Biochemistry (Moscow), Vol. 68, No. 8, 2003, pp. 827-837. Translated from Biokhimiya, Vol. 68, No. 8, 2003, pp. 1013-1025.

REVIEW

Copper Chaperones, Intracellular Copper Trafficking Proteins. Function, Structure, and Mechanism of Action

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Received November 12, 2002
Revision received March 20, 2003

Abstract—This review summarizes findings on a new family of small cytoplasmic proteins called copper chaperones. The copper chaperones bind and deliver copper ions to intracellular compartments and insert the copper into the active sites of specific partners, copper-dependent enzymes. Three types of copper chaperones have been found in eukaryotes. Their three-dimensional structures have been determined, intracellular target proteins identified, and mechanisms of action have been revealed. The Atx1 copper chaperone binds Cu(I) and interacts directly with the copper-binding domains of a P-type ATPase copper transporter, its physiological partner. The copper chaperone CCS delivers Cu(I) to Cu,Zn-superoxide dismutase 1. Cox17 and Cox11 proteins serve as copper chaperones for cytochrome *c* oxidase, a copper-dependent enzyme.

Key words: copper chaperones, P-type ATPase copper transporter, Cu,Zn-superoxide dismutase, cytochrome *c* oxidase, copper transport

Many enzymes employ transition metal ions as specific cofactors. However, the mechanisms by which metal ions are transported to various cells and intracellular structures and the mechanisms of insertion of metal ions into enzymes are just being unraveled. In recent years a new family of cytosolic, soluble, low-molecular-weight metal-receptor proteins, called "metallochaperones", has been found to act in the intracellular trafficking of metal ions [1, 2]. By their functions, metallochaperones, the metal-trafficking proteins, are different from molecular chaperones participating in protein folding [3, 4].

Copper ions act as cofactors in many enzymes [5, 6]. Such enzymes as Cu,Zn superoxide dismutase (SOD; EC 1.15.1.1), cytochrome *c* oxidase (EC 1.9.3.1), amino oxidase (EC 1.4.3.6), dopamine β-hydroxylase (EC 1.14.17.1), peptidylglycine α-hydroxylating monooxygenase (EC 1.14.17.3), tyrosinase (EC 1.14.18.1), lysyl oxidase (EC 1.4.3.13), and many others need copper ions for catalytic activity [7, 8]. *In vitro*, many copper-dependent enzymes acquire copper ions without auxiliary proteins. For example, SOD1 accepts copper ions from the milieu and binds them with an extraordinarily high affinity ($K_{dis} \approx 10^{-15}$ M) [9]. Previously, it was suggested that *in vivo* copper enzymes accepted their essential cofactor by capture of free copper ions or from copper complexes with low-molecular-weight ligands, such as glutathione, in the cytoplasm. However, free copper ions are toxic for organisms even at low concentrations. Free Cu(I) and Cu(II) ions in the cytoplasm can catalyze autooxidation of proteins, lipids, and nucleic acids [10, 11]. Actually, intracellular free copper concentration is extremely low due to overcapacity of the cytoplasm to bind free copper excess for detoxification, including copper chelation by metallothionein (a low-molecular-weight, cysteine-rich protein that binds heavy metal ions and participates in copper homeostasis). As it has been shown for yeast cells, the intracellular free copper concentration is $\approx 10^{-18}$ M, that is less than one atom of copper per cell [2]. Thus, formation of active copper-dependent enzymes has to take place with participation of additional agents delivering copper ions to these enzymes.

COPPER TRANSPORT ACROSS THE PLASMA MEMBRANE

It is suggested that numerous copper ions carriers, including ceruloplasmin, copper complexes with albumin and histidine, as well as a high-molecular-weight protein—transcuprein—can deliver copper ions into cells

Abbreviations: Atx1) copper chaperone for Ccc2; Ccc2) yeast P-type ATPase copper transporter; CCS) copper chaperone for SOD1; SOD1) Cu,Zn-superoxide dismutase 1; COX) cytochrome *c* oxidase.

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[12, 13]. Penetration of copper ions into cells is generally rapid and does not depend on the intracellular ATP level [14], which can reflect a passive copper transport across the cellular membrane [15, 16]. Some amino acids [17], chloride and hydrocarbonate ions [18], as well as other agents, as shown on cell cultures, are capable of stimulating copper uptake by cells.

An accessible model for investigation of eukaryotic copper transport is the yeast *Saccharomyces cerevisiae*. Cu(II) ion uptake by yeast cells is accompanied by reduction of Cu(II) to Cu(I) by one of the metalloreductases of the plasma membrane [19]. In mammals, as shown for rat liver cells, Cu(II) ions are also reduced by a membrane reductase, using NADH as the electron donor [20]. Subsequent transport of Cu(I) ions across yeast plasma membrane is carried out by Ctr1, Ctr2, and Ctr3 proteins (Fig. 1) (Ctr stands for Cu transport) [14, 21]. Transmembrane Cu(I) transporters, analogous to yeast Ctr1, have been identified for humans (hCtr) (Fig. 2) [22, 23], mice [21], and the plant *Arabidopsis thaliana* (Copt1) [24].

For all transmembrane Ctr proteins it is typical to have a structural motif MXXM (where M and X refer to Met and any amino acid, respectively) repeated 11 times, which binds in a specific way with Cu(I) ions and transports them across yeast plasma membrane [14, 25].

