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1 **Handling of nutrient copper in the bacterial envelope**

2

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21 **Abstract**

22

23 In bacteria, copper (Cu) is often recognised for its potential toxicity and its antibacterial
24 activity is now considered a key component of the mammalian innate immune system. Cu
25 ions bound in weak sites can catalyse harmful redox reactions while Cu ions in strong but
26 adventitious sites can disrupt protein or enzyme function. For these reasons, the outward
27 transport of Cu from bacteria has received significant attention. Yet, Cu is also a bacterial
28 nutrient, required as a cofactor by enzymes that catalyse electron transfer processes, for
29 instance in aerobic and anaerobic respiration. To date, the inward flow of this metal ion as a
30 nutrient and its insertion into target cuproenzymes remain poorly defined. Here we revisit the
31 available evidence related to bacterial nutrient Cu trafficking and identify gaps in knowledge.
32 Particularly intriguing is the evidence that bacterial cuproenzymes do not always require
33 auxiliary metallochaperones to insert nutrient Cu into their active sites. This review outlines
34 our effort to consolidate the available experimental data using an established energy-driven
35 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],

61 *i.e.* removal of excess Cu from the cell under conditions of Cu surplus, when the cellular Cu
62 buffer is "full". By contrast, trafficking of nutrient copper, particularly when the buffer is
63 "empty", is less understood.
64
65 Cuproenzymes are thought to have evolved after the appearance of atmospheric O_2[13] and so
66 they are typically involved in reactions with oxygen and oxygen-containing species. In
67 prokaryotes, Cu is a major nutrient for aerobic respiration (*via* haem-Cu oxidases in the
68 electron transport chain), anaerobic respiration (*via* nitrous oxide reductases and Cu-
69 containing nitrite reductases in the denitrification pathway), and removal of toxic reactive
70 oxygen species (*via* Cu,Zn-superoxide dismutase). Intriguingly, the Cu-dependent enzymes
71 in the aforementioned pathways are all localised to the bacterial envelope (*i.e.* in the
72 periplasm of Gram-negative bacteria or on the surface of Gram-positive bacteria). Indeed,
73 with the exception of plastocyanin and cytochrome oxidase in Cyanobacteria, cuproproteins
74 are not known to exist inside the bacterial cytoplasm. This apparent compartmentalisation of
75 Cu to the extracytoplasmic space may represent a mechanism for balancing the physiological
76 advantages of using nutrient Cu in catalysis while protecting against its potential toxicity.
77 Indeed, Cu is generally considered to be more toxic in the cytoplasm and thus must be
78 buffered at a lower availability (*i.e.* bound by higher affinity or lower energy sites in the
79 buffer) relative to the extracytoplasmic space.
80
81 Metals in the bacterial envelope are readily exchangeable with the extracellular environment,
82 for example *via* passive diffusion across outer membrane porins in Gram-negative
83 organisms[14]. Hence, fine control of metalation in this compartment may be more challenging
84 than in the cytoplasm. This is considered particularly problematic for metalloproteins that are
85 translocated *via* the Sec general secretory pathway and thus are folded (and metalated) in the

86 extracytoplasmic space.[15] By contrast, metalloproteins that are Tat substrates fold inside the
87 cytoplasm and, at least in some cases, obtain their cognate metal prior to secretion. In the
88 case of Cu, recent examination of the periplasmic multicopper oxidase CueO from
89 *Escherichia coli* demonstrated that removal of the Tat signal sequence and expression of
90 CueO in the cytoplasm led to isolation of only the *apo*-enzyme.[16] In fact, all bacterial
91 cuproproteins for which the steps of Cu insertion have been identified (detailed in this
92 review) are thought to become metalated outside the cytoplasm, regardless of the
93 translocation mechanism of the protein scaffold. One explanation is that the Cu affinities of
94 these cuproenzymes are compatible with the buffered availability of Cu in the bacterial
95 envelope but incompatible with that of the cytoplasm. In addition, the oxidation state of Cu in
96 the buffer and the oxidation state preferred by the enzyme might further define
97 extracytoplasmic metalation of cuproproteins.
98
99 What is the source of nutrient Cu for cuproenzymes in the bacterial envelope? In the simplest
100 model, a buffered pool of Cu in the extracytoplasmic space acts as the Cu supplier. The
101 molecular nature of this Cu buffer is presently unknown. It has been long assumed that thiols
102 like glutathione (GSH) buffer Cu in the cytoplasm.[17] There is also evidence that GSH is
103 exported to the periplasm of Gram-negative organisms[18], and so it can presumably also buffer
104 Cu in this compartment. However, the affinity of GSH for Cu is orders of magnitudes weaker
105 when compared to those of bacterial Cu sensors in the cytoplasm[19,20] or nutrient Cu
106 metallochaperones in the periplasm[21]. These relative values suggest that glutathione would
107 constitute a high energy buffer, filled only when an excess of Cu is available. By contrast, the
108 identity of the low energy or high affinity buffer that contributes to normal Cu nutrition is
109 unknown. Nevertheless, metalloproteomics examination of periplasmic extracts from

110 *Salmonella enterica* sv. Typhimurium[22] and *Synechocystis*[23] indicated that periplasmic Cu is
111 bound either to Cu metallochaperones or to unidentified low molecular weight proteins.
112
113 Regardless of the precise identity of the extracytoplasmic Cu buffer, it is presumably filled by
114 Cu from the extracellular environment (Figure 2). This exchange of Cu may occur *via* passive
115 diffusion through porins[14,24] or other unidentified mechanisms[25]. Active uptake of Cu is also
116 known to occur, for example *via* TonB-dependent receptors[26] or *via* classical siderophores[27]
117 and Cu-binding metallophores ("chalkophores") such as yersiniabactin and methanobactin[28-
118 30]. Once the buffer is filled by Cu, provided that the affinities of the cuproenzymes are higher
119 than the affinity of the buffer, Cu will flow down the thermodynamic gradient and ultimately
120 insert into target enzymes (Figure 1b). Yet, there is now mounting evidence that Cu-
121 exporting P-type ATPases embedded in the cytoplasmic membrane are involved in
122 metalating extracytoplasmic cuproproteins.[23,31,32] The implication is that nutrient Cu ions are
123 trafficked through the cytoplasm *en route* to the extracytoplasmic targets and, if so, this must
124 be a vital process for Cu homeostasis. When combined with the dearth of known cytoplasmic
125 Cu importers, this apparently circuitous routing of Cu is one of the most puzzling aspects of
126 nutrient Cu handling in bacteria.
127
128 Our research groups have studied bacterial Cu tolerance for several years and have recently
129 begun to investigate nutrient Cu handling, specifically in pathogenic *Neisseria*. This
130 prompted us to revisit existing literature related to bacterial nutrient Cu trafficking and
131 identify gaps in knowledge. We were particularly intrigued by the evidence that bacterial
132 cuproenzymes do not always require auxiliary metallochaperones to insert nutrient Cu into
133 their active sites. This review outlines our effort to consolidate the available experimental
134 data by expanding an established energy-driven model for Cu trafficking[2]. We focus on four

