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Review

Cellular copper management—a draft user's guide

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This article is dedicated to Fausto Calderazzo—a great maestro of general chemistry, an illustrious scientist, and a man of outstanding morality who has inspired my life.

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ABSTRACT

Copper is essential to many organisms as a cofactor for many proteins and enzymes involved in key biological processes such as respiration and protection from oxidative stress. However, as copper is potentially toxic to living systems, regulatory mechanisms have evolved for its acquisition, trafficking, and release. These mechanisms, whose malfunction is typically associated with severe cellular damage, rely on the concerted action of protein systems that implement mechanisms for copper homeostasis and usage. The ensemble of copper proteins in given organisms can now be predicted with bioinformatics methods from an analysis of amino acid sequences. This work has endeavored to study the copper binding sites in these proteins, and to classify them based on their structural features. When associated with information on occurrence throughout the domains of life and intracellular localization, some generalized perspectives on copper management emerge that may provide a basis for the creation of models of cellular copper metabolism within a systems framework.

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1. Introduction

Copper is essential for most living systems in all kingdoms, as it is a cofactor for several physiologically critical proteins and enzymes, including cytochrome *c* oxidase (COX) and copper–zinc superoxide dismutase (SOD) [1]. However, copper can also be toxic to cells, due to its redox activity and its affinity for binding sites that should be occupied by other metals. Nature has therefore evolved complex homeostatic and protective mechanisms to tackle the challenge of controlling cellular copper concentration and speciation [2,3]. Throughout the biological world, organisms must overcome similar challenges related to copper management in order to achieve a wide range of goals, from the simple efflux of unwanted copper in certain prokaryotes to the often complicated and widespread use of copper in higher-order eukaryotes.

Copper handling is estimated to involve almost half of the copper proteome of a given organism [4]. Current knowledge indicates that these proteins work in concert to regulate and mediate copper uptake, distribution and efflux by sensing copper levels, delivering copper to specific target proteins, and pumping copper across the membranes of cells and cellular compartments [5–8]. There is increasing awareness that impairment of these mechanisms can lead to severe alterations in copper metabolism and the subsequent development of disease [9–12], thus motivating a quest to understand the complex molecular machinery responsible for copper management.

The ensemble of copper proteins in given organisms can now be predicted with bioinformatics methods from an analysis of amino acid sequences [13]. A considerable amount of structural data is also now available on a range of these copper proteins. Structural information can be used to obtain clues on the structure–function relationships that exist for given classes of copper proteins, not only with regard to long-known enzymes and electron transfer proteins, but also to more recently characterized proteins involved in copper homeostasis. This picture can be substantially enriched by integrating information on the cellular compartments where copper proteins are localized and on the organisms which possess them, allowing copper proteins to be viewed and examined in the context of living cells.

The present work recounts a systematic analysis of copper sites in proteins with known structures, and their categorization into a relatively small number of structural motifs. This may prove useful in predicting the ability of individual proteins to bind copper, as well as provide hints on their function. These structural sets have been associated with information on their cellular localization, and individual function when known. This permits the description of the basic components of the copper utilization systems acting in living organisms. The analysis of the occurrence of copper proteins in 57 representative organisms has provided a platform on which to discuss how these components may work together in biological systems, suggesting potential model pathways for copper management throughout the three domains of life (archaea, bacteria and eukaryotes).

2. Methods

All available copper protein structures were downloaded by querying the Protein Data Bank (PDB) [14] for entries containing at least one copper atom. Copper sites in these proteins were identified by taking all the copper atoms in the corresponding PDB files, and considering copper atoms at a distance of less than 5.0 Å from one another as belonging to the same site. For each site, copper-binding residues were defined as those having a non-hydrogen atom at a distance of less than 3.0 Å from any copper atom in the site.

Copper sites were grouped based on the SCOP classification [15] of the protein domains containing them. In the SCOP database, proteins with known structures are hierarchically classified into families (grouping together proteins with a clear evolutionary relationship), superfamilies (grouping together proteins with probable common evolutionary origin) and folds (grouping together proteins with major structural similarity) [16]. For this work, each copper site was assigned to the SCOP family (corresponding to a four-digit code) of the protein domain containing the copper-binding residues of the site defined as above, and copper sites assigned to the same SCOP family were grouped together. The SSM server (http://www.ebi.ac.uk/msd-srv/ssm) [17], which compares protein structures with the whole SCOP archive, was used to assign sites of proteins that have not yet been included in the SCOP database. In cases where SSM searches with default settings did not match any SCOP-assigned protein, the site was left unassigned. The relevant literature was examined to annotate the functions of grouped copper sites and to identify non-physiological copper sites, such as sites in metalloproteins where copper has been substituted for the native metal ion (e.g., carbonic anhydrase, PDB code 1rzc) [18], or non-specific sites due to adventitious binding of copper to the protein (e.g., thioredoxin, PDB code 2trx) [19].

Representative copper sites were selected by dividing each group into subgroups of sites found in proteins sharing at least 50% sequence identity, and then choosing the PDB structure in each subgroup with the highest resolution. The protein clusters at 50% sequence identity were taken from the PDB (ftp://ftp.wwpdb.org/pub/pdb/derived_data/NR). For each representative copper site, a copper-binding pattern was defined by mapping the copper-binding residues onto the protein sequence. Copper-binding patterns are represented as sequence motifs AX(n)BX(m)C..., where A, B, C... are the copper-binding residues and n, m... are the number of residues in between two subsequent copper-binding residues. Each copper-binding residue was also associated to the secondary structure element in which it is found in the PDB structure, as taken from the secondary structure section of the PDB file (see http://www.wwpdb.org/documentation/format23/sect5.html for the format of this section). For each group of copper sites, a single variable pattern was then defined by manually aligning the copper-binding patterns of all the representative sites in the group, and calculating the percent occurrence of different secondary structures for each copper-binding residue (e.g., if a given copper-binding residue is found in a helix in three out of four representative structures, and in a loop in the fourth, then its associated secondary structure is 75% helix and 25% loop). This combined information was visualized as sequence logos [20] where the one-letter codes of copper-binding residues are stacked on top of each other for each position in the pattern, and the height of each letter is made proportional to its frequency, distinguishing between the same residues in different secondary structures.

The InterPro (http://www.ebi.ac.uk/interpro) [21] and UniProt (http://www.uniprot.org) [22] databases, in combination with literature mining, were used to annotate the sub-cellular localization of the copper proteins relevant to this work (i.e., which have a PDB structure available). In cases where information on the sub-cellular localization could not be retrieved, it was predicted using the SubLoc server (http://www.bioinfo.tsinghua.edu.cn/SubLoc) [23]. The occurrence of homologues of these copper proteins was evaluated in 57 representative organisms using the results of bioinformatics predictions of copper proteomes previously made by our group and available in the literature [4].

3. Results

As of August 2008, the entire PDB contained 617 structure entries with at least one copper atom for a total of 1059 copper sites. As described in the Methods section, copper sites found in proteins of the same SCOP family were grouped together, and one or

Table 1
Summary of the copper proteins analyzed in this work, grouped based on the type of copper site they contain. This table reports (i) the category in which the proteins were classified, (ii) the SCOP family to which the proteins belong, (iii) the name, (iv) the PDB code of the selected representatives (see Section 2 for details on how representatives were selected), (v) the general function of the proteins, (vi) the number of copper ions in the site, and (vii), (viii), (ix), and (x) the subcellular localization(s) of the proteins in archaea, Gram-positive bacteria, Gram-negative bacteria and eukaryotes, respectively.

Category	SCOP family	Proteins	PDB codes	Functions	# of Cu	Archaea	Gram + bacteria	Gram – bacteria	Eukaryotes
Cu(I) sites									
Transcription factors	DNA-binding N-terminal domain of transcription activators (a.6.1.3)	CueR	1q05	Transcription regulation	1		Cytoplasm	Cytoplasm	
	Not classified	CsoR	2hh7	Transcription regulation	1	–	Cytoplasm	Cytoplasm	–
Copper transport proteins	HMA, heavy metal-associated domain (d.58.17.1)	Atx1 HAH1 CopZ Ccc2[a] ATP7A[b] ATP7A[b] ATP7A[b] CopA[c]	1fd8 1fee 1k0v 1fvs 1kvj 1s6u 1yjv 1kqk	Copper transport	1	Cytoplasm, membrane	Cytoplasm, membrane	Cytoplasm, membrane	Cytoplasm, membrane
	COX17-like (a.17.1.2)	Cox17	2rnb	COX assembly	1				Mitochondrial intermembrane space
	Glutathione peroxidase-like (c.47.1.10)	Sco1	2gqm	COX assembly	1	Extracellular (membrane-anchored)	Extracellular (membrane-anchored)	Periplasm (membrane-anchored)	Mitochondrial intermembrane space (membrane-anchored)
	Metallothionein (g.46.1.1)	Cu-MT	1rju	Copper storage	8	–	–[d]	–	Cytoplasm
Periplasmic copper transport proteins	Copper resistance protein C (CopC, PcoC) (b.1.18.17)	CopC[e]	2c9q	Copper transport	1		Membrane	Periplasm	
	DR1885-like metal-binding protein (b.2.10.1)	DR1885	1x9l	COX assembly	1	Cytoplasm	Cytoplasm	Periplasm	
	Not classified	CusF	2vb2	Copper transport	1	–	–	Periplasm	–
Complex catalytic sites	Molybdenum cofactor-binding domain (d.133.1.1)	CODH	1n62	Enzymatic catalysis	1[f]		Cytoplasm	Cytoplasm	
	Acetyl-CoA synthase (e.26.1.3)	CODH/ACS	1mjg	Enzymatic catalysis	1[g]	Cytoplasm	Cytoplasm	Cytoplasm	
Sites where Cu changes oxidation state									
Type 1 -like sites	Plastocyanin-azurin-like (b.6.1.1)	Pseudoazurin Stellacyanin Plastocyanin Plastocyanin Auracyanin Mavicyanin Umecyanin Auracyanin A Rusticyanin Phytocyanin Azurin II Plastocyanin Amicyanin	1bqk 1jer 1kdj 1plc 1qhq 1ws8 1x9u 2aan 2cak 2cbp 2ccw 2gim 2ov0	Electron transfer	1	Extracellular	Extracellular	Periplasm, thylakoid	Membrane, chloroplast thylakoid
	Multidomain cupredoxins (b.6.1.3)	Ascorbate ox. CotA Nitrite red. CueO Nitrite red. Fet3P Nitrite red.	1aoz 1gsk 1kbv 1kv7 1snr 1zpu 2dv6	Enzymatic catalysis	1	Cytoplasm, extracellular	Cytoplasm, spore coat, extracellular	Periplasm, extracellular	Membrane, extracellular

