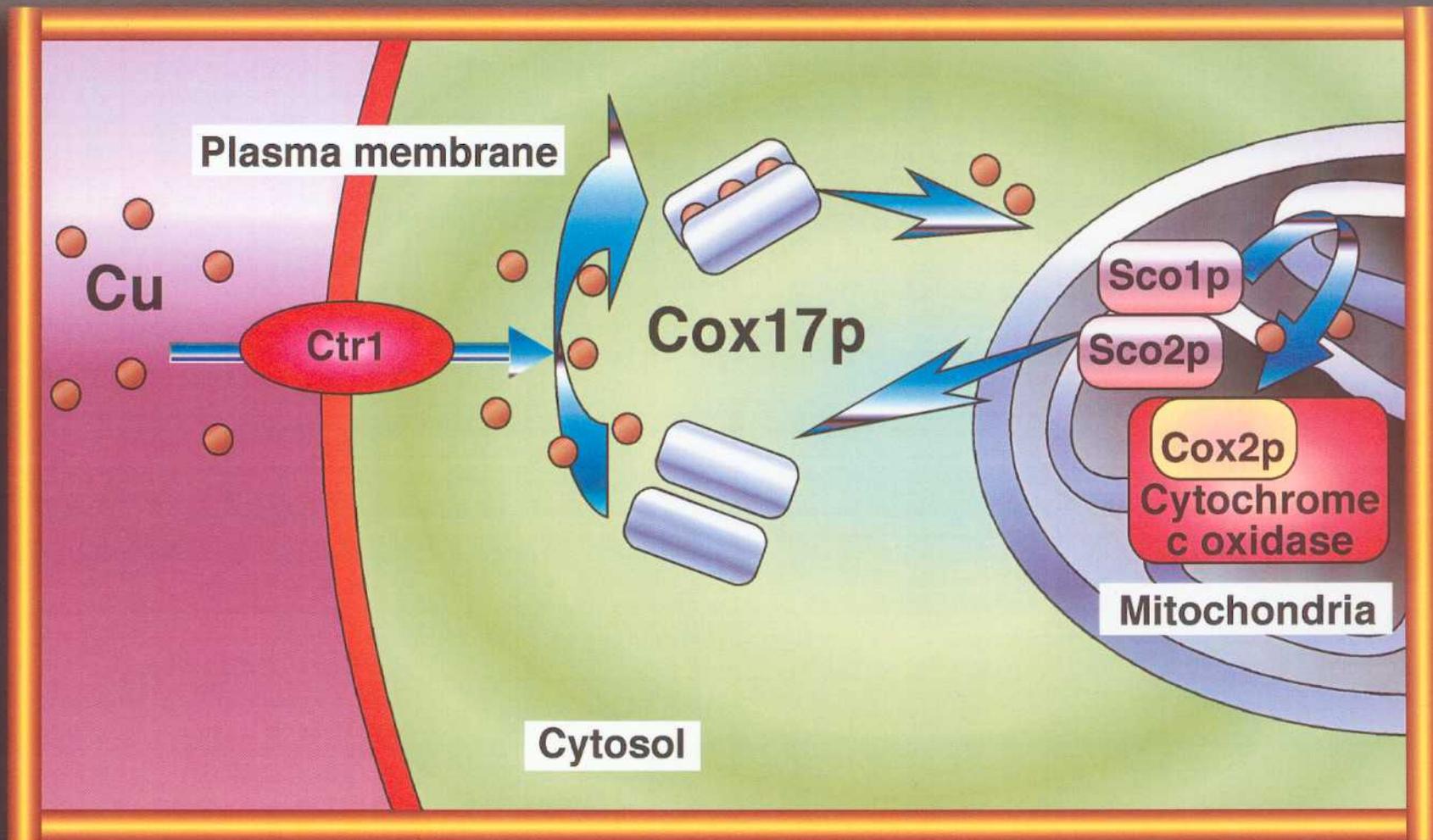


Fig. I-1. Copper trafficking pathways in yeast (O'Halloran and Culotta, 2000).