135 major families of bacterial cuproenzymes: (1) nitrous reductases, (2) nitrite reductases, (3)
136 Cu,Zn-superoxide dismutases, and (4) haem-Cu oxidases, and pay particular attention to the
137 precise steps of Cu insertion. The genomic context and genetic distribution, structural
138 features and properties of the Cu centres in the enzymes (and in the associated
139 metallochaperones), as well as kinetic properties of these enzymes are already subjects of
140 numerous excellent reviews and so will not be covered in detail.
141
142 **Cu insertion into nitrous oxide reductases**
143
144 Assembling a denitrification pathway is a Cu-expensive process since it involves at least one
145 multicopper enzyme, namely nitrous oxide reductase (N_2OR or NosZ), which catalyses the
146 reduction of N_2O to N_2. NosZ homologues are classified as typical or atypical, distinguished
147 by two key biochemical characteristics: (i) an additional haem *c* binding site is present near
148 the C-terminus in atypical NosZ but is absent in typical NosZ; and (ii) translocation of
149 atypical NosZ is Sec-dependent while that of typical NosZ is Tat-dependent.[33] Both types of
150 NosZ contain 6 Cu atoms per monomer (12 Cu per functional homodimer), arranged into one
151 tetranuclear Cu_Z copper-sulfur (Cu_4S_2) cluster that binds and activates N_2O during catalysis,
152 and one binuclear mixed-valent Cu_A centre that acts as the site of electron entry.[34-37]
153
154 Consistent with its high demand for Cu, NosZ activity in denitrifying organisms is greatly
155 influenced by extracellular Cu levels.[38-40] During conditions of Cu deficiency, NosZ activity
156 decreases and N_2O accumulates. This Cu-dependent regulation of NosZ occurs at the post-
157 translational level, *i.e.* by modulating occupancy of the Cu centres. Growth in Cu-deficient
158 conditions leads to production of NosZ in an inactive form. However, N_2O reductase activity
159 is restored by addition of exogenous Cu without the need for new protein synthesis.[39] In

160 bacteria possessing the typical NosZ, increases in extracellular Cu levels also induce the
161 expression of *nos*Z. This requires at least one factor, the flavoprotein NosR, although the
162 molecular details are yet to be elucidated.[40,41] The *nosR* gene is not found in genomes
163 encoding atypical NosZ[33], and whether Cu regulates *nosZ* transcription in these organisms is
164 unknown.
165
166 The current models for Cu_Z and Cu_A biogenesis suggest that these Cu centres are assembled
167 in the periplasm following secretion of the protein, for both the typical and atypical NosZ,
168 regardless of the translocation mechanism (Figure 2). Homologous expression of NosZ in the
169 cytoplasm results in the production of neither the Cu_Z nor the Cu_A centre.[42] Assembly of Cu_Z
170 *in vivo* requires NosDFY, an ABC-type transporter that may transport sulfur (Figure 2)[43],
171 although this is yet to be confirmed experimentally. This requirement for NosDFY appears to
172 be obligate and the genetic clustering of *nosZ* with *nosDFY* is absolutely conserved in all
173 sequenced genomes that are currently available.[33] N_2O respiration is abolished if any of the
174 *nosDFY* genes is mutated and this defect is not restored by addition of extracellular Cu.[44,45] In
175 addition, NosZ isolated from *nosDFY*-deficient strains contains only the Cu_A centre[46-48],
176 indicating that NosDFY may not be required to assist Cu_A assembly.
177
178 Insertion of nutrient Cu into the Cu_Z cluster *in vivo* is likely facilitated by NosL, a small
179 lipoprotein that is anchored to the outer membrane (Figure 2). The soluble periplasmic
180 domain of NosL binds one Cu(I) ion *in vitro* but its affinity has not been determined.[49] The
181 Cu ligands include one Cys and one Met, presumably from a conserved Cys-X-Met motif
182 near the N-terminus.[50,51] The third ligand, likely from a His residue, is yet to be identified,
183 and no obvious candidate is found from analysis of amino acid sequences. Whether NosL
184 delivers Cu(I) to NosDFY or directly to NosZ, whether metalation is coupled to sulfur

185 insertion, and whether NosL assists in assembly of the Cu_A centre are yet to be established.
186 None of the *nos* cluster genes appears to be essential for Cu_A assembly. Nevertheless,
187 denitrifying organisms often possess additional Cu metallochaperones like Sco and PCu_AC
188 (described below), which may metalate the Cu_A sites in NosZ, but this remains to be
189 elucidated.
190
191 Unlike NosDFY, NosL appears to be dispensable for Cu_Z assembly. Although fitness
192 analyses of a mutant library suggest that *nosL* is essential for denitrification in *Pseudomonas*
193 *stutzeri*[52], mutational inactivation of *nosL* in this organism does not yield an obvious defect in
194 N_2O reductase activity.[53] Likewise, heterologous expression of NosZ in its active form in the
195 periplasm of a nondenitrifying host does not necessitate the co-expression of NosL.[46]
196 Furthermore, *nosL* is absent from the *nos* gene cluster in many genomes and this absence
197 does not correlate with the type of NosZ (typical or atypical) or the NosZ translocation
198 mechanism.[33]
199
200 How does Cu_Z obtain nutrient Cu in the absence of NosL? There is a proposal that other Cu
201 metallochaperones such as PCu_AC (described below) can compensate, although this is yet to
202 be tested experimentally. An alternative, and arguably simpler, hypothesis is that the Cu_Z site
203 acquires Cu directly from the extracytoplasmic Cu buffer (Figure 2). This reaction is
204 thermodynamically favourable ("downhill" or exergonic) as long as the affinity of the Cu_Z
205 scaffold for Cu is higher than the affinity of the buffer (*i.e.* the bound Cu ion in Cu_Z is lower
206 in energy or more stable than is Cu in the extracytoplasmic buffer) (Figure 1b). NosL may
207 provide an "intermediate buffer" (with intermediate Cu affinities) that lowers the overall
208 energy barrier for the transfer of Cu from the extracytoplasmic buffer to the Cu_Z scaffold,
209 with Cu-NosL acting as a reaction intermediate (Figure 1c). In this scenario, the absence of

