Handling of nutrient copper in the bacterial envelope

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Abstract

In bacteria, copper (Cu) is often recognised for its potential toxicity and its antibacterial activity is now considered a key component of the mammalian innate immune system. Cu ions bound in weak sites can catalyse harmful redox reactions while Cu ions in strong but adventitious sites can disrupt protein or enzyme function. For these reasons, the outward transport of Cu from bacteria has received significant attention. Yet, Cu is also a bacterial nutrient, required as a cofactor by enzymes that catalyse electron transfer processes, for instance in aerobic and anaerobic respiration. To date, the inward flow of this metal ion as a nutrient and its insertion into target cuproenzymes remain poorly defined. Here we revisit the available evidence related to bacterial nutrient Cu trafficking and identify gaps in knowledge. Particularly intriguing is the evidence that bacterial cuproenzymes do not always require auxiliary metallochaperones to insert nutrient Cu into their active sites. This review outlines our effort to consolidate the available experimental data using an established energy-driven model for metalation.

Introduction: the challenge of handling nutrient copper

Approximately half of enzymes and a third of all proteins require metals to function – an often overlooked dimension of bacterial physiology and nutrition. Understanding the way in which the correct metal ion is inserted into enzymes and proteins still represents a major challenge in bioinorganic chemistry. *In vitro*, these biomolecules prefer the same relative order for metals that follows the Irving-Williams series. However, it is now understood that these universal preferences can, in general, be overcome *in vivo* because molecules within the crowded intracellular milieu buffer the available concentrations of metals in the inverse order.[1] Tighter binding metals like copper (Cu) and zinc (Zn) are buffered at lower concentrations (and hence are less available), while weaker binding metals like manganese (Mn) are buffered at higher concentrations (and hence are more available).[1] Once inside cells, metals flow down a thermodynamic gradient, *i.e.* from weaker (high energy) to tighter (low energy) sites in the buffer, *via* a series of stochastic, associative exchange reactions (Figure 1a).[2] A metalloprotein ultimately acquires the correct metal as long as its affinity for this metal is higher than the affinity of the buffer (*i.e.* the metal-protein complex is more stable than is the metal-buffer complex) (Figure 1b).[3,4]

Cu sits at the top of the Irving-Williams series and hence metalation of cuproenzymes is normally an endergonic or thermodynamically favourable process. By the same principle, Cu can also partition into stable sites in the wrong protein, leading to enzyme inactivation and bacterial poisoning. To minimise mis-metalation, cells employ metallochaperones that are thought to shuttle (or "chaperone") the Cu ion from import pumps to target cuproproteins. Such pathways are well described for the eukaryotic cytosol and organelles.[5-9] For prokaryotes, discussions of Cu homeostasis have revolved mainly around Cu tolerance[10-12],

i.e. removal of excess Cu from the cell under conditions of Cu surplus, when the cellular Cu buffer is "full". By contrast, trafficking of nutrient copper, particularly when the buffer is "empty", is less understood.

Cuproenzymes are thought to have evolved after the appearance of atmospheric O_2[13] and so they are typically involved in reactions with oxygen and oxygen-containing species. In prokaryotes, Cu is a major nutrient for aerobic respiration (*via* haem-Cu oxidases in the electron transport chain), anaerobic respiration (*via* nitrous oxide reductases and Cu-containing nitrite reductases in the denitrification pathway), and removal of toxic reactive oxygen species (*via* Cu,Zn-superoxide dismutase). Intriguingly, the Cu-dependent enzymes in the aforementioned pathways are all localised to the bacterial envelope (*i.e.* in the periplasm of Gram-negative bacteria or on the surface of Gram-positive bacteria). Indeed, with the exception of plastocyanin and cytochrome oxidase in Cyanobacteria, cuproproteins are not known to exist inside the bacterial cytoplasm. This apparent compartmentalisation of Cu to the extracytoplasmic space may represent a mechanism for balancing the physiological advantages of using nutrient Cu in catalysis while protecting against its potential toxicity. Indeed, Cu is generally considered to be more toxic in the cytoplasm and thus must be buffered at a lower availability (*i.e.* bound by higher affinity or lower energy sites in the buffer) relative to the extracytoplasmic space.

Metals in the bacterial envelope are readily exchangeable with the extracellular environment, for example *via* passive diffusion across outer membrane porins in Gram-negative organisms[14]. Hence, fine control of metalation in this compartment may be more challenging than in the cytoplasm. This is considered particularly problematic for metalloproteins that are translocated *via* the Sec general secretory pathway and thus are folded (and metalated) in the

extracytoplasmic space.[15] By contrast, metalloproteins that are Tat substrates fold inside the cytoplasm and, at least in some cases, obtain their cognate metal prior to secretion. In the case of Cu, recent examination of the periplasmic multicopper oxidase CueO from *Escherichia coli* demonstrated that removal of the Tat signal sequence and expression of CueO in the cytoplasm led to isolation of only the *apo*-enzyme.[16] In fact, all bacterial cuproproteins for which the steps of Cu insertion have been identified (detailed in this review) are thought to become metalated outside the cytoplasm, regardless of the translocation mechanism of the protein scaffold. One explanation is that the Cu affinities of these cuproenzymes are compatible with the buffered availability of Cu in the bacterial envelope but incompatible with that of the cytoplasm. In addition, the oxidation state of Cu in the buffer and the oxidation state preferred by the enzyme might further define extracytoplasmic metalation of cuproproteins.

What is the source of nutrient Cu for cuproenzymes in the bacterial envelope? In the simplest model, a buffered pool of Cu in the extracytoplasmic space acts as the Cu supplier. The molecular nature of this Cu buffer is presently unknown. It has been long assumed that thiols like glutathione (GSH) buffer Cu in the cytoplasm.[17] There is also evidence that GSH is exported to the periplasm of Gram-negative organisms[18], and so it can presumably also buffer Cu in this compartment. However, the affinity of GSH for Cu is orders of magnitudes weaker when compared to those of bacterial Cu sensors in the cytoplasm[19,20] or nutrient Cu metallochaperones in the periplasm[21]. These relative values suggest that glutathione would constitute a high energy buffer, filled only when an excess of Cu is available. By contrast, the identity of the low energy or high affinity buffer that contributes to normal Cu nutrition is unknown. Nevertheless, metalloproteomics examination of periplasmic extracts from

Salmonella enterica sv. Typhimurium[22] and *Synechocystis*[23] indicated that periplasmic Cu is bound either to Cu metallochaperones or to unidentified low molecular weight proteins.

Regardless of the precise identity of the extracytoplasmic Cu buffer, it is presumably filled by Cu from the extracellular environment (Figure 2). This exchange of Cu may occur *via* passive diffusion through porins[14,24] or other unidentified mechanisms[25]. Active uptake of Cu is also known to occur, for example *via* TonB-dependent receptors[26] or *via* classical siderophores[27] and Cu-binding metallophores ("chalkophores") such as yersiniabactin and methanobactin[28-30]. Once the buffer is filled by Cu, provided that the affinities of the cuproenzymes are higher than the affinity of the buffer, Cu will flow down the thermodynamic gradient and ultimately insert into target enzymes (Figure 1b). Yet, there is now mounting evidence that Cu-exporting P-type ATPases embedded in the cytoplasmic membrane are involved in metalating extracytoplasmic cuproproteins.[23,31,32] The implication is that nutrient Cu ions are trafficked through the cytoplasm *en route* to the extracytoplasmic targets and, if so, this must be a vital process for Cu homeostasis. When combined with the dearth of known cytoplasmic Cu importers, this apparently circuitous routing of Cu is one of the most puzzling aspects of nutrient Cu handling in bacteria.

