The mechanisms underlying the biogenesis of the structurally unique, binuclear Cu\(^{1.5+}\)–Cu\(^{1+}\) redox center (Cu\(_A\)) on subunit II (CoxB) of cytochrome oxidases have been a long-standing mystery. Here, we reconstituted the CoxB-Cu\(_A\) complex in vitro from apo-CoxB and the holo-forms of the copper transfer chaperones Sco and PcuC. A previously unknown, highly stable Sco-Cu\(^{2+}\)-CoxB complex was shown to be rapidly formed as the first intermediate in the pathway. Moreover, our structural data revealed that PcuC has two copper-binding sites, one each for Cu\(^{1+}\) and Cu\(^{2+}\), and that only PcuC-Cu\(^{1+}\)-Cu\(^{2+}\) can release CoxB-Cu\(^{2+}\) from the Sco-Cu\(^{2+}\)-CoxB complex. The CoxB-Cu\(_A\) center was then formed quantitatively by transfer of Cu\(^{1+}\) from a second equivalent of PcuC-Cu\(^{1+}\)-Cu\(^{2+}\) to CoxB-Cu\(_A\). This metalation pathway is consistent with all available in vivo data and identifies the sources of the Cu ions required for Cu\(_A\) center formation and the order of their delivery to CoxB.

**INTRODUCTION**

Cytochrome oxidase (Cox; EC 1.9.3.1.) is the terminal enzyme of the respiratory chain in mitochondria of eukaryotes and in many aerobic bacteria and archaea (1, 2). Located in the inner mitochondrial membrane and in the cytoplasmic membrane of prokaryotes, it receives electrons from reduced cytochrome c and transfers them to molecular oxygen as the final electron acceptor. Oxygen reduction is coupled to proton translocation (3), and the resulting electrochemical membrane potential drives adenosine 5’-triphosphate (ATP) synthase. The aa3-type Cox is the most thoroughly studied cytochrome oxidase (4–6). Although its overall subunit composition varies among different organisms, the catalytically active core is highly conserved, consisting of a membrane-integral subunit I (CoxA in bacteria; Cox1 in mitochondria) and a membrane-anchored subunit II (CoxB or COX2) with a globular domain protruding into the mitochondrial intermembrane space or the periplasm of Gram-negative bacteria. A single electron from reduced cytochrome c is transferred to the exposed, binuclear Cu\(_A\) center of the globular CoxB domain and passed on to a low-spin heme a site and a high-spin heme a\(_{3}\)-Cu\(_B\) center, the latter being the active site for O\(_2\) reduction to H\(_2\)O (7).

In this study, we elucidate the reaction mechanisms underlying the biogenesis of the Cu\(_A\) center of CoxB. The term Cu\(_A\) refers to a mixed-valence, binuclear copper site, with the unpaired electron delocalized between two adjacent Cu ions in the globular CoxB domain (Cu\(^{1.5+}\)–Cu\(^{1+}\), oxidized form) (8–10). Incidentally, the Cu\(_A\) architecture occurs not only on CoxB but also on a few other metalloenzymes (11, 12). In CoxB, the two copper atoms are typically coordinated by the nitrogen atoms of two histidine imidazoles, the sulfur atoms of one methionine and two bridging cysteine thiols, and a protein backbone carbonyl oxygen (usually from a glutamate residue) present in the highly conserved amino acid sequence motif H-X\(_{34}\)-C\(_{X}\)-E\(_{X}\)-C\(_{X}\)-H\(_{X}\)-M (ligands underlined) (6, 8). It seems compelling to assume that the binuclear Cu\(_A\) center originates from one Cu\(^{1+}\) ion and one Cu\(^{2+}\) ion (10), but how Cu\(_A\) assembly is achieved is still not fully understood. Part of the uncertainty is due, on the one hand, to the substantial diversity between organisms under investigation, which precludes the formulation of a unified assembly pathway. On the other hand, the peculiar topology and cellular location of CoxB-Cu\(_A\) might ask for different mechanisms of Cu incorporation, depending on whether assembly takes place in the mitochondrial intermembrane space, in the periplasm of Gram-negative bacteria, or on the extracellular surface of the cytoplasmic membrane in Gram-positive bacteria and in archaea. In all of these environments, however, Cu-binding metallochaperones (13) play a decisive role (see below). In general, these cuproproteins ensure coordinated and controlled Cu ion delivery to cytochrome oxidase, and prevent formation of reactive oxygen species or displacement of other transition metals from essential enzymes by free intracellular Cu ions (14, 15).

At this point, it is pertinent to introduce the organism studied in the authors’ laboratories: *Bradyrhizobium diazoefficiens* (formerly called *Bradyrhizobium japonicum* strain USDA 110) (16), a Gram-negative \(\alpha\)-proteobacterium and, hence, one of the closest extant relatives of mitochondria. This trait allows interesting comparisons of related processes in a prokaryote versus the eukaryotic organelle. Respiration in this bacterium is highly flexible and diverse, as it has up to eight different oxygen reductases that are probably used for growth under different oxygen regimes (17). In cells growing strictly aerobically, the aa3-type cytochrome oxidase is the most prevalent oxygen reductase.

Regarding Cu\(_A\) formation, *B. diazoefficiens* was the first organism in which a specific thioredoxin-like reductase (TlpA) was found to satisfy an obligate requirement before metalation, i.e., the need to keep the two Cu-bridging cysteine ligands of the periplasmic CoxB domain in the reduced (dithiol) state (18). Just like CoxB, TlpA is anchored to the cytoplasmic membrane, and its thioredoxin-like domain faces the periplasm. Only reduced CoxB can serve as a target for copper insertion. In addition to TlpA, two different copper chaperones were identified previously in various organisms, including *B. diazoefficiens*, to be involved in Cu ion trafficking to CoxB, namely, Sco and PcuC (13, 19, 20). Sco is phylogenetically the more widespread of the two, occurring both
in mitochondria and in many aerobic prokaryotes (alternative names are SCO1, SenC, PrcC, and YpmQ) (21, 22). PcuC, originally discovered in Deinococcus radiodurans and Thermus thermophilus [therein named PcuAC (23, 24)], was recognized as a second important Cu chaperone for Cux center formation in several bacteria, but not in mitochondria, in which COX17 (13) may act as a functional equivalent.

Akin to CoxB and TlpA, all known ScoI proteins are tethered to the membrane and have a periplasmic, thioredoxin-like domain. However, unlike the thioredoxin domain of TlpA with its Cys-Val-Pro-Cys (CVPC) active-site motif, the thioredoxin domain of ScoI carries a conserved CxxC motif, in which the two cysteines form the copper-binding site together with a more distant histidine (25–27). If these two vicinal cysteines are oxidized to a disulfide bridge, they need to be reduced by TlpA in B. diazoefficiens before Cu can bind (18, 28). In organisms that lack a TlpA-like protein, it was argued that ScoI itself or a second Sco homolog [e.g., SCO2 in mitochondria (13, 29–31)] might fulfill this reductase function. While B. diazoefficiens ScoI (19) readily associates with Cu2+, the bonding of Cu1+ to other Sco-like proteins was reported (13, 32–34), and a redox change in the course of Cu transfer was uncovered for the Bacillus subtilis ScoI-like protein (35). Binding and delivery of both Cu2+ and Cu1+ ions by Sco proteins might be an option in those organisms that lack a second Cu1+-specific chaperone such as PcuC (PCuAC). Where tested (20, 23, 36, 37), the specificity of the latter is the highest for Cu1+. PcuC is unrelated to ScoI in both amino acid sequence and structure. It has a cupredoxin fold carrying a novel type of copper-binding site with the consensus motif Hx9-Mx5-Hx3-M, in which the two histidines and two methionines act as Cu1+ ligands (23, 24). Unlike the membrane-anchored ScoI, PcuC is a soluble protein in the bacterial periplasm. Null mutations in scop- and pcuC-like genes in B. diazoefficiens cause a strongly diminished activity of reduced CoxB·CuA. They can be taken as fingerprints for these two metalloproteins (20, 38).

