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ORIGINAL ARTICLE

The dual personality of ionic copper in biology

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Abstract Biological copper is mainly involved in electron transport to catalyse essential oxido-reduction processes. It is an essential trace element which is extremely toxic because exchangeable intracellular copper is Cu(I) which generates reactive oxygen species. To handle this paradox the evolution has led to a fine homeostasis in which copper ions are never free. Intracellular Cu(I) instead is bound to numerous proteins forming specific cascades towards its targets.

Keywords Copper · Biology · Homeostasis · Toxicity

Introduction

Since 25 years, significant advances were accomplished at the inorganic chemistry and biology interface. The bioinorganic chemistry has developed as a new field, the main purpose of which was to understand the functions and mechanisms of metals in biology. Metal ions play a fundamental role in living organisms in activating enzymes and stabilizing Zn(II)-fingers in transcriptional factors. As a consequence, intracellular disorders in the metabolism of metals are implicated in numerous diseases including microbial infections, cancer and neurodegenerative diseases.

Dedicated to Prof Jack Harrowfield and Dr Jacques Vicens on the celebration of their 65th birthday.

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An important facet of the role of transition metals in biology relies on their capacity to adopt several ionic forms thus allowing electron transfers necessary for the oxido-reduction processes. Because of the redox potentials of iron and copper, these two metals are often encountered in metallo-enzyme active sites. In the following, we will focus on biological copper to give a glimpse of the importance of this metal in the cell. Copper is a trace element essential for living systems where its main role is to exchange electrons in cuproenzymes. For instance, it is required for cellular functions such as respiration, protection against oxidative stress, pigment formation, neuro-transmitter biosynthesis, peptide amidation, iron transport, as well as connective tissue maturation [1].

The extracellular circulating copper is Cu(II), whereas the available intracellular copper is Cu(I). Those changes in oxidation state make copper a paradoxical trace element. As a matter of fact the Cu(I) $\leftrightarrow$ Cu(II) reversible transition transforms copper in a toxic compound through a Fenton-like reaction which generates deleterious free radicals contributing to oxidative damage to cellular components. Because of this dual role, copper being at the same time essential and toxic, all living organisms have developed mechanisms to accurately tune its homeostasis. In particular, due to its instability, intracellular Cu(I) is not free but bound to storage proteins or to metallochaperones which thus participate to its trafficking and delivery to proteins needing copper for their activity.

Evolution

Some 2.7×10^9 years ago, before the advent of atmospheric oxygen, the atmosphere of the earth was highly

reducing containing H_2O, H_2S, NH_3 and CH_4. In this environment biological systems preferentially used soluble Fe(II) and Mn(II) ions rather than copper which is found mainly as poorly soluble sulphite, oxide or carbonate (Cu_2S, Cu_2O, $Cu_2CO_3(OH)_2$).

When oxygen appeared in the atmosphere, thanks to the photosynthetic activity of cyanobacteria, this transition from reducing to oxidizing conditions favored a change in all metal oxidation states. Due to this evolution, copper has been released from the insoluble Cu_2S as soluble Cu(II) ions and became a prevalent metal in biology [2]. In the anaerobic atmosphere, the enzymes worked at low redox potential (Fe–S proteins from −0.8 to +0.4 V). The presence of oxygen and availability copper induced an evolution towards cuproenzymes working at higher potentials (between 0.25 and 0.75 V). The control of copper metabolism in the presence of oxygen became a key factor in the evolution of aerobic organisms [3].