		Phenox. synth. Ceruloplasmin Laccase Laccase	2g23 2j5w 2q9o 2qt6						
	Periplasmic domain of cytochrome *c* oxidase subunit II (b.6.1.2)	COX COX COX	1v54 2cua 2gsm	Enzymatic catalysis	2[h]	Membrane	Membrane	Inner membrane	Mitochondrial inner membrane
	Nitrosocyanin (b.6.1.4)[i]	N_2O reductase	1fwx	Enzymatic catalysis	2[h]	–	–	Periplasm	–
	Nitrosocyanin (b.6.1.4)[i]	Nitrosocyanin	1iby	Electron transfer (?)	1	–	–	Periplasm	–
Type 2-like sites	Cu, Zn superoxide dismutase-like (b.1.8.1)	SOD SOD SOD SOD	1pzs 1q0e 2aqm 2v0a	Enzymatic catalysis	1[j]	Extracellular	Extracellular	Periplasm	Cytoplasm, mitochondrial intermembrane space, extracellular
	Amine oxidase catalytic domain (b.30.2.1)	Amine oxidase Amine oxidase Amine oxidase Amine oxidase Amine oxidase Lysyl oxidase	1ksi 1oac 1tu5 1w6g 2oqe 1w7c	Enzymatic catalysis	1[k]	–	–	Periplasm	Extracellular
	Quercetin 2,3-dioxygenase-like (b.82.1.5)	Quercetin diox.	1juh	Enzymatic catalysis	1	–	–	–	Extracellular
	Peptidylglycine alpha-hydroxylating monooxygenase PHM (b. 121.1.2)	PHM[l] PHM[l]	1sdw 1sdw	Enzymatic catalysis	1	–	–	–	Extracellular
	Galactose oxidase, central domain (b.69.1.1)	Galactose ox.	1gof	Enzymatic catalysis	1	–	–	–	Extracellular
	Not classified	pMMO[m]	1yew	Enzymatic catalysis	1	–	–	Inner membrane	–
	Multidomain cupredoxins (b.6.1.3)n	Nitrite red. Nitrite red. Nitrite red.	1kbv 1snr 2dv6	Enzymatic catalysis	1	Extracellular	–	Periplasm	Extracellular
	Multidomain cupredoxins (b.6.1.3)[n]	Ascorbate ox. CotA CueO Fet3P Phenox. synth. Ceruloplasmin Laccase Laccase	1aoz 1gsk 1kv7 1zpu 2g23 2j5w 2q9o 2qt6	Enzymatic catalysis	3	Cytoplasm, extracellular	Cytoplasm, spore coat, extracellular	Periplasm, extracellular	Membrane, extracellular
	Nitrous oxide reductase, N-terminal domain (b.69.3.1)	N_2O reductase	1fwx	Enzymatic catalysis	4[o]	–	–	Periplasm	–
Type 3-like sites	Hemocyanin middle domain (a.86.1.1)	Hemocyanin Hemocyanin Hemocyanin Hemocyanin	1hc1 1js8 1lla 1lnl	Oxygen transport	2	–	–	–	Extracellular
	Catechol oxidase (a.86.1.2)	Catechol ox.	2p3x	Enzymatic catalysis	2				Chloroplast thylakoid
	Not classified	Tyrosinase	1wx3	Enzymatic catalysis	2	–	Membrane	Membrane	Membrane

Table 1 (*Continued*)

Category	SCOP family	Proteins	PDB codes	Functions	# of Cu	Archaea	Gram + bacteria	Gram – bacteria	Eukaryotes
Membrane sites	Cytochrome *c* oxidase subunit I-like (f. 24.1.1)	COX COX COX COX	1fft 1v54 1xme 2gsm	Enzymatic catalysis	1[p]	Membrane	Membrane	Inner membrane	Mitochondrial inner membrane
Cu(II) sites	S100 proteins (a.39.1.2)	S100A12	1odb	Immune response	1	–	–	–	Cytoplasm, extracellular
	Copper resistance protein C (CopC, PcoC) (b.1.18.17)	CopC[e]	2c9q	Copper transport	1		Membrane	Periplasm	
	Not classified	APP	2fk1	Unknown[q]	1	–	–	–	Membrane, extracellular
Other sites	YhcH-like (b.82.2.7)	YhcH	1s4c	Unknown	1	–	Cytoplasm	Cytoplasm	–
	Not classified	MopE	2vov	Unknown	1	–	–	Outer membrane	–

[a] The structure refers to the first of the two HMA domains contained in Ccc2.
[b] The structures refer to the first, second and sixth of the six HMA domains contained in ATP7A, respectively.
[c] The structure refers to the second of the two HMA domains contained in CopA.
[d] A copper-binding metallothionein has recently been identified in *Mycobacterium tuberculosis* [56].
[e] CopC has both a Cu(I) and a Cu(II) site.
[f] Copper is part of a cluster that also contains molybdenum and inorganic sulfur.
[g] Copper is part of a cluster (termed A-cluster) that also contains nickel and an FeS center.
[h] Cu_A sites, where the oxidized Cu(II)–Cu(I) species is a fully delocalized mixed-valence pair with two $Cu^{1.5+}$ ions.
[i] Nitrous oxide reductase and nitrosocyanin have been placed into different groups although they are classified in the same SCOP family.
[j] Copper is part of a dinuclear site that also contains zinc.
[k] The active site contains a trihydroxyphenylalanine quinone (TPQ) cofactor which originates from a Tyr ligand.
[l] PHM has two distinct type 2 sites.
[m] pMMO may also contain a dinuclear copper site, whose occurrence, however, is uncertain.
[n] The mononuclear sites of nitrite reductases and the trinuclear sites of multicopper oxidases have been placed in different groups.
[o] CuZ site, which also contains inorganic sulfur.
[p] CuB site, which also contains a heme group.
[q] APP has been implicated in a variety of processes in addition to copper homeostasis, including neurogenesis, neuronal regeneration, apoptosis and transcriptional regulation.

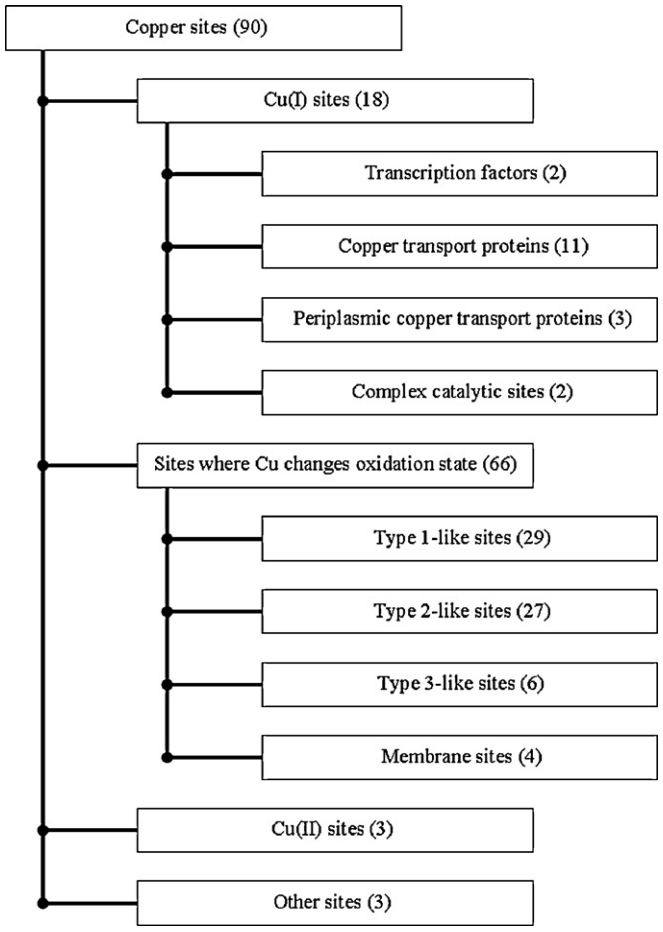

Fig. 1. General divisions of copper sites. Copper sites are primarily grouped by the oxidation state of copper, and then by broad functional and structural categories. The numbers in parentheses indicate the number of representative copper sites in the group.

more representative copper sites were selected from each group to account for the variation in copper-binding patterns within a group. This procedure resulted in the definition of 35 groups comprising a total of 90 representative copper sites. Each group was annotated with information on (i) the number of copper atoms in the site, (ii) the proteins containing the representative sites of the group, (iii) the functions of these proteins, (iv) their SCOP family, and (v) their intracellular localization in archaea, bacteria (Gram-positive and Gram-negative) and eukaryotes (Table 1). These groups were further classified into broader categories based on structural and functional similarities, and most generally on the oxidation state of copper (i.e., Cu(I), Cu(II), or sites where Cu changes oxidation state) as shown in Table 1 and Fig. 1.

The copper-binding residues of representative sites were examined with regard to the patterns in which they occur in the protein's amino acid sequence (e.g., CX(2)C for two Cys ligands separated by two residues) and the secondary structure elements in which they are located (i.e., helix, sheet, or loop). As described in Section 2, a combined pattern was defined for each group to simultaneously describe the copper-binding patterns of all of the representative sites of the group. These combined patterns are diagrammatically depicted in Figs. 2–7, where the one-letter codes of copper ligands are colored according to their secondary structure (helix, sheet, and loop are shown by red, cyan, and yellow, respectively) and the vertical size of the letters represents the percent occurrence of a given ligand in a given secondary structure. The percent occurrences of specific copper-binding residues and secondary structures in selected categories of copper sites are shown in Fig. 8.

Based on the above grouping, the occurrence of copper proteins in 57 representative organisms spanning the entire tree of life (5 eukaryotes, 12 archaea and 40 bacteria), as inferred by bioinformatics predictions, was analyzed (Fig. 9). A list of the 57 organisms including their taxonomic classification is given in Table S1.

4. Discussion

Copper has a dual nature as an essential yet dangerous metal ion; it is a necessary cofactor for many proteins and enzymes in a variety of key biological processes, but it can also be highly toxic. Copper's toxicity is partly due to its potential for engaging in oxidation–reduction reactions, which can drive the creation of reactive free radicals that can seriously damage cellular components. One of the major challenges involved in copper management by living organisms is therefore the safe exploitation of copper's redox states. Copper can also be dangerous because it has a high binding affinity for metal sites in proteins, and may occupy these sites in place of the physiological metal. Copper trafficking and distribution in cells must therefore be closely coordinated to ensure proper and safe usage. Copper uptake also constitutes a substantial physiological challenge, as extracellular copper is primarily available in the oxidized Cu(II) form, while intracellular copper appears to be maintained in the Cu(I) state [24].

When these challenges are framed within the environmental context of various cellular compartments, a framework for functional needs begins to emerge. Organisms have evolved a number of strategic mechanisms to effectively manage and exploit copper, which sometimes involve complex systems of proteins. A survey of these systems reveals similarities among copper proteins in terms of copper-binding motifs and localization that can be used to narrate some generalized mechanistic stories regarding copper handling. However, the actual existence, use, and localization of these proteins changes among organisms based on their particular situations and needs; in fact, some individual organisms do not have any copper proteins at all.

The three "chapters" of this "copper user's guide" regard three major divisions of copper proteins, i.e. Cu(I)-binding, Cu(II)–Cu(I)-cycling, and Cu(II)-binding. Cu(I)-binding proteins are typically involved in homeostasis and trafficking, while Cu(II)–Cu(I)-cycling proteins are used to drive processes such as catalysis and electron transfer. The biological functions of Cu(II)-binding proteins, if any, still remain largely unknown. In the following sections, these proteins are used as the basis to discuss copper management and utilization, attempting to establish connections between the structural features of their copper sites and their involvement in these processes, and between their occurrence in cells and the various approaches taken by living organisms to accomplish the aforementioned goals.