Figure 1 (C and D) depicts the characteristic continuous wave electron paramagnetic resonance (cw EPR) spectra of ScoI·Cu2+ and CoxB·CuA. They rise to a highly symmetric CuA center reminiscent of Fe2S2 clusters (11). Copper-ligand distances (Fig. 1B) are as 2.4 Å; and Cu1 and Cu2 to S of Cys233, 2.3 Å each, giving rise to a highly symmetric CuB center reminiscent of Fe3S4 clusters (11). The high-resolution structure solved here revealed the environment of the delocalized mixed-valence (Cu1+/Cu2+) binuclear CoxB-CuA center in exquisite detail. The two copper ions are coordinated by the highly conserved copper-binding motif (Hx3-CxEEXCX3HXXXM). The distance between the two copper ions is 2.5 Å, which is in agreement with previously published CuB center structures (42, 43). Copper-ligand distances (Fig. 1B) are as follows: Cu1–Nδ1His237 and Cu2–Nδ1His194, 2.0 Å each; Cu2–S Met230, 2.4 Å; and Cu1 and Cu2 to S of Cys229 and Cys33, 2.3 Å each, giving rise to a highly symmetric CuA center reminiscent of Fe3S4 clusters (11). Comparison of the CoxB-CuA structure with the previously determined apo-CoxB structure (PDB ID: 4TXV) (18) revealed a major structural rearrangement upon CuA center formation, in which the loop segment 228–239 that adopts an open conformation in apo-CoxB closes over the binuclear CuA center in the CoxB-CuA structure (fig S1E).

Figure 1 (C and D) depicts the characteristic continuous wave electron paramagnetic resonance (cw EPR) spectra of ScoI·Cu2+ and CoxB·CuA. They can be taken as fingerprints for these two metalloproteins that reflect the local electronic structure. ScoI·Cu2+ shows a well-resolved EPR spectrum, with three of four copper hyperfine lines clearly visible on the low-field side. Their center and splitting is described by a

**RESULTS**

**High-resolution crystal structures of ScoIox, ScoI-Cu2+,** and CoxB-CuA

As a prelude to this study, we attempted to solve the x-ray structures of all relevant B. diazoefficiens copper proteins (ScoI, PcuC, and CoxB) to confirm that they showed the same overall fold and Cu-binding site architecture as their closest homologs with known three-dimensional structure. For this purpose, we produced and purified the soluble periplasmic domains of ScoI and CoxB (18.0 and 15.6 kDa, respectively) and soluble, mature PcuC (15.7 kDa). We succeeded in determining the x-ray structures of the periplasmic ScoI domain in its oxidized state [both cysteines in the Cu2+-binding site linked by a disulfide bond, no Cu2+ bound; Protein Data Bank (PDB) ID: 4WBJ] and in the reduced state in complex with Cu2+ (PDB ID: 4WRB) with very high resolutions of 1.3 and 1.4 Å, respectively (Fig. 1A, fig. S1A, and table S1). Likewise, the x-ray structure of the periplasmic CoxB domain containing the binuclear CuA center of cytochrome c oxidase (CoxB-CuA) (PDB ID: 4W9Z) was solved with the highest resolution (1.3 Å) reported thus far for a protein harboring a CuA center (Fig. 1B and table S1). In contrast, our attempts to crystallize apo-PcuC and holo-PcuC failed, which was possibly caused by the flexible, C-terminal extension of PcuC (see below). We therefore used the solved nuclear magnetic resonance (NMR) structure of the holo core of PCuAC from T. thermophilus (PDB ID: 2K6Z) (24) as a model for B. diazoefficiens PcuC, although PCuAC from T. thermophilus lacks the C-terminal extension of B. diazoefficiens PcuC (figs. S1F and S2; see also below).

ScoI displays a characteristic thioredoxin-like fold, composed of six α helices and nine β strands with a β-hairpin extension between α helix 4 and β strand 8 (26, 41). The CXXX...H motif of ScoI with the Cu2+-coordinating cysteine pair C74/C78 is located in the loop segment 71–80 between strand β3 and helix α1. The Cu2+ ion in the ScoI-Cu2+ complex shows a distorted, squared planar coordination sphere. The Cu–S of Cys74 and Cu–S of Cys78 distances in the complex are both 2.1 Å, the Cu1–Ne2 of His162 distance is 2.2 Å, and a water molecule is the fourth Cu2+ ligand (Fig. 1A). Superposition of the ScoI-Cu2+ structure with that of oxidized apo-ScoI showed that no major conformational changes occurred upon Cu2+ binding, and only small local structural differences around the Cys74–Cys78 disulfide bond of oxidized apo-ScoI were detected (fig S1B). These two structures might differ from the structure of reduced apo-ScoI, in which the His-containing loop possibly adopts a different conformation.

Our solved x-ray structure of the periplasmic CoxB-CuA domain is virtually identical to the previously reported structure of holo-subunit II in the aa3-type cytochrome c oxidase from Paracoccus denitrificans (PDB ID: 3H83) (fig. S1, C and D) (42). The high-resolution structure solved here revealed the environment of the delocalized mixed-valence (Cu1+/Cu2+) binuclear CoxB-CuA center in exquisite detail. The two copper ions are coordinated by the highly conserved copper-binding motif (Hx3-CxEEXCX3HXXXM). The distance between the two copper ions is 2.5 Å, which is in agreement with previously published CuB center structures (42, 43). Copper-ligand distances (Fig. 1B) are as follows: Cu1–Nδ1His237 and Cu2–Nδ1His194, 2.0 Å each; Cu2–S Met230, 2.4 Å; and Cu1 and Cu2 to S of Cys229 and Cys33, 2.3 Å each, giving rise to a highly symmetric CuA center reminiscent of Fe3S4 clusters (11). Comparison of the CoxB-CuA structure with the previously determined apo-CoxB structure (PDB ID: 4TXV) (18) revealed a major structural rearrangement upon CuA center formation, in which the loop segment 228–239 that adopts an open conformation in apo-CoxB closes over the binuclear CuA center in the CoxB-CuA structure (fig S1E).

Figure 1 (C and D) depicts the characteristic continuous wave electron paramagnetic resonance (cw EPR) spectra of ScoI·Cu2+ and CoxB·CuA. They can be taken as fingerprints for these two metalloproteins that reflect the local electronic structure. ScoI·Cu2+ shows a well-resolved EPR spectrum, with three of four copper hyperfine lines clearly visible on the low-field side. Their center and splitting is described by a
The value of $g_z = 2.158$ and a hyperfine coupling of $A_z = 523$ MHz, indicative of a coordination by two sulfur ligands (44). CoxB-CuA shows a spectrum typical (45) for a CuA site with a small and barely resolved hyperfine structure in the low-field region around a $g_z$ value of 2.198.