Copper chemistry

Copper is an exception in the first transition metal series since it does not share the common electronic characteristics of this series of 10 elements. Transition elements have a partially filled d sub-shell and exist under several different oxidation states which in turn, results in the possibility to form numerous complexes. Copper has a full d sub-shell ($3d^{10}$) plus a single 4s electron and can switch between two ionic forms : Cu(I) with a full d sub-shell ($3d^{10}$) and Cu(II) with partially filled d sub-shell ($3d^9$). The relative stabilities of the Cu(I) and Cu(II) states can be evaluated from their redox potentials [4]:

$$Cu^+ + e^- = Cu, \quad E^0 = 0.52 \text{ V and}$$
$$Cu^{2+} + e^- = Cu^+, \quad E^0 = 0.153 \text{ V}$$

The fact that within a cuproprotein, copper can donate or accept one electron at mild potential makes it an appropriate cofactor in a number of biological redox reactions. The binding of copper ions to ligands is governed by the "hard and soft acid/base" association, according to Pearson's classification [5]. The highly polarisable Cu(I) is a soft acid, it forms complexes by covalent binding to soft bases. In proteins, soft bases are found in three amino acids. Cu(I) can coordinate thiols from cysteines (Cys), thioether from methionines (Met) and imidazole nitrogen in histidines (His). The number of Cu(I) coordinations can be 2, 3 or 4, and the binding can adopt a linear, trigonal or tetrahedral coordination geometry. Cu(II) is borderline, between soft and hard acids, less polarisable than Cu(I) and forms also ionic interactions. Its number of coordinations can be 4,5 or 6 with an octahedral geometry [6–8].

Therefore, the biological functions of copper are closely related to its chemical properties as a transition metal. It is found in all organisms, bacteria, yeast, plants, mammals and humans. Some important cuproenzymes are listed in Table 1.

Copper toxicity

As stated above, the possibility for copper to adopt two oxidation states transforms this essential metal into a cytotoxic compound. Free Cu(I) is one of the most toxic trace elements because it induces a Fenton like reaction,

Table 1 Some bacterial and mammalian cuproenzymes

Enzyme/protein	Function	Consequences of loss or deficience
Cu/Zn Superoxyde dismutase	Antioxidant defense (superoxyde dismutation)	Oxidative stress, hepatocellular carcinoma, neurodegeneration
Cytochrome c oxidase	Mitochondrial oxidative phosphorylation	Respiratory deficiency, encephalopathy, cardiac failure
Ceruloplasmin	Ferroxidase: Fe loading onto transferrin	Anaemia, Neurodegeneration, Diabetes
Tyrosinase	Melanin synthesis	Loss of pigmentation: albinism
Lysyl oxidase	Covalent cross-linking of collagen and elastin	Arterial aneurysms, cardiovascular dysfunction [9]
Peptidylglycine α amidating mono-oxygenase	Activation of peptides with α terminal glycine	Endocrine dysfunction, lethality [10]
Dopamine β hydrolase	Norepinephrine synthesis	Hypoglycaemia, hypotension
Coagulation Factors V and VIII	Blood clotting	Haemophilia
Hephaestin	Ferroxidase	Anaemia, impaired iron absorption [11]
Nitrous oxide reductase	Reduction of N_2O/N_2 in denitrification pathway of bacteria	Respiratory deficiency, imbalance in nitrogen cycle

creating the highly reactive hydroxyl radical $OH^{\bullet}$ according to the reaction:

$$H_2O_2 + Cu^{+} \rightarrow OH^{-} + OH^{\bullet} + Cu^{2+}$$

$OH^{\bullet}$ (half-life 1 ns) instantaneously reacts with all the cellular components, sugars, proteins, membrane lipids and nucleic acids, therefore inhibiting the cell machinery.

Additionally, copper may manifest its toxicity by displacing other metal ions in a number of structural or catalytic motifs. For example, the replacement of Zn^{2+} by Cu^{2+} in the DNA zinc-finger domain of the human estrogen receptor totally inactivates the receptor [12]. This apparent contradiction between "absolutely required and highly toxic" is rationalized by the presence of a number of small proteins which bind Cu(I) ions. These small proteins allow the storage and the transport of copper ions through cellular compartments as stable and non-toxic complexes. However, agriculture takes advantage of this toxicity, as copper is used as a parasiticide.