4.1. Limited or no copper management

Some organisms do not have mechanisms in place to deal with copper at all, as evidenced by the fact that five of the 57 organisms analyzed (*Nanoarchaeum equitans*, *Mesoplasma florum*, *Onion yellows phytoplasma*, *Chlamydia trachomatis* and *Borrelia burgdorferi*) do not possess any copper proteins (Fig. 9). According to the NCBI database (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi), all of these are obligate host-associated microbes, which may have lost all genes involved in copper metabolism as an adaptation to their lifestyle, in which many molecules can be obtained from the host. Copper proteins, however, are possibly lacking altogether

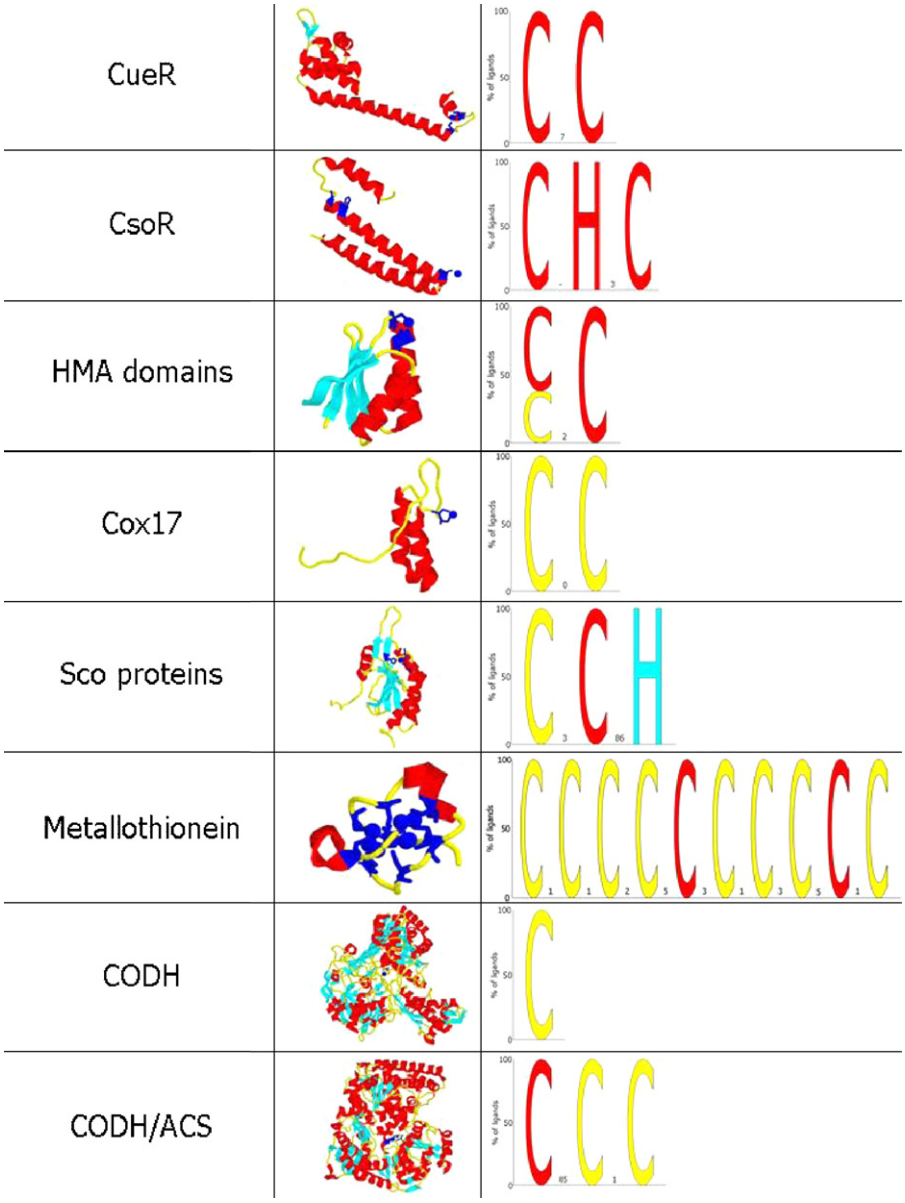

Fig. 2. Cu(I) sites of transcription factors (CueR and CsoR), copper transport proteins (HMA domains, Cox17, Sco proteins and metallothionein) and CODH and CODH/ACS enzymes (where copper is part of a Cu–S–Mo cluster and an FeS–Cu–Ni cluster, respectively). The cartoon structure of a representative protein (with helices in red, sheets in cyan, loops in yellow, copper-binding residues as blue sticks and copper atoms as blue spheres) and the copper-binding pattern of each group are shown.

also in the free-living archaea *Methanocaldococcus jannaschii* and *Methanopyrus kandleri*, because the only copper protein homologue found in these organisms is a bifunctional carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) which is likely to be a nickel-dependent rather than a copper-dependent enzyme [25].

4.2. Management of Cu(I): structures of Cu(I)-binding proteins

In 18 out of the 90 representative copper sites, copper is bound in the reduced Cu(I) state, and is kept as such. With the exception of two complex catalytic sites in which copper is found together with other metal centers (molybdenum in CODH, iron and nickel in CODH/ACS), proteins that bind Cu(I) are proteins involved in copper homeostasis and trafficking. In Fig. 1, the latter are categorized into transcription factors, copper transporters and periplasmic copper transporters.

4.2.1. Transcription factors

The copper-binding transcription factors CueR and CsoR are all-alpha proteins, and bind Cu(I) with two Cys ligands (plus one additional His in the case of CsoR) located in two different alpha-helices (Figs. 2 and 8). Helices are separated by a short loop in CueR and belong to two distinct protein chains in CsoR, where the copper site is found at the interface between two subunits of a homodimer. Therefore, although it occurs with different modalities, copper binding in both of these proteins involves two helices acting as a clamp. This binding mode may be a common theme underlying the sensing mechanisms of these factors, both of which are multimeric (CueR is a dimer, and CsoR is a tetramer) and undergo a copper-driven rearrangement of their quaternary structure that affects their interaction with DNA. In particular, copper binding to CsoR is thought to stabilize a tetramer conformation that, at variance with the copper-free form, cannot bind DNA in a form functional for transcription repression [26]. In CueR, on the other hand, copper binding is though to promote a dimer conformation that

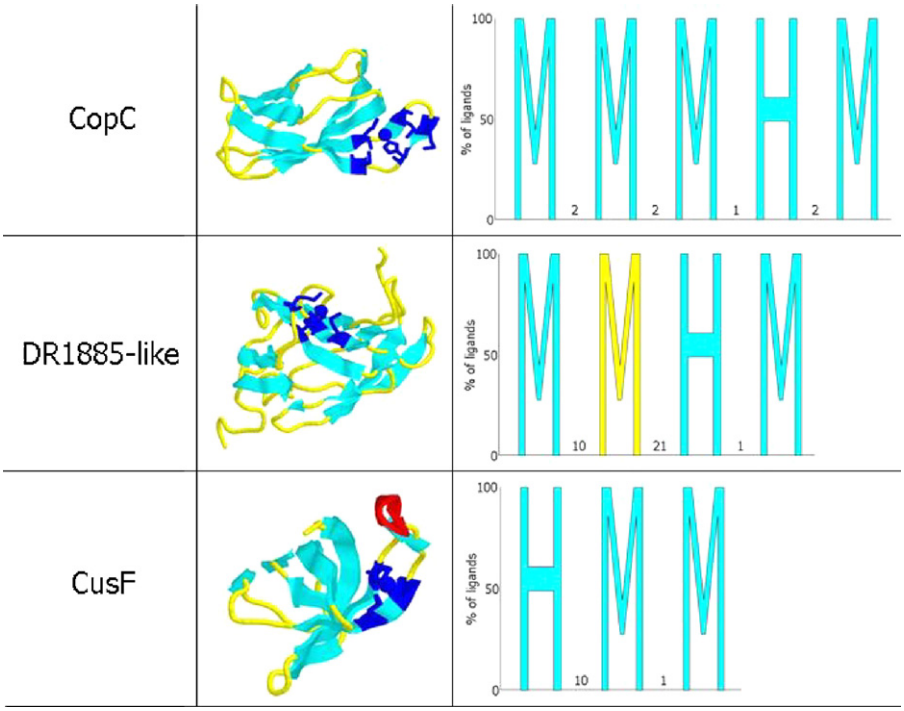

Fig. 3. Cu(I) sites of periplasmic copper transport proteins (CopC, DR1885-like and CusF). The cartoon structure of a representative protein (with helices in red, sheets in cyan, loops in yellow, copper-binding residues as blue sticks and copper atoms as blue spheres) and the copper-binding pattern of each group are shown.

is able to bind and distort DNA, allowing transcription activation [27].

4.2.2. Copper transporters

Proteins categorized as copper transporters include a number of proteins that, while structurally unrelated, use (at least) two spatially close Cys to bind copper. These include (i) single-domain copper chaperones (e.g., Atx1 [28]) and multi-domain copper-transporting ATPases (e.g., Ccc2 [29]) that share a conserved copper-binding domain which has been termed heavy-metal-associated (HMA), (ii) Cox17, (iii) Sco proteins, and (iv) metallothionein. Together, these proteins bind Cu(I) almost exclusively (97%) with Cys ligands, which are located in helices (52%) or in loops (47%) (Fig. 8).

In particular, the HMA domain found in Atx1-like and Ccc2-like proteins comprises a beta-sheet and two alpha-helices packed in a ferredoxin-like fold, and binds Cu(I) by two Cys ligands provided by the helical region of the domain (Fig. 2). The two Cys ligands are most commonly located on the same helix in a strictly conserved CX(2)C pattern (Fig. 2), thus forming a relatively rigid binding site. This binding mode is compatible with a copper transfer process that involves limited structural rearrangements in the protein partners, where re-orientation of Cys side-chains is sufficient to achieve the optimal geometry for the copper exchange reaction [30].

Cox17 is an all-alpha protein characterized by the presence of an alpha-hairpin motif, and binds Cu(I) by two Cys ligands that are both located in a loop (Fig. 2), suggesting that the copper transfer process in which it is involved requires a relatively more flexible copper site. The site plasticity of Cox17 may depend on the fact that this protein delivers copper to two other, different COX assembly factors (i.e., Cox11 and Sco1 [31]).

Sco proteins have a thioredoxin-like fold and also employ two Cys ligands for binding copper, one of which is found in a helix and one in a loop. Furthermore, Sco proteins exhibit an additional His ligand located in a beta-sheet (Fig. 2). This appears to be at variance with HMA domains and Cox17, where Cys are not found in combination with other ligands. However, it is worth noting that in both Cox17 [32] and Atx1 [33] a conserved Lys residue is located close to the copper center, most likely contributing to stabilize the overall negative charge resulting from Cu(I) binding to two cysteinate anions [34]. It is thus conceivable that Lys and His residues are to some extent equivalent in the sites of copper transporters, where they may generally act as "gating switches", stabilizing and protecting the site when copper is bound (even without directly coordinating the metal ion), and moving away from the site when copper must be acquired, or released. This functional similarity between His and Lys is also supported by the observation that a Cys-Cys-His set of copper ligands occurs in a cyanobacterial Atx1 homolog [35], for which however no PDB structure of the copper-bound protein is available.