Our research groups have studied bacterial Cu tolerance for several years and have recently begun to investigate nutrient Cu handling, specifically in pathogenic *Neisseria*. This prompted us to revisit existing literature related to bacterial nutrient Cu trafficking and identify gaps in knowledge. We were particularly intrigued by the evidence that bacterial cuproenzymes do not always require auxiliary metallochaperones to insert nutrient Cu into their active sites. This review outlines our effort to consolidate the available experimental data by expanding an established energy-driven model for Cu trafficking[2]. We focus on four

major families of bacterial cuproenzymes: (1) nitrous reductases, (2) nitrite reductases, (3) Cu,Zn-superoxide dismutases, and (4) haem-Cu oxidases, and pay particular attention to the precise steps of Cu insertion. The genomic context and genetic distribution, structural features and properties of the Cu centres in the enzymes (and in the associated metallochaperones), as well as kinetic properties of these enzymes are already subjects of numerous excellent reviews and so will not be covered in detail.

Cu insertion into nitrous oxide reductases

Assembling a denitrification pathway is a Cu-expensive process since it involves at least one multicopper enzyme, namely nitrous oxide reductase (N_2OR or NosZ), which catalyses the reduction of N_2O to N_2. NosZ homologues are classified as typical or atypical, distinguished by two key biochemical characteristics: (i) an additional haem *c* binding site is present near the C-terminus in atypical NosZ but is absent in typical NosZ; and (ii) translocation of atypical NosZ is Sec-dependent while that of typical NosZ is Tat-dependent.[33] Both types of NosZ contain 6 Cu atoms per monomer (12 Cu per functional homodimer), arranged into one tetranuclear Cu_Z copper-sulfur (Cu_4S_2) cluster that binds and activates N_2O during catalysis, and one binuclear mixed-valent Cu_A centre that acts as the site of electron entry.[34-37]

Consistent with its high demand for Cu, NosZ activity in denitrifying organisms is greatly influenced by extracellular Cu levels.[38-40] During conditions of Cu deficiency, NosZ activity decreases and N_2O accumulates. This Cu-dependent regulation of NosZ occurs at the post-translational level, *i.e.* by modulating occupancy of the Cu centres. Growth in Cu-deficient conditions leads to production of NosZ in an inactive form. However, N_2O reductase activity is restored by addition of exogenous Cu without the need for new protein synthesis.[39] In

bacteria possessing the typical NosZ, increases in extracellular Cu levels also induce the expression of *nosZ*. This requires at least one factor, the flavoprotein NosR, although the molecular details are yet to be elucidated.[40,41] The *nosR* gene is not found in genomes encoding atypical NosZ[33], and whether Cu regulates *nosZ* transcription in these organisms is unknown.

The current models for Cu_Z and Cu_A biogenesis suggest that these Cu centres are assembled in the periplasm following secretion of the protein, for both the typical and atypical NosZ, regardless of the translocation mechanism (Figure 2). Homologous expression of NosZ in the cytoplasm results in the production of neither the Cu_Z nor the Cu_A centre.[42] Assembly of Cu_Z *in vivo* requires NosDFY, an ABC-type transporter that may transport sulfur (Figure 2)[43], although this is yet to be confirmed experimentally. This requirement for NosDFY appears to be obligate and the genetic clustering of *nosZ* with *nosDFY* is absolutely conserved in all sequenced genomes that are currently available.[33] N_2O respiration is abolished if any of the *nosDFY* genes is mutated and this defect is not restored by addition of extracellular Cu.[44,45] In addition, NosZ isolated from *nosDFY*-deficient strains contains only the Cu_A centre[46-48], indicating that NosDFY may not be required to assist Cu_A assembly.

Insertion of nutrient Cu into the Cu_Z cluster *in vivo* is likely facilitated by NosL, a small lipoprotein that is anchored to the outer membrane (Figure 2). The soluble periplasmic domain of NosL binds one Cu(I) ion *in vitro* but its affinity has not been determined.[49] The Cu ligands include one Cys and one Met, presumably from a conserved Cys-X-Met motif near the N-terminus.[50,51] The third ligand, likely from a His residue, is yet to be identified, and no obvious candidate is found from analysis of amino acid sequences. Whether NosL delivers Cu(I) to NosDFY or directly to NosZ, whether metalation is coupled to sulfur

insertion, and whether NosL assists in assembly of the Cu_A centre are yet to be established. None of the *nos* cluster genes appears to be essential for Cu_A assembly. Nevertheless, denitrifying organisms often possess additional Cu metallochaperones like Sco and PCu_AC (described below), which may metalate the Cu_A sites in NosZ, but this remains to be elucidated.

Unlike NosDFY, NosL appears to be dispensable for Cu_Z assembly. Although fitness analyses of a mutant library suggest that *nosL* is essential for denitrification in *Pseudomonas stutzeri*[52], mutational inactivation of *nosL* in this organism does not yield an obvious defect in N_2O reductase activity.[53] Likewise, heterologous expression of NosZ in its active form in the periplasm of a nondenitrifying host does not necessitate the co-expression of NosL.[46] Furthermore, *nosL* is absent from the *nos* gene cluster in many genomes and this absence does not correlate with the type of NosZ (typical or atypical) or the NosZ translocation mechanism.[33]

How does Cu_Z obtain nutrient Cu in the absence of NosL? There is a proposal that other Cu metallochaperones such as PCu_AC (described below) can compensate, although this is yet to be tested experimentally. An alternative, and arguably simpler, hypothesis is that the Cu_Z site acquires Cu directly from the extracytoplasmic Cu buffer (Figure 2). This reaction is thermodynamically favourable ("downhill" or exergonic) as long as the affinity of the Cu_Z scaffold for Cu is higher than the affinity of the buffer (*i.e.* the bound Cu ion in Cu_Z is lower in energy or more stable than is Cu in the extracytoplasmic buffer) (Figure 1b). NosL may provide an "intermediate buffer" (with intermediate Cu affinities) that lowers the overall energy barrier for the transfer of Cu from the extracytoplasmic buffer to the Cu_Z scaffold, with Cu-NosL acting as a reaction intermediate (Figure 1c). In this scenario, the absence of

NosL would not affect the Cu occupancy of NosZ, provided that the buffered Cu availability is sufficiently high (*i.e.* Cu is bound by high energy or low affinity sites in the buffer) and the barrier for Cu transfer to NosZ is sufficiently low. NosL would become more important in Cu-deficient conditions, when the buffered Cu availability decreases (*i.e.* Cu is bound by low energy or high affinity sites in the buffer) and thus, presumably, the barrier for onward Cu transfer to NosZ increases (Figure 1d).

Regardless of the precise role for NosL, the question remains: what is the source of the buffered Cu in the extracytoplasmic space? In the simplest model, this buffer is filled directly by Cu from the extracellular environment (Figure 2). In some, but not all, denitrifying Gram-negative organisms, N_2O respiration during conditions of Cu limitation requires NosA, a TonB-dependent receptor that may increase uptake of Cu into the periplasm (Figure 2).[26,46,54,55] Intriguingly, there is also evidence that the extracytoplasmic pool of Cu is filled by supply from the cytoplasm. NosZ activity *in vivo* was shown to depend on CtpA, a P-type ATPase that resembles known bacterial Cu-efflux transporters (Figure 2).[31] Mutation of *ctpA* leads to decreased NosZ activity but enzyme activity is restored by addition of Cu to the extracellular medium. This exogenous Cu presumably fills the extracytoplasmic Cu buffer, which in turn metalates NosZ (Figure 2). If direct metalation of NosZ by the extracytoplasmic Cu buffer is possible in the Δ*ctpA* mutant, why nutrient Cu must first be routed through the cytoplasm in the wild type organism appears a major conundrum.