INTRACELLULAR COPPER TRANSPORTING PROTEINS

In 1997 low-molecular-weight proteins were found, which, by mechanisms still unknown, bind with Cu(I) ions ($K_{dis} < 10^{-20}$ M) transferred into the cell and deliver

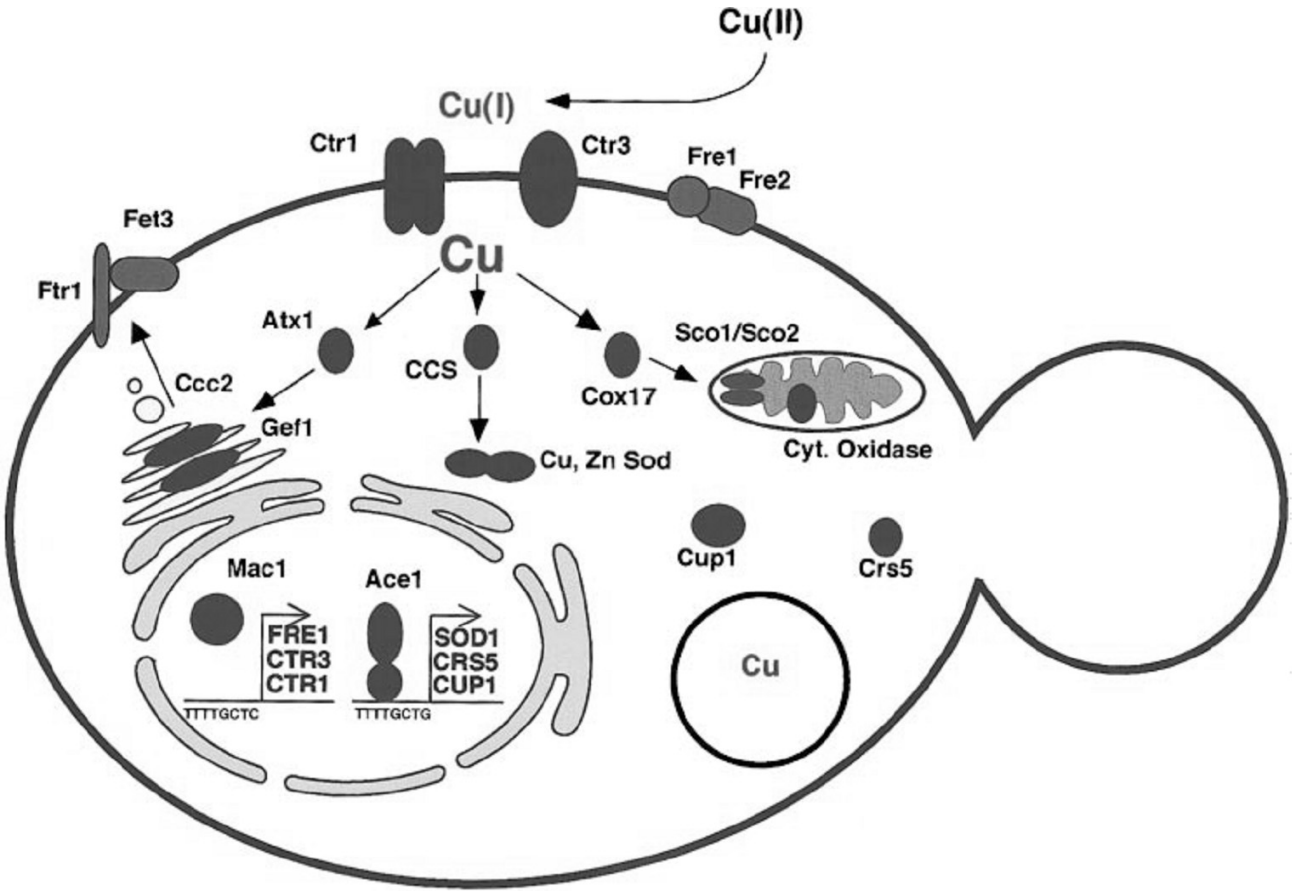

Fig. 1. Copper transport and distribution in *Saccharomyces cerevisiae* cells [25]. Copper is first reduced from Cu(II) to Cu(I) by cell surface reductases Fre1/Fre2 prior to uptake. High affinity Cu(I) ion uptake is mediated by the Ctr1 and Ctr3 proteins. Within the cell Cu(I) ions are bound to the copper chaperones Atx1, Cox17, and CCS for specific delivery to Golgi complex, mitochondria, and Cu,Zn-SOD1, respectively. Within the Golgi complex, Cu-ATPase (Ccc2) accepts Cu(I) from Atx1, followed by incorporation of Cu(I) into the multicopper ferroxidase, Fet3. Fet3 forms a complex with the iron permease Ftr1, and both proteins are responsible for high-affinity iron uptake. In mitochondria Cu(I) ions delivered by Cox17 are incorporated into cytochrome *c* oxidase (COX), a process that requires the integral inner-membrane protein Sco1 and possibly its homolog Sco2. CCS delivers Cu(I) specifically to Cu,Zn-SOD1 in the cytosol.

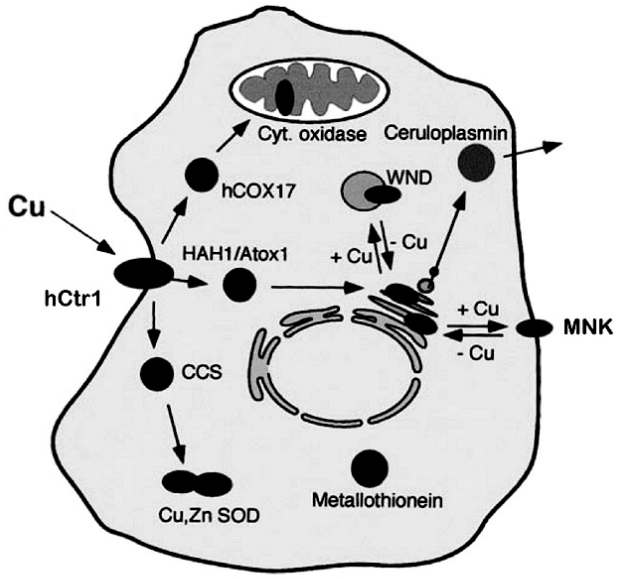

Fig. 2. Model for human copper uptake and distribution at the cellular level [25]. Tissue uptake of copper is likely to be mediated by the hCtr1 Cu-transporter. Once transported by hCtr1, the low-molecular-weight cytoplasmic Cu-chaperones hCox17, Hah1(hAtx1), and CCS distribute copper ions to specific cellular compartments for the incorporation of Cu(I) into Cu-requiring proteins. hCox17, Hah1, and CCS deliver Cu(I) to mitochondria, Golgi compartment, and Cu,Zn-SOD1, respectively.

them to target proteins, performing a chaperone-like role for copper-dependent enzymes (Figs. 1 and 2) [1]. Atx1, the originally identified copper chaperone from yeast, binds with copper ions and delivers them to its physiological partner protein, copper ATPase [1]. Copper chaperone CCS delivers copper to its specific target in the cytoplasm—SOD1 [1, 2]. Proteins Cox17 and Cox11 are copper chaperones for COX [26, 27].