210 NosL would not affect the Cu occupancy of NosZ, provided that the buffered Cu availability
211 is sufficiently high (*i.e.* Cu is bound by high energy or low affinity sites in the buffer) and the
212 barrier for Cu transfer to NosZ is sufficiently low. NosL would become more important in
213 Cu-deficient conditions, when the buffered Cu availability decreases (*i.e.* Cu is bound by low
214 energy or high affinity sites in the buffer) and thus, presumably, the barrier for onward Cu
215 transfer to NosZ increases (Figure 1d).
216
217 Regardless of the precise role for NosL, the question remains: what is the source of the
218 buffered Cu in the extracytoplasmic space? In the simplest model, this buffer is filled directly
219 by Cu from the extracellular environment (Figure 2). In some, but not all, denitrifying Gram-
220 negative organisms, N_2O respiration during conditions of Cu limitation requires NosA, a
221 TonB-dependent receptor that may increase uptake of Cu into the periplasm (Figure
222 2).[26,46,54,55] Intriguingly, there is also evidence that the extracytoplasmic pool of Cu is filled
223 by supply from the cytoplasm. NosZ activity *in vivo* was shown to depend on CtpA, a P-type
224 ATPase that resembles known bacterial Cu-efflux transporters (Figure 2).[31] Mutation of *ctpA*
225 leads to decreased NosZ activity but enzyme activity is restored by addition of Cu to the
226 extracellular medium. This exogenous Cu presumably fills the extracytoplasmic Cu buffer,
227 which in turn metalates NosZ (Figure 2). If direct metalation of NosZ by the
228 extracytoplasmic Cu buffer is possible in the Δ*ctpA* mutant, why nutrient Cu must first be
229 routed through the cytoplasm in the wild type organism appears a major conundrum.
230
231 **Cu insertion into Cu-containing nitrite reductases**
232
233 Cu-dependent nitrite reductase (Cu-NIR), usually called NirK, catalyses the reduction of
234 NO_2^- to N_2O. This enzyme contains a total of 2 Cu centres per monomer (6 Cu per functional

235 homotrimer): one T1 “blue” Cu centre that acts as the site for electron entry and one T2 Cu
236 centre that acts as the active site for NO_2^- binding and reduction.[56] NirK is sometimes co-
237 encoded in the genome with NirV, a protein of unknown function that does not appear to bind
238 Cu.[57,58] Only a minority of NirK homologues carry the Tat signal sequence, with most
239 thought to be translocated *via* the Sec or other nonspecific secretory pathways.[59]
240
241 T1 and T2 Cu centres are readily reconstituted by Cu salts *in vitro* and so insertion of Cu into
242 NirK *in vivo* was previously assumed to require no accessory metallochaperones. However, a
243 recent genetic screen identified that a soluble periplasmic Cu-binding protein, AccA, is
244 required for metalating NirK (AniA) in pathogenic *Neisseria* (Figure 3).[60] AccA is a
245 homologue of PCu_AC, a metallochaperone that is thought to aid assembly of Cu_A and Cu_B
246 centres in haem-Cu oxidases[61-64] (described below). Like PCu_AC, AccA binds one Cu(I) ion
247 with a high apparent affinity[60], although precise quantification is still awaited. Conserved
248 Met and His residues are likely involved in binding Cu(I). AccA also binds one additional Cu
249 ion in the Cu(II) oxidation state.[60] Several candidate ligands for Cu(II) are present in the His-
250 and Met-rich C-terminus but their identities are yet to be determined. Programmes in our
251 research groups are currently ongoing to determine which of the two bound Cu ions in AccA
252 is loaded to which of the two Cu sites in AniA.
253
254 Mutants lacking *accA* generate wild type amounts of AniA but fail to reduce NO_2^-,
255 suggesting that AniA is produced in the *apo*- or incorrectly metalated form. Consistent with
256 this view, reduction of NO_2^- resumes, albeit only partially, upon addition of Cu salts into the
257 extracellular media.[60] Assuming that no other unidentified Cu trafficking pathway
258 compensates for AccA, the observed recovery of AniA activity by exogenous Cu is
259 consistent with the proposal that that this enzyme is metalated directly by a buffered Cu pool

260 in the periplasm (Figure 3). This reaction is energetically downhill as long as the affinity of
261 AniA for Cu is higher than the affinity of the buffer (*i.e.* the bound Cu in AniA is more stable
262 or less energetic) (Figure 1b). As hypothesised earlier for NosL, the function of AccA may be
263 to act as an intermediate buffer that lowers the overall energy barrier for Cu exchange and
264 thus functionally "catalyses" the transfer of Cu from the buffer to the T1 and/or T2 sites of
265 AniA (Figure 1c). In the absence of AccA, provided that the buffered Cu availability is
266 sufficiently high (*i.e.* Cu is bound by high energy or low affinity sites in the buffer), the
267 barrier for onward Cu transfer decreases, and AniA becomes metalated (Figure 1e).
268
269 There is evidence that deletion of the AccA homologue PCu_AC in *Bradyrhizobium japonicum*
270 also leads to transient accumulation of NO_2^-, implying a defect in NirK activity.[65] Hence,
271 although PCu_AC primarily aids assembly of Cu_A centres (discussed below), the possibility
272 that this metallochaperone inserts nutrient Cu into multiple cuproenzymes, including NirK,
273 should not be disregarded. In the energy-driven model, the role of PCu_AC is facilitative rather
274 than obligatory (Figure 1c). This model can rationalise why not all genomes that encode a
275 NirK[59] possess a PCu_AC or AccA, and conversely, why the presence of *pcu_AC* in denitrifying
276 organisms does not exclusively correlate with the presence of *nirK*[40] or even with Cu_A
277 centres[66].
278
279 Like pathogenic *Neisseria*, some NirK-containing organisms also possess the
280 metallochaperone Sco.[66,67] Together with PCu_AC, Sco is thought to facilitate assembly of Cu_A
281 and Cu_B centres in haem-Cu respiratory oxidases (described below). Whether Sco is required
282 for inserting Cu into T1 and T2 sites of NirK is not known. Likewise, whether a Cu importer
283 such as NosA from *P. stutzeri* or a Cu-exporting P-type ATPase such as CopA in pathogenic
284 *Neisseria* is involved in metalating AniA or NirK is yet to be examined (Figure 3).