Cu insertion into Cu-containing nitrite reductases

Cu-dependent nitrite reductase (Cu-NIR), usually called NirK, catalyses the reduction of NO_2^- to N_2O. This enzyme contains a total of 2 Cu centres per monomer (6 Cu per functional

homotrimer): one T1 "blue" Cu centre that acts as the site for electron entry and one T2 Cu centre that acts as the active site for NO_2^- binding and reduction.[56] NirK is sometimes co-encoded in the genome with NirV, a protein of unknown function that does not appear to bind Cu.[57,58] Only a minority of NirK homologues carry the Tat signal sequence, with most thought to be translocated *via* the Sec or other nonspecific secretory pathways.[59]

T1 and T2 Cu centres are readily reconstituted by Cu salts *in vitro* and so insertion of Cu into NirK *in vivo* was previously assumed to require no accessory metallochaperones. However, a recent genetic screen identified that a soluble periplasmic Cu-binding protein, AccA, is required for metalating NirK (AniA) in pathogenic *Neisseria* (Figure 3).[60] AccA is a homologue of PCu_AC, a metallochaperone that is thought to aid assembly of Cu_A and Cu_B centres in haem-Cu oxidases[61-64] (described below). Like PCu_AC, AccA binds one Cu(I) ion with a high apparent affinity[60], although precise quantification is still awaited. Conserved Met and His residues are likely involved in binding Cu(I). AccA also binds one additional Cu ion in the Cu(II) oxidation state.[60] Several candidate ligands for Cu(II) are present in the His- and Met-rich C-terminus but their identities are yet to be determined. Programmes in our research groups are currently ongoing to determine which of the two bound Cu ions in AccA is loaded to which of the two Cu sites in AniA.

Mutants lacking *accA* generate wild type amounts of AniA but fail to reduce NO_2^-, suggesting that AniA is produced in the *apo*- or incorrectly metalated form. Consistent with this view, reduction of NO_2^- resumes, albeit only partially, upon addition of Cu salts into the extracellular media.[60] Assuming that no other unidentified Cu trafficking pathway compensates for AccA, the observed recovery of AniA activity by exogenous Cu is consistent with the proposal that that this enzyme is metalated directly by a buffered Cu pool

in the periplasm (Figure 3). This reaction is energetically downhill as long as the affinity of AniA for Cu is higher than the affinity of the buffer (*i.e.* the bound Cu in AniA is more stable or less energetic) (Figure 1b). As hypothesised earlier for NosL, the function of AccA may be to act as an intermediate buffer that lowers the overall energy barrier for Cu exchange and thus functionally "catalyses" the transfer of Cu from the buffer to the T1 and/or T2 sites of AniA (Figure 1c). In the absence of AccA, provided that the buffered Cu availability is sufficiently high (*i.e.* Cu is bound by high energy or low affinity sites in the buffer), the barrier for onward Cu transfer decreases, and AniA becomes metalated (Figure 1e).

There is evidence that deletion of the AccA homologue PCu_AC in *Bradyrhizobium japonicum* also leads to transient accumulation of NO_2^-, implying a defect in NirK activity.[65] Hence, although PCu_AC primarily aids assembly of Cu_A centres (discussed below), the possibility that this metallochaperone inserts nutrient Cu into multiple cuproenzymes, including NirK, should not be disregarded. In the energy-driven model, the role of PCu_AC is facilitative rather than obligatory (Figure 1c). This model can rationalise why not all genomes that encode a NirK[59] possess a PCu_AC or AccA, and conversely, why the presence of *pcu$_A$C* in denitrifying organisms does not exclusively correlate with the presence of *nirK*[40] or even with Cu_A centres[66].

Like pathogenic *Neisseria*, some NirK-containing organisms also possess the metallochaperone Sco.[66,67] Together with PCu_AC, Sco is thought to facilitate assembly of Cu_A and Cu_B centres in haem-Cu respiratory oxidases (described below). Whether Sco is required for inserting Cu into T1 and T2 sites of NirK is not known. Likewise, whether a Cu importer such as NosA from *P. stutzeri* or a Cu-exporting P-type ATPase such as CopA in pathogenic *Neisseria* is involved in metalating AniA or NirK is yet to be examined (Figure 3).

Cu insertion into Cu,Zn-superoxide dismutase

The Cu,Zn-superoxide dismutase (SodC) is noted for its distribution among pathogenic bacteria[68] and is often considered a virulence factor owing to its ability to detoxify the superoxide anion during phagocytosis.[69-72] This enzyme contains one solvent-exposed T2 Cu centre (2 Cu per functional homodimer) in the active site. Like the other bacterial cuproenzymes discussed in this review, SodC is invariably localised to the extracytoplasmic space, either in its soluble (*e.g.* in the Gram-negative periplasm) or anchored form (*e.g.* on the surfaces of Gram-positive bacteria[73] or on the outer membrane of some Gram-negative bacteria). It was previously assumed that SodC was secreted *via* the Sec pathway but it is now proposed that the hydrophobic signal sequence of SodC may interact with the Tat translocase.[74]

The T2 centre in SodC assembles spontaneously *in vitro* without the need for assembly factors.[75] Based on studies with *Salmonella enterica* sv. Typhimurium, metalation of SodC *in vivo* likely involves, but does not absolutely require, a soluble periplasmic metallochaperone named CueP (Figure 4).[32,76] Homologues of CueP are found in both Gram-positive and Gram-negative bacteria[77], but certainly not in all SodC producers (*e.g. E. coli*). Deletion of *cueP* impairs, but does not completely eliminate, the activities of the two SodC homologues in *Salmonella,* SodCI and SodCII.[32,76] The reduction in enzyme activities correlates with decreased occupancy of the T2 Cu site, at least for SodCII.[32] However, enzyme activity and/or Cu occupancy are restored *in vivo* by supplementing the culture medium with Cu salts or *in vitro* by addition of Cu into the cell-free extracts.[32,76] Purified CueP binds one Cu(I) ion

309 with high affinity using a combination of Cys and His ligands[77,78] and it is indeed able to
310 deliver this bound Cu to purified SodC *in vitro*[32].
311
312 We noted that CueP is the first example of a bacterial Cu metallochaperone that participates
313 in *both* Cu nutrition and Cu tolerance, and hence contributes fully to bacterial Cu
314 homeostasis. Low basal amounts of CueP are produced during normal growth conditions but
315 high amounts of this protein are generated during conditions of Cu surplus.[22,79] Upregulation
316 of *cueP* expression by Cu requires both the cytoplasmic Cu sensor CueR and CpxRA, which
317 controls transcriptional responses to envelope stress.[79] It has been proposed that CueP
318 contributes to Cu tolerance by binding and sequestering excess Cu(I) in the periplasm.
319 Consistent with this idea, CueP has been identified as a major Cu store in the *Salmonella*
320 periplasm[22] and the Δ*cueP* mutant is Cu-sensitive[77].
321
322 How does CueP balance its seemingly dual role? The energy-driven model posits that as long
323 as the T2 Cu site in SodC is more stable (*i.e.* higher in affinity or lower in energy) than is the
324 periplasmic Cu buffer, SodC will acquire Cu directly from this buffer (Figure 1b). Here,
325 CueP acts as an intermediate buffer that lowers the energy barrier for Cu transfer,
326 analogously to the other extracytoplasmic Cu metallochaperones described earlier (Figure
327 1c). Consistent with this role as a functional catalyst (or Cu "insertase"), only low amounts of
328 CueP need to be present. In the absence of CueP, the barrier for metalation of SodC is likely
329 overcome by supplying excess extracellular Cu, which saturates the low energy (high
330 affinity) sites and starts to fill the high energy (low affinity) sites in the periplasmic Cu buffer
331 (Figure 1e). This idea that SodC may acquire Cu directly from the periplasmic fluid has
332 indeed been postulated previously.[80]
333