According to available data, each copper-dependent enzyme in the cell is supplied with copper ions by a specific copper chaperone [28]. Within one cell copper chaperone of one type cannot functionally substitute for a copper chaperone of another type. However, it is probable that copper chaperones delivering copper ions to the same protein in cells of different types are analogous.

Atx1-type copper chaperones as copper ion carriers for copper-transporting ATPases. In 1995, a gene expressing a protein with antioxidant activity was discovered in *S. cerevisiae* cells that lacked SOD1 activity [29]. The protein was designated as antioxidant 1 (Atx1). However, *in vitro* it was revealed that antioxidant activity appears to result from stoichiometric consumption of superoxide by Cu-Atx1 complex and causes inactivation of the protein [30]. Study of physiological activity of Atx1 has shown that this protein functions as a copper shuttle to Cu-ATPase which is, in turn, located in the plasma membrane [1] and pumps copper ions delivered by Atx1 into the Golgi complex for insertion into copper enzymes [1, 31]. Thereafter proteins homologous to Atx1 were identified in a variety of eukaryotes including humans, mice, rats, sheep, dogs, and plants [28, 29, 32-35]. The only sufficiently studied copper chaperone in prokaryotes is the copper chaperone CopZ from *Enterococcus hirae* and *Bacillus subtilis* delivering Cu(I) to Cu-ATPases, CopA and CopB [36-38]. Atx1-like proteins from various phylogenetic sources are 8-kD proteins consisting of approximately 70 amino acids [39-41].

Copper transporting ATPases, to which Atx1-type copper chaperones deliver copper ions, are members of a large family of proteins that use energy from ATP hydrolysis to drive membrane transport of ions [10]. Cu-ATPase expressed in yeast participates in copper inclusion into copper-dependent oxidase Fet3 involved in iron uptake and oxidation (Fig. 1) [42]. Human copper chaperone Hah1 (hAtx1), homologous to yeast copper chaperone yAtx1, delivers copper ions to Cu-ATPases, ATP7A and ATP7B, which pump them into the lumen of the Golgi complex for subsequent insertion into ceruloplasmin (Fig. 2) [33, 43]. In humans, inherited mutations in ATP7A and ATP7B are responsible for Menkes and Wilson diseases, specific human disorders of copper metabolism [14, 44]. Menkes disease is characterized by a failure to pass copper ions across the intestinal mucous, that leads to copper deficiency in peripheral organs and tissues and causes impairment of nervous system and connective tissue functions. In contrast, Wilson disease, or hepatolenticular degeneration, is characterized by accumulation of toxic amounts of copper, predominantly in liver and in brain, with accompanying cirrhosis and neurodegeneration. Copper transport to ATP7A and ATP7B by copper chaperone Hah1 occurs both in the course of normal copper metabolism and in response to a high influx of copper under copper metabolism disturbance. Identified in roots, leaves, and inflorescence of the plant *A. thaliana*, Atx1-like copper chaperone Cch delivers Cu(I) to Golgi-complex-located Cu-ATPase Ran1 (Responsive to Antagonist), providing for formation of functional ethylene receptors and modulating plant growth in response to ethylene, as well as regulating copper content in senescent leaves [45, 46].

Understanding of mechanisms of copper binding with Atx1-like copper chaperones, recognition of appropriate copper chaperones by Cu-transporting ATPases, and transfer of copper ions between chaperone and ATPase is based on structural studies of these chaperones and copper-binding domains of Cu-ATPases. It has been established that copper-trafficking pathway proteins, including Atx1-like copper chaperones [33], Cu-transporting ATPases [47-49], copper-dependent oxidase Fet3 and ceruloplasmin [50, 51] are highly conserved in various eukaryotes from yeast to humans [50-53].

Crystallographic and NMR-studies reveal that yAtx1 and hAtx1 possess a βαββαβ ferredoxin-like fold [54], where four β-strands and two α-helices are connected by loop regions with a solvent-exposed metal-binding site (Fig. 3) [55, 56]. By contrast, the iron-sulfur clusters in the structurally similar ferredoxins and the catalytic copper sites in oxidases and oxygenases are buried in the protein molecule [57]. The chaperone metal binding site is thus more suited to copper delivery rather than to a catalytic or electron-transfer function. The presence of multiple domains with Atx1-like secondary structure and MXCXXC copper-binding sites is characteristic for P-type Cu-transporting ATPases [58].