Copper metabolism

On average the adult human body contains 110 mg of copper and the daily intake is 1–3 mg which meets body needs. About 15% of the copper taken up from the diet are retained. The remaining 85% are excreted and because the only physiological mechanism for copper elimination is the biliary excretion, liver plays a central role in copper homeostasis. Copper is absorbed via the intestinal epithelium into the blood circulation, where it is bound to albumine, transcupreine or histidine and forms an exchangeable pool of Cu(II). Rapidly removed from the circulation, copper enters the liver from where it is dispatched in the whole body. The liver is a central organ in copper metabolism as it receives all dietary copper and regulates the whole body copper content by excretion in the bile.

Cellular copper homeostasis

Cellular copper homeostasis requires a delicate balance between uptake, distribution, storage and export. This maintenance requires a specific family of soluble copper binding proteins that drive copper towards its intracellular targets. Because yeast cells represent the simplest eukaryotic organism and is easy to manipulate, most of the proteins involved in copper homeostasis were first identified in *Saccharomyces cerevisiae*.

In yeast, copper is taken up by transporters of the Ctr family which possess a high affinity for Cu(I). At the plasma membrane and prior to uptake, reduction of Cu(II) occurs through the reductases Fre1 and Fre2, which also reduce Fe(III) in Fe(II) to allow iron uptake by Ftr1. After reduction, Cu(I) enters the cell via Ctr1 and Ctr3, two membrane proteins transporting Cu(I) via a strictly conserved Met-XXX-Met copper binding motif. This motif, located in the second transmembrane helix, participates in Cu(I)-coordination during its transport [13]. Ctr1 and Ctr3 have a micromolar affinity for Cu(I) and it has been proposed that Cu(I) uptake is coupled to a K^{+} efflux with a 1:2 stoichiometry [14]. In case of copper deficiency a genic regulation enhances Ctr1 and Ctr3 transcription thus facilitating copper uptake. When copper is too abundant, the Ctr1 and Ctr3 transporters undergo endocytosis and degradation thus abolishing copper uptake [15]. In humans, the molecular pathway for copper uptake is not yet well understood although hCtr1, the homologue to Ctr1, has been found in many tissues. However, in suckling mice, Ctr1 has been found in the intestinal epithelium, that is a propitious situation for copper uptake, but this localization disappears in adult mice [16].

Copper sequestration

To prevent Cu(I) toxicity, there is virtually no free copper ion available in a cell [17]. The free Cu(I) concentration does not exceed 10^{-18} M, that is less than one atom per cell. Upon entering the cytosol, all Cu(I) is quickly complexed to millimolar glutathione (GSH), a molecule which contains one cysteine (γ-Glu-Cys-Gly). The Cu(I)-GSH complex can donate Cu(I) to various cytosolic proteins, such as metallothioneins. The latter are cysteine-rich proteins possessing several Cys-XX-Cys and/or Cys-X-Cys sequences. The repeated sequences confer to metallothioneins the capacity to bind many atoms of Cu(I) coordinated by 2–3 thiols, for instance Cup1 from yeast binds 8 Cu(I) with 10 cysteines [18].

Copper distribution and transport in the cell

Cu(I) bound to glutathione is available for another family of thiol-rich proteins called metallo-chaperones. The function of a metallochaperone is to deliver Cu(I) to another protein which is its specific target. Three copper chaperones have been identified in human and yeast. In the cytosol, Ccs contains a Met-X-Cys-XX-Cys copper binding motif and specifically delivers Cu(I) to the Cu/Zn superoxide dismutase SOD1 [19]. This enzyme has a key role in the defense against oxidative stress, converting the superoxide in H_2O_2 and O_2, using copper as cofactor. Cu(I) also enters the mitochondria via Cox 17 another cysteine-rich chaperone. Cox17 governs the incorporation of Cu(I) into Cco, the cytochrome c-oxidase assembly [20, 21]. Cco is the terminal oxidase that performs the four-electron-reduction of one oxygen molecule in two water molecules.