**Scol-Cu$^{2+}$ forms a stable 1:1 complex with reduced CoxB**

In the initial design of Cu transfer experiments, we started out with the naive idea that mature CoxB-CuA might be formed simply by mixing Scol-Cu$^{2+}$ with apo-CoxB, because formation of a disulfide bond in either CoxB or Scol could potentially provide the reducing equivalents for generation of the Cu$^{2+}$ ions necessary for CuA center formation (10). However, titration of reduced CoxB (40 μM) with increasing amounts of Scol-Cu$^{2+}$ did not yield CoxB-CuA. Instead, analytical gel filtration of the reaction products demonstrated that a previously unknown binary Scol-Cu$^{2+}$-CoxB complex (labeled CP in Fig. 1E) was quantitatively formed when Scol-Cu$^{2+}$ was mixed with equimolar amounts of CoxB, which eluted...
at the retention time expected for this 33.6-kDa complex (Fig. 1E). By contrast, mixing apo-Scol with CoxB or Scol-Cu2+ with oxidized CoxB did not result in the formation of a complex. The Scol-Cu2+-CoxB complex was then purified on a preparative scale and further characterized. Its cw EPR Cu2+ spectrum features a copper hyperfine coupling of $A_2 = 465$ MHz centered around $g = 2.135$ that is consistent with a four-sulfur ligand coordination (44) and clearly distinct from that of Scol-Cu2+ and CoxB-Cu4, showing the unique Cu2+ coordination sphere in the complex (Fig. 1C, gray) (44). In addition, the Scol-Cu2+-CoxB complex exhibited a specific diagnostic absorbance maximum at 520 nm (manifesting its pinkish color in solution), which allowed us to confirm its 1:1 stoichiometry by absorbance titration (constant CoxB concentration of 15 µM, 0 to 5 molar equivalents (meq) of Scol-Cu2+; Fig. 1F). The sharp kink in the titration profile indicates that Scol-Cu2+-CoxB is a high-affinity complex with a dissociation constant ($K_D$) below $10^{-7}$ M (Fig. 1F).

Stopped-flow absorbance kinetics of formation of Scol-Cu2+-CoxB, recorded at 520 nm after mixing of CoxB (25 µM) with 1.5-, 3-, or 5-fold excess of Scol-Cu2+, revealed rapid quantitative Scol-Cu2+-CoxB formation within 10 s (Fig. 1G). The reaction mechanism, however, proved to be complex. Among several kinetic models tested, the best global fits were obtained for a mechanism with reversible formation of an encounter complex [Scol-Cu2+-CoxB]*, followed by intramolecular re-arrangement of the encounter complex to the final Scol-Cu2+-CoxB complex (reaction scheme 1)

$$\text{Scol-Cu}^{2+} + \text{CoxB} \xrightarrow{k_1} \text{[Scol-Cu}^{2+}\cdot\text{CoxB]}^* \xrightarrow{k_2} \xrightarrow{k_3} \text{[Scol-Cu}^{2+}\cdot\text{CoxB]}$$

(1)

The deduced rate constants $k_1$ to $k_3$ are given in the figure legend (Fig. 1G). Specifically, the fast rate constant with which the encounter complex is formed ($k_1 = 1.5 \pm 0.014 \times 10^5$ M$^{-1}$ s$^{-1}$) provides a strong hint that the reaction between Scol-Cu2+ and CoxB is highly specific, as it is clearly above the threshold of $10^3$ M$^{-1}$ s$^{-1}$ for rate constants of formation of physiologically relevant protein-protein complexes.

**PcuC has a Cu1+-specific binding site and a second Cu2+-specific binding site**

Apart from the Cu2+-chaperone Scol, the periplasmic, Cu1+-specific chaperone PcuC is considered the second candidate protein acting as Cu transfer catalyst for CuA center biogenesis in *B. diazoefficiens* (20). Its homologs PCuA from *T. thermophilus* (TthPCuA,C) and *Rhodobacter sphaeroides* were shown to assist in the metalation of the CuA site of their $b_{33}$ and $a_{33}$ oxidases, respectively (24, 38). The NMR structure of the TthPCuA,C-Cu1+ complex (PDB ID: 2K6Z) revealed a β-sheet fold harboring a conserved H(M)X$_3$MX$_3$HXM motif to which Cu1+ is bound (fig. S1F) (24). Like most other α-proteobacterial members of this chaperone family, *B. diazoefficiens* PcuC, however, differs from TthPCuA in that a 23-residue extension is fused to the C terminus of its folded core domain. This extension contains a strikingly high content of typical copper-binding residues (five Met and two His residues). Although these residues are not conserved in the C-terminal extensions of other PcuC members, they share a high Met and His content with *B. diazoefficiens* PcuC (fig. S2). To test if the C-terminal extension *B. diazoefficiens* PcuC is also involved in copper binding and to determine its Cu ion specificity, PcuC was loaded with 4 meq of either Cu2+ or Cu1+ under anoxic conditions. After removal of excess copper by gel filtration, the samples were analyzed by electrospray ionization (ESI) mass spectrometry. With an expected mass increase relative to apo-PcuC (15,689.5 Da) of 63.5 Da per bound Cu ion, the measured masses of 15,813 Da for the holo-proteins showed that PcuC has two Cu-binding sites and is able to bind either two Cu1+ ions or two Cu2+ ions (Fig. 2A).

To determine the specificity of PcuC for Cu1+/Cu2+, we next recorded Cu2+-specific cw EPR spectra of PcuC after incubation with either only Cu1+ or only Cu2+ or 1:1 mixtures of Cu1+ and Cu2+ and desalting under anaerobic conditions. The EPR spectra of PcuC-Cu2+-Cu2+ and PcuC-Cu1+-Cu1+ served as reference spectra (Fig. 2B). PcuC-Cu1+-Cu1+ proved to be highly sensitive to oxidation, and the complete absence of Cu1+ in the EPR spectra could only be established in the presence of the reductant dithionite (Fig. 2B). Notably, identical EPR spectra were obtained for PcuC incubated with a 1.5- or 3-fold molar excess of the Cu1+/Cu2+ mixture, from which a Cu2+ content of ~1.3 Cu2+ per PcuC polypeptide could be deduced after normalization to identical protein concentrations (Fig. 2B). Within experimental error, this result indicated that PcuC has a Cu1+- and a Cu2+-specific binding site and selects one Cu1+ and one Cu2+ from solutions with excess Cu1+ and Cu2+. The presence of a highly specific Cu1+-specific binding site in PcuC that protects the bound Cu1+ from oxidation is supported by the observation that only one of the two Cu1+ ions proved to be oxidation insensitive when PcuC-Cu1+-Cu1+ was stored in the absence of dithionite (Fig. 2B). Together, the results indicate that the PcuC-Cu1+-Cu2+ complex is the thermodynamically preferred metalation state of the chaperone.

As Cu1+ is spectroscopically silent in EPR spectra, we measured the distance between the Cu1+ ions in PcuC-Cu2+-Cu2+ using a four-pulse ultra-wideband double electron-electron resonance (DEER) experiment (46, 47). The results showed that the two Cu2+ ions have a broad distance distribution (up to 4 nm), with most distances in the range between 1.6 and 2.6 nm (Fig. 2C and fig. S3). We assume that the distance between Cu1+ and Cu2+ in the PcuC-Cu1+-Cu2+ complex is in the same range because the Cu1+-specific binding site of PcuC can also be occupied by Cu2+, albeit with lower affinity (fig. S4).

**PcuC has a high-affinity Cu1+-binding site in its folded core domain and a Cu2+-specific binding site in its C-terminal 23-residue extension**

PcuC contains 10 methionines. Five Met residues are predicted to be located in the structured PcuC core domain, of which at least two are part of the conserved Cu1+-binding motif (see above). The other five Met residues are located in the C-terminal PcuC extension (fig. S2). To identify the Cu1+- and Cu2+-specific binding sites of PcuC, we labeled 10 Met residues with Met-(methyl-13C) and measured changes in the 13C methyl NMR resonances upon binding of Cu1+ and Cu2+. To assign Met signals from the folded PcuC core and the C-terminal extension, we first compared the Met-(methyl-13C) resonances of full-length apo-PcuC with those of a Met-(methyl-13C)-labeled, truncated variant, apo-PcuC ΔC, lacking the C-terminal 23 residues. Superposition of the 2D [13C,1H]-HMBC NMR spectra of apo-PcuC and apo-PcuC ΔC identified the well-dispersed resonances of the five Met residues in the folded core, while the very low chemical shift dispersion of the five Met residues in the C-terminal extension indicated a lack of defined tertiary structure in this segment of apo-PcuC (Fig. 3A).