285

286 Cu insertion into Cu,Zn-superoxide dismutase

287

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

298

299 The T2 centre in SodC assembles spontaneously *in vitro* without the need for assembly
300 factors.[75] Based on studies with *Salmonella enterica* sv. Typhimurium, metalation of SodC *in*
301 *vivo* likely involves, but does not absolutely require, a soluble periplasmic metallochaperone
302 named CueP (Figure 4).[32,76] Homologues of CueP are found in both Gram-positive and
303 Gram-negative bacteria[77], but certainly not in all SodC producers (*e.g. E. coli*). Deletion of
304 *cueP* impairs, but does not completely eliminate, the activities of the two SodC homologues
305 in *Salmonella*, SodCI and SodCII.[32,76] The reduction in enzyme activities correlates with
306 decreased occupancy of the T2 Cu site, at least for SodCII.[32] However, enzyme activity
307 and/or Cu occupancy are restored *in vivo* by supplementing the culture medium with Cu salts
308 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

334 Even during conditions of Cu stress, *i.e.* when the low affinity and high energy sites in the
335 buffer become full, SodC remains energetically downhill from the buffer, and so this enzyme
336 will continue to be metalated correctly. However, adventitious protein sites may now also
337 become downhill from the “full” buffer and subsequently mis-metalated by Cu (Figure 5).
338 Increasing the amounts of CueP under these conditions will generate alternative stable but,
339 more importantly, specific sites for Cu binding. Thus, the excess Cu can “drain” from less
340 stable (*i.e.* lower in affinity or more energetic) sites in the original buffer or in mismetalated
341 proteins to the more stable (*i.e.* higher in affinity or less energetic) site in CueP (Figure 5c).
342 In this model, CueP essentially directs or regulates the flow of Cu down the thermodynamic
343 gradient, both during conditions of normal Cu nutrition and conditions of Cu stress. This
344 “intermediate buffering” function for Cu metallochaperones during Cu homeostasis has
345 indeed been postulated previously[81-83] although it has not been tested for the
346 metallochaperones highlighted in this review. The challenge for bacteria is to control
347 production of CueP such that it does not start to drain Cu from SodC as a consequence of
348 mass action (*e.g.* see equation in Figure 1). There is experimental evidence that correct
349 amounts of CueP are indeed important. Expression of *cueP* from a CpxR-independent
350 promoter leads to growth defects in the presence and absence of added Cu.[79]
351
352 Another mechanism to maintain SodC in its metalated form may involve control of the
353 oxidation state of Cu. The Δ*cueP* mutant is Cu-sensitive only during anaerobic growth
354 conditions.[77] In the presence of O_2, Cu(I) is removed from the buffer *via* oxidation to Cu(II)
355 by the cuprous oxidase CueO[84,85], and thus additional buffering of Cu(I) by CueP may not be
356 necessary. In addition, Cu in the resting form of SodC exists in the Cu(II) state. *In vitro*, this
357 bound Cu(II) ion does not re-partition into *apo*-CueP.[32] Thus, overexpression of CueP *in vivo*
358 is unlikely to lead to de-metalation of SodC, at least under aerobic growth conditions, when

359 SodC activity is essential[86]. During anaerobic growth, when SodC is not required,
360 extracytoplasmic reductants may reduce the Cu(II) ion in SodC and subsequent back-transfer
361 of Cu(I) to CueP is plausible, although not yet demonstrated.
362
363 Metalation of SodC *in vivo* also depends, at least partly, on outward transport of Cu from the
364 cytoplasm to the periplasm *via* either one of the two, functionally redundant, Cu efflux
365 pumps in *S.* Typhimurium, CopA and GolT (Figure 4). SodC isolated from mutant bacteria
366 lacking both P-type ATPases contains only the Zn centre but readily acquires Cu upon
367 addition of Cu(II) salts into cell-free extracts.[32] This finding may further highlight the
368 importance of the correct oxidation state for Cu. CopA and GolT transport Cu in the reduced
369 Cu(I) form, and the relative affinities of the periplasmic domains of the P-type ATPases, the
370 periplasmic buffer, CueP, and SodC for Cu(I) may be ordered such that metalation of SodC
371 with Cu(I) is thermodynamically favourable. However, as already mentioned earlier, the T2
372 site in SodC is also competent to acquire Cu(II), at least *in vitro.*[32] In vivo, one possibility is
373 that the buffered Cu(II) availabilities (or energies) in the periplasm are low and hence
374 insertion of Cu(II) into SodC may be a thermodynamically uphill or unfavourable process.
375 Measurements of the affinities of CueP and SodC each for Cu(I) and Cu(II), and comparisons
376 with the buffered availabilities of Cu(I) and Cu(II) in the periplasm would be informative.
377
378 **Cu insertion into haem-Cu respiratory oxidases**
379
380 Haem-Cu respiratory oxidases are transmembrane, multi-subunit, multi-haem enzymes that
381 catalyse the terminal step in the electron transport chain, namely the conversion of molecular
382 oxygen to water. All members of this enzyme superfamily contain a mononuclear Cu_B centre
383 that is spin-coupled to a haem. This is the site of O_2 binding and reduction, and it is

384 embedded deep within the transmembrane structures. Transfer of electrons from a
385 cytochrome or quinol typically involves a relay of haem cofactors and, in some cytochrome *c*
386 oxidases, a dinuclear Cu_A centre that is housed within a soluble periplasmic subunit. As
387 anticipated from the complex enzyme architecture, assembly of haem-Cu oxidases likely
388 requires a modular process that is synchronised both temporally and spatially, along with
389 checkpoints that prevent folding of empty Cu sites into the mature but nonfunctional
390 complex.[87]
391

392 Of interest in this review are the precise steps of Cu insertion into Cu_B and Cu_A. These
393 processes are most studied for mitochondrial cytochrome *c* oxidase (COX) in eukaryotes.[88,89]
394 Given the endosymbiotic bacterial origin of mitochondria, the mechanisms for metalation of
395 mitochondrial COX and bacterial haem-Cu oxidases likely share some universal features.
396 The bacterial metallochaperones involved in Cu_B and Cu_A assembly, namely Sco[64,90-94],
397 PCu_AC[61-63,65,95], or Cox11p[96-100], are, again, localised to the extracytoplasmic space (Figure
398 6). These metallochaperones are structurally and functionally analogous to their eukaryotic
399 counterparts (PCu_AC acts as a functional Cox17 homologue). However, unlike the eukaryotic
400 system, the precise contribution of each protein in the assembly of bacterial Cu_A *vs.* Cu_B
401 centres and the sequence of Cu insertion events remain poorly defined and, bafflingly, appear
402 to be organism-dependent.
403

404 Part of the confusion can perhaps be ascribed to the varied genomic distributions of these
405 metallochaperones. For instance, it is generally agreed that assembly of bacterial Cu_A centres
406 *in vivo* involves both Sco and PCu_AC (Figure 6).[21,63] There is indeed evidence that Sco and
407 PCu_AC form a transient complex *in vitro* and *in vivo*.[63] However, the genes encoding these
408 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 $HX_6MX_{21}HXM$
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

434 PCu_AC acts as the Cu metallochaperone (or Cu donor) while Sco acts as a reductase that
435 maintains either the Cu ion or the Cu_A cysteine ligands in the reduced forms (Figure 6).[64]
436 Upstream reductases such as TlpA may provide the reducing power *in vivo* (Figure 6).[104,105]
437