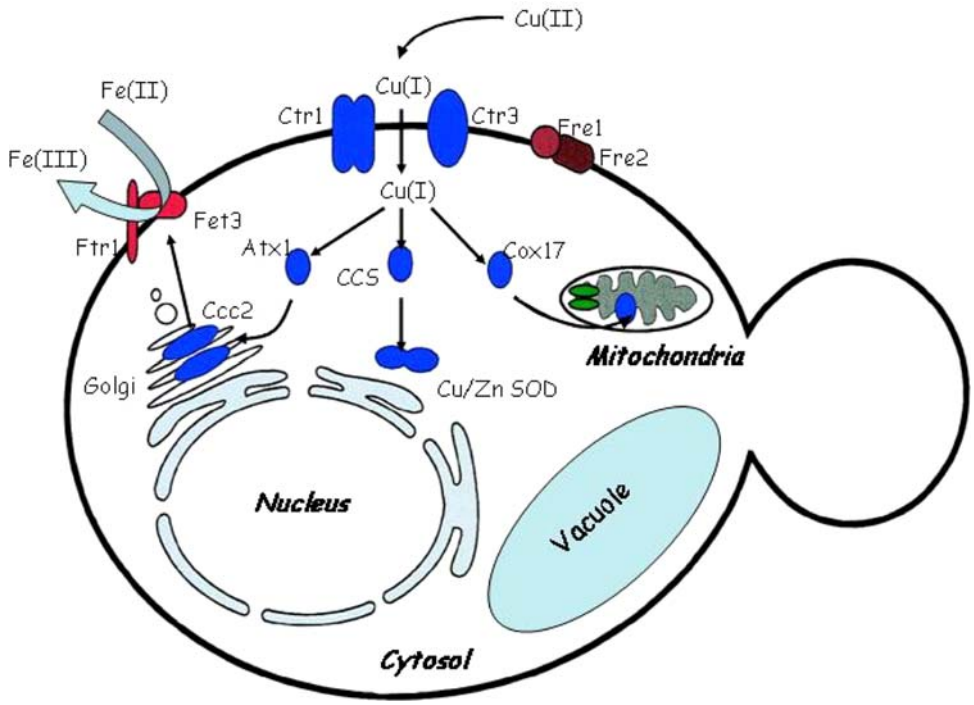

Fig. 1 Copper metabolism in yeast. Prior to uptake, copper ions are reduced by the plasma membrane reductases Fre1 et Fre2. Reduced copper is then transported by two membrane proteins Ctr1 and Ctr3 of micromolar affinity. Within the cell, copper is bound and dispatched among copper chaperones Ccs, Cox17 and Atx1 for copper delivery to specific targets. In the cytosol, Ccs delivers copper to SOD1; in the mitochondria, copper delivered by Cox17 is incorporated in the cytochrome-c oxidase assembly; copper bound to Atx1 is transferred to Ccc2 which in turn, transports copper into the Golgi where it is incorporated in the multicopper ferroxydase, Fet3. Ftr1, an iron permease forms with Fet3 a complex which migrates to the plasma membrane for iron uptake

The third chaperone, called Atx1 in yeast or Atox1 in humans, delivers Cu(I) to the Golgi apparatus. This organelle is the place from where newly synthesized proteins are sent to the plasma membrane or secreted out of the cell. Any of these enzymes that needs copper will incorporate its cofactor in the Golgi. In yeast, Atx1 delivers Cu(I) to Ccc2, a membrane protein which transfers Cu(I) across the membrane into the Golgi. The same delivery pathway is found in mammalian cells, which express two different proteins equivalent to Ccc2, named ATP7A and ATP7B. Atx1 and Atox1 contain the same Met-X-Cys-XX-Cys functional motif as Ccs, a motif which is also present in the amino-terminal domain of Ccc2, ATPA and ATP7B. Cu(I) is exchanged from the Met-X-Cys-XX-Cys motif of the chaperone to the same motif on the membrane protein [22, 23]. In yeast, Atx1 delivers Cu(I) to Ccc2 which transfers it across the Golgi membrane. In the lumen, Cu(I) incorporation in the ferroxidase Fet3 is required for the Fet3/Ftr1 complex to be sent to the plasma membrane (Fig. 1). In other words, copper is necessary for iron homeostasis and this property is verified in most living organisms. It should be emphasized here the differences between the Cu(I) binding site of the chaperones, which participate to Cu(I) transfer across the cell, and the co-factor site in cuproenzymes (Fet3, SOD1, Cco). The Met-X-Cys-XX-Cys site is found at the surface of the proteins to facilitate exchanges, whereas the catalytic site is generally embedded in the enzyme.