Next, we prepared NMR samples of PcuC and PcuC ΔC in the presence of Cu1+ and/or Cu2+ and, when applicable, removed excess Cu ions by gel filtration under anoxic conditions before recording the NMR spectra. We expected that resonances of Met residues involved in Cu1+ binding would be shifted, whereas the signals of Met residues contributing to Cu2+ binding would be quenched due to the paramagnetic properties of Cu2+. Figure 3B shows that four of five Met signals from
the PcuC core were shifted in the presence of 1 meq of Cu$^{1+}$, with two signals shifting by more than 4 ppm in the $^{13}$C dimension, while the Met peaks from the C-terminal extension were not affected by Cu$^{1+}$. Figure 3C shows the analogous NMR experiments in the presence of 1 meq of Cu$^{2+}$ relative to PcuC, where Cu$^{2+}$ binding only quenched the Met peaks from the C-terminal extension but left the resonances from the core domain unaffected. In addition, a small fraction of the resonances in the C-terminal extension remained visible, which was likely caused by a small excess of PcuC over Cu$^{2+}$ in the sample. These resonances were significantly broadened compared to apo-PcuC, indicating fast, dynamic exchange of Cu$^{2+}$ ions bound to the C-terminal extension, which was confirmed by NMR measurements recording Cu$^{2+}$ binding to a synthetic 23-residue peptide corresponding to the C-terminal PcuC extension (fig. S3), is therefore affected by trace length and noise, and was not interpreted.

The fast and specific formation of the stable ScoI-Cu$^{2+}$-CoxB complex from ScoI-Cu$^{2+}$ and CoxB (Fig. 1, E to G) indicated that ScoI-Cu$^{2+}$-CoxB is an obligatory intermediate in CoxB-CuA biogenesis. Therefore, we tested whether apo-PcuC or PcuC in its different metalation states did not react with the ScoI-Cu$^{2+}$-CoxB complex. In the presence of excess PcuC-Cu$^{1+}$ over ScoI-Cu$^{2+}$-CoxB, the reductant dithionite showed that Cu$^{1+}$ bound to the C-terminal PcuC extension was oxidation sensitive (leading to quenching of the C-terminal Met signals), whereas Cu$^{1+}$ bound to the PcuC core was protected against air oxidation (Fig. 3, D and F), which may have been caused by the marginal air permeability of the top cover of the NMR tube. Together, our NMR experiments on PcuC (Fig. 3) are fully consistent with the mass spectrometry and EPR results shown in Fig. 2 (A and B) and demonstrate that (i) the primary Cu$^{1+}$-binding site is located in the PcuC core, (ii) the primary Cu$^{2+}$-binding site is in the C-terminal PcuC extension, and (iii) the C-terminal PcuC extension can also bind Cu$^{1+}$.

### Two molar equivalents of PcuC-Cu$^{1+}$-Cu$^{2+}$ are required for quantitative formation of the CoxB-CuA center from the ScoI-Cu$^{2+}$-CoxB complex

The fast and specific formation of the stable ScoI-Cu$^{2+}$-CoxB complex from ScoI-Cu$^{2+}$ and CoxB (Fig. 1, E to G) indicated that ScoI-Cu$^{2+}$-CoxB is an obligatory intermediate in CoxB-CuA biogenesis. Therefore, we tested whether apo-PcuC or PcuC in its different metalation states would be able to dissolve the ScoI-Cu$^{2+}$-CoxB complex and/or form CoxB-CuA. For this purpose, the ScoI-Cu$^{2+}$-CoxB complex was incubated overnight under anaerobic conditions with different amounts (0 to 3 meq) of either apo-PcuC, PcuC-Cu$^{1+}$, or PcuC-Cu$^{2+}$-Cu$^{2+}$, and the reaction products were separated by analytical gel filtration, in which PcuC and ScoI exhibited identical retention times but could be well separated from ScoI-Cu$^{2+}$-CoxB and apo-CoxB/CoxB-CuA (Fig. 4, A and B).

Figure 4C shows that apo-PcuC did not react with the ScoI-Cu$^{2+}$-CoxB complex. In the presence of excess PcuC-Cu$^{1+}$ over ScoI-Cu$^{2+}$-CoxB,
only a small fraction of CoxB was released from the ScoI-Cu²⁺-CoxB complex, but no CoxB-Cu₃ was formed (Fig. 4, D and E). Recording the gel filtration profiles at both 280 and 813 nm (the CoxB-Cu₃ specific absorbance maximum; see fig. S6) demonstrated the complete absence of Cu₃ in the fraction of released CoxB molecules (Fig. 4E). Therefore, Cu²⁺ cannot be transferred directly from its primary binding site in the PcuC core domain to CoxB. The inability of PcuC-Cu²⁺ of transferring the Cu²⁺ from the core domain was additionally confirmed with titration experiments using the Cu²⁺ indicator dye bathocuproine disulfonate (BCS), which forms a high-affinity Cu²⁺(BCS)₂ complex with Cu²⁺, with a characteristic absorbance maximum at 483 nm (fig. S7) (48, 49). BCS failed to extract Cu²⁺ from PcuC-Cu²⁺ or PcuC-Cu²⁺ ΔC and only extracted the more weakly bound Cu²⁺ at the C-terminal PcuC extension from PcuC-Cu²⁺. Release of Cu²⁺
from the PcuC core domain could only be detected in triple variants of PcuC or PcuC ΔC, in which the Cu$^{1+}$ chelating residues His$^{79}$, His$^{113}$, and Met$^{115}$ of the core domain had been replaced by alanines (fig. S7, A to C). These results are consistent with the observation that a mutant B. diazoefficiens strain harboring only PcuC ΔC shows strongly decreased cytochrome oxidase activity compared with the wild type, although cytochrome oxidase activity was not completely absent in the strain harboring the PcuC ΔHHHM triple variant (fig. S8).