In experiments on yeast copper chaperone expressed in *Escherichia coli*, Pufahl et al. [1] investigated the Cu oxidation state in Atx1 by EPR and X-ray absorption spectroscopy (XAS). Addition of $CuSO_4$ to purified apo-Atx1 in the presence of excess thiol reductant yielded a complex with a copper/protein ratio 0.6 to 0.8. No EPR signal was observed in Cu-Atx1 at 77 K, suggesting the absence of Cu(II) in a mononuclear copper complex. XAS experiments have also indicated that the bulk copper, bound with protein, is Cu(I). Although Cu(I) ions in aqueous solutions are not stable and undergo auto-oxidation or disproportionation, the solution of Cu(I)-AtxI complex was stable at neutral pH for at least 30 min [1]. As a rule, monovalent copper ions in proteins are found either as polynuclear metal thiolate clusters, as in metallothionein [59], or in the environment of His_2Cys, as in blue copper proteins [60]. In contrast, extended X-ray absorption fine structure (EXAFS) measurements reveal that in Atx1 Cu(I) ion is coordinated to the two cysteine residues present in the amino acid sequence MXCXXC and is weakly bound to a third sulfur atom, possibly from an adjacent Atx1 molecule [1]. The Cu-binding site in Atx1 is flexible and can participate in formation of Cu(I) complexes with different coordination number [55]. At the same time, the copper chaperone fold provides formation of a copper-binding site in which bound Cu(I) ion is protected both from oxidants and from capture by excess of competing thiols such as glutathione [1]. This type of copper coordination environment is unprecedented in copper proteins. However, as follows from structural studies of copper chaperones (Atx1, CCS, bacterial Cu-chaperone CopZ), as well as from the metal-binding domains of copper transporting Cu-ATPases, this type of copper coordination environment is a common feature of copper trafficking proteins [9, 39]. NMR investigations of structures of Atx1 apo-form and Cu(I)-Atx1 complex have shown that protein-bound Cu(I) ions have an effect on the conformation of the Atx1 molecule [40], promoting interaction with the target protein.

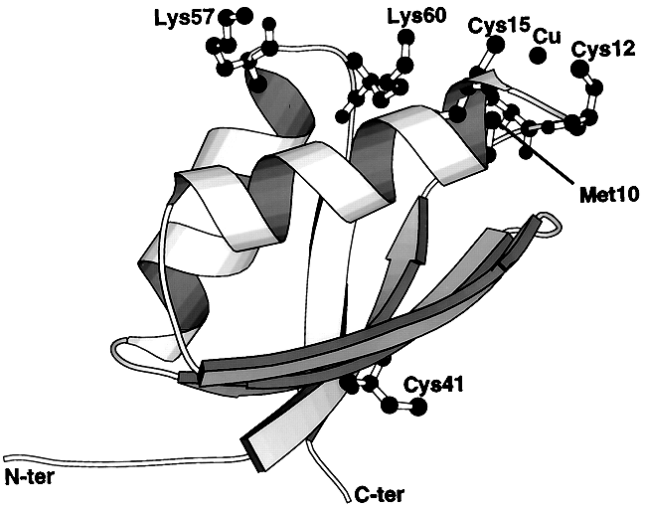

Fig. 3. Three-dimensional structure of recombinant Hah1 (hAtx1) [43]. Individual residues are numbered according to the wild type Hah1 sequence [33].

It has been established that on encountering copper acceptor ATPase, Cu(I)-Atx1 complex associates with the cytosolic Atx1-like ATPase domain [1]. Positively charged lysine residues on the surface of Atx1 and negatively charged amino acid residues on the surface of the Atx1-like segment of ATPase provide electrostatic interactions between copper chaperone and ATPase [30, 55]. Mutation of lysine residues on the surface of Atx1 greatly reduces the copper-dependent complex formation between Atx1 and ATPase. It is suggested that as a result of juxtaposition of the protein surfaces in addition to electrostatic interactions between the two proteins, hydrophobic interactions and hydrogen bonding arise. Formation of the complex between Atx1 and ATPase produces a certain orientation of the Cu-binding loops in which copper ion forms a bridge between two proteins (Fig. 4) [61]. Meanwhile, copper-coordinated cysteines of Atx1 are sequentially replaced with cysteines of the Cu-binding domains of ATPase, and copper ion moves from Atx1 to ATPase (Fig. 4) [1].

Copper exchange rates in cysteine sites of copper containing proteins such as Cu-thionein are generally slow due to the strength of the thiolate bonds with copper ion [62]. However, *in vitro* experiments show that incubation of copper-bound form of the chaperone with apo-form of Cu-ATPase (apo-Ccc2a) is accompanied by rapid Cu(I) equilibration between the two proteins [61]

$$\text{Cu-Atx1} + \text{apo-Ccc2a} \rightleftarrows \text{apo-Atx1} + \text{Cu-Ccc2a}$$

with an equilibrium constant $K_{exchange} = 1.4$ [56].

It is generally believed that Atx1 stimulates exchange between yet-to-be-identified copper donors and Cu-binding sites of specific acceptor-target, such as