438 As mentioned earlier, not all bacterial haem-Cu oxidases contain a Cu_A centre. However, in
439 contrast to the relative wealth of information available for Cu_A, current understanding of Cu_B
440 assembly remains limited, mainly because the location of Cu_B deep within a transmembrane
441 domain has largely precluded *in vitro* studies. Nevertheless, as discussed below, there is
442 mounting *in vivo* evidence that Sco and PCu_AC are also involved in Cu_B assembly, at least in
443 some organisms. The mechanism may parallel that for Cu_A although the precise details still
444 need investigation. In addition, the bacterial homologue of mitochondrial Cox11, Cox11p,
445 has been implicated in forming bacterial Cu_B centres *in vivo*.[98,100] Whether bacterial Cox11p
446 coordinates its function with PCu_AC and/or Sco is unknown.
447

448 Mutation of *sco*, *pcu$_A$C*, or both, typically, but not always[60], leads to decreases, but not
449 complete losses, in the activities of haem-Cu oxidases, regardless of whether the specific
450 oxidase contains only Cu_B (*e.g.* *cbb*$_3$ oxidase) or both Cu_B and Cu_A (*e.g.* cytochrome *aa*$_3$ and
451 *ba*$_3$ oxidases)[64,65,91,95,99,106,107]. Deletion of *sco* typically produces the stronger
452 phenotype.[106,108] The defects in oxidase activities correlate with decreases in the amounts of
453 mature subunits formed *in vivo* but these can be overcome, at least partially, by
454 supplementing the extracellular medium with Cu salts.[63-65,91,95,106-108]
455

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

459 metalation with Cu *in vitro* is coupled to reduction (either of the Cu ion or of the Cu_A or Cu_B
460 ligands), general extracytoplasmic reductases may provide this reducing power *in vivo*. This
461 scenario is plausible for Cu_A because this centre is readily assembled *in vitro* in the presence
462 of Cu salts and reductants.[34,109] Provided that the thermodynamic gradients (*i.e.* buffered Cu
463 availabilities, affinities of the metallochaperones, and affinities of the Cu_A scaffold) *in vivo*
464 are appropriately setup for the exergonic Cu transfer from the buffer into the empty Cu_A sites,
465 Cu will insert (Figure 1b and Figure 1c). For Cu_B, metalation may be combined with folding
466 checkpoints to avoid accidental incorporation of the non-metalated sites in the mature
467 complex. Indeed, potential roles in Cu insertion for other accessory components such as
468 CcoG, CcoH, and CcoS (at least for the *cbb*$_3$ oxidase) have been proposed, which may reflect
469 their role in regulating such maturation checkpoints but mechanistic data are currently
470 lacking.[93,110,111]
471
472 Like the other extracytoplasmic cuproenzymes described in this review, haem-Cu oxidases
473 also appear to utilise nutrient Cu that has been routed *via* the cytoplasm, first *via* a major
474 facilitator superfamily (MFS)-type transporter named CcoA that putatively imports Cu into
475 the cytoplasm[112,113] and subsequently *via* a Cu efflux pump (CcoI or CtpA)[31,111,114,115] (Figure
476 6). Deletion of each of these transporters leads to decreases in the activities of Cu_B and/or
477 Cu_A-containing cytochrome oxidase activities, but these are, to some extent, alleviated by
478 supplementation with Cu salts. This apparent routing of Cu through the intracytoplasmic
479 compartment to metalate an extracytoplasmic cuproenzyme is one of the least understood
480 aspects of nutrient Cu trafficking but, if it does occur, must represent a vital process in
481 bacterial Cu homeostasis.
482

483 **Outlook and perspectives: The need for systems approaches to examine nutrient Cu**
484 **handling in bacteria**
485
486 Among the six, first-row *d*-block transition metal ions that are considered as bacterial
487 nutrients (Mn, Fe, Co, Ni, Cu, Zn), Cu is often highlighted for its potential toxicity. Cu ions
488 bound in weak or high energy or unstable sites can catalyse harmful redox reactions, while
489 Cu ions in strong, low energy or stable but non-native (adventitious) sites (mismetalation)
490 can disrupt protein or enzyme function. While the outward transport of Cu as a bacterial
491 poison has received significant attention from the metallomics community, inward flow of
492 this metal ion as a bacterial nutrient remains less defined. Confounding this issue, known
493 bacterial Cu importers and Cu-binding metallophores are still exceedingly rare and, as
494 described in this review, while they are relatively more common, nutrient Cu
495 metallochaperones are often functionally redundant.
496
497 The apparent redundancy of Cu metallochaperones may be rationalised by the energy-driven
498 model, in which target cuproenzymes obtain nutrient Cu directly from a buffered Cu pool *via*
499 "downhill" or exergonic associative exchange reactions (Figure 1b). It is our view that this
500 model can universally rationalise all the available experimental evidence for the metalation of
501 cuproenzymes in different bacterial organisms. In this model, the *apo*-metallochaperones can
502 be considered as intermediate buffers or functional catalysts that lower the energy barrier for
503 Cu transfer regulate the flow of Cu down the thermodynamic gradient (Figure 1c). The Cu-
504 bound form of the metallochaperone thus represents a thermodynamic local minimum that
505 limits "sideway" flows of Cu into adventitious sites (Figure 1c). Hence, these
506 metallochaperones are not obligate components for Cu homeostasis but are nonetheless able
507 to provide alternative and more efficient routes for metalation during Cu nutrition,

508 particularly when extracellular Cu is limiting, and for preventing (or correcting)
509 mismetalation during Cu poisoning. This "intermediate buffering" function for Cu
510 metallochaperones has indeed been proposed previously[81-83] but how these
511 metallochaperones lower the energy barrier for Cu transfer remains to be determined.
512

513 A key advantage of this model is that, in organisms where the metallochaperone is absent,
514 there is no need to describe elaborate backup or compensatory mechanisms. Instead, the main
515 considerations would be the oxidation state of Cu, as well as the relative amounts and Cu
516 affinities of the target cuproenzymes, of the metallochaperones, and of the extracytoplasmic
517 buffer. Differences in these properties may explain why periplasmic cuproproteins do not
518 acquire Cu when expressed homologously in the cytoplasm. If the affinities of the
519 cytoplasmic buffer for Cu are higher than the affinities of the cuproenzymes (*i.e.* bound Cu in
520 the buffer is less energetic or more stable), transfer of Cu out of the buffer would be
521 thermodynamically uphill or endergonic. Hence, knowledge of the relative tunings of
522 extracytoplasmic buffer components compared to the cytoplasm becomes equally important.
523