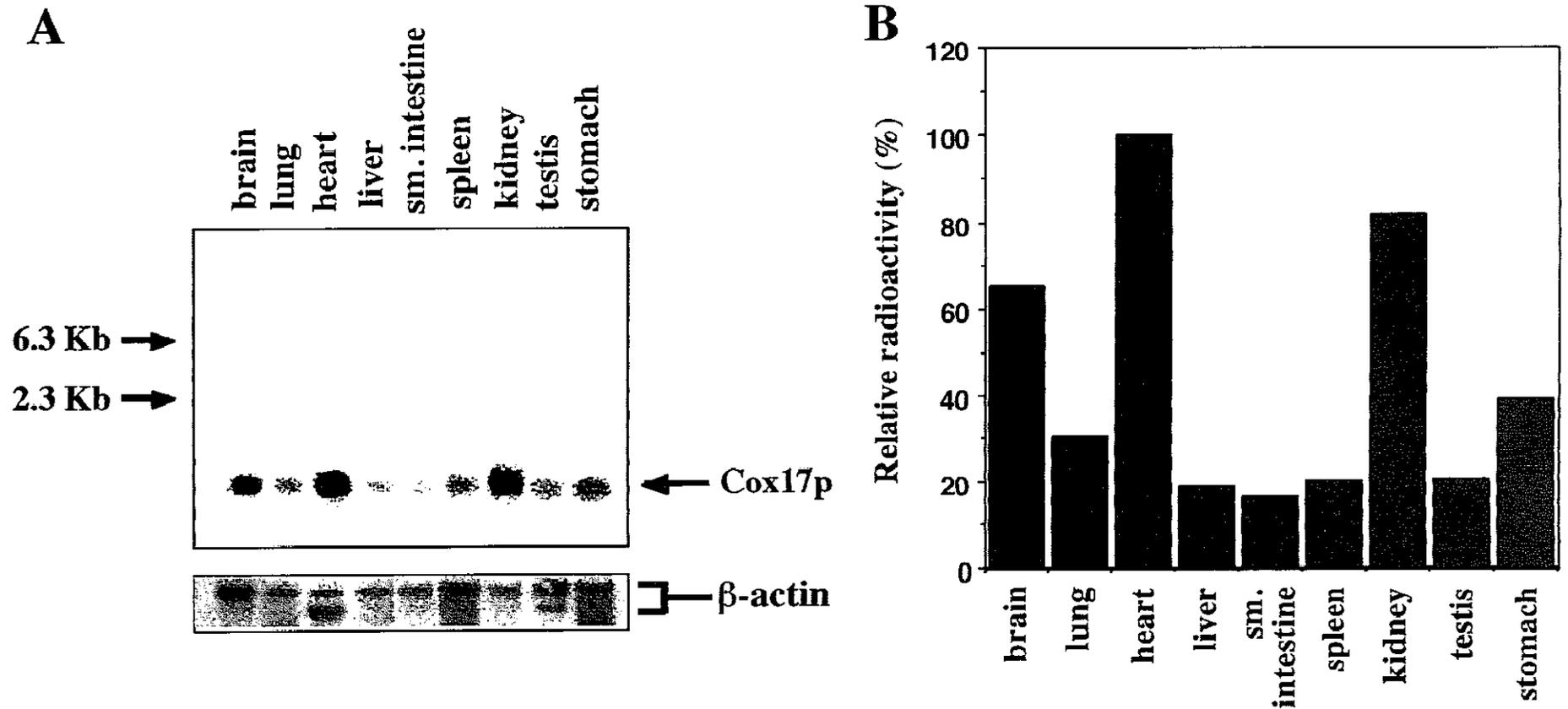


**Fig. I-2. The current model of cytosol-mitochondria copper trafficking in yeast.** The copper is reduced by one of plasma membrane reductases. And then, the reduced copper is transported across the plasma membrane by the high affinity copper transporter, Ctr1. Cox17p guides the copper from cytosol to the mitochondrial intermembrane space for insertion into cytochrome c oxidase, the terminal oxidase of the respiratory chain.

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Mouse	MPGLAAASPAPPEAQEKKPLK <b>PCCAC</b> PETKKARDACII--EKGEEHCGHLIEAHKECMRALGFKI	63
Rat	MPGLAAASPAPPEAQEKKPLK <b>PCCAC</b> PETKKARDACII--EKGEEHCGHLIEAHKECMRALGFKI	63
Porcine	PGLAAAI <b>PAPPE</b> SQEKKPLK <b>PCCAC</b> PETKKARDACII--EKGEEHCGHLIEAHKECMRALGFKI	62
Human	MPGL <b>VDS</b> NPAPPE <b>S</b> QEKKPLK <b>PCCAC</b> PETKKARDACII--EKGEEHCGHLIEAHKECMRALGFKI	63
Yeast	MTETDKK <b>QEQEN</b> HAECEDKP-K <b>PCCVCKPEKEER</b> TCIL <b>FN</b> QD <b>SEKCKEFIEKY</b> KECM <b>KGYGF</b> EVPSAN	68

**Fig. I-3. Comparison of amino acid sequence of mouse, rat, porcine, human and yeast Cox17p.** Black letters indicate identical residues and red letters indicate conserved substitutions. The putative copper binding motif (KXCCXC) is boxed.



**Fig. I-4. Northern blot analysis of mouse tissue Cox17p mRNAs (Kako *et al.*, 2000).** (A) Expression of mouse Cox17p mRNA in tissues. Representative Northern blots of Cox17p and  $\beta$ -actin mRNAs. The positions of the 28S (6.3 kb) and 18S (2.3 kb) rRNA markers are shown. (B) Quantification of Cox17p mRNA levels from the Northern blots. After the radioactivity (PSL) of the Cox17p and  $\beta$ -actin bands in each lane is measured, the Cox17p/ $\beta$ -actin ratios are calculated. The values shown in this figure are the averages of four calculated values from four independent experiments. The heart mRNA value is expressed as 100%.