Even during conditions of Cu stress, *i.e.* when the low affinity and high energy sites in the buffer become full, SodC remains energetically downhill from the buffer, and so this enzyme will continue to be metalated correctly. However, adventitious protein sites may now also become downhill from the "full" buffer and subsequently mis-metalated by Cu (Figure 5). Increasing the amounts of CueP under these conditions will generate alternative stable but, more importantly, specific sites for Cu binding. Thus, the excess Cu can "drain" from less stable (*i.e.* lower in affinity or more energetic) sites in the original buffer or in mismetalated proteins to the more stable (*i.e.* higher in affinity or less energetic) site in CueP (Figure 5c). In this model, CueP essentially directs or regulates the flow of Cu down the thermodynamic gradient, both during conditions of normal Cu nutrition and conditions of Cu stress. This "intermediate buffering" function for Cu metallochaperones during Cu homeostasis has indeed been postulated previously[81-83] although it has not been tested for the metallochaperones highlighted in this review. The challenge for bacteria is to control production of CueP such that it does not start to drain Cu from SodC as a consequence of mass action (*e.g.* see equation in Figure 1). There is experimental evidence that correct amounts of CueP are indeed important. Expression of *cueP* from a CpxR-independent promoter leads to growth defects in the presence and absence of added Cu.[79]

Another mechanism to maintain SodC in its metalated form may involve control of the oxidation state of Cu. The Δ*cueP* mutant is Cu-sensitive only during anaerobic growth conditions.[77] In the presence of O_2, Cu(I) is removed from the buffer *via* oxidation to Cu(II) by the cuprous oxidase CueO[84,85], and thus additional buffering of Cu(I) by CueP may not be necessary. In addition, Cu in the resting form of SodC exists in the Cu(II) state. *In vitro*, this bound Cu(II) ion does not re-partition into *apo*-CueP.[32] Thus, overexpression of CueP *in vivo* is unlikely to lead to de-metalation of SodC, at least under aerobic growth conditions, when

SodC activity is essential[86]. During anaerobic growth, when SodC is not required, extracytoplasmic reductants may reduce the Cu(II) ion in SodC and subsequent back-transfer of Cu(I) to CueP is plausible, although not yet demonstrated.

Metalation of SodC *in vivo* also depends, at least partly, on outward transport of Cu from the cytoplasm to the periplasm *via* either one of the two, functionally redundant, Cu efflux pumps in *S.* Typhimurium, CopA and GolT (Figure 4). SodC isolated from mutant bacteria lacking both P-type ATPases contains only the Zn centre but readily acquires Cu upon addition of Cu(II) salts into cell-free extracts.[32] This finding may further highlight the importance of the correct oxidation state for Cu. CopA and GolT transport Cu in the reduced Cu(I) form, and the relative affinities of the periplasmic domains of the P-type ATPases, the periplasmic buffer, CueP, and SodC for Cu(I) may be ordered such that metalation of SodC with Cu(I) is thermodynamically favourable. However, as already mentioned earlier, the T2 site in SodC is also competent to acquire Cu(II), at least *in vitro*.[32] In vivo, one possibility is that the buffered Cu(II) availabilities (or energies) in the periplasm are low and hence insertion of Cu(II) into SodC may be a thermodynamically uphill or unfavourable process. Measurements of the affinities of CueP and SodC each for Cu(I) and Cu(II), and comparisons with the buffered availabilities of Cu(I) and Cu(II) in the periplasm would be informative.

Cu insertion into haem-Cu respiratory oxidases

Haem-Cu respiratory oxidases are transmembrane, multi-subunit, multi-haem enzymes that catalyse the terminal step in the electron transport chain, namely the conversion of molecular oxygen to water. All members of this enzyme superfamily contain a mononuclear Cu_B centre that is spin-coupled to a haem. This is the site of O_2 binding and reduction, and it is

embedded deep within the transmembrane structures. Transfer of electrons from a cytochrome or quinol typically involves a relay of haem cofactors and, in some cytochrome *c* oxidases, a dinuclear Cu_A centre that is housed within a soluble periplasmic subunit. As anticipated from the complex enzyme architecture, assembly of haem-Cu oxidases likely requires a modular process that is synchronised both temporally and spatially, along with checkpoints that prevent folding of empty Cu sites into the mature but nonfunctional complex.[87]

Of interest in this review are the precise steps of Cu insertion into Cu_B and Cu_A. These processes are most studied for mitochondrial cytochrome *c* oxidase (COX) in eukaryotes.[88,89] Given the endosymbiotic bacterial origin of mitochondria, the mechanisms for metalation of mitochondrial COX and bacterial haem-Cu oxidases likely share some universal features. The bacterial metallochaperones involved in Cu_B and Cu_A assembly, namely Sco[64,90-94], PCu_AC[61-63,65,95], or Cox11p[96-100], are, again, localised to the extracytoplasmic space (Figure 6). These metallochaperones are structurally and functionally analogous to their eukaryotic counterparts (PCu_AC acts as a functional Cox17 homologue). However, unlike the eukaryotic system, the precise contribution of each protein in the assembly of bacterial Cu_A *vs.* Cu_B centres and the sequence of Cu insertion events remain poorly defined and, bafflingly, appear to be organism-dependent.

Part of the confusion can perhaps be ascribed to the varied genomic distributions of these metallochaperones. For instance, it is generally agreed that assembly of bacterial Cu_A centres *in vivo* involves both Sco and PCu_AC (Figure 6).[21,63] There is indeed evidence that Sco and PCu_AC form a transient complex *in vitro* and *in vivo*.[63] However, the genes encoding these proteins are not always adjacent to each other in bacterial genomes.[101] Moreover, *sco* and

409 *pcu$_A$C* are not always in close proximity with genes encoding Cu$_A$-containing oxidases. In
410 some organisms, *sco* or *pcu$_A$C* is instead associated with the *nos* cluster for nitrous reductase,
411 *nirK* for nitrite reductase, putative operons for Cu homeostasis[65], other cuproenzyme genes,
412 or genes with unknown functions.[101] In addition, Sco and PCu$_A$C homologues are present in
413 bacteria that do not possess Cu$_A$ (*e.g.* pathogenic *Neisseria*)[66,90,102] and, in some organisms,
414 multiple, functionally distinct homologues can exist[67].
415
416 The current model for prokaryotes, which parallels that for eukaryotes, suggests that
417 metalation of the Cu$_A$ site is coupled to redox processes. The lipoprotein Sco contains a
418 soluble, periplasmic thioredoxin-like domain and a conserved Cys-X-X-X-Cys motif, and
419 thus it is not surprising that this protein displays thiol-disulfide reductase activity *in vitro*.
420 Along with one additional His residue, the Cys thiols in Sco bind one Cu(I) ion with high
421 affinity. This site also binds Cu(II) with an affinity that is higher than that for Cu(I).[103]
422 However, this Cu(II) ion is kinetically more inert than the bound Cu(I) ion and hence,
423 exchange of Cu from Sco to its partners would occur only upon reduction to Cu(I).[103] PCu$_A$C
424 displays a characteristic cupredoxin fold and binds one Cu(I) ion with high affinity using a
425 combination of Met and His ligands (total of four) from a conserved HX$_6$MX$_{21}$HXM
426 motif.[62,65,95] Some homologues of PCu$_A$C also bind Cu(II) *in vitro* but, in most cases, this
427 binding is accompanied by reduction to Cu(I).[21,60,62,95]
428
429 Insertion of Cu into the Cu$_A$ site *in vitro* does not require Sco, as long as the Cys ligands for
430 Cu$_A$ are present in their reduced forms.[61] *In vivo*, the oxygen-rich environment of the
431 periplasm may promote oxidation of these Cys ligands. Under these aerobic conditions, *in*
432 *vitro* experiments have shown that the Cu$_A$ site is metalated only when both PCu$_A$C and Sco
433 are present, and only when Sco is provided in a reduced form.[61] It is thus hypothesised that