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

533 operate under the control of cytoplasmic Cu sensors that activate transcription only when the
534 cytoplasmic buffer is "full". Under these conditions, buffered Cu availability outside the
535 cytoplasm is presumably also elevated and indeed multiple extracytoplasmic components of
536 Cu tolerance are usually produced.[116] Why, then, is extracytoplasmic buffered Cu not used
537 directly as the source of nutrient Cu? Is this related to the oxidation state of the metal? What
538 is the contribution of Cu storage proteins like Csp, which can be present in the cytosol or the
539 periplasm[117]? Systems measurements of the buffered Cu availabilities, and comparisons
540 between the cytoplasm and the periplasm, even if technically challenging, may prove
541 illuminating. These can build on recent pioneering efforts by others in the metallomics
542 community to decipher bacterial Cu (and metal) homeostasis.[1,3,4,81,118,119]
543

544 **Conflicts of interest**

545

546 We declare no conflict of interest.

547

548 **Acknowledgement**

549

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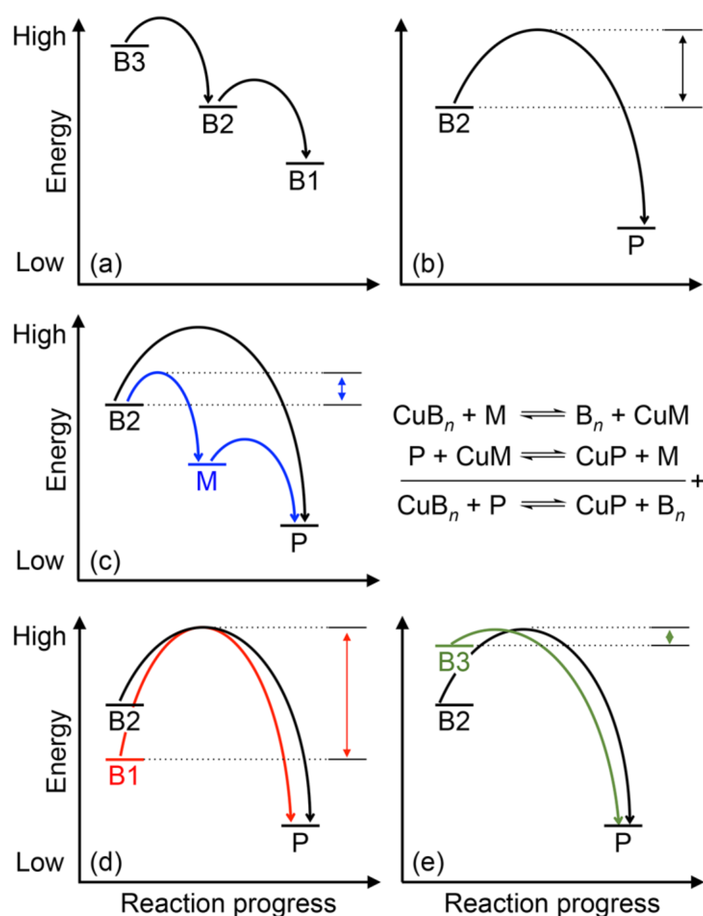
559

560 **FIGURES**

561



562

563

564 **Figure 1.** General energy-driven model for the insertion of Cu into cuproenzymes. The

565 relative energy for each Cu-binding site, whether in the buffer (B1, B2, B3), cuproprotein (P),

566 or metallochaperone (M) is shown. Curved arrows represent the forward transfer of Cu from

567 one binding site to another while double-headed arrows represent the energy barrier that must

568 be overcome. Several scenarios are depicted: **(a)** Upon entry into cells, Cu fills the buffer by

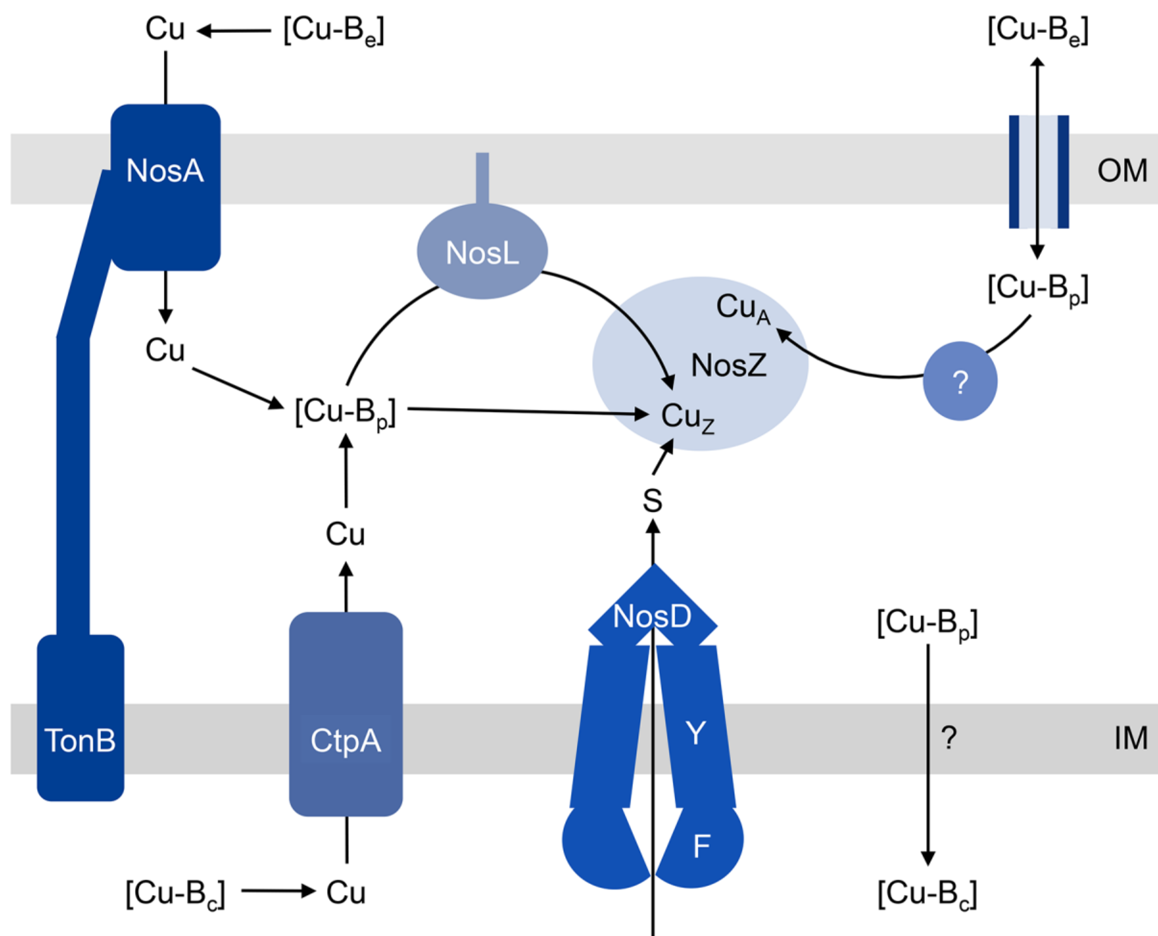
569 stepwise transfer from high energy or low affinity sites (denoted as B3) to low energy or high