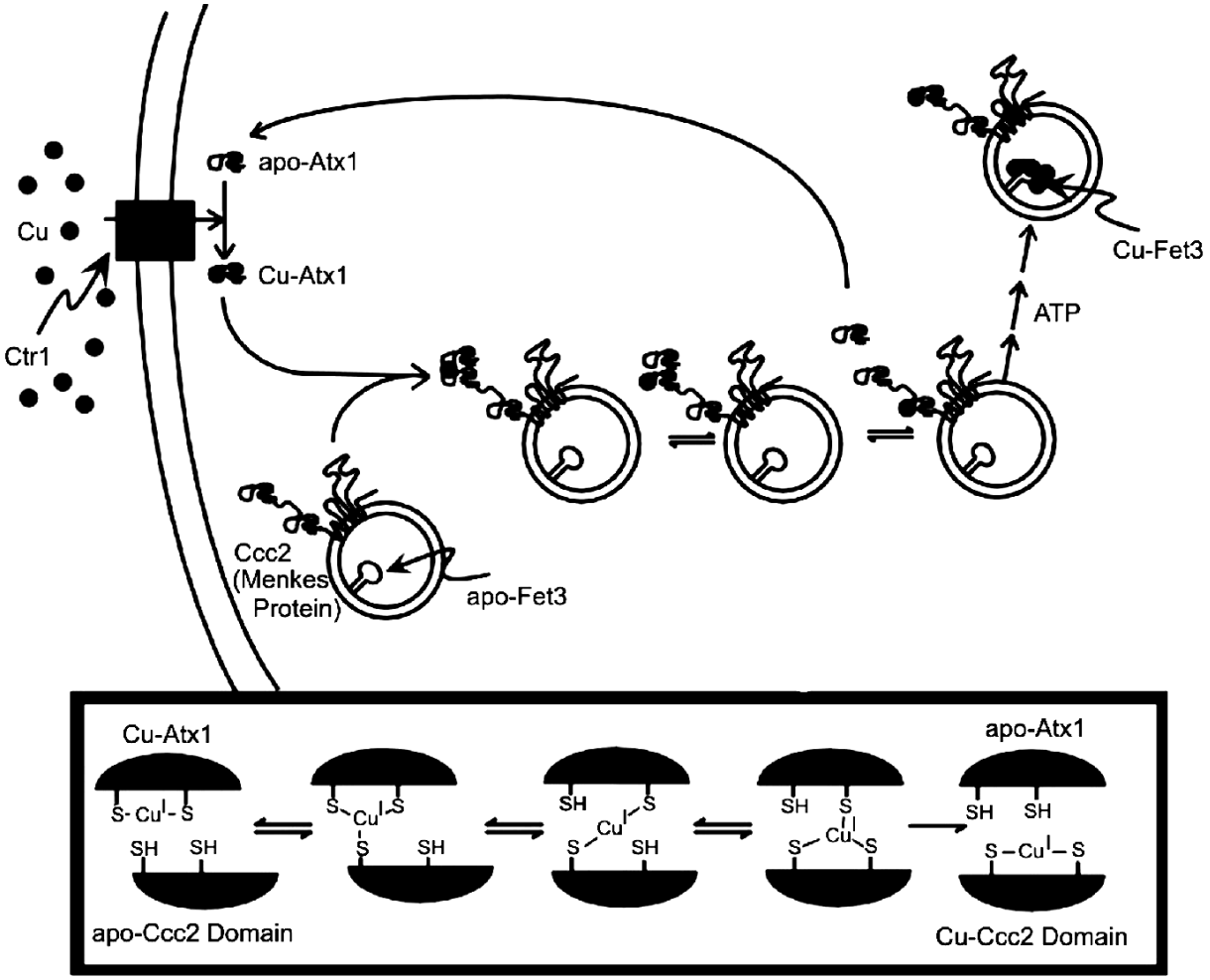

Fig. 4. Proposed pathway for the intracellular transfer of Cu(I) by Atx1 [1]. Cu(I) transfer for incorporation into the vesicular multicopper oxidase Fet3 requires the proteins Ctr1 and Ccc2 (Cu-ATPase). Cytoplasmic Cu(I)-Atx1, but not apo-Atx1, associates with the N-terminal domain of Ccc2 and Cu(I) is transferred to the latter. Inset: a proposed mechanism for the exchange of Cu(I) involving two- and three-coordinate Cu-bridged intermediates. The human homologs of Atx1 (Hah1), Ccc2 (Menkes and Wilson proteins), and Fet3 (ceruloplasmin) probably employ similar mechanisms.

Cu-ATPases [61]. Further copper transfer is provided for Cu-ATPase domains which as a result of ATP hydrolysis deliver Cu(I) to compartments available to copper-dependent oxidases and other apo-proteins—copper ion acceptors.

Thus, Atx1-like Cu-chaperones and their physiological partner Cu-ATPases from several phylogenetic kingdoms recognize each other via electrostatic and hydrogen bonding as well as hydrophobic interactions, in a manner that precisely orients the copper-binding polypeptide chains for rapid copper transfer. Copper ions are transferred immediately from Cu(I)-Atx1 into copper-binding sites of Atx1-like domains of Cu-transporter [1, 30].

Copper chaperone CCS as a copper ion carrier to superoxide dismutase. SOD1, a cytosolic Cu,Zn-dependent enzyme that catalyzes disproportionation of O_2^- to H_2O_2 and O_2, serves as a key antioxidant enzyme in eukaryotic cells [63]. The reaction is accompanied by the redox cycling of the bound copper ion Cu(II). SOD1 binds copper ions with high affinity *in vitro* and *in vivo*. However, under physiological conditions, owing to low concentration of intracellular copper ions, copper insertion into the enzyme requires a copper chaperone [2, 64, 65]. So far, Cu-chaperones (CCS) delivering copper ions to SOD1 have been identified and characterized in both yeast (yCCS) and humans (hCCS). Homologous proteins have also been found in plants and insects [66, 67]. In yeast cells devoid of CCS and with expression of SOD1 on a normal level, the newly synthesized enzyme lacks copper and does not show catalytic activity [64, 68].

CCS-like copper chaperones are 26- to 30-kD proteins and, unlike single-domain proteins Atx1 and Cox17, consist of three functionally distinct domains: a small N-terminal domain I, a middle 16-kD domain II, and a small C-terminal domain III (Fig. 5) [66, 69, 70]. The N-terminal domain I is highly homologous with copper chaperone Atx1, including the MXCXXC copper-binding motif [66]. Crystallographic investigations revealed that domain I of CCS has the same folding as Atx1 [69]. Besides, in the CCS crystal the two cysteines are linked by a disulfide bond similar to that observed in apo-form of Atx1 [55]. These data indicate that domain I is capable of binding copper ions. However, despite similarity of domain I of CCS and Atx1, mutagenesis experiments on *S. cerevisiae* show that substitution of CCS domain I for Atx1 leads to loss of the CCS chaperone activity [66].

Domain II, the central domain of CCS, is homologous to SOD1 [66, 69, 71]. Crystallographic investigations of yeast and human CCS reveal that, similar to SOD1, domain II from both yCCS and hCCS comprises an eight-stranded β barrel structure (Fig. 5) [69, 70, 72]. As shown for human CCS, the homology between domain II and SOD1 is so strong that a single mutation in domain II is sufficient to turn CCS into a molecule with SOD-like activity [73].

The C-terminal domain III comprises 27 amino acid residues with a sequence unique for CCS copper chaperone. This domain has not been structurally characterized. However, *in vivo* domain III is crucial for copper insertion into SOD1 [66, 74]. Crystallographic studies reveal that domain III is located in the vicinity of domain I [72], and both domains bind Cu(I) [66, 67, 75]. Ability of domain III to bind copper is evidenced by the presence of a CXC sequence motif, in which both cysteine residues are necessary for binding copper and activation of SOD1 [66].