PCu_AC acts as the Cu metallochaperone (or Cu donor) while Sco acts as a reductase that maintains either the Cu ion or the Cu_A cysteine ligands in the reduced forms (Figure 6).[64] Upstream reductases such as TlpA may provide the reducing power *in vivo* (Figure 6).[104,105]

As mentioned earlier, not all bacterial haem-Cu oxidases contain a Cu_A centre. However, in contrast to the relative wealth of information available for Cu_A, current understanding of Cu_B assembly remains limited, mainly because the location of Cu_B deep within a transmembrane domain has largely precluded *in vitro* studies. Nevertheless, as discussed below, there is mounting *in vivo* evidence that Sco and PCu_AC are also involved in Cu_B assembly, at least in some organisms. The mechanism may parallel that for Cu_A although the precise details still need investigation. In addition, the bacterial homologue of mitochondrial Cox11, Cox11p, has been implicated in forming bacterial Cu_B centres *in vivo.*[98,100] Whether bacterial Cox11p coordinates its function with PCu_AC and/or Sco is unknown.

Mutation of *sco*, *pcu$_A$C*, or both, typically, but not always[60], leads to decreases, but not complete losses, in the activities of haem-Cu oxidases, regardless of whether the specific oxidase contains only Cu_B (*e.g. cbb*$_3$ oxidase) or both Cu_B and Cu_A (*e.g.* cytochrome *aa*$_3$ and *ba*$_3$ oxidases)[64,65,91,95,99,106,107]. Deletion of *sco* typically produces the stronger phenotype.[106,108] The defects in oxidase activities correlate with decreases in the amounts of mature subunits formed *in vivo* but these can be overcome, at least partially, by supplementing the extracellular medium with Cu salts.[63-65,91,95,106-108]

All of the abovementioned experimental data are again consistent with the model that the Cu sites in haem-Cu oxidases can acquire Cu directly from the extracytoplasmic Cu buffer (Figure 6) as long as the energy of this buffer is sufficiently high (Figure 1b). Although

metalation with Cu *in vitro* is coupled to reduction (either of the Cu ion or of the Cu_A or Cu_B ligands), general extracytoplasmic reductases may provide this reducing power *in vivo*. This scenario is plausible for Cu_A because this centre is readily assembled *in vitro* in the presence of Cu salts and reductants.[34,109] Provided that the thermodynamic gradients (*i.e.* buffered Cu availabilities, affinities of the metallochaperones, and affinities of the Cu_A scaffold) *in vivo* are appropriately setup for the exergonic Cu transfer from the buffer into the empty Cu_A sites, Cu will insert (Figure 1b and Figure 1c). For Cu_B, metalation may be combined with folding checkpoints to avoid accidental incorporation of the non-metalated sites in the mature complex. Indeed, potential roles in Cu insertion for other accessory components such as CcoG, CcoH, and CcoS (at least for the cbb_3 oxidase) have been proposed, which may reflect their role in regulating such maturation checkpoints but mechanistic data are currently lacking.[93,110,111]

Like the other extracytoplasmic cuproenzymes described in this review, haem-Cu oxidases also appear to utilise nutrient Cu that has been routed *via* the cytoplasm, first *via* a major facilitator superfamily (MFS)-type transporter named CcoA that putatively imports Cu into the cytoplasm[112,113] and subsequently *via* a Cu efflux pump (CcoI or CtpA)[31,111,114,115] (Figure 6). Deletion of each of these transporters leads to decreases in the activities of Cu_B and/or Cu_A-containing cytochrome oxidase activities, but these are, to some extent, alleviated by supplementation with Cu salts. This apparent routing of Cu through the intracytoplasmic compartment to metalate an extracytoplasmic cuproenzyme is one of the least understood aspects of nutrient Cu trafficking but, if it does occur, must represent a vital process in bacterial Cu homeostasis.

Outlook and perspectives: The need for systems approaches to examine nutrient Cu handling in bacteria

Among the six, first-row *d*-block transition metal ions that are considered as bacterial nutrients (Mn, Fe, Co, Ni, Cu, Zn), Cu is often highlighted for its potential toxicity. Cu ions bound in weak or high energy or unstable sites can catalyse harmful redox reactions, while Cu ions in strong, low energy or stable but non-native (adventitious) sites (mismetalation) can disrupt protein or enzyme function. While the outward transport of Cu as a bacterial poison has received significant attention from the metallomics community, inward flow of this metal ion as a bacterial nutrient remains less defined. Confounding this issue, known bacterial Cu importers and Cu-binding metallophores are still exceedingly rare and, as described in this review, while they are relatively more common, nutrient Cu metallochaperones are often functionally redundant.

The apparent redundancy of Cu metallochaperones may be rationalised by the energy-driven model, in which target cuproenzymes obtain nutrient Cu directly from a buffered Cu pool *via* "downhill" or exergonic associative exchange reactions (Figure 1b). It is our view that this model can universally rationalise all the available experimental evidence for the metalation of cuproenzymes in different bacterial organisms. In this model, the *apo*-metallochaperones can be considered as intermediate buffers or functional catalysts that lower the energy barrier for Cu transfer regulate the flow of Cu down the thermodynamic gradient (Figure 1c). The Cu-bound form of the metallochaperone thus represents a thermodynamic local minimum that limits "sideway" flows of Cu into adventitious sites (Figure 1c). Hence, these metallochaperones are not obligate components for Cu homeostasis but are nonetheless able to provide alternative and more efficient routes for metalation during Cu nutrition,

particularly when extracellular Cu is limiting, and for preventing (or correcting) mismetalation during Cu poisoning. This "intermediate buffering" function for Cu metallochaperones has indeed been proposed previously[81-83] but how these metallochaperones lower the energy barrier for Cu transfer remains to be determined.

A key advantage of this model is that, in organisms where the metallochaperone is absent, there is no need to describe elaborate backup or compensatory mechanisms. Instead, the main considerations would be the oxidation state of Cu, as well as the relative amounts and Cu affinities of the target cuproenzymes, of the metallochaperones, and of the extracytoplasmic buffer. Differences in these properties may explain why periplasmic cuproproteins do not acquire Cu when expressed homologously in the cytoplasm. If the affinities of the cytoplasmic buffer for Cu are higher than the affinities of the cuproenzymes (*i.e.* bound Cu in the buffer is less energetic or more stable), transfer of Cu out of the buffer would be thermodynamically uphill or endergonic. Hence, knowledge of the relative tunings of extracytoplasmic buffer components compared to the cytoplasm becomes equally important.