570 affinity sites (denoted as B1) in the buffer through stochastic exchange reactions. **(b)** Direct

571 transfer of Cu from the buffer (in this example the mid-affinity or mid-energy site B2) to a

572 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



581

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

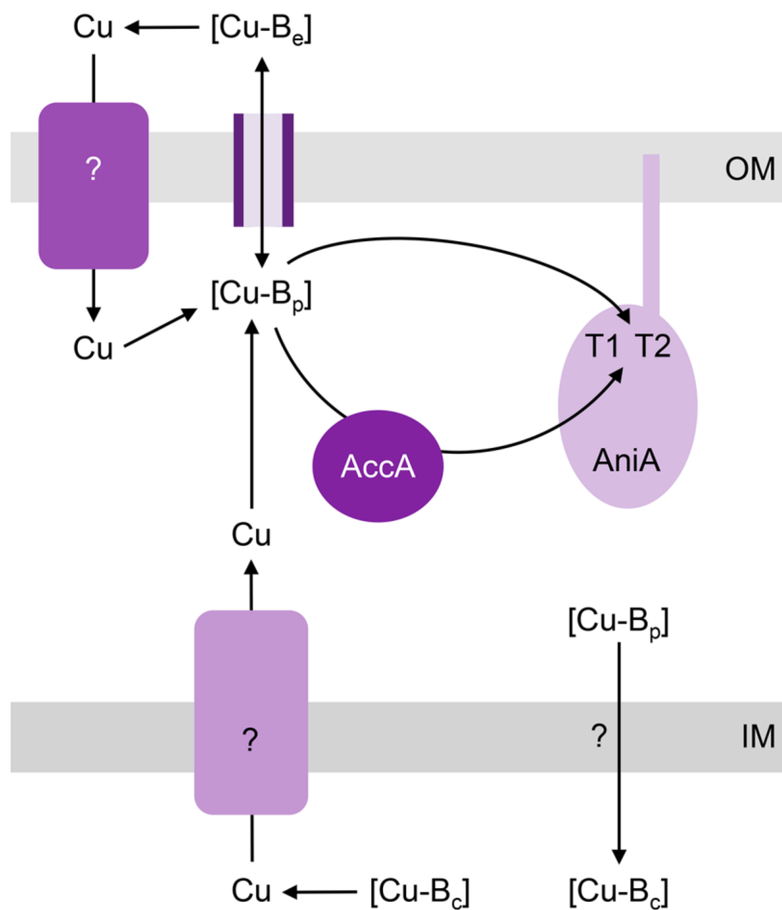
588 unknown. The Cu_Z site in NosZ acquires Cu from [Cu-B_p] either directly or *via* the

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.

592



593

594 **Figure 3.** General model for the insertion of Cu into AniA (NirK). [Cu-B_e], [Cu-B_c], and

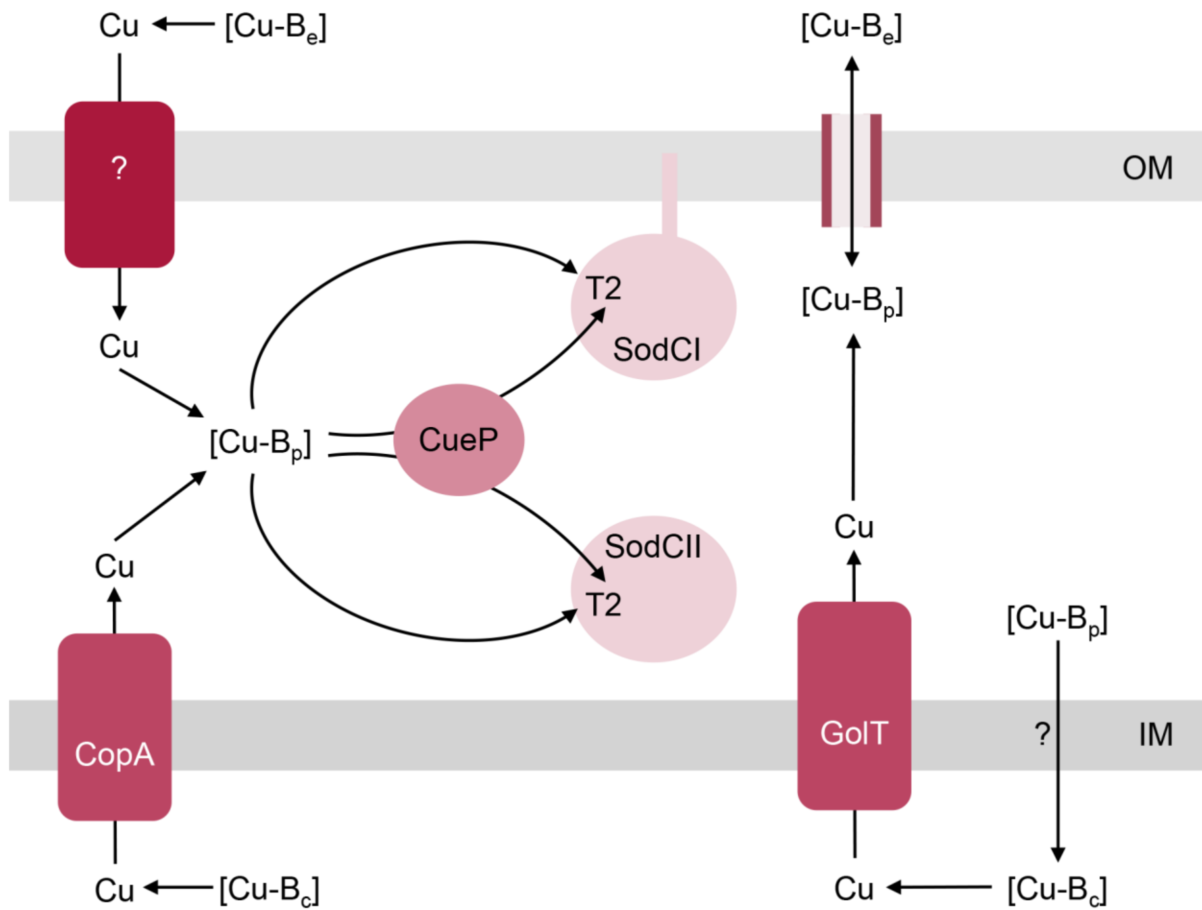
595 [Cu-B_p] are buffered pools of Cu in the extracellular space, cytoplasm, and periplasm,

596 respectively. The T1 and T2 sites in AniA acquire Cu from [Cu-B_p] either directly or *via* the

597 metallochaperone AccA (PCu_AC). [Cu-B_p] is likely filled by [Cu-B_e] *via* direct exchange

598 across outer membrane porins. Whether an outer membrane importer or a cytoplasmic

599 exporter is involved in filling [Cu-B_p] is yet to be determined.



600

601

602 **Figure 4.** General model for the insertion of Cu into SodC. [Cu-B_e], [Cu-B_c], and [Cu-B_p] are

603 buffered pools of Cu in the extracellular space, cytoplasm, and periplasm, respectively.

604 [Cu-B_p] is likely filled by [Cu-B_e] *via* direct exchange across outer membrane porins.

605 Whether an outer membrane importer is involved in this process is yet to be established. [Cu-

606 B_p] is also filled by [Cu-B_c] *via* the P-type ATPases CopA or GolT. How [Cu-B_c] is

607 generated is unknown. The T2 Cu site in SodC acquires Cu from [Cu-B_p] either directly or

608 *via* the metallochaperone CueP.

609

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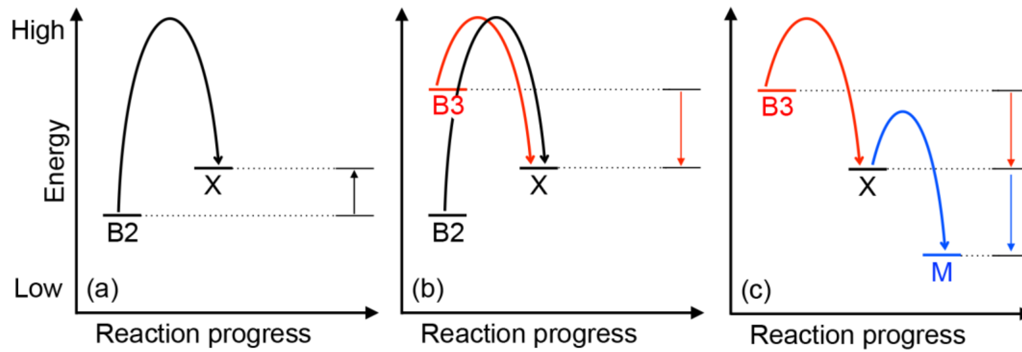
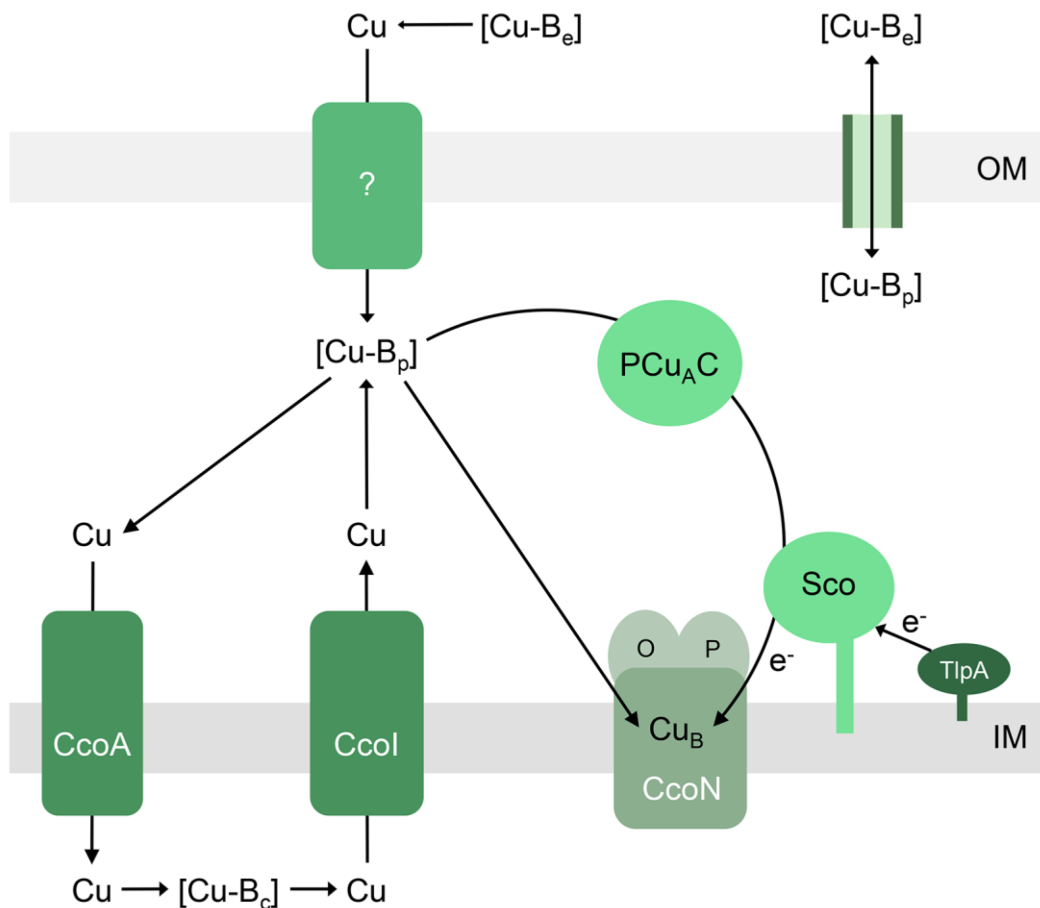


611

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

627



628

629

630 **Figure 6.** General model for the insertion of Cu into the Cu_B site into cytochrome *cbb*$_3$

631 oxidase. Only the active site subunits CcoNOP are shown. This model may broadly apply to

632 insertion of Cu into the Cu_A site in other haem-Cu oxidases. [Cu-B$_e$], [Cu-B$_c$], and [Cu-B$_p$]

633 are buffered pools of Cu in the extracellular space, cytoplasm, and periplasm, respectively.

634 The Cu_B site in CcoN (and/or Cu_A site in other haem-Cu oxidases) may obtain nutrient Cu

635 directly [Cu-B$_p$] or *via* the periplasmic Cu metallochaperones PCu$_A$C and Sco. Based on

636 studies on Cu_A assembly, Sco may also act as a thiol-disulfide reductase that maintains either

637 the Cu ion or the Cu_B (or Cu_A) Cys ligands in their reduced forms. Upstream reductases such

638 as TlpA may provide the reducing equivalents. Supply of Cu to [Cu-B$_p$] could occur by direct

639 exchange across outer membrane porins or *via* an as yet unidentified importer. The MFS

640 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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