Metallothionein represents a singular case among the proteins classified as copper transporters, in that it has a random coil structure with the loops enfolding a cluster of eight Cu(I) ions. Nonetheless, it also fulfils the general rules depicted above for Cu(I) coordination, indicating a prevalence of Cys residues in helices and/or in loops. The copper cluster is in fact coordinated by a total of 10 Cys residues, mostly located in loops (Fig. 2). Cu(I) ions are sequestered into the core of the polypeptide, supporting a role for metallothionein in copper detoxification and long-term storage [36]. Six of the eight copper ions are three-coordinated whereas the other two are two-coordinated, suggesting that the latter are bound in a relatively more labile fashion and may be transferred to other proteins [36]. It is worth mentioning here that the above criterion for Cu(I) coordination, as far as protein ligands are considered, is also applied in the complex catalytic sites of CODH and CODH/ACS, where Cu(I)-binding residues are exclusively Cys located in loops or in helices (Fig. 2).

4.2.3. Periplasmic copper transporters

Proteins categorized as periplasmic copper transporters are Cu(I)-binding proteins located in the periplasm of Gram-negative

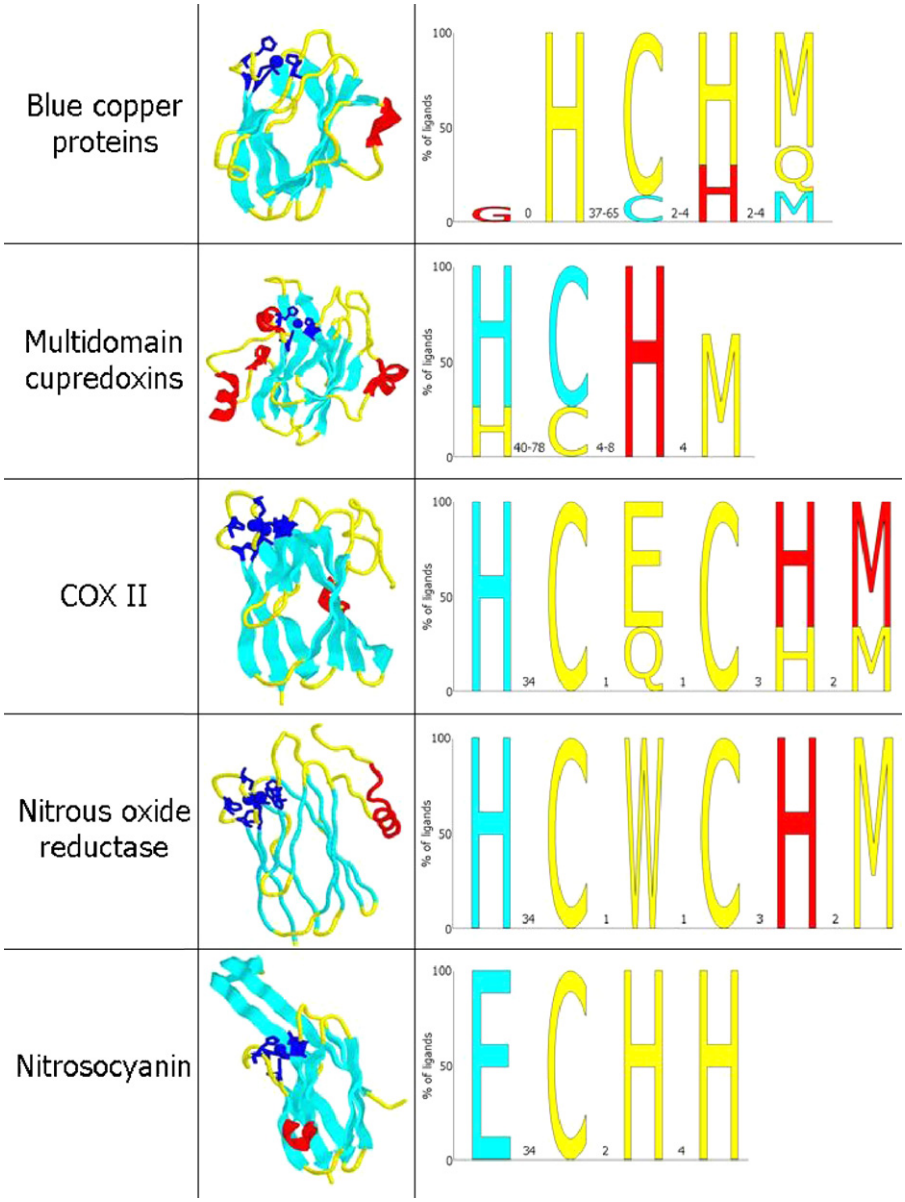

Fig. 4. Type 1-like copper sites, including the mononuclear blue sites of blue copper proteins (plastocyanin-azurin-like in Table 1) and multidomain cupredoxins, the dinuclear Cu_A sites of COX and nitrous oxide reductase, and the mononuclear red site of nitrosocyanin. The cartoon structure of a representative protein (with helices in red, sheets in cyan, loops in yellow, copper-binding residues as blue sticks and copper atoms as blue spheres) and the copper-binding pattern of each group are shown.

bacteria, including (i) CopC, which also has a Cu(II)-binding site [37], (ii) the COX assembly factor DR1885, and (iii) CusF. These proteins are strikingly different from the other copper transporters discussed above: they all exhibit beta-sandwich folds (which are instead widespread in copper-dependent enzymes, as described later), and employ a combination of Met and His to bind copper (Figs. 3 and 8). In particular, copper-binding residues comprise one His and two or three Met residues, with at least two ligands very close in the sequence (1–2 amino acids) (Fig. 3). The existence of such dissimilar types of copper transporters indicates that, in the course of evolution, organisms have developed a number of customized solutions to bind copper. In particular, the use of Met in the place of Cys is seemingly aimed at preserving S-donor ligands with high affinity for Cu(I) while increasing their resistance to oxidation. These different solutions depend on the necessity to cope with copper in cellular environments with distinct redox properties, as discussed in the following sections.

4.3. Management of Cu(I): copper homeostasis and trafficking

4.3.1. Copper efflux

"Basic" copper management systems begin with proteins that bind Cu(I); indeed, the first requirement for copper management appears to be the removal of unwanted copper from the cell. Atx1-like and Ccc2-like proteins are found throughout the domains of Life. In some organisms, Ccc2-like P-type ATPases and Atx1-like transporters form a "minimal" set of copper proteins, most probably comprising a basic copper efflux system in organisms which do not need copper to survive, but are able to remove it from the cell through the action of a carrier (the Atx1-like protein) that delivers copper to a pump (the Ccc2-like protein) for export across the membrane. Such organisms include *Archaeoglobus fulgidus*, *Thermoplasma acidophilum* and *Thermotoga maritima*, as well as *Streptococcus pyogenes*, *Thermoanaerobacter tengcongensis* and *Chlorobium tepidum*. The latter three also

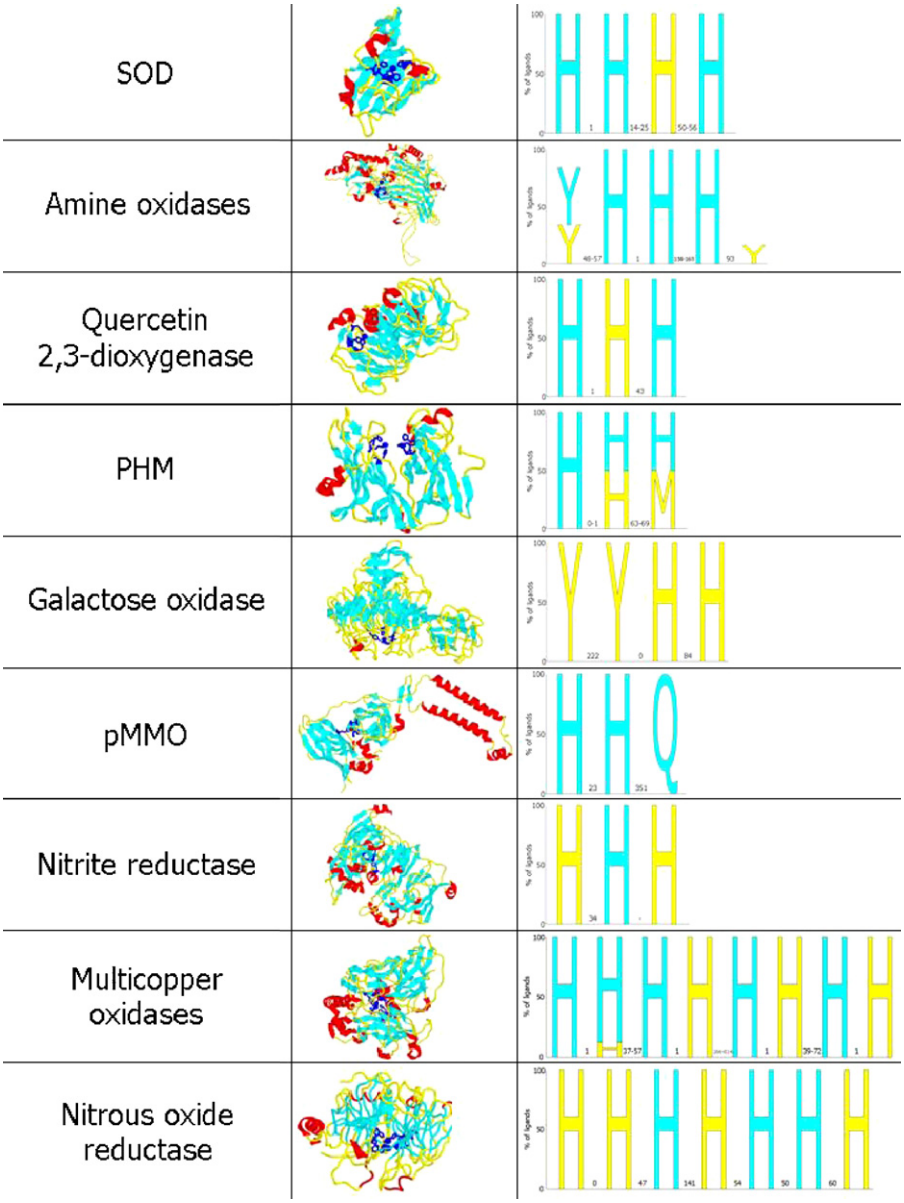

Fig. 5. Type 2-like copper sites, including the dinuclear Cu–Zn site of SOD, the mononuclear sites of amine oxidase (where Cu is coupled to TPQ), quercetin 2,3-dioxygenase, PHM, galactose oxidase, pMMO and nitrite reductase, the trinuclear site of multicopper oxidases (which belong to multidomain cupredoxins, together with nitrite reductase), and the tetranuclear Cu_Z site (which also contains S) of nitrous oxide reductase. The cartoon structure of a representative protein (with helices in red, sheets in cyan, loops in yellow, copper-binding residues as blue sticks and copper atoms as blue spheres) and the copper-binding pattern of each group are shown.

have copper-responsive transcription factors (CueR or CsoR homologues), which presumably activate the expression of the efflux system. CueR and CsoR are found in the cytoplasm of Gram-negative and Gram-positive bacteria. The other three organisms may have different transcriptional activators, or may constitutively express the efflux system at some level. Similarly, a copper transporter that is not homologous to Atx1 may be present in *Pyrococcus abyssi* and *Methanothermobacter thermoautotrophicus*, where only a Ccc2-like pump has been identified. On the other hand, only CueR and CsoR homologues have been detected in *Bifidobacterium longum*, which could rely on an alternative efflux system. It is worth noting that Atx1-like and Ccc2-like proteins bind Cu(I), although copper in the extracellular environment predominantly occurs as Cu(II). Therefore, potentially toxic copper that accidentally enters the cell must be reduced in order for this efflux system to function. In *Archaeoglobus fulgidus*, copper reduction can be performed by the Atx1-like transporter itself (called CopZ) through a redox-active, [2Fe–2S]-containing domain fused to the Atx1-like copper-binding domain [38].