The hypothesis that the cytoplasm supplies nutrient Cu to the extracytoplasmic space highlights a critical gap in knowledge. The extracytoplasmic space is largely contiguous with the extracellular environment. During conditions of environmental Cu deficiency, the Cu buffer could spontaneously drain, *e.g. via* diffusion through outer membrane porins, although this may be offset by outer membrane Cu uptake receptors or chalkophores, if they are present. By contrast, the cytoplasm is encapsulated within an impermeable lipid bilayer. While this appears to be a sensible solution for maintaining a stable supply of nutrient Cu, the sequence of events is unclear. How Cu fills the cytoplasmic buffer in the first place still needs investigation. Moreover, some Cu exporters (*e.g.* CopA and GolT from *S.* Typhimurium)

operate under the control of cytoplasmic Cu sensors that activate transcription only when the cytoplasmic buffer is "full". Under these conditions, buffered Cu availability outside the cytoplasm is presumably also elevated and indeed multiple extracytoplasmic components of Cu tolerance are usually produced.[116] Why, then, is extracytoplasmic buffered Cu not used directly as the source of nutrient Cu? Is this related to the oxidation state of the metal? What is the contribution of Cu storage proteins like Csp, which can be present in the cytosol or the periplasm[117]? Systems measurements of the buffered Cu availabilities, and comparisons between the cytoplasm and the periplasm, even if technically challenging, may prove illuminating. These can build on recent pioneering efforts by others in the metallomics community to decipher bacterial Cu (and metal) homeostasis.[1,3,4,81,118,119]

Conflicts of interest

We declare no conflict of interest.

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FIGURES

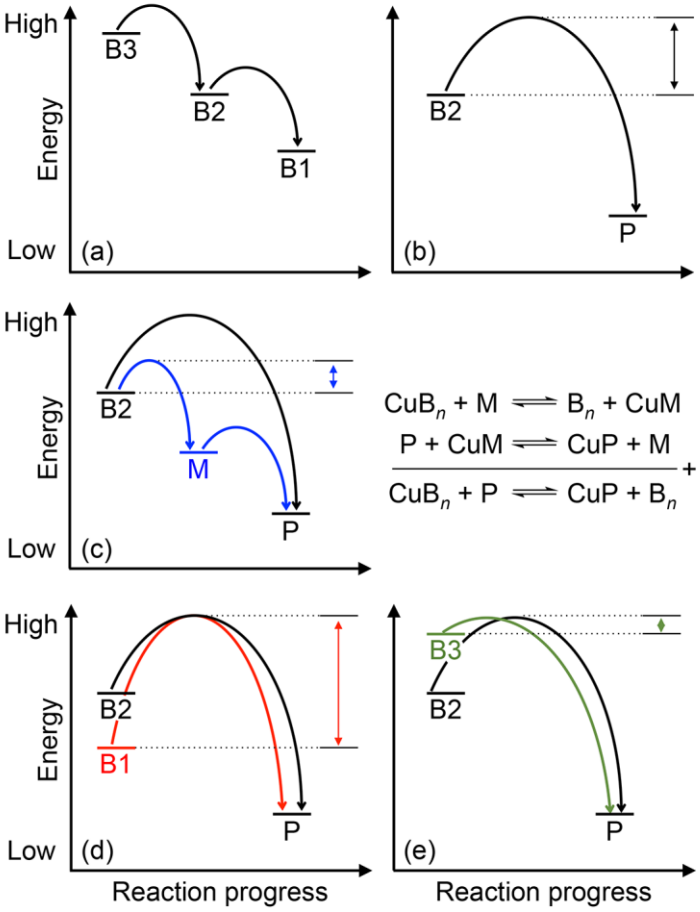
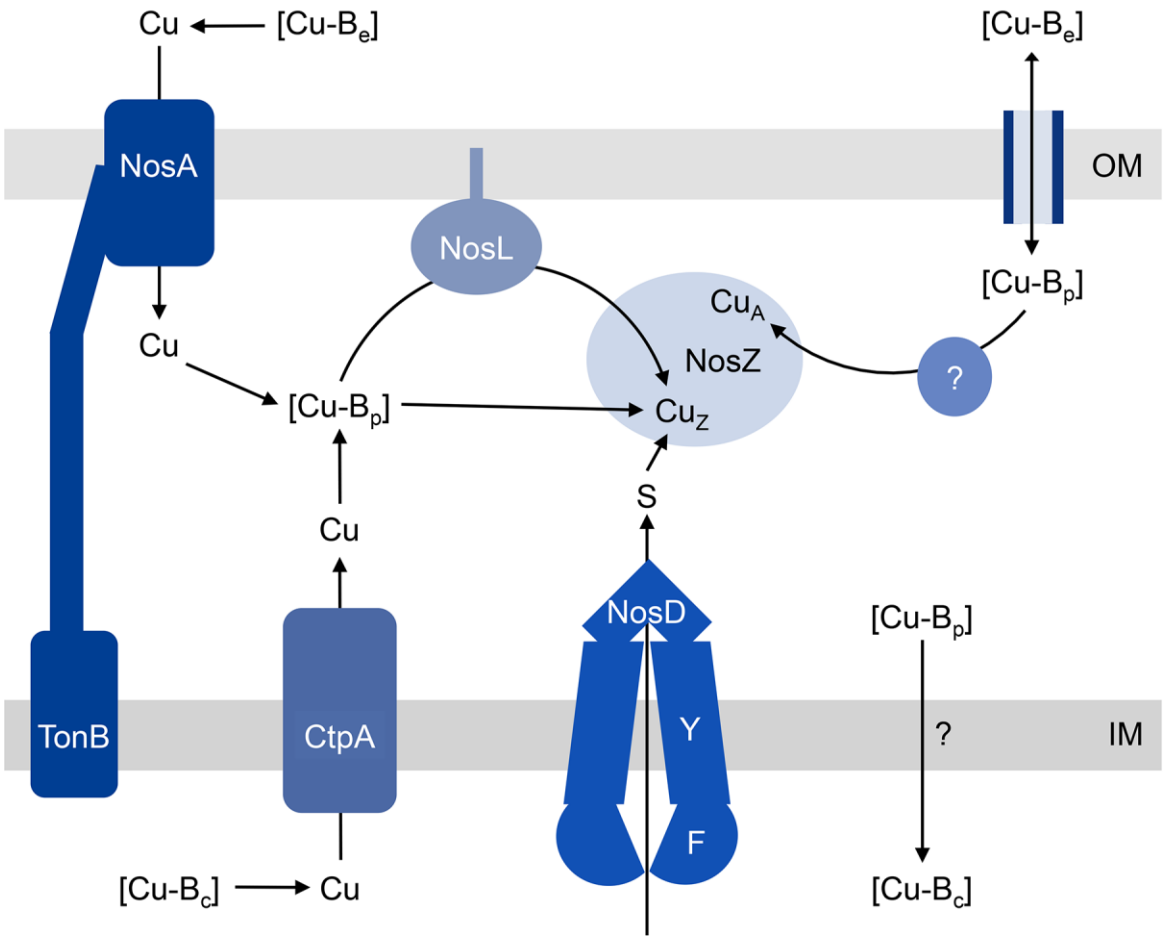


Figure 1. General energy-driven model for the insertion of Cu into cuproenzymes. The relative energy for each Cu-binding site, whether in the buffer (B1, B2, B3), cuproprotein (P), or metallochaperone (M) is shown. Curved arrows represent the forward transfer of Cu from one binding site to another while double-headed arrows represent the energy barrier that must be overcome. Several scenarios are depicted: **(a)** Upon entry into cells, Cu fills the buffer by stepwise transfer from high energy or low affinity sites (denoted as B3) to low energy or high affinity sites (denoted as B1) in the buffer through stochastic exchange reactions. **(b)** Direct transfer of Cu from the buffer (in this example the mid-affinity or mid-energy site B2) to a cuproprotein (P). **(c)** Transfer of Cu from the mid-affinity buffer (B2) to a cuproprotein (P)