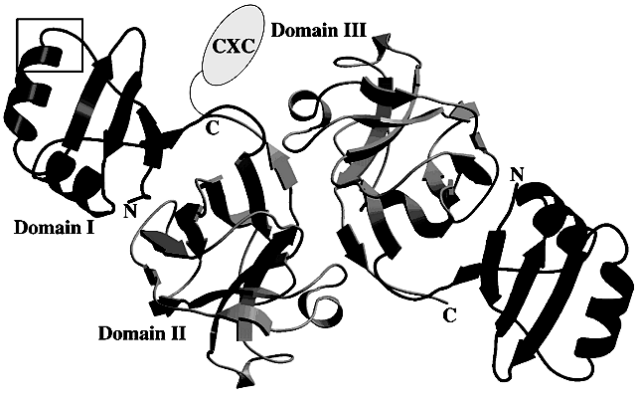

Fig. 5. Structure of yCCS [57]. The location of the MT/HCXXC sequence motif in domain I is denoted by a box. Domain III was disordered in the crystal structure and is represented by an oval. The dimer interface is formed exclusively by residues belonging to domain II.

Determination of spatial structures of SOD1 [76] and CCS [69, 72] is a key to understanding the molecular mechanism of copper transfer to SOD1 by CCS copper chaperone. Both SOD1 and hCCS molecules exist as homodimers in solution (yCCS is dimeric only in the presence of copper), and formation of transient heterodimer or heterotetramer (i.e., a dimer of dimers) complexes between SOD1 and CCS may precede copper transfer [66, 69, 71, 73]. The nature of the intermediate complexes was determined for a mutant form of SOD1 in which one of four copper binding histidine residues had been replaced with phenylalanine [77]. By immunoprecipitation and gel electrophoresis it has been established that the mutant form of SOD1 cannot accept copper, but is able to form a stable complex with apo- and Cu-CCS. A complex isolated by gel filtration is a heterodimer containing one ion of copper and one ion of zinc. Fluorescence anisotropy measurements corroborate the formation of the dimeric complexes and indicate that the presence of a copper ion enhances significantly the stability of the dimer.

From results of genetic and biochemical experiments with yeast CCS, Wong et al. [65] have suggested that each of the three domains of yeast copper chaperone plays a certain role in SOD1 activation. According to the data of these authors, the yeast peptide, corresponding to domain III of CCS, binds Cu(I) ion and probably functions independently of domain I, which acquires copper from the cell. However, most researchers suppose that both domains I and III bind metal ions simultaneously [67, 74, 75].

It has been proposed that the process of copper ion transfer occurs via ligand exchange in the coordination sphere of copper ion in CCS domain III and the SOD1 active site by a "pivot, insert, and release" mechanism (Fig. 6) [74]. By this mechanism, domain III acquires copper ion from domain I, then pivots toward the adjacent apo-SOD1 and releases copper into the enzyme active site [74]. Results of biochemical investigations on the human CCS protein suggest that copper ion transfer proceeds in various stages, in each of them hCCS being in conformation favorable to delivery of copper to SOD1 [74]. The first stage is that of "copper acquisition state", wherein CCS, most likely domain I, obtains copper from a yet to be identified donor. The second is the "transiting state", in which CCS protects the copper ion from copper-binding scavengers in the intracellular environment. In the absence of the donor and target enzyme the copper ion is most likely bound by the cysteine residues of domains I and III. Finally, a "copper insertion state" includes the binding of chaperone to the target protein and subsequent release of the copper ion to domain III and then into the SOD1 active site.

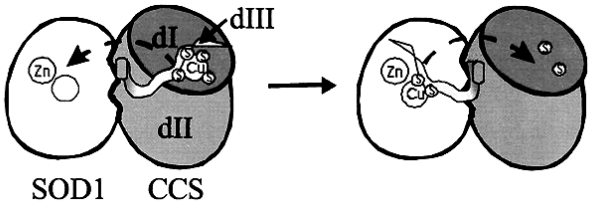

Fig. 6. Proposed mechanism of Cu(I) ion transfer from CCS to apo-SOD1 [74].

It has recently been established that functions of CCS are not limited to recognition of a partner protein and copper transfer. Investigations of the hCCS/hSOD1 system have demonstrated that copper ions added to SOD1 denatured with organic solvents cannot reactivate the enzyme. At the same time, in the presence of CCS the SOD1 activity was restored completely [74]. It is evident from these data that human copper chaperone can participate in SOD1 folding.

It is known that mutations in SOD1, including mutation in one of the copper-binding sites, cause a serious neurological disease (familial amyotrophic lateral sclerosis, FALS) [78]. Analysis of several *S. cerevisiae* mutants with expressed typical FALS-SOD1 mutant forms revealed that *in vivo* all yeast mutants were able to bind copper and possessed the SOD-activity [78]. Although the presence of CCS facilitated copper binding by SOD1 in all the investigated mutant yeasts [78], it has been shown that FALS development in mutant-SOD1 mice does not depend on the enzyme copper loading [79]. Since yCCS binds tightly with mutant-SOD1 forms, it is supposed that the interaction of CCS with less tightly folded SOD1-mutant forms plays a protective role in the cell [78, 80].

Copper chaperones—copper ion carriers to cytochrome *c* oxidase. COX is the key enzyme of the respiratory chain of eukaryotic mitochondria that catalyzes reduction of molecular oxygen to water. COX is a multisubunit enzyme embedded in the inner mitochondrial membrane, consisting of 12 subunits in yeast and of 13 subunits in humans. In aerobic bacteria, COX consists of 2-4 subunits and is embedded in the plasma membrane [81-83]. In the eukaryotes, the three largest subunits (COX1, COX2, and COX3) are synthesized in mitochondria and form the catalytic core. A number of subunits, which are found in the eukaryotic COX and are additional to the bacterial COX, are probably involved in the assembly and regulation of the enzyme [84-86].