There are three organisms (*Fusobacterium nucleatum*, *Bacteroides thetaiotaomicron* and *Desulfotalea psychrophila*) that, in addition to Atx1-like and Ccc2-like proteins, only possess a homologue of the poorly characterized YhcH protein [39]. Given that YhcH is highly similar to quercetin dioxygenase [40] (Fig. 10), which in some organisms is an iron-dependent enzyme, it is possible that YhcH homologues do not always bind copper. Therefore also these organisms, similarly to those mentioned above, may not require copper though being capable of exporting it from the cell. On this assumption, a total of 19 out of 57 organisms do not need copper for their metabolism. They are typically host-associated, or adapted to extreme environments, and are mainly (14 out of 19) anaerobes (Fig. 9).

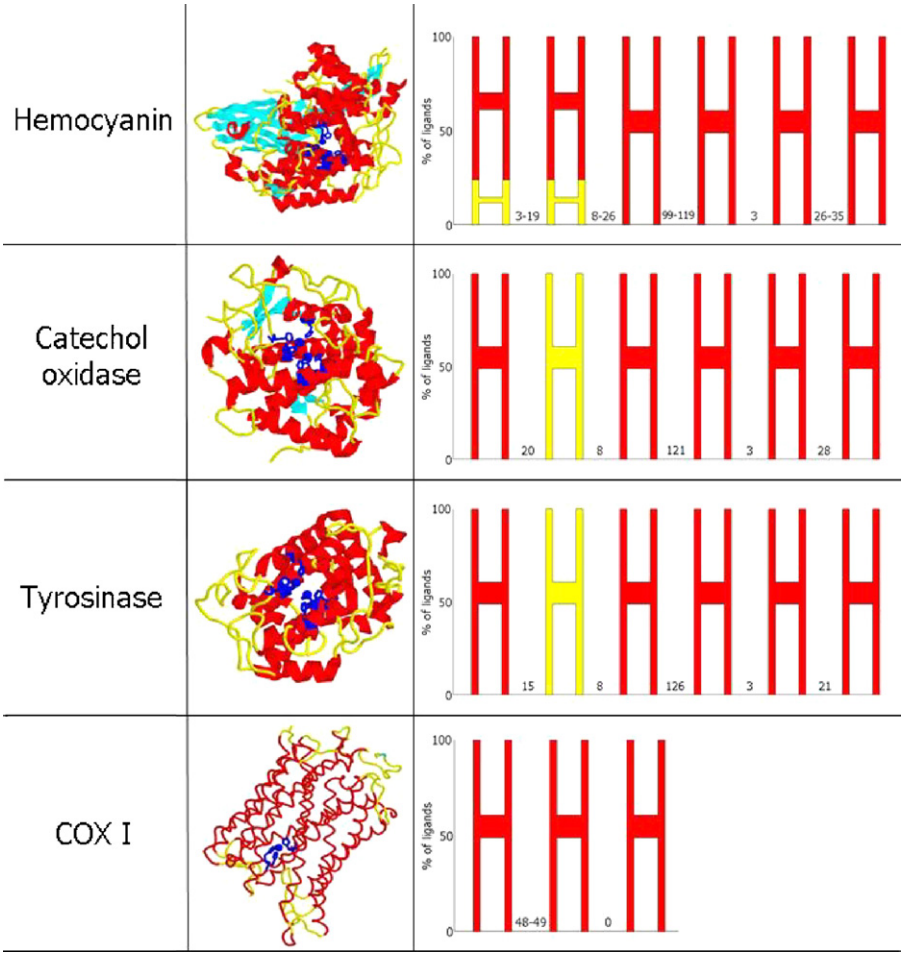

Fig. 6. Type 3-like copper sites of hemocyanin, catechol oxidase and tyrosinase, and Cu_B site of COX, categorized as a membrane site. The cartoon structure of a representative protein (with helices in red, sheets in cyan, loops in yellow, copper-binding residues as blue sticks and copper atoms as blue spheres) and the copper-binding pattern of each group are shown.

4.3.2. Copper uptake and distribution

In archaea and Gram-positive bacteria, which have a single cellular membrane, all known copper-dependent proteins are located outside the cytoplasm. These include proteins embedded in the plasma membrane and protruding in the extracellular space, such as COX, and secreted proteins, such as *Streptomyces coelicolor* tyrosinase [41]. Therefore, these organisms appear to prevent possible danger deriving from copper utilization by restricting it to processes that take place outside of the intracellular space. Given this fact, the hypothesis arises that the incorporation of copper into these proteins also occurs outside the cell membrane, subsequent to their export in the apo form. Proteins would thus acquire copper directly from the environment, in the form of Cu(II). This hypothesis, however, is unlikely for a number of reasons. In general, a simple thermodynamic process whereby metalloproteins selectively acquire the correct metal ion and exclude all others based on their binding affinities appears to be inefficient and potentially dangerous [42]. Furthermore, there are data suggesting that the incorporation of copper must occur prior to protein export at least in the cases of the multicopper oxidase CotA from *Bacillus subtilis* [43] and *Streptomyces* tyrosinase [41,44]. Therefore, a more likely hypothesis is that Atx1-like/Ccc2-like (or similar) systems present in these organisms (although *Pyrobaculum aerophilum* apparently lacks Atx1-like transporters) function not only in copper efflux, similarly to the organisms that do not have a requirement for copper, but also in copper uptake. As discussed above, the entry of copper into the cell would be coupled to its reduction from Cu(II) to Cu(I) for being transported and eventually incorporated into copper-dependent proteins. The incorporation of copper may not necessarily take place in the cytoplasm, but may also involve membrane-anchored chaperones (such as the Sco homologue of *Bacillus subtilis* [45]) that load copper in the cytoplasm and deliver it outside. The controlled entry of copper into the cytoplasm would also presumably allow the cell to regulate copper uptake in concert with the expression of copper-dependent proteins. In both *Bacillus subtilis* and *Streptomyces coelicolor* the CopC transporter, which can bind both Cu(I) and Cu(II), occurs as a fusion protein with a copper pump called CopD. This suggests that copper uptake in Gram-positive bacteria may involve the binding of environmental Cu(II) to the Cu(II)-binding site of CopC, and the subsequent reduction of Cu(II) to Cu(I) (by a mechanism to be determined), followed by intra-molecular transfer to the Cu(I)-binding site of CopC, and finally the internalization of Cu(I) through CopD.

The model discussed above for archaea and Gram-positive bacteria can be transferred with a few changes to Gram-negative bacteria, as these organisms also utilize copper solely outside of the cytoplasm. Gram-negative bacteria have two membranes, and all known copper-dependent proteins are located in the periplasmic space between the inner and the outer membrane, either soluble (such as SOD) or embedded in the inner membrane (such as COX). In these cells, therefore, the hypothesis that copper is imported into the cytoplasm in concert with its reduction from Cu(II) to Cu(I) implies that environmental Cu(II) first crosses the

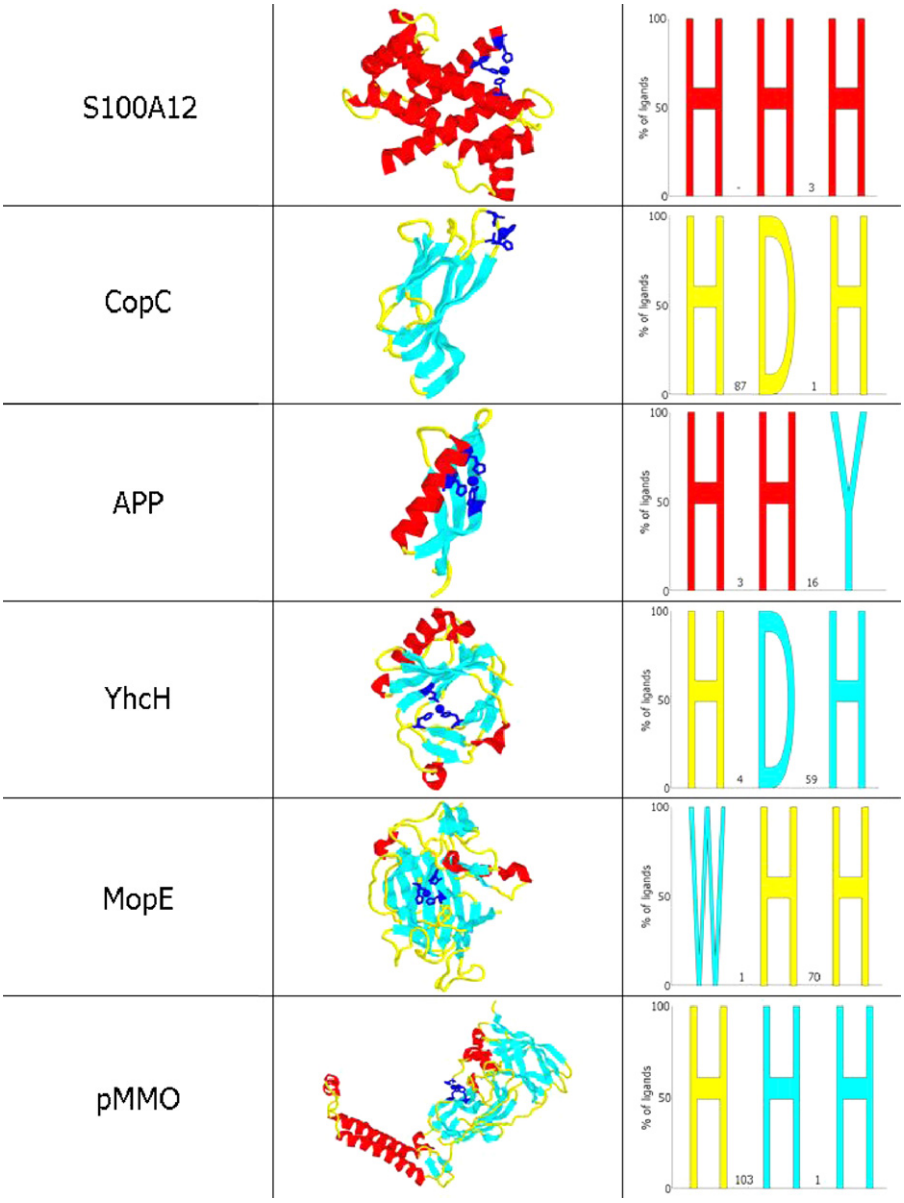

Fig. 7. Cu(II) sites of S100A12, CopC and APP, and uncategorized sites of YhcH, MopE and pMMO. The cartoon structure of a representative protein (with helices in red, sheets in cyan, loops in yellow, copper-binding residues as blue sticks and copper atoms as blue spheres) and the copper-binding pattern of each group are shown. In MopE, the Trp residue shown in the pattern is modified to kynurenine for binding copper.