573 *via* a metallochaperone (M). Equations representing these equilibria are shown on the right.
574 **(d)** During conditions of Cu starvation, low affinity or high energy sites in the buffer (B3)
575 start to empty, leaving only Cu that is bound in high affinity or low energy buffer sites (B1).
576 Onward transfer of Cu from this low energy buffer to the cuproprotein (P) is shown with a
577 high energy barrier. **(e)** During conditions of Cu stress, the excess Cu starts to fill the weaker
578 sites in the buffer start (B3). Onward transfer from this high energy buffer to the cuproprotein
579 (P) is shown, requiring a lower activation energy.
580



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582

583 **Figure 2.** General model for the insertion of Cu into NosZ. [Cu-B_e], [Cu-B_c], and [Cu-B_p] are
584 buffered pools of Cu in the extracellular space, cytoplasm, and periplasm, respectively. In
585 this model, [Cu-B_p] is filled by [Cu-B_e] either *via* the TonB-dependent receptor NosA or
586 possibly *via* direct exchange across outer membrane porins in NosA-deficient organisms.
587 [Cu-B_p] is also filled by [Cu-B_c] *via* the P-type ATPase CtpA. How [Cu-B_c] is generated is
588 unknown. The Cu_Z site in NosZ acquires Cu from [Cu-B_p] either directly or *via* the
589 metallochaperone NosL, and this process is likely coupled with insertion of sulfur (S) by
590 NosDFY. How the Cu_A site obtains Cu is unknown but this process likely resembles
591 mechanisms for Cu_A assembly in haem-Cu oxidases.

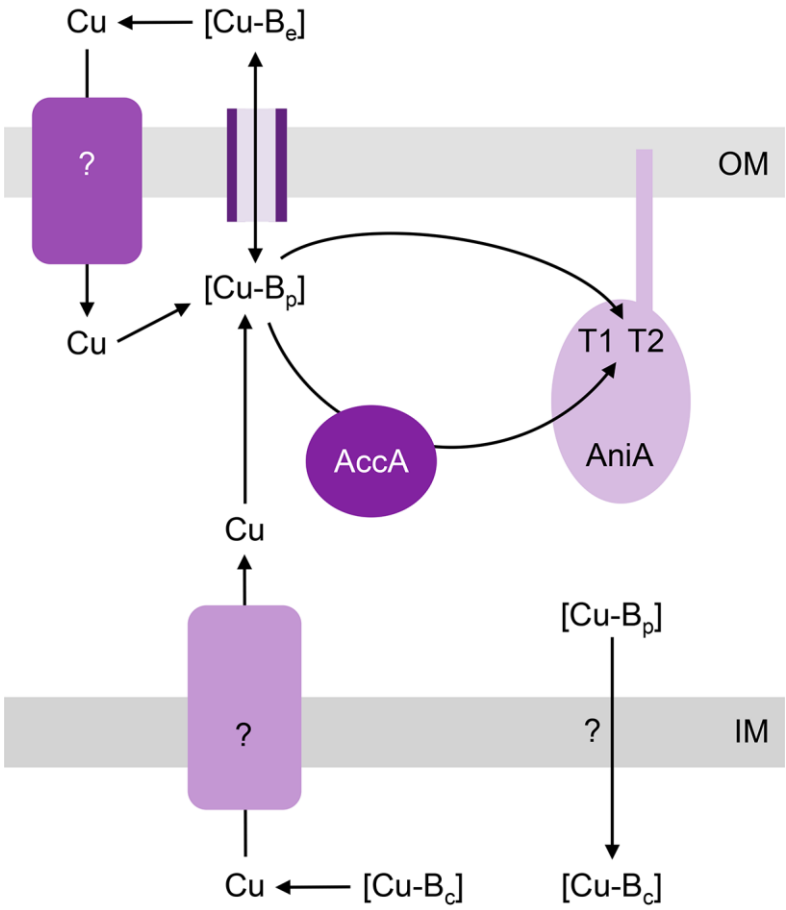

Figure 3. General model for the insertion of Cu into AniA (NirK). [Cu-B_e], [Cu-B_c], and [Cu-B_p] are buffered pools of Cu in the extracellular space, cytoplasm, and periplasm, respectively. The T1 and T2 sites in AniA acquire Cu from [Cu-B_p] either directly or *via* the metallochaperone AccA (PCu_AC). [Cu-B_p] is likely filled by [Cu-B_e] *via* direct exchange across outer membrane porins. Whether an outer membrane importer or a cytoplasmic exporter is involved in filling [Cu-B_p] is yet to be determined.

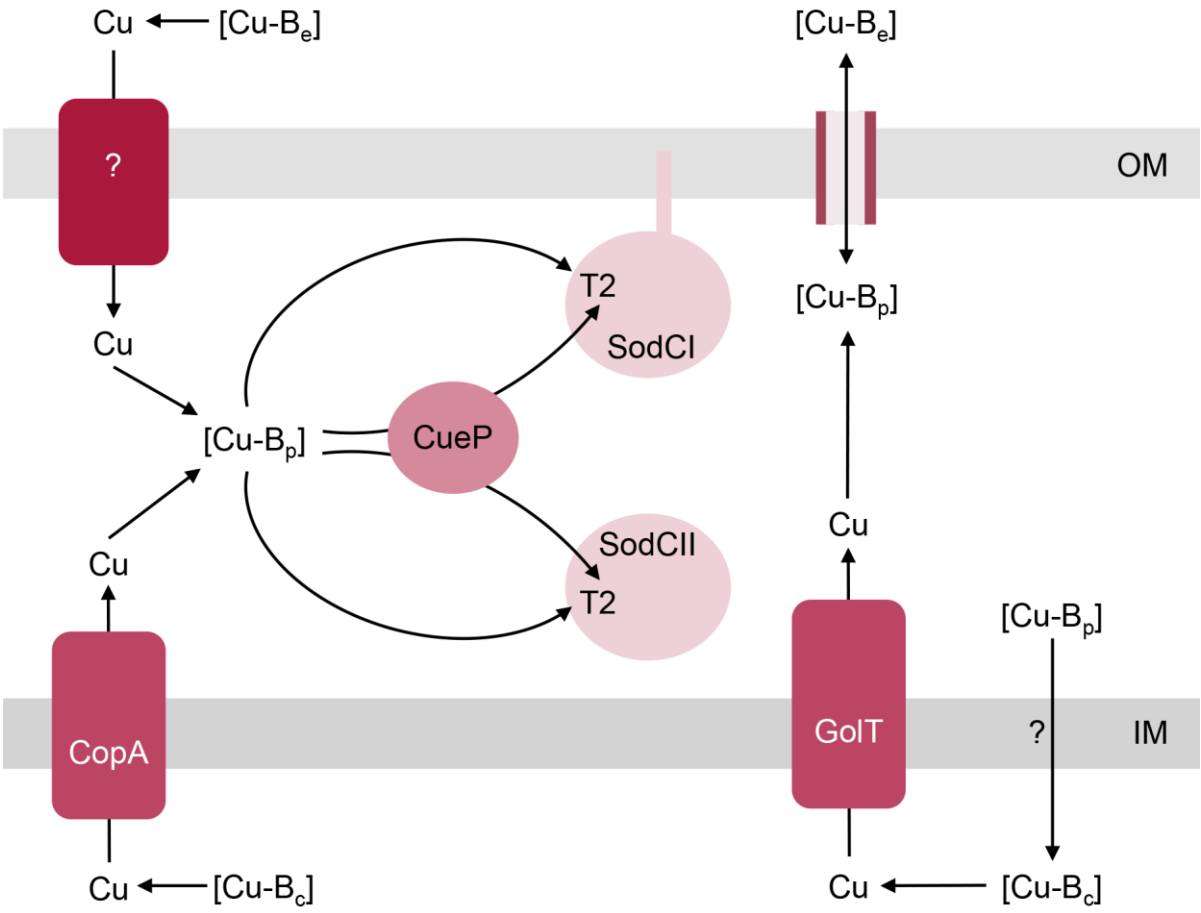

Figure 4. General model for the insertion of Cu into SodC. [Cu-B_e], [Cu-B_c], and [Cu-B_p] are buffered pools of Cu in the extracellular space, cytoplasm, and periplasm, respectively. [Cu-B_p] is likely filled by [Cu-B_e] *via* direct exchange across outer membrane porins. Whether an outer membrane importer is involved in this process is yet to be established. [Cu-B_p] is also filled by [Cu-B_c] *via* the P-type ATPases CopA or GolT. How [Cu-B_c] is generated is unknown. The T2 Cu site in SodC acquires Cu from [Cu-B_p] either directly or *via* the metallochaperone CueP.