COX contains several cofactors, including three copper ions, localized in subunits 1 and 2. Two copper ions form a binuclear Cu_A center located within the C-terminal hydrophilic domain of subunit 2 in the intermembrane space. The third copper ion, localized in subunit 1, forms a mononuclear center Cu_B, buried in the inner mitochondrial membrane [87]. Because both subunits, COX1 and COX2, are synthesized inside the mitochondria, copper ions essential to the enzyme catalytic activity have to be imported from the cytoplasm. The mechanism by which copper ions are incorporated into COX is not yet established. However, proteins (Cox17, Sco1, Sco2, and Cox11) have been discovered that participate in copper ion delivery and insertion into the enzyme (Fig. 7) [26, 88-91]. One of such proteins is the metallochaperone Cox17. Cox17, an 8-kD protein containing six conserved cysteine residues, is a unique protein for all eukaryotes and is homologous with metallothionein [92]. In yeast, mutation of Cox17 leads to inhibition of the COX enzymatic activity and as a consequence to a respiratory defect [26, 93]. Restoration of the yeast respiration after addition of copper in the form of Cu(II), to the yeast growth media led to the proposal that Cox17 functions in copper ion trafficking to COX. Because the SOD1 activity does not change with this mutation, it is suggested that the copper-delivering function of Cox17 to COX is specific. In yeast, Cox17 is localized to the cytosol and the intermitochondrial membrane space [94] and may act as a shuttle to transfer copper ions between two compartments.

Recombinant purified form of Cox17 from *E. coli* binds three Cu(I) ions per monomer in a polynuclear cuprous-thiolate cluster [95]. Such clusters exhibit predominantly trigonal Cu(I) coordination. Polynuclear copper clusters in Cox17 resemble the copper clusters in

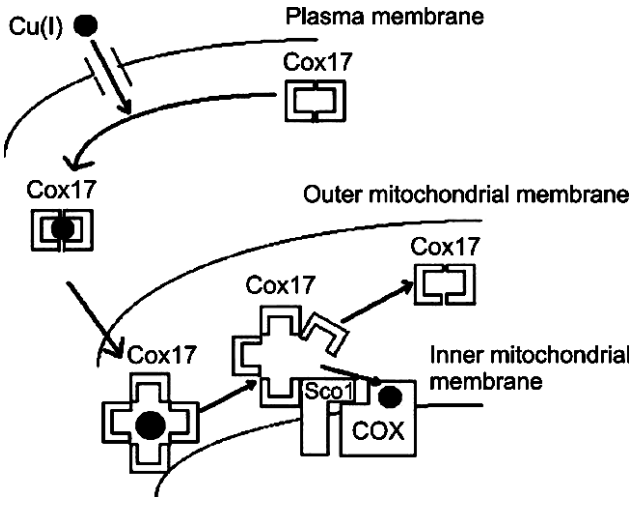

Fig. 7. A model for Cu(I) ion delivery to the mitochondria by Cox17 [88]. Co-metallochaperone, Sco1, participates in the COX assembly.

Cu-thionein in pH stability and luminescent properties. However, the Cu-Cox17 clusters are distinct from the polycopper clusters in Cu-thionein in their higher ability for ligand exchange. Three of the seven cysteine residues found in a CCXC motif are not essential for copper binding, yet are deemed as critical for the functions of the chaperone [96].

In vivo the monomeric Cu-Cox17 complex, as it follows from investigations of Winge and coauthors [90], exists in a dimer/tetramer equilibrium. The tetrameric form of Cu-Cox17 is predominantly localized within the intermitochondrial membrane space, whereas the dimeric form of Cu-Cox17 is in the cytosol. Substitution of three cysteine residues in the Cox17 molecule by serine prevents dimerization and tetramerization of the protein and abolishes its functional activity. However, Cu(I) ion binding is largely unaffected. These data suggest that the oligomeric form of Cox17 is functionally important [90].

The research data indicate that Sco1 and Sco2 proteins localized to the inner mitochondrial membrane perform an important role in copper delivery and COX activation [97, 98]. Sco1-deficient yeast cells do not show any COX-activity, which is not restored by the addition of high concentrations of copper ions into the yeast cultivation medium [98]. It has been proposed that functions of both Sco1 and Sco2 proteins consist of copper ion transfer to COX, whereas Cox17 delivers copper to Sco1 and Sco2 [98].

Two alternative models have been proposed to explain the role of Sco1 in copper delivery to COX. According to the first model, Sco1 transfers copper ions directly from Cox17 to COX [98]. According to the second model, Sco1 is required for reduction of the cysteine residues in the copper-binding center of COX subunit 2 [99]. In both cases, a direct interaction between Sco1 and COX2 is expected, and copper transfer is accompanied by the formation of a number of intermediates.

Sco1 is a 30-kD protein of the mitochondrial membrane [97]. Topological studies show that Sco1 is anchored in the inner membrane by a transmembrane segment [94]. The major C-terminal portion of the protein protrudes into the intermembrane space. It has been shown that the mutant yeast *S. cerevisiae*, lacking the SCO1 gene is characterized by a rapid proteolytic degradation of subunits newly synthesized in mitochondria, especially of enzyme subunit 2 [100]. The latter does not exclude that Sco1 is involved in the assembly of COX [101].

Data obtained from size-exclusion chromatography of yeast mitochondrial lysates demonstrate that Sco1 is most likely oligomeric *in vivo* [95]. It has been shown that isolated C-terminal domain of Sco1 binds one Cu(I) ion per monomer [90]. Determination of the protein structure by XAS reveals that each Cu(I) ion in Sco1 is coordinated via three ligands. Two ligands are cysteine residues, present in a conserved motif CXXXC, and the third ligand is a conserved histidine residue [90]. It has been found that Sco1 and COX subunit 2 are homologous, including the copper-binding cysteine ligands of the CXXXC motif [94, 99]. On this basis, it has been proposed that CXXXC motif in Sco1 interacts directly with subunit 2 and transfers a copper ion unto this subunit. The mutation of any of the three amino acid residues of the copper-binding center impairs the function of Sco1, resulting in a nonactive COX form [102]. Thus, the function of Sco1 correlates with Cu(I) ion binding and its insertion into the binuclear Cu_A center of subunit 2 [90].