outer membrane in this form. Cu(II) may move freely across the outer membrane, or may be actively transported by divalent metal ion pumps. Whatever the mechanism of entry, however, all Cu(II) that enters the periplasm should be shuttled to the cytoplasm to avoid the presence of unbound periplasmic Cu(II). In the oxidizing environment of the periplasm, Cu(II) is not expected to engage in dangerous redox chemistry, yet it can still cause damage by occupying incorrect binding sites. Copper shuttling across the periplasm could be mediated by the CopC–CopD system described above for Gram-positive bacteria, which in Gram-negative bacteria involves a soluble CopC transporter. CopC homologues, however, appear to be present in only a few organisms (Fig. 9); other mechanisms may therefore contribute to the safe transit of copper to the cytoplasm. One possibility is that the reduction of Cu(II) to Cu(I) is necessary for copper to pass through not only the inner, but also the outer membrane. If so, Atx1-like/Ccc2-like systems (which are generally also widespread throughout Gram-negative bacteria, see Fig. 9) would control the access of copper, in the Cu(I) form, not only from the periplasm to the cytoplasm, but also from the extracellular space to the periplasm. Furthermore, similarly to Gram-positive bacteria, they would also function in Cu(I) efflux from the cytoplasm. In this framework, copper would be present in the periplasm (in part or exclusively) in the Cu(I) form, coming either from the extracellular space or from the cytoplasm. All periplasmic Cu(I) is expected to be bound to proteins, in that, at variance with Cu(II), it is capable of generating harmful superoxide radicals. These proteins may vary depending on the destination of the copper they carry. For instance, copper exiting from the cytoplasm can be loaded onto carriers that target efflux pumps on the outer membrane (such as CusF [46]) or onto carriers that target specific copper-dependent proteins (such as the DR1885-like COX assembly factor of *Thermus thermophilus* [47]) depending on whether copper must be removed from the cell or delivered to periplasmic proteins, respectively. It is conceivable that the expression of these various carriers depends on the amount of copper available to the cell. Similarly, copper entering from the extracellular space can be loaded onto carriers that target the inner

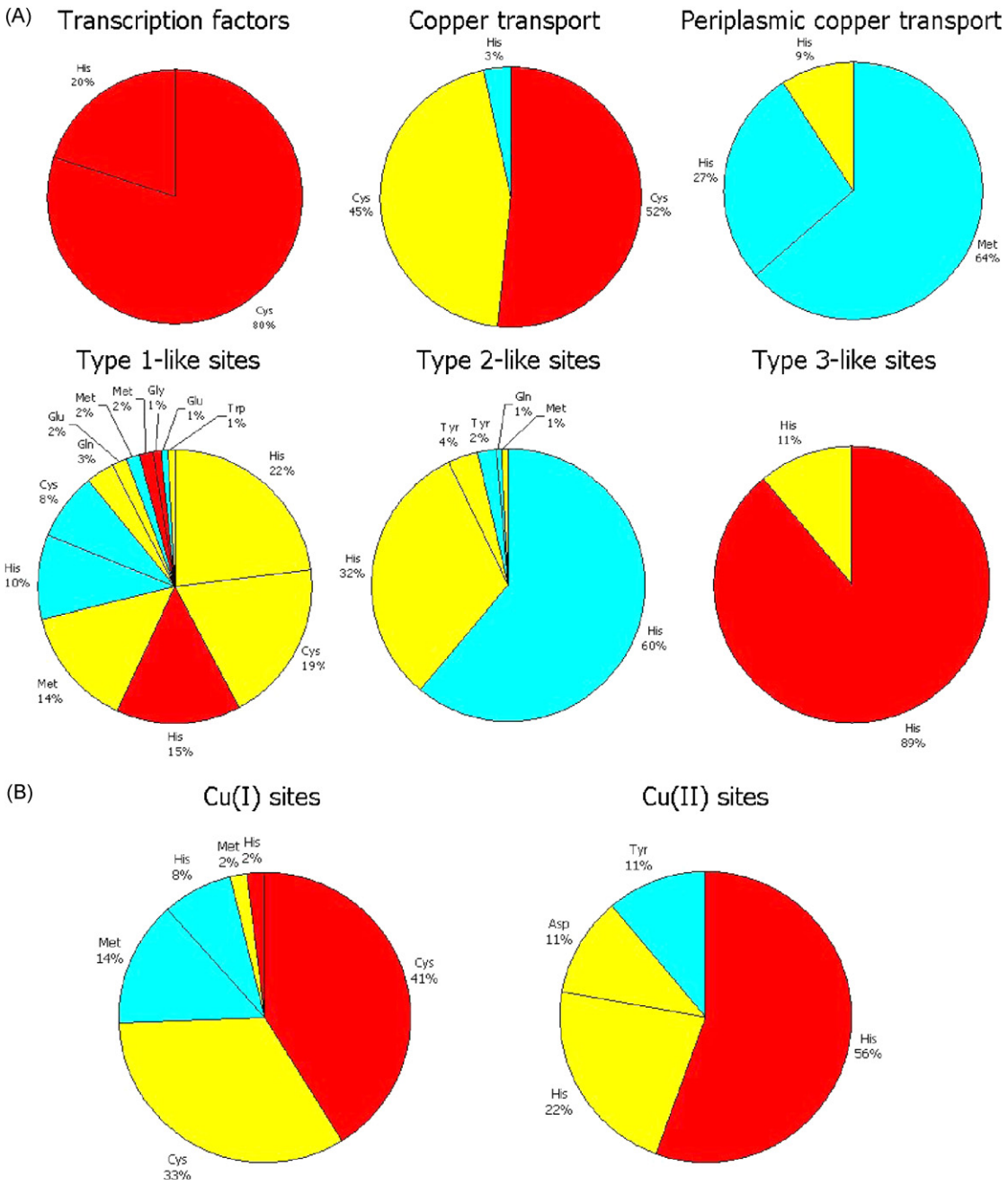

Fig. 8. Pie charts showing the relative occurrences of specific copper-binding residues and secondary structures (with helices in red, sheets in cyan, and loops in yellow) in selected categories of copper sites (**A**) and in all Cu(I) and Cu(II) sites (**B**).

membrane (such as CopC) for import into the cytoplasm or, as above, onto carriers that target specific copper-dependent proteins. The former pathway is necessary for copper-dependent proteins that incorporate the metal ion prior to localization in the periplasm, while the latter may represent a shortcut for copper-dependent proteins that incorporate the metal ion after being exported to the periplasm in the apo form. It is worth noting that this shortcut has no parallel in cells with a single membrane, so the "long" pathway involving the transit of copper through the cytoplasm may be the only one that is active for proteins that obtain the metal in the periplasm as well. A "long" pathway is known to be active in cyanobacteria for delivering copper to copper-dependent proteins (COX and plastocyanin) located in thylakoids, which are the membrane-bound compartments of cyanobacterial cells where the photosynthetic and respiratory chains are located. It has been established that at least plastocyanin is imported into the lumen of thylakoids in the apo form, and that an Atx1-Ccc2 system is responsible for transferring cytoplasmic Cu(I) to these compartments [48,49].

When analyzing the cellular localization and the source organisms of Cu(I)-binding copper transporters, it results that the His-Met binding mode is practically exclusive of bacterial periplasmic proteins. This may depend on the necessity to bind copper in an oxidizing environment, where Cys pairs may be oxidized to disulfides. Proteins localized to reducing environments, instead, use Cys to coordinate Cu(I). This holds for cytoplasmic proteins, both exclusive of prokaryotes such as CueR and CsoR and ubiquitous such as Atx1-like proteins, as well as for mitochondrial proteins such as Cox17. In this respect, the copper-binding capability of prokaryotic Sco proteins appears to be an anomaly, in that they represent the

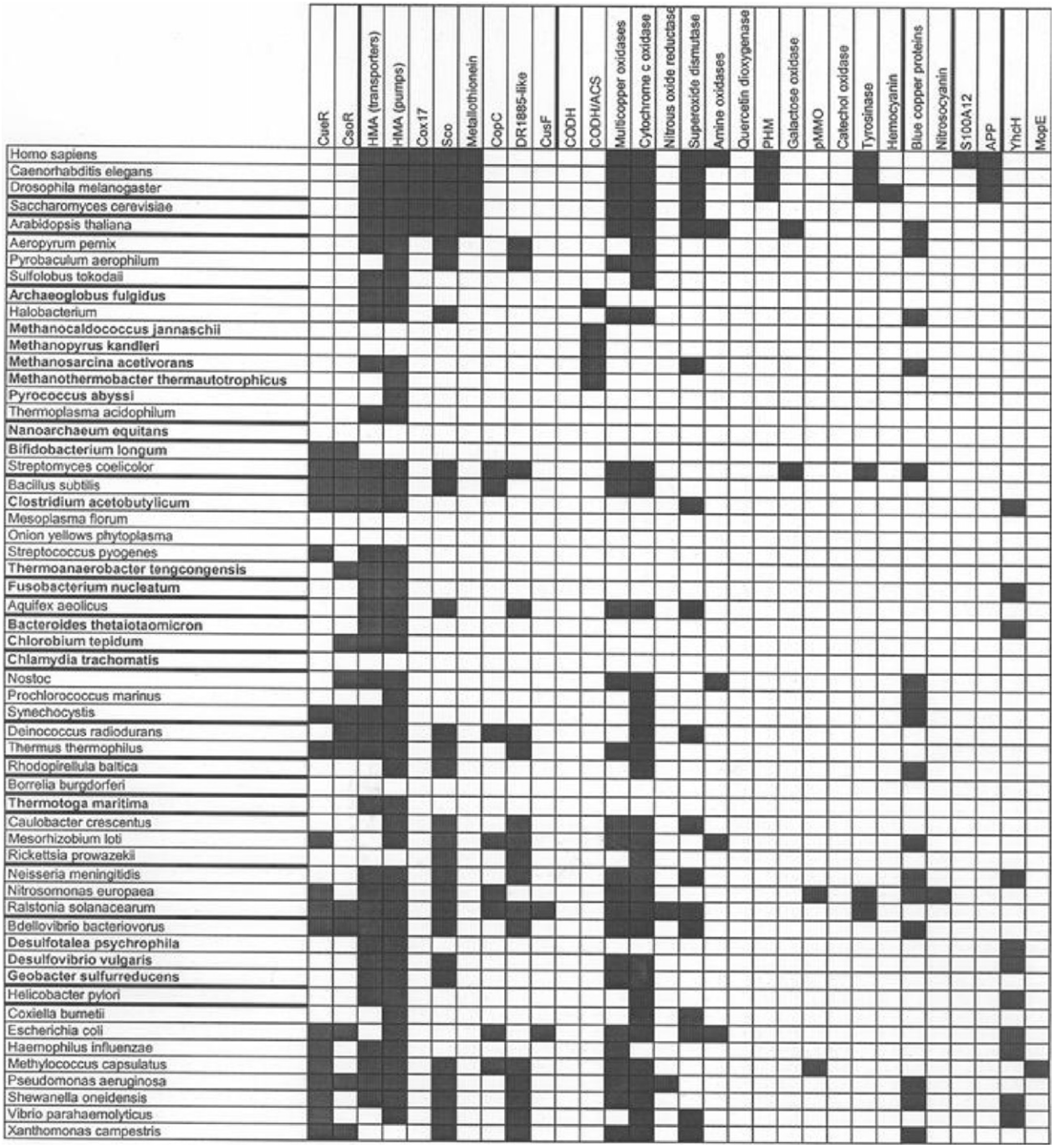

Fig. 9. Matrix showing the occurrence of homologues of the copper proteins listed in Table 1 (columns) in 57 representative organisms spanning the entire tree of life (rows), as inferred from bioinformatics predictions made in [4]. Copper proteins are sorted and grouped (thick lines) according to their function as shown in Table 1. Organisms are grouped (thick lines) according to their taxonomic classification (see Table S1). Anaerobic organisms are shown in bold.

only example of copper coordination by Cys in the periplasm. This observation raises the possibility that prokaryotic Sco proteins do not bind copper *in vivo*. Recent work on the biogenesis of ba_3 oxidase from *Thermus thermophilus* in fact supports a role for Sco as a thiol-disulfide reductase in this process, rather than as a copper chaperone [47]. A similar role has also recently been proposed for human Sco2, which would act to oxidize the copper-coordinating Cys in Sco1 during COX maturation [50]. Human Sco1 and Sco2 are both essential for COX assembly because mutations in either one lead to respiratory deficiencies associated with various clinical phenotypes [51], yet their individual functions are still not understood in detail. Two Sco proteins also occur in yeast where, however, only Sco1 is required for COX maturation, and the physiological role of Sco2 is unclear [52,53].