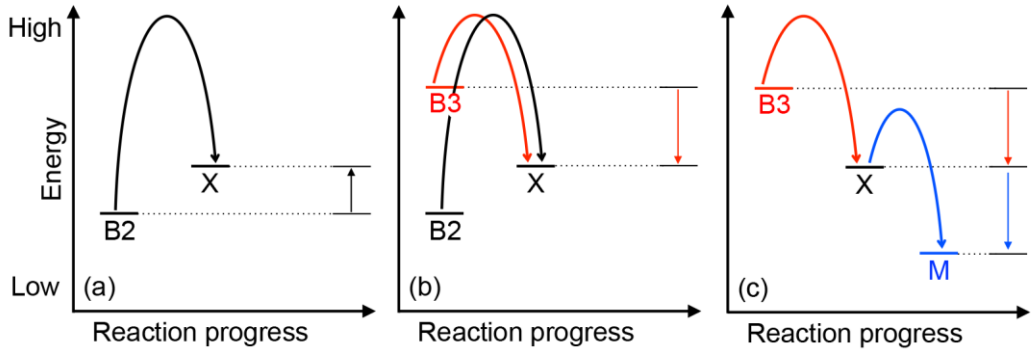

Figure 5. A general energy-driven model for the insertion of Cu into the wrong proteins (mismetalation). The relative energy for each Cu-binding site, whether in the buffer (B2, B3), Cu-binding metallochaperone (M), or a non-native adventitious protein (X) is shown. Curved arrows represent the forward transfer of Cu from one binding site to another. Several scenarios are depicted: **(a)** Protein X, which is not a Cu-binding protein, binds Cu with an affinity that is weaker than that of the B2 buffer (*i.e.* the Cu-X complex is less stable or is more energetic than is the Cu-B2 complex). Hence, during normal Cu conditions, Cu transfer from buffer B2 to protein X is thermodynamically unfavourable (straight upward arrows), and X is not mismetalated by Cu. **(b)** During conditions of Cu stress, excess Cu enters cells and begins to fill low affinity or high energy sites in the buffer (B3). If this site is sufficiently high in energy, Cu will transfer out of the buffer into protein X, causing mismetalation. This transfer of Cu is now thermodynamically downhill and favourable (straight downward arrows). **(c)** Expression of a Cu-binding metallochaperone (M) during Cu stress conditions provides alternative, high-affinity or low energy but, more importantly, specific sites for Cu. Cu is thus transferred out of protein X and mismetalation is alleviated.

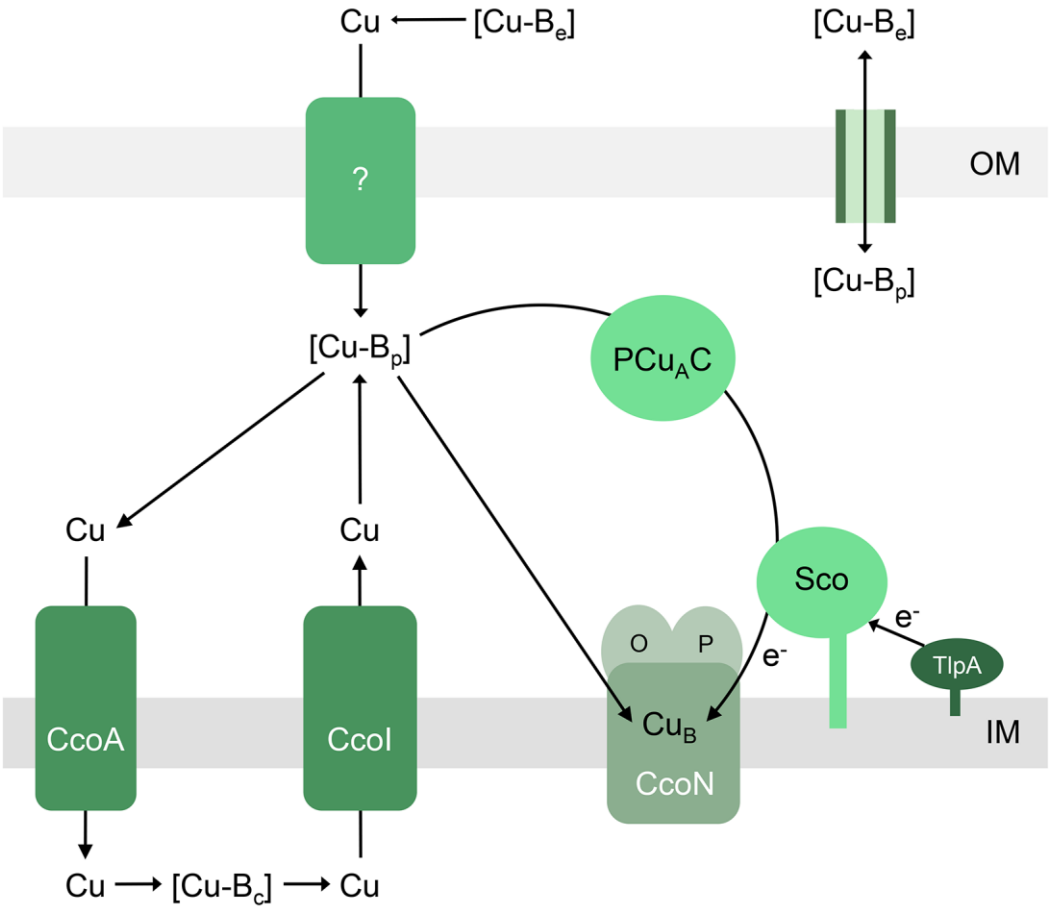

Figure 6. General model for the insertion of Cu into the Cu_B site into cytochrome *cbb*$_3$ oxidase. Only the active site subunits CcoNOP are shown. This model may broadly apply to insertion of Cu into the Cu_A site in other haem-Cu oxidases. [Cu-B_e], [Cu-B_c], and [Cu-B_p] are buffered pools of Cu in the extracellular space, cytoplasm, and periplasm, respectively. The Cu_B site in CcoN (and/or Cu_A site in other haem-Cu oxidases) may obtain nutrient Cu directly [Cu-B_p] or *via* the periplasmic Cu metallochaperones PCu_AC and Sco. Based on studies on Cu_A assembly, Sco may also act as a thiol-disulfide reductase that maintains either the Cu ion or the Cu_B (or Cu_A) Cys ligands in their reduced forms. Upstream reductases such as TlpA may provide the reducing equivalents. Supply of Cu to [Cu-B_p] could occur by direct exchange across outer membrane porins or *via* an as yet unidentified importer. The MFS transporter CcoA supplies Cu to [Cu-B_c], with reduction from Cu^{2+} to Cu^{+} occurring either

641 during transit or spontaneously in the reducing environment of the cytoplasm. Cu is routed

642 back to the periplasm to fill [Cu-B_p] *via* the P-type ATPase CcoI.

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