The discovery of a significant similarity of the C-terminal portion of Sco1 to peroxiredoxins and to bacterial thiol:disulfide oxidoreductase led to the development of an alternative reaction mechanism, according to which Sco1 keeps cysteine residues in the copper binding center of COX2 in reduced state, to allow insertion of copper from Cu-Cox17 [89, 99]. However, an arginine residue, conserved among peroxiredoxins and involved in the formation and stabilization of a reactive thiolate intermediate, is not present among the members of the Sco1 protein family. It is proposed that in *S. cerevisiae* the role of arginine in the copper binding center is performed by a histidine residue at the respective position [99].

Genome analyses of a number of organisms from bacteria to humans revealed a number of genes with significant homology to SCO1 gene of *S. cerevisiae* [103-105]. In the yeast *S. cerevisiae* a protein Sco2, homologous to Sco1, was identified [106]. In humans two homologs of Sco2, localized in mitochondria, have been detected [104, 105]. It is suggested that both human and yeast Sco2 proteins act as copper chaperones transporting copper ions to the binuclear center Cu_A on COX subunit 2, but the mechanism of action remains unclear.

Mutations of one of the Sco2 genes cause fatal infantile mitochondrial disorder connected with COX deficiency in brain, heart, and striated muscle. This disorder is characterized by encephalopathy and hypertrophic cardiomyopathy [107]. Structure of Sco2 proteins is conserved in humans [105], and lack of those results in COX deficiency and infant death within a few months of birth [107, 108]. To elucidate the molecular basis of pathogenesis of Sco2 defects in humans, genetic and biochemical studies on tissues, myoblasts, and fibroblasts from affected patients, as well as on a recombinant human C-terminal Sco2 segment (22 kD), bearing the putative CXXXC copper-binding motif were performed [109]. Recombinant Sco2 was shown to bind copper with a 1 : 1 stoichiometry. Immunoblotting analysis of fibroblasts and myoblasts reveals severely reduced Sco2 content in patients suffering from encephalopathy and hypertrophic cardiomyopathy.

Winge and coauthors [91] have recently published data on newly discovered protein Cox11, essential for yeast respiratory growth. It was demonstrated that Cox11 binds copper and participates in the assembly of the Cu_B

Copper chaperones and their target proteins

Copper chaperone	Source	Intracellular target protein	Reference
yAtx1 (yeast Antioxidant 1)	*Saccharomyces cerevisiae*	Ccc2 (Cu-ATPase), transmembrane copper ion carrier to copper-dependent oxidase Fet3	1, 30, 31, 42
hAtx1 (human Antioxidant 1)	human	Cu-ATPases (ATP7A and ATP7B), transmembrane copper ion carriers to ceruloplasmin	33, 43
Cch (Copper chaperone)	*Arabidopsis thaliana*	Cu-ATPase Ran1 (Responsive to antagonist)	45, 46
EhCopZ BsCopZ	*Enterococcus hirae* *Bacillus subtilis*	Cu-ATPases (CopA and CopB) and CopY (Copper responsive repressor)	36-39
yCCS (yeast Copper Chaperone for SOD)	*Saccharomyces cerevisiae*	Cu,Zn-SOD1	1, 2, 66, 69, 70
hCCS (human Copper Chaperone for SOD)	human		
Cox17 and Cox11	*Saccharomyces cerevisiae*	Sco1 and Sco2, transmembrane copper ion carriers to COX	26, 27, 98

site of COX. The soluble C-terminal domains of Cox11 form dimers that coordinate one Cu(I) ion per monomer via three thiolate ligands. The two Cu(I) ions in the dimer exist in a binuclear cluster and appear to be ligated by three conserved cysteine residues. Mutation of any of these cysteine residues reduces Cu(I) binding and causes reduction in the COX catalytic activity and respiratory incompetence. Thus, the cysteine residues important for Cu(I) binding correlate with *in vivo* function, suggesting that Cu(I) binding is of importance in Cox11 function.

Data on intracellular copper ion carriers, copper chaperones, and their target proteins are summarized in the table below.

The structural, biophysical, and biochemical studies of copper chaperones performed to date have provided the first steps toward understanding intracellular copper delivery on the molecular level. At present, it is not clear how the copper chaperones initially bind copper ions. Possible mechanisms include interactions with membrane transporters of copper ions, with proteins such as metallothionein or yet to be identified factors, and with small intracellular chelators such as glutathione [57]. Besides, properties and mechanism of action of copper chaperones have not been sufficiently studied. From research data it has emerged that Cu(I), rather than Cu(II), is employed in intracellular copper trafficking, and all the copper chaperones characterized to date preferentially stabilize and exchange Cu(I) [110]. Very low intracellular free copper concentrations serve in favor of direct interaction of copper chaperone with a target enzyme. The crystal structures of Atx1 and CCS have revealed that each chaperone is structurally similar to its partner protein. Nevertheless, it remains unresolved whether other metallochaperones exist. At present, proteins involved in nickel [111], iron [112], and molybdenum [113] cofactor delivery and insertion have been identified alongside iron–sulfur [114] and iron–molybdenum [115] complexes. In all likelihood, enzyme target recognition by chaperones and metal ions transfer to oligomeric metalloenzymes might occur by chaperone-mediated mechanisms similar to those proposed for CCS. Extending knowledge of metallochaperone structure, function, and mechanism of action is one of the relevant problems for present-day biochemistry.

This study was funded by the Russian Foundation for Basic Research (grants 02-04-48704 and 00-15-97787) and the Program "Physicochemical Biology" of the Presidium of the Russian Academy of Sciences.

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