All organisms possess ion motive pumps which are specific proteins called P-type ATPases. They are involved in the active transport of a large set of ionic species through biological membranes. The ion transport is tightly coupled to the reaction of ATP hydrolysis that provides the energy required for the ion to move against its electrochemical gradient. The energy is provided by the transfer of the ATP γ-phosphate to the protein (ATP stands for adenosine triphosphate). The Cu(I)-ATPases comprise three main parts. The first part, at the N-terminus, consists in a variable number of copper binding domains bearing the Met-X-Cys-XX-Cys sequences which receive Cu(I) from the chaperone. The second part is the catalytic unit where the phosphoryl transfer occurs between ATP and the protein. The last part is the membrane domain which represents the ionic pathway. It is made of eight transmembrane spans organized as a bundle, containing the Cu(I) transport site, a Cys-Pro-Cys motif found in all Cu(I)-ATPases (Pro stands for proline). The molecular mechanism by which Cu(I) crosses the membrane is still a matter of debate but there is a consensus about the following scenario. The Cu(I) ion bound at the *N*-terminus is transferred to the membrane site

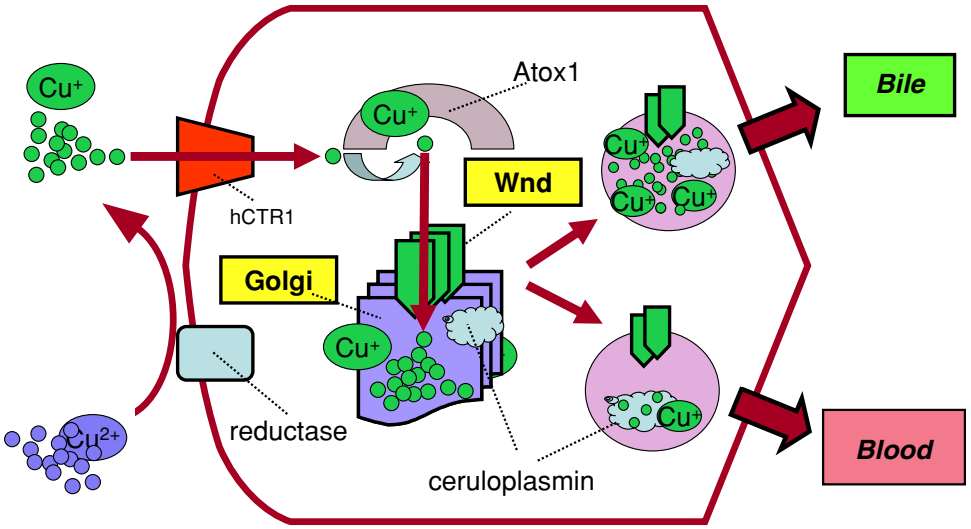

Fig. 2 Model for copper homeostasis in hepatocytes. Reduced copper enters hepatocytes via the hCtr1 high affinity copper transporters. At variance with yeast, the reductases have not been clearly identified; at the moment, the Steap proteins are good candidates. In the cytosol, the pathways are similar to those described in Fig. 1 and not reported here. At normal or low copper ATP7B (Wnd) located in the Golgi apparatus receives copper from Atox1 to deliver it to ceruloplasmin. Ceruloplasmin is then excreted into the blood for copper redistribution in all tissues. When copper is in excess, vesicles formed from Golgi apparatus and containing large amounts of copper, drive copper towards the biliary canaliculus for detoxification

[24]. This step allows the phosphoryl transfer reaction to take place at the catalytic site and the energy is used to dissociate Cu(I) towards the other side of the membrane, the Golgi lumen. Copper is now available for newly synthesized proteins on their way to the plasma membrane.