The model described for bacteria is also substantially valid for unicellular eukaryotes such as *Saccharomyces cerevisiae*. In eukaryotic cells as well, in fact, environmental copper is imported into the cytoplasm exclusively as Cu(I), and is sorted by Atx1-like/Ccc2-like systems to specialized structures such as the Golgi apparatus (for delivery to copper-dependent proteins such as Fet3p) or vesicles (for accumulation and efflux) [2,7]. Furthermore, the large majority of copper-dependent proteins are located outside the cytoplasm in eukaryotes as well, with the conspicuous exception of SOD. In eukaryotic cells, SOD is mostly found in the cytoplasm, where it

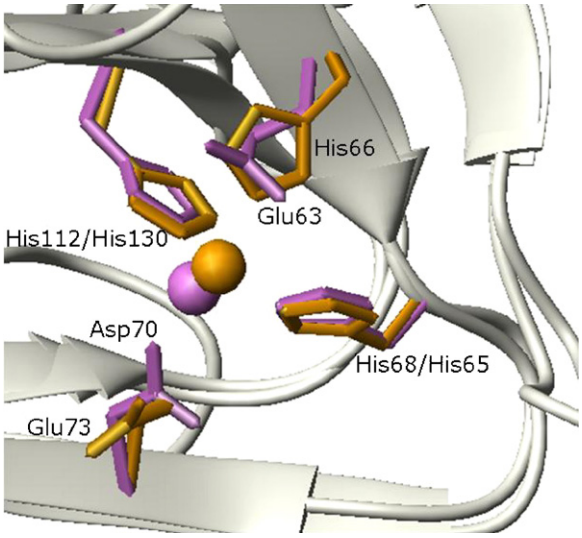

Fig. 10. Superimposition of the copper site of quercetin 2,3-dioxygenase (PDB code 1juh [101], orange) with the copper site of the YhcH protein (PDB code 1s4c [39], violet). The His66 ligand of 1juh overlaps with Glu63, which is not a ligand in 1s4c.

receives copper from a specific chaperone called Ccs [54]. A fraction (approximately 1–5%) of SOD is also localized to the intermembrane space (IMS) of mitochondria [55]. Another distinctive feature of eukaryotic cells with no equivalent in prokaryotes (possibly apart from *Mycobacterium tuberculosis* [56]) is the copper detoxification system consisting of metallothioneins. It is possible that certain metallothioneins can also bind Cu(II) that may accidentally enter the cell, and that they have multiple roles in addition to metal scavenging, including storage and buffering.

A crucial sub-cellular organelle of eukaryotic cells to which copper must be delivered is the mitochondrion, where COX is assembled [57,58]. In addition, mitochondrial copper recruitment is required for incorporation into the fraction of SOD localized to the IMS (see above), which is imported to this compartment in a metal-free form [59]. Given the endosymbiotic origin of mitochondria from free-living Gram-negative bacteria [60], a similarity may still exist between the mechanism by which copper enters mitochondria (which also have two membranes) and that by which environmental copper enters the periplasm of bacterial cells. If the latter involves the uptake of Cu(II), it seems unlikely that mitochondria have conserved this mechanism, as cytoplasmic Cu(I) should then be oxidized in order to cross the outer mitochondrial membrane. Rather, mitochondria may have conserved the bacterial machinery for the import of Cu(I), deprived of the components responsible for copper reduction. Alternatively, eukaryotes may have evolved a specific Cu(I)-transporting system. It would be important to elucidate the molecular machinery responsible for copper translocation across the outer membrane of Gram-negative bacteria, because it could provide key information to also understand mitochondrial copper import. With the assumption that copper trafficking in mitochondria resembles that in Gram-negative bacteria, copper entering the IMS of the mitochondrion as Cu(I) may or may not cross the inner membrane depending on how copper is inserted into COX and SOD. Current knowledge of the assembly of these enzymes [61] indicates that copper is delivered in the IMS to both COX1 (from Cox11) and COX2 (from Sco1), as well as to SOD (from Ccs), suggesting that the shortcut pathway hypothesized above for bacteria may be active in mitochondria. On the other hand, it has been proposed that a pool of copper exists within the mitochondrial matrix as a low molecular weight Cu(I) complex [62], whose formation would thus require that Cu(I) also crosses the inner membrane. It is worth noting that this comparison between mitochondria and bacteria may be deceptive, as their common ancestry lies very far in the past, and major adaptations have occurred throughout mitochondrial evolution. An example of such changes is provided by Cox17, which is only found in the IMS of eukaryotic mitochondria and acts as a copper donor to both Cox11 and Sco1 [31]. The evolution of Cox17 is linked to the evolution of the periplasmic space, which is an oxidizing environment where only His- and Met-containing Cu(I)-binding proteins are present, into the IMS, where variable redox conditions allow the existence and regulation of Cys-containing Cu(I)-binding transporters subject to redox control [63].

4.4. Exploitation of copper's redox states: structures of Cu(I)–Cu(II)-cycling proteins

In the majority of representative copper sites (66 out of 90), copper cycles between the Cu(I) and Cu(II) oxidation states to perform one function from among electron transfer, catalysis and oxygen binding. A possible exception is represented by the copper center of amine oxidase, which may remain in the Cu(II) state during the entire catalytic cycle [64]. Many of these copper sites have been extensively investigated over the years, and a wealth of data on their spectroscopic, physicochemical and biochemical properties are available in the literature. They are traditionally classified based on their spectroscopic features into type 1, type 2 and type 3 centers [65]. Type 1 centers, which are also called "blue" because of their intense blue color, and type 2 centers are mononuclear sites functioning in electron transfer and in the catalysis of reactions which most often involve oxygen species, respectively. Type 3 centers are dinuclear, antiferromagnetically coupled sites involved in either catalysis or in dioxygen binding.

4.4.1. Electron transfer

All known copper sites that perform electron transfer (type 1-like sites in Fig. 1) are found in protein domains with the cupredoxin fold, consisting of a beta-sandwich with seven strands in two sheets arranged in a Greek-key beta-barrel. Proteins containing these sites include: (i) "blue" copper proteins such as plastocyanin, (ii) multi-copper oxidases such as laccase, (iii) COX, (iv) nitrous oxide reductase, and (v) nitrosocyanin. (i) and (ii) contain classical, mononuclear "blue" sites, (iii) and (iv) contain dinuclear CuA sites, and (v) contains a mononuclear "red" site whose function has not been clearly defined. These sites are invariably located at one end of the beta-barrel and are clearly structurally, and most likely evolutionarily, related [66]. Copper coordination in these sites is characterized by the combination of His (47%), Cys (27%) and Met (18%) ligands (Fig. 8) which are found in conserved sequence patterns (Fig. 4). Copper ligands are predominantly (61%) located in the loops protruding from the beta-barrel, but can also be found at the end of the strands forming the beta-barrel (21%) or in short alpha-helices (18%). It is conceivable that such specific structural variations, sometimes together with the usage of less common ligands (e.g., Gln instead of Met in some blue copper proteins) are responsible for tuning the redox potential of copper.

4.4.2. Enzymatic catalysis

The large majority of copper sites that perform enzymatic catalysis are classified in Fig. 1 as type 2-like sites. In addition to classical, mononuclear type 2 sites, these sites include the tri-copper sites of multi-copper oxidases and the tetra-copper site (called CuZ) of nitrous oxide reductase. Mononuclear type 2 sites are found in several enzymes, including (i) SOD, (ii) amine oxidases, (iii) quercetin dioxygenase, (iv) peptidylglycine alpha-hydroxylating monooxygenase (PHM), which contains two distinct type 2 sites, (v) galactose oxidase, and (vi) particulate methane monooxygenase (pMMO). Additionally, a type 2 site is found in nitrite reductase, at the inter-

face between two cupredoxin domains. Irrespective of the number of copper ions bound, copper ligands in these sites are prevalently (63%) located in beta-sheets, and the large majority (92%) of them are His (Fig. 8). The prevalence of beta-sheets stems from the fact that the proteins that contain these sites are all classified in SCOP as all-beta proteins, i.e., proteins where beta-sheets are the only, or the largely prevalent, secondary structure elements. However, most of these proteins have different SCOP folds, and, at variance with type 1-like sites, their copper sites are not obviously related to one another. The only two proteins with the same fold are galactose oxidase and nitrous oxide reductase, and their copper sites are found in remarkably similar positions, though the former binds a single copper ion and the latter binds a four-copper cluster. Consistent with this fold variability, type 2-like sites are not associated to general sequence patterns, although some analogies can be recognized. For instance, several of these sites are characterized by two His ligands that are located on the same beta-strand and are embedded in a HX(1)H (or rarely a HH) pattern, and the same pattern is found repeated four times in the trinuclear sites of multi-copper oxidases (Fig. 5). It is also worth mentioning that the coordination of copper may change during the redox cycle: in the catalytic mechanism of copper-zinc SOD, for example, the histidine that bridges copper and zinc in the oxidized form of the enzyme is released from copper coordination upon reduction [67].

Other copper sites that are involved in catalysis and are unrelated to type 2-like sites are the dinuclear sites of catechol oxidase and tyrosinase, which are classified in SCOP as all-alpha proteins. These sites are clearly structurally related to the type 3 site of hemocyanin; they have therefore been grouped together as type 3-like sites, though in hemocyanin copper is employed to bind and carry oxygen (Figs. 1 and 6). Copper ligands in type 3-like sites are exclusively His, which, consistent with the prevalently helical structure of the proteins, are mostly (89%) found in alpha-helices (Fig. 8). Similar features are displayed by the CuB site of COX, where copper is also bound by His located in alpha-helices (Fig. 6). Nevertheless, this site is found within the trans-membrane region of COX; it has therefore been categorized as a "membrane" site (Fig. 1).