In contrast to mixing Sco1-Cu$^{2+}$-CoxB with apo-PcuC or PcuC-Cu$^{1+}$, which did not yield CoxB-Cu$_{A}$, we essentially achieved complete (97% yield) CoxB-Cu$_{A}$ formation from the Sco1-Cu$^{2+}$-CoxB complex when PcuC-Cu$^{1+}$ was added to the complex (fig. 5). Unexpectedly, however, titration of Sco1-Cu$^{2+}$-CoxB with PcuC-Cu$^{1+}$-Cu$^{2+}$ revealed that a twofold excess of PcuC-Cu$^{1+}$-Cu$^{2+}$ over Sco1-Cu$^{2+}$-CoxB was required for quantitative CoxB-Cu$_{A}$ formation. Figure 5A shows the two-step reaction mechanism deduced from our titration experiments. In the first step (I) of CoxB-Cu$_{A}$ formation, the first equivalent of PcuC-Cu$^{1+}$-Cu$^{2+}$ dissociates the Sco1-Cu$^{2+}$-CoxB complex to CoxB-Cu$^{2+}$, Sco1-Cu$^{1+}$, and PcuC-Cu$_{A}$ (fig. 5, B, C, and F, and fig. S9A), but CoxB-Cu$_{A}$ is not quantitatively formed yet. Thus, only PcuC-Cu$^{1+}$-Cu$^{2+}$ can release CoxB-Cu$^{2+}$ from the Sco1-Cu$^{2+}$-CoxB complex, confirming that Cu$^{1+}$ bound to the PcuC core domain cannot be transferred to CoxB. In the second reaction step (II), Cu$^{1+}$ is then transferred from the second PcuC-Cu$^{1+}$-Cu$^{2+}$ equivalent to CoxB-Cu$^{2+}$, yielding the genuine CoxB-Cu$_{A}$, center with its characteristic absorbance maxima at 367, 479, and 813 nm (fig. 5D and fig. S6). Strikingly, the results indicated that only copper ions bound to the C-terminal PcuC extension can be donated to CoxB and that intramolecular transfer of a single electron from Cu$^{1+}$ in the PcuC core to Cu$^{2+}$ in the C-terminal PcuC extension occurred before the resulting Cu$^{3+}$ in the C-terminal extension was transferred to CoxB-Cu$^{2+}$ in step II. Intramolecular electron transfer from Cu$^{1+}$ to Cu$^{2+}$ in PcuC-Cu$^{1+}$-Cu$^{2+}$ is supported by the following observations: First, the two bound copper ions are sufficiently close (1.6 to 2.6 nm, fig. 2C) to allow sufficiently fast electron transfer (50, 51). Second, our NMR titration experiments showed that the PcuC core can also bind Cu$^{2+}$ instead of Cu$^{1+}$ (fig. S4) and that the C-terminal PcuC extension can also bind Cu$^{1+}$ instead of Cu$^{2+}$ (fig. 3D). Both findings are strict requirements for intramolecular electron transfer and consistent with a dynamic electron transfer equilibrium in which the PcuC state with Cu$^{3+}$ in the core and Cu$^{2+}$ in the C-terminal extension is thermodynamically favored. Recent studies on the PcuC homolog PccA from Rhodobacter capsulatus had shown that the core domain of PccA can also bind either Cu$^{2+}$ or Cu$^{3+}$, in accordance with our own findings, albeit a second Cu-binding site in its C-terminal domain.

**Fig. 4.** Neither apo-PcuC nor PcuC-Cu$^{1+}$ can release CoxB from the Sco1-Cu$^{2+}$-CoxB complex. (A) SDS-polyacrylamide gel (20%) of the purified protein components used in the titration experiments shown here and in Fig. 5. (C to E) Titration of Sco1-Cu$^{2+}$-CoxB with apo-PcuC or PcuC-Cu$^{1+}$ at pH 7.0 and 25°C, analyzed by analytical gel filtration. Eluted proteins were detected via their absorbance at 280 nm. The Sco1-Cu$^{2+}$-CoxB complex (20 μM) was kept constant in all titration experiments and mixed with 0.25 to 3 meq of apo-PcuC or PcuC-Cu$^{1+}$, and the reaction products were separated by gel filtration. (B) Gel filtration profiles of the individual components and controls used in the titration experiments shown here and in Figs. 1E and 5, demonstrating that (i) PcuC and Sco1 exhibit the same retention time but can be well separated from Sco1-Cu$^{2+}$-CoxB and CoxB and (ii) no complex is formed between apo-Sco1 and CoxB and between Sco1-Cu$^{2+}$ and oxidized CoxB (CoxB$_{ox}$). (C) Titration of Sco1-Cu$^{2+}$-CoxB with apo-PcuC. Neither dissociation of Sco1-Cu$^{2+}$-CoxB nor CoxB-Cu$_{A}$ formation could be observed. (D) Titration of Sco1-Cu$^{2+}$-CoxB with PcuC-Cu$^{1+}$, showing that only a tiny fraction of Sco1-Cu$^{2+}$-CoxB dissociated at high excess of PcuC-Cu$^{1+}$. (E) Titration experiment from (D) but with protein detection at the CoxB-Cu$_{A}$ absorbance maximum of 813 nm instead of 280 nm, showing that no CoxB-Cu$_{A}$ was formed. Purified CoxB-Cu$_{A}$ (20 μM) was used as reference for 100% CoxB-Cu$_{A}$ formation (magenta peak).
Fig. 5. Two equivalents of PcuC·Cu\textsuperscript{2+}·Cu\textsuperscript{2+} are required for conversion of ScoI·Cu\textsuperscript{2+}·CoxB to CoxB·Cu\textsubscript{A}.

(A) Mechanism of in vitro formation of CoxB·Cu\textsubscript{A} (97% yield), deduced from the experiments shown in (B to H). (B and F) Titration of ScoI·Cu\textsuperscript{2+}·CoxB (20 μM) with PcuC·Cu\textsuperscript{1+}·Cu\textsuperscript{2+} at pH 7.0 and 25°C, analyzed by gel filtration (protein detection at 280 nm). ScoI·Cu\textsuperscript{2+}·CoxB dissociation was completed after addition of one PcuC·Cu\textsuperscript{1+}·Cu\textsuperscript{2+} equivalent. (C) Gel filtration runs from (B) but with protein detection at the CoxB·Cu\textsubscript{A}–specific absorbance maximum at 813 nm. CoxB·Cu\textsubscript{A} formation only reached its maximum after addition of two PcuC·Cu\textsuperscript{1+}·Cu\textsuperscript{2+} equivalents to ScoI·Cu\textsuperscript{2+}·CoxB. (D) Absorption spectra of the isolated CoxB·Cu\textsubscript{A} peaks from (B) and (C), showing that two equivalents of PcuC·Cu\textsuperscript{1+}·Cu\textsuperscript{2+} added to ScoI·Cu\textsuperscript{2+}·CoxB quantitatively reconstituted the CoxB·Cu\textsubscript{A} center (absorbance maxima at 367, 479, and 813 nm). (E) CoxB·Cu\textsubscript{A} peak areas from (C) plotted against the added equivalents of PcuC·Cu\textsuperscript{1+}·Cu\textsuperscript{2+}. (G) Kinetics of complex dissociation. (H) Detection of cytochrome oxidase activity in B. diazoefficiens wild type (WT; positive control) and deletion mutants deficient in ScoI or PcuC. A coxB deletion mutant served as a negative control. Bacteria were grown aerobically in peptone–salt–yeast extract medium with or without 50 μM CuCl\textsubscript{2}. Identical amounts of cells were spotted onto a filter paper soaked with the indicator dye TMPD that reacts to indophenol blue when CoxB is active.
extension that is 10 residues shorter than that of PcuC had not been reported (37). Third, as predicted by the mechanism in Fig. 5A, an NMR titration experiment in which Met- (methyl- 13C) labeled PcuC-Cu1+-Cu2+ was mixed with different amounts of ScoI-Cu2+ confirmed that the intensities of resonances specific to the Cu1+-bound PcuC core domain only started to decrease when more than one equivalent of PcuC-Cu1+-Cu2+ was added to ScoI-Cu1+-Cu2+ (fig. S9, A to D). This intensity decrease of Cu1+-bound core domain resonances was not accompanied by simultaneous appearance of resonances of the apo-core domain (fig. S9E), in agreement with the appearance of Cu1+ in the core domain as a consequence of intramolecular electron transfer.

Notably, complete dissociation of the ScoI-Cu2+-CoxB complex was already achieved when only a single PcuC-Cu1+-Cu2+ equivalent was added to the complex (Fig. 5F). Stopped-flow absorbance kinetics of this reaction, recorded via the decrease in the absorbance of the complex at 520 nm, showed that the reaction is comparably fast, with a rate constant of ~700 M−1 s−1 (Fig. 5G). Figure 5 (C to E) shows that about 50% of the CoxB-Cu2+ molecules that had been generated after complex dissociation by one equivalent of PcuC-Cu1+-Cu2+ (reaction I) reacted further to CoxB-CuA after overnight incubation. We interpret this result such that CoxB-CuA can also be formed when ScoI-Cu2+-CoxB reacts with only one PcuC-Cu1+-Cu2+ equivalent but only very slowly (half-life above 10 hours). This alternative mechanism would require the regeneration of PcuC-Cu1+-Cu2+, the only species capable of donating Cu1+ to CoxB-Cu2+, by Cu2+ transfer from ScoI-Cu2+ to PcuC·Cu1+ (fig. S10A). This reaction is energetically unfavorable and makes CoxB-CuA formation with only one equivalent of PcuC-Cu1+-Cu2+ so slow that this alternative pathway is most likely not physiologically relevant. Last, we also attempted to record formation of CoxB-CuA, from CoxB-Cu2+ and PcuC-Cu1+-Cu2+ (reaction II in Fig. 5A) directly. This experiment, however, proved to be unfeasible, because we could not obtain stable preparations of CoxB-Cu2+.

Specifically, a mixture of the disulfide bonded form of CoxB (CoBoxCys2 disulfide between the Cu-coordinating CoxB cysteines) and CoxB-CuA was formed very rapidly when apo-CoxB was mixed with Cu2+ in vitro according to reaction scheme 2, which previously had also been reported for the CoxB homolog from T. thermophilus (see also Materials and Methods) (10)

\[
2 \text{apo-CoxB} + 3 \text{Cu}^{2+} \rightarrow \text{CoxB-CuA} + \text{CoBoxCys2} + \text{Cu}^{1+} + 2 \text{H}^{+}
\]

Although this reaction generates CoxB-CuA with a maximum yield of 50%, it is an artificial in vitro reaction, because there is no free Cu2+ in the cell (14, 52, 53). Therefore, we believe that the CoxB-Cu2+ complexes in the reaction scheme of Fig. 5A are only formed transiently in ScoI/PcuC-dependent CoxB-CuA formation and readily react with PcuC-Cu1+-Cu2+ to CoxB-CuA (reaction II in Fig. 5A). This was independently confirmed by cw EPR spectra that also demonstrated nearly complete formation (89%) of CoxB-CuA when ScoI-Cu2+-CoxB was mixed with two equivalents of PcuC-Cu1+-Cu2+ (fig. S11). Moreover, the data obtained from these cw EPR spectra revealed that ScoI retained a Cu2+ ion during CuA site formation (fig. S11), which is in agreement with the proposed reaction mechanism. Cu2+ was, however, slowly released from ScoI with increasing incubation time (fig. S11, B and C), most likely due to disulfide bond formation between the oxidation-prone active-site cysteines of ScoI (fig. S12).

Both chaperones, ScoI and PcuC, are required to form the CuA center of cytochrome oxidase in vivo

Our data presented so far showed that both ScoI-Cu2+ and PcuC-Cu1+-Cu2+ are required for quantitative formation of the CoxB-CuA center in vitro. In addition, apo-CoxB reacted with PcuC-Cu1+-Cu2+ alone (no ScoI present) to CoxB-CuA with only ~50% yield (fig. S13). Thus, our in vitro results predicted that both ScoI and PcuC are required for efficient formation of the CuA center in the CoxB subunit of aa3-type cytochrome oxidase in vivo. To test this, we generated B. diazoefficiens deletion mutants deficient in PcuC (ΔpcuABCDE), ScoI (ΔscoI), or CoxB (ΔcoxB). The strains were grown either under Cu-limiting conditions or in the presence of excess Cu2+ (50 μM CuCl2) in the medium. Cell extracts were tested for aa3-type cytochrome oxidase activity with the specific redox indicator dye tetramethyl-p-phenylenediamine (TMPD). The ΔpcuC and ΔscoI deletion mutants, like the CoxB deletion mutant ΔcoxB (negative control), both lacked cytochrome oxidase activity when grown under Cu-limiting conditions (Fig. 5H). Hence, the absence of one of the two copper chaperones could not be compensated by the other chaperone, and cytochrome oxidase activity in the ΔpcuC and ΔscoI mutants could only be restored with artificially high Cu2+ concentrations in the medium (Fig. 5H).

DISCUSSION

The electronically coupled di-copper CuA center on subunit II (CoxB) of cytochrome c oxidase is unique because of its highly symmetric architecture and its peculiar extracytoplasmic membrane topology. Its location in an oxidizing compartment (bacterial periplasm or mitochondrial intermembrane space) demands an inherently complex, multifactorial assembly process. Research of different laboratories using disparate model organisms had shown that a disulfide reductase for CoxB and two chaperones for Cu delivery (ScoI- and PcuC-like) are involved in CoxB-CuA biogenesis. Although a variety of scenarios for CuA formation had been proposed [reviewed in (54)], our mechanistic understanding of how the assembly factors cooperate remained incomplete. Previous genetic and physiological investigations in vivo (19, 30–34) were limited by the difficulty to assign specific functions to individual CuA biogenesis factors because of their intimate interactions and interdependencies. In the present study, we have taken a rigorously different approach by reconstituting CoxB-CuA assembly in vitro using the purified B. diazoefficiens CoxB protein as target and the purified, copper-loaded metallochaperones ScoI-Cu2+ and PcuC-Cu1+-Cu2+ for copper delivery. A detailed and comprehensive mechanism of CuA assembly has emerged, which is summarized and illustrated in Fig. 6. The entire pathway of copper insertion into CoxB can be dissected in three steps (Fig. 6).

In step I, ScoI-Cu2+ forms a 1:1 complex with apo-CoxB (Fig. 6). The ScoI-Cu2+-CoxB complex not only formed very rapidly but proved to be remarkably stable against dissociation and oxidation of the active-site cysteine pairs in ScoI and CoxB. By contrast, we noticed a relatively fast Cu2+ loss from isolated ScoI-Cu2+ (fig. S12), most likely due to Cu2+-mediated oxidation of the ScoI cysteine pair, similar to oxidation of the CoxB thiol pair (10) by Cu2+. In addition, the periplasmic domain of apo-CoxB proved to be only marginally stable and aggregated unspecifically at concentrations above 100 μM. These results strongly support the notion that ScoI-Cu2+-CoxB is an obligatory intermediate in CuA center formation. Besides its stabilizing function for CoxB and ScoI, it may also function as temporary Cu2+ depository preventing Cu-provoked oxidative stress. The stability of ScoI-Cu2+-CoxB is
reminiscent of PmoD from methane-oxidizing microbes, a homodimeric copper-binding protein forming a bimolecular CuA-like site at the subunit/subunit interface in which the two Cu-bridging cysteines might be similarly protected from oxidation (12). In the B. diazoefficiens ScoI·Cu2+·CoxB complex, the Cu2+ ion is most likely coordinated by four cysteines (one cysteine pair from each protein), giving rise to a previously unknown, pink metal center with its characteristic absorbance and EPR spectra (Fig. 1C and fig. S6).

Step II in CoxB-CuA biogenesis is the dissociation of the ScoI·Cu2+·CoxB complex by PcuC·Cu1+·Cu2+ and the transfer of Cu2+ from the C-terminal PcuC-extension to ScoI, and step III is the transfer of Cu1+ from a second equivalent of PcuC·Cu1+·Cu2+ to CoxB·Cu2+ (Fig. 6). The following discoveries, based on a combination of mass spectrometry, EPR and NMR spectroscopy, titration experiments, and stopped-flow kinetics, proved to be critical for establishing these reactions.

First, we demonstrated that PcuC binds one Cu1+ and one Cu2+ ion. In addition to the previously described Cu1+-binding site in the PcuC core domain (24), a Cu2+ ion specifically binds to the C-terminal PcuC extension. A Cu2+-binding site is most likely also present in the Met- and His-rich C-terminal extensions of other PcuC-like proteins but may have been missed in previous studies due to the lack of obvious sequence conservation (fig. S2). Our results on B. diazoefficiens PcuC, together with previously reported results on T. thermophilus PcuAC (24), indicate that PCuAC/PcuC-like proteins can be divided into two functionally distinct groups, namely, PCuAC domains with and without His/Met-rich C-terminal extension. Among the 920 representative

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**Fig. 6. Model for copper trafficking to the CoxB subunit of aa3-type cytochrome c oxidase in the periplasm of B. diazoefficiens.** CuA center formation in subunit II (CoxB) of aa3 oxidase of B. diazoefficiens requires soluble periplasmic PcuC (violet) and membrane-anchored ScoI (red). The periplasmic thioredoxin-like reductase TlpA maintains the active-site cysteine pairs of ScoI and CoxB in the reduced (dithiol) state that is required for Cu2+ binding. The Cu2+-specific chaperone ScoI readily reacts with apo-CoxB to a stable ScoI-Cu2+-CoxB complex (reaction I). The preferred metalation state of PcuC has a Cu1+ ion bound to the folded PcuC core domain and a Cu2+ ion bound to its flexible, C-terminal extension. PcuC-Cu1+·Cu2+ specifically reacts with the ScoI-Cu2+-CoxB complex and releases CoxB-Cu2+ from the complex (reaction II). A second equivalent of PcuC-Cu1+·Cu2+ then transfers Cu1+ to CoxB-Cu2+, and the CoxB-CuA center is formed (reaction III). As only Cu ions bound to the C-terminal PcuC extension can be transferred to CoxB, donation of Cu1+ to CoxB·Cu2+ requires intramolecular transfer of an electron from Cu1+ in the core domain to Cu2+ at the C-terminal PcuC extension. The overall reaction scheme at the bottom shows that, formally, ScoI·Cu2+ is not consumed in ScoI/PcuC-mediated CoxB-CuA biogenesis, indicating that catalytic amounts of ScoI relative to CoxB might be sufficient for efficient CoxB-CuA center formation in vivo.
proteomes in the Pfam database with 15% co-membership threshold (http://pfam.xfam.org/) (55), 674 representative proteomes contain a PCuAC-like protein domain. Of these, only 4% (including PCuAC from T. thermophilus) lack a C-terminal extension, while the other members have a C-terminal extension with median length of 20 residues in which Met and His residues are enriched two- and fourfold, respectively, relative to their average abundance pattern in proteins. It thus appears as if PCuAC/PcuC-like proteins with a Met/His-rich C-terminal extension that might harbor a second Cu-binding site are rather frequent, and that the mechanism of PcuC function in B. diazoefficiens, as characterized in our study, could apply to a number of other PcuC homologs. Some PcuC-like proteins (e.g., that of P. denitrificans) lack His or Met residues in their C-terminal region but have additional Met or His residues near their N terminus to potentially form a second Cu-binding site (UniProtKB: A1BAG4).

Second, we showed that only the Cu ion (Cu²⁺ or Cu¹⁺) bound to the C-terminal PcuC extension can be transferred to CoxB and that the C-terminal PcuC extension is strictly required for the dissociation of ScoI-Cu²⁺-CoxB by PcuC-Cu¹⁺-Cu²⁺. The intrinsic flexibility of the C-terminal PcuC extension (Figs. 2C and 3A) might enable PcuC-Cu¹⁺, Cu²⁺ to target the ScoI/CoxB interface. This reaction is highly specific: ScoI-Cu²⁺, CoxB could be dissociated under physiological buffer conditions only with PcuC-Cu¹⁺, Cu²⁺, which readily explains why PcuC is indispensable for CoxB·Cu₄₄ formation in vivo under Cu-limiting conditions (Fig. 5H and fig. S8).

Third, our data suggest that a single electron can be transferred from Cu²⁺ in the PcuC core to the Cu²⁺ at the C-terminal extension, which gives PcuC the unique ability to transfer either Cu¹⁺ or Cu²⁺ to its substrates. A putative redox reaction in the course of Cu transfer from either Sco- or PcuC-like chaerones has been a matter of debate for years (54). Notably, Abriata et al. (24) quantitatively reconstituted the Cu₄ center in cytochrome oxidase subunit II of T. thermophilus by addition of two equivalents of PCuAC-Cu²⁺, in which aerobic oxidation of one of the two Cu¹⁺ ions to Cu²⁺ led to the formation of the mixed-valence Cu₄ center. In our B. diazoefficiens system, we could not achieve complete CoxB·Cu₄ formation by incubating apo-CoxB with excess PcuC-Cu¹⁺,Cu²⁺ (fig. S13). Together with the observation that both PcuC and ScoI are required for the formation of a functional terminal oxidase in vivo (Fig. 5H), this rather excludes the mechanism of CoxB-Cu₄ formation in T. thermophilus for B. diazoefficiens. Instead, the results indicate that there are distinct mechanisms of Cu₄ center biogenesis in bacteria and that pathways of Cu₄ center formation analogous to those in T. thermophilus or B. diazoefficiens can be distinguished via the presence or absence of a Met/His-rich C-terminal PcuC extension in the respective proteome.

We are fully aware that the three-step mechanism of CoxB-Cu₄ formation in B. diazoefficiens depicted in Fig. 6 is a simplified reaction scheme that does not address the mechanisms of loading of ScoI with Cu²⁺ and PcuC with Cu¹⁺/Cu²⁺ in vivo, which certainly involves further upstream Cu uptake and delivery systems [reviewed in (14, 56)]. To which extent the mechanism established here for CoxB-Cu₄ assembly in B. diazoefficiens can be extrapolated to other organisms remains another open question. In any case, our results allow us to make predictions as to whether bacterial organisms having ScoI- and PcuC-like metallochaperones might share the Cu₄ assembly mechanism with that of B. diazoefficiens. The length of the C-terminal extension in the respective PcuC orthologs and the number of Met/His residues therein could serve as conspicuous indicators. The predictions then ought to be validated by in vivo and in vitro experiments analogous to those reported in this study. Even in mitochondria that have ScoI/Sco2 but lack a PcuC homolog, the first step—formation of a complex between ScoI-Cu¹⁺ and COX2 or ScoI-Cu²⁺ and COX2 analogous to ScoI-Cu²⁺/CoxB—ought to be considered and examined, because there is ample evidence for physical interactions between Sco and cytochrome oxidase subunit II (COX2) (13, 54). If a Cu-bridged ScoI-COX2 complex were formed in mitochondria, a promising candidate to resolve that complex in a PcuC-like manner would be the mitochondrial Cu chaperone COX17. Cu transfer from COX17 to at least Sco1 has already been demonstrated (57).

Last, we would like to point to another interesting aspect of ScoI- and PcuC-assisted CoxB-Cu₄ formation in B. diazoefficiens as depicted in Fig. 6: The total reaction (sum of individual steps I to III) shows that ScoI-Cu²⁺ is not consumed during CoxB-Cu₄ formation and therefore can be considered, at least formally, a catalyst. Consequently, stoichiometric amounts of ScoI relative to CoxB might be sufficient for quantitative CoxB-Cu₄ formation in vivo.

MATERIALS AND METHODS
Expression plasmids
Bacterial expression plasmids for the production of the periplasmic domains of CoxB (residues 128 to 265) and ScoI (residues 34 to 196) (18) and the expression plasmid for PcuC (for residues, see fig. S3) (20) were described before. Plasmid variants were constructed using standard molecular cloning techniques. Detailed information on the individual plasmids including the protein-encoding nucleotide sequences is available from the authors on request.

Protein production and purification
For expression of CoxB in inclusion bodies and the cyttoplasmic expression of ScoI and PcuC under T7 promoter/lac operator control, Escherichia coli BL21 (DE3) carrying the corresponding expression plasmid was grown at 37°C in 2YT medium containing ampicillin (100 μg/ml) until an OD₆₀₀ (optical density at 600 nm) of 0.6 had been reached. Expression was induced by the addition of isopropyl-β-D-thiogalactoside (final concentration, 0.1 mM), and cells were further grown at 30°C for 4 hours and harvested by centrifugation. Proteins were purified according to the following protocols:

CoxB: Cells were suspended in 100 mM tris-HCl (pH 8.0) and 1 mM EDTA (3 ml/g wet cells), mixed with deoxyribonuclease I (DNase I) (50 μg/ml final concentration), and lysed with a microfluidizer (M-110L, Microfluidics, Westwood, MA). After the addition of 0.5 volume of 60 mM EDTA-NaOH (pH 7.0), 1.5 M NaCl, and 6% (v/v) Triton X-100, the lysate was stirred at 4°C for 1 hour. The CoxB inclusion bodies were harvested by centrifugation (30 min, 47,850g, 4°C) and washed five times at 4°C with 100 mM tris-HCl (pH 8.0) and 20 mM EDTA to remove Triton X-100. The inclusion bodies were solubilized in 100 mM tris-HCl (pH 8.0), 6 M guanidinium chloride, 1 mM EDTA, and 100 mM dithiothreitol (DTT) (20 ml/g of inclusion bodies) at room temperature under stirring for 2 hours. Insoluble material was removed by centrifugation (30 min, 142,159g, 20°C). CoxB was refolded at room temperature from the supernatant by rapid, 100-fold dilution with refolding buffer [0.5 M arginine, 1 mM EDTA, 10 mM DTT, and 20 mM tris-HCl (pH 8.0)] and stirring for 1 hour. Refolded and reduced CoxB was concentrated to 4°C to ca. 1.5 mg/ml and dialyzed against 2 × 10 volumes of 20 mM tris-HCl (pH 8.5), 1 mM EDTA, 5 mM DTT, and 1 × 10 volumes of 20 mM tris-HCl (pH 8.5). Precipitated protein was removed by centrifugation, and the supernatant was applied at 4°C to
a Resource Q column (GE Healthcare) equilibrated with 20 mM tris-HCl (pH 8.5). CoxB was eluted with a linear NaCl gradient (0 to 1.0 M). Fractions containing pure CoxB according to SDS–polyacrylamide gel electrophoresis (PAGE) were combined; supplemented with 5 mM DTT, 1 mM EDTA, and 5% glycerol; frozen in liquid nitrogen; and stored at −20°C until further use. The final yield of purified CoxB was 60 mg/liter of bacterial culture.

ScoI: Cells were suspended at 4°C in 50 mM acetic acid–NaOH, 1 mM EDTA, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), DNase I (50 µg/ml), and complete protease inhibitor mix (1 tablet/60 ml, Roche Applied Science) (3 ml/g wet cells) and lysed with a microfluidizer (M-110L, Microfluidics). After centrifugation, the supernatant was filtered (0.2-μm pore size) and loaded onto an SP Sepharose column (GE Healthcare) equilibrated with 50 mM acetic acid–NaOH, 1 mM EDTA, and 2 mM DTT. ScoI was eluted with a linear NaCl gradient (0 to 0.5 M). ScoI-containing fractions were pooled, dialyzed at 4°C against 20 mM tris-HCl (pH 9.0), and loaded onto a Source 30Q column (GE Healthcare) equilibrated with the same buffer. ScoI was eluted with a linear NaCl gradient (0 to 0.5 M). ScoI-containing fractions were pooled, concentrated, and loaded onto a Superdex 75 (HiLoad 26/60) column (GE Healthcare) equilibrated with 20 mM tris-Cl (pH 8.5), 0.3 M NaCl, 5 mM DTT, and 0.5 mM EDTA. Fractions containing pure ScoI according to SDS–PAGE were combined, frozen in liquid nitrogen, and stored at −20°C until further use. The final yield of purified ScoI was 30 mg/liter of bacterial culture.

PcuC variants: PcuC and PcuC ΔC were produced as fusion proteins with N-terminal His10 tag, cleavable with tobacco etch virus (TEV) protease. Cells were suspended at 4°C in 50 mM Hepes–NaOH (pH 7.5), 300 mM NaCl, 1 mM PMSF, complete protease inhibitor mix (1 tablet/60 ml), and DNase I (50 µg/ml) (3 ml/g wet cells) and lysed with a microfluidizer (M-110L, Microfluidics). PcuC variants were purified from the soluble fractions of the lysates by application to prepacked Ni2+-NTA (nitrioltriacetic acid) columns (GE Healthcare) equilibrated with 50 mM Hepes–NaOH (pH 7.5), 300 mM NaCl, and 10 mM imidazole. The proteins were eluted with a linear imidazole gradient (10 to 250 mM). PcuC-containing fractions were pooled, and the His10-tags were removed by TEV protease (40 µg/ml; protease/substrate ratio, 1:25) cleavage during overnight dialysis at 4°C against 50 mM Hepes–NaOH (pH 7.5) and 300 mM NaCl. The cleaved His10-tag and TEV protease [His10-tagged variant (50)] were removed by loading the sample onto a Ni2+-NTA column. Flow-through fractions containing PcuC were pooled and incubated with 50 mM EDTA overnight, concentrated, and loaded onto a Superdex 75 (HiLoad 16/60) column (GE Healthcare) equilibrated with 20 mM Mops–NaOH (pH 7.0) and 50 mM NaCl. apo-PcuC-containing fractions were pooled, frozen in liquid nitrogen, and stored at −20°C until further use. The final yield of purified apo–PcuC and apo–PcuC ΔC was 26 and 10 mg/liter of bacterial culture, respectively.

**Copper loading of ScoI, CoxB, and PcuC**

As the active-site cysteine pairs of ScoI and CoxB need to be in the dithiol form for copper binding, both proteins were treated with reducing agents before loading with Cu ions. ScoI was incubated overnight at 4°C with 10 mM DTT, 20 mM tris-Cl (pH 8.5), 0.3 M NaCl, and 0.5 mM EDTA. CoxB (20 mM) was incubated with 5 to 10 mM tris(2-carboxyethyl)phosphine (TCEP) overnight at 4°C in 20 mM Mops–NaOH (pH 7.0) and 50 mM NaCl. Reduced proteins were buffer-exchanged against 20 mM Mops–NaOH (pH 6.5), and concentrations were determined via their absorbance at 280 nm. For loading of ScoI with Cu2+, reduced apo–ScoI was mixed with 1.5 mg of CuCl2 and incubated for 15 min at room temperature. For preparation of CoxB–CuA, reduced apo–CoxB (10 to 20 µM) was mixed with 3 mg of CuCl2 and incubated for 5 min at room temperature. The CoxB–CuA–specific pink color formed immediately after mixing. Cu-loaded proteins were desalted and buffer-exchanged against 20 mM Mops–NaOH (pH 7.0) by gel filtration to remove excess, unbound Cu ions and subjected to a final purification step by hydrophobic chromatography. The ScoI–Cu2+ and CoxB–CuA solutions were supplemented with 1 M ammonium sulfate and 1.5 M ammonium sulfate, respectively, and the apo–proteins were separated from the apo–proteins on Butyl Sepharose 4 Fast Flow columns (GE Healthcare). ScoI–Cu2+ and CoxB–CuA were eluted with a linear gradient from 1 or 1.5 M to 0 M ammonium sulfate, respectively. Separation from the apo–protein was monitored using the ScoI–Cu2+–specific absorbance at 360 nm or the CoxB–CuA–specific absorbance at 479 nm. Fractions containing the apo–proteins were