Copper balance in the body

In humans, the two Cu(I)-ATPases ATP7A and ATPB are similar to Ccc2 except for the *N*-terminus which contains six metal binding domains (Met-X-Cys-XX-Cys) instead of two in Ccc2. They are localized in the Golgi membrane for incorporation of Cu(I) into cuproenzymes (as Fet3 in yeast) such as ceruloplasmin, tyrosinase, lysyl oxidases (Table 1). Among these cuproenzymes, ceruloplasmin which binds 6–8 copper ions is secreted into the blood to dispatch copper in all tissues. In addition to their function in delivering copper to cuproenzymes, when intracellular copper level is too high for the cell, ATP7A and ATP7B translocate in vesicles formed from the Golgi membrane to fuse with the plasma membrane [25]. This allows pumping of excess copper out of the cells.

Cells belonging to epithelia, such as intestine (enterocytes) or liver (hepatocytes), are polarized. This property describes the fact that epithelia are barriers between the external medium (intestine lumen or biliary canaliculus) and the body internal medium. In an epithelial cell, the apical membrane, i.e. the portion of the membrane that is in contact with the external medium, contains proteins that are not found in the basolateral membrane, i.e. the rest of the cell membrane. In enterocytes, ATP7A migrates from the Golgi to the basolateral membrane to ensure copper uptake by the body. In hepatocytes, ATP7B delivers copper through the apical membrane to the biliary canaliculi to ensure detoxification of the body.

ATP7A is expressed in all tissues, particularly in the brain and the heart, except for the liver where ATP7B is predominant. The latter is also expressed in the brain, although in small amounts. Both genes were discovered in 1993 by independent laboratories and since then, hundreds of mutations were identified. The importance of ATP7A and ATP7B in copper homeostasis is illustrated by the two genetic diseases arising from these mutations, which promote a dysfunction of the ATPases and are sources of severe diseases, the Menkes syndrome for ATP7A and the Wilson disease for ATP7B [26]. The Menkes syndrome results in copper accumulation in intestinal cells and a copper deficiency in blood. As a consequence, essential cuproenzymes lack their cofactor and death usually occurs during early childhood. The only known therapy consists in a treatment with copper histidine [27]. The Wilson disease in contrast results in copper overload in the liver and brain with risks of cirrhosis and neurological problems as main consequences. This disease is fatal in absence of treatment. To fight copper toxicosis, patients limit ingestion of dietary copper and take copper chelators. Another therapy consists in taking Zn(II) to stimulate the amount of metallothioneins [28]. Figure 2 illustrates copper metabolism in hepatocytes.

ATP7A and ATP7B have a similar role in regulating cell copper homeostasis. They pump Cu(I) into the Golgi where it is incorporated into secreted enzymes as cofactor. Copper balance in the body is ensured by ATP7A which allows copper assimilation across the intestine and by ATP7B which carries out copper excretion in the bile.

Conclusion

Although many aspects of copper homeostasis remain unclear in mammals, *Saccharomyces cerevisiae* has been fruitfully used to get the general idea of copper homeostasis at the cellular level. This knowledge has opened the way for researches on the role of copper in a number of inflammatory and/or neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson's, prion or Alzheimer's disease … [29]. This kind of research is now very active in the biological and medical communities. Herein, in addition to an overview on copper homeostasis, we wanted to describe the cascade of molecular events allowing the cell to handle the metabolism of a toxic ion.

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