Some iron proteins contain dinuclear sites embedded in a helix bundle which resemble type 3 copper centers. Also in the case of iron, these sites can either have a catalytic function, such as in class I ribonucleotide reductases [68], or bind dioxygen, such as in hemerythrin [69]. Likewise, a number of iron enzymes exist that have all-β structures related to the aforementioned type 2 copper enzymes (e.g., isopenicillin N synthase [70]). These observations are in agreement with the hypothesis that copper has in part replaced iron during the course of evolution [71].

4.5. Exploitation of copper's redox states: electron transfer and enzymatic catalysis

The repertoires of copper enzymes and electron transfer proteins in copper-dependent organisms are of various sizes, and, as a minimum, may consist solely of COX, which is the only copper enzyme in *Sulfolobus tokodaii* and in the host-associated human pathogens *Rickettsia prowazekii* and *Helicobacter pylori* (which also has a YhcH homologue). In any case, all copper-dependent organisms face the problem that copper must not just be kept far away from cells, but must be acquired from the environment at the level that is necessary for their copper enzymes and electron transfer proteins to function.

The four anaerobic organisms that utilize copper are *Desulfovibrio vulgaris*, *Geobacter sulfurreducens*, *Methanosarcina acetivorans* and *Clostridium acetobutylicum*. In particular, COX occurs in both *Desulfovibrio vulgaris* and *Geobacter sulfurreducens*, which, although classified as strict anaerobes (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi), have been shown to be capable of surviving and growing under oxic conditions [72,73]. COX is also by far the most widespread copper enzyme in the 34 copper-dependent aerobic organisms (it occurs in 33, the only exception being *Haemophilus influenzae*), followed by multicopper oxidases (occurring in 24) and SOD (occurring in 15).

The only free-living aerobic organism that does not need copper is *Thermoplasma acidophilum*, which can perform oxygen respiration by the non-copper-containing cytochrome *bd* quinol oxidase [74,75]. On the other hand, the majority of the organisms that have a requirement for copper are aerobic, or facultative aerobic (34 out of 38). These data are in agreement with the hypothesis that copper utilization by cells is evolutionarily linked to the rise of atmospheric oxygen levels [76].

4.6. Use of Cu(II): structures of Cu(II)-binding proteins

Three proteins are known to bind Cu(II): CopC, the S100A12 protein and the Alzheimer's amyloid precursor protein (APP) (Fig. 7). Very different residues are used in these proteins as compared to Cu(I)-binding sites (Fig. 8): in Cu(I)-binding sites, ligands occur as 74% Cys, 16% Met, and 10% His; in Cu(II)-binding sites, instead, there are 78% His, 11% Asp, and 11% Tyr. Specifically, Cys (in helices and loops) and Met (in sheets and loops) are only used to bind Cu(I), and Asp (in loops) and Tyr (in sheets) are used only to bind Cu(II). His is used to bind both Cu(I) and Cu(II), but is much more common in Cu(II)-binding sites and is found in helices and loops but not in sheets. His is rare in Cu(I)-binding sites, where it is primarily found in sheets.

4.7. Use of Cu(II): copper used extracellularly?

CopC is a bacterial protein which is capable of binding Cu(I) and Cu(II) at two different sites [37]. This feature renders CopC unique among copper transporters, which contain Cu(I) sites only. As previously discussed, bacteria may have evolved this protein to efficiently couple Cu(II) scavenging from the environment with its reduction to Cu(I) for import. In Gram-negative bacteria, CopC may also work in the detoxification of excess copper from the periplasm, as it has been implicated in conferring copper resistance to certain strains of bacteria such as *Pseudomonas syringae* [77] and *Escherichia coli* [78].

APP is a eukaryotic trans-membrane protein which is ubiquitously expressed in human tissues and is known to play a central role in the development of Alzheimer's disease, although its physiological function remains elusive [79]. It contains an extracellular domain, where Cu(II) is bound and is reduced to Cu(I) *in vitro* [80]. The relevance of this copper reduction activity to the normal cellular function of APP and to the onset mechanism of Alzheimer's disease is unclear. In multicellular eukaryotes, there is a further level of contextual complexity in copper management due to the presence of extracellular, yet internal to the organism, environments where many copper-dependent proteins are located (e.g., ceruloplasmin in blood plasma). These organisms thus have the additional problem of conveying and managing copper in these environments. It is possible that proteins such as APP have a role in this extracellular homeostasis, about which very little is known. It has been suggested, for example, that APP may reduce extracellular Cu(II) for intracellular copper uptake [81]. On the other hand, it has long been proposed that the reduction of Cu(II) to Cu(I) may be a primary mechanism by which APP contributes to neurological damage [82]. Whatever the precise role of APP, it is conceivable that it mediates the toxic effects of altered levels of extracellular Cu(II), resulting from the breakdown of the homeostatic mechanisms disrupted in neurodegenerative diseases [83].

S100A12 is a member of the family of S100 proteins, which are regulatory proteins involved in the calcium signal transduction pathway [84]. It is almost exclusively expressed in certain immune system cells (i.e., neutrophil granulocytes), and has been proposed to have a role in the immune response and inflammatory processes [85]. At variance with APP, the Cu(II)-binding properties of S100A12 are somehow surprising, because this protein is predominantly localized in the cytoplasm, where copper is expected to occur exclusively in the reduced form. S100A12 would thus represent the sole cytoplasmic protein that binds Cu(II), possibly together with SOD. It should be noted, however, that the catalytic mechanism of SOD is such that Cu(I)- and Cu(II)-containing resting states are perfectly equivalent [86]; it is therefore possible that SOD in the cytoplasm occurs predominantly in the Cu(I)-containing form, in agreement with its (positive) redox potential and the reducing environment in which it is found.

Indeed, the significance of copper binding by S100A12 *in vivo* still has to be elucidated: there are observations that indicate that the copper-binding site can also be occupied by zinc, which may thus be the physiologically relevant metal [87]. On the other hand, copper-binding properties of possible physiological importance have been reported for several other S100 proteins, including S100b, which has been suggested to function in copper detoxification [88], S100A5, for which a dual role in copper transport and sequestration has been proposed [89], and S100A13, which is involved in the copper-dependent cellular export of interleukin-1α, a potent proinflammatory cytokine, and of fibroblast growth factor-1, which plays a central role in a variety of biological processes such as angiogenesis, tissue regeneration and inflammation [90–92]. In this latter case, protein export is based on the formation of multi-protein complexes containing S100A13 and Cu(II), which may be translocated from the extracellular space and directly conveyed by S100A13 to the site of complex formation, occurring at the inner leaflet of the cell membrane [93].

The proposed mechanism of action of S100A13 implies that some highly specialized cells such as those of the immune system may, under certain conditions, contain Cu(II) in the cytoplasm, at least transiently. This may also be the case for S100A12, which is known to be secreted into the extracellular compartment in response to cell stress [85]. In particular, S100A12 is released by neutrophil granulocytes as a result of the immune response against microorganisms, and the copper-mediated generation of reactive oxygen species may be one of the mechanisms by which it exerts its antimicrobial activity [94]. While it is possible, therefore, that S100A12 binds Cu(II) only after being secreted, copper loading of S100A12 may also occur within the cell, perhaps only shortly before its release. Again, changes in the level of extracellular Cu(II) may represent an indicator of the particular conditions triggering S100A12 secretion, though it is unclear whether this should be considered a cause or an effect.

The above cases of APP and S100A12 illustrate the challenges inherent in investigating the mechanisms of copper management in multicellular organisms. These organisms consist of differentiated cells with specialized functions organized into tissues and organs, which are all linked by complex extracellular communication networks. A system-level description of copper trafficking in these organisms thus involves accounting for the specific requirements and functions of different cell types, as well as for the constraints imposed by the overall body morphology. In humans, for example, dietary copper is absorbed by the intestinal epithelium, exported into the blood and delivered primarily to the liver (which is also responsible for copper excretion from the body through the bile and subsequently feces) and eventually to other tissues [95]. The molecular pathways underlying copper transport and regulation on this whole-body scale, however, are poorly understood. While intracellular pathways of copper distribution are thought to be the same in all cell types, copper trafficking to and from specific organs and tissues may occur via different routes [96]. For example, it has recently been shown that copper transport into the brain is mainly achieved through the blood–brain barrier as a free ion [97]. Therefore, in order to answer the many open questions regarding copper management in higher organisms, it is necessary to obtain information on which copper proteins contribute to the determination of the specific characteristics of distinct cell types and extracellular fluids. Emerging metalloproteomics techniques are promising for the provision of this type of information [98–100].

5. Concluding remarks

Copper sites in proteins are found in a limited number of structural motifs, which obey relatively simple rules. These rules, in conjunction with information on the cellular localization of proteins, can be used to predict metal-binding and functional properties of copper proteins, thus helping to define the molecular components responsible for cellular copper homeostasis.

When analyzing the occurrence of copper proteins in living species in this context, it appears that the large majority of copper-containing enzymes and electron transfer proteins are found outside the cytoplasm in both eukaryotes and prokaryotes. In prokaryotes, they are mostly membrane-bound proteins facing the periplasm (in Gram-negative bacteria) or the extracellular space (in Gram-positive bacteria), while in eukaryotes they are mostly found in the extracellular space or in specific compartments such as organelles and vesicles. The most conspicuous exception to this rule is SOD, which is primarily found in the cytoplasm. Conversely, proteins that are involved in copper trafficking and homeostasis are found in the cytoplasm of both eukaryotes and prokaryotes, which also contain them in the periplasm. These proteins all bind Cu(I), and CopC can bind both Cu(I) and Cu(II). A few eukaryotic Cu(II)-binding proteins may also be found in the cytoplasm, such as S100A12.

By considering similarities and differences between types of cells and their copper requirements, it is possible to speculate on simple models for copper handling by archaea, Gram-positive and Gram-negative bacteria, and eukaryotes. These models share common features representing evolutionarily conserved mechanisms of copper uptake, distribution, and removal. The general picture that emerges is that all types of cells may need to reduce Cu(II) to Cu(I) for uptake. Copper levels in the cells are then regulated through a relatively limited battery of Cu(I) carriers and sensing proteins in the cytoplasm (and in the periplasm). Copper sites of these proteins contain either Cys in helices and loops, which may render them subject to thiol:disulfide redox equilibria, or a combination of His and Met in beta-sheets and loops (periplasmic proteins). Different types of these proteins interact with uptake and/or export pumps, and with nascent or apo target proteins which require copper to function. Copper-dependent proteins are typically confined to specific compartments or to the extracellular space, and are expected to release copper only upon protein degradation.

These models are limited by the many gaps that still exist in the known batteries of copper proteins in living organisms. Further structural information is thus needed to extend our knowledge of the functions of these proteins and will help to refine these rules towards a comprehensive description of the mechanisms of copper homeostasis in cells. For example, very little is known with regard to the distinctive features of copper management in different tissues and organs and in the extracellular compartments of multicellular organisms. Proteomics and metalloproteomics data are eagerly awaited to gain a deeper insight

into the copper proteins acting in these environments, for which no counterpart exists in unicellular prokaryotes and eukaryotes. Furthermore, interactomics data are needed to provide information on the interaction networks involving copper proteins, whose detailed description is crucial to achieve a system-level picture of the various interlinked physiological processes affected by copper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ccr.2009.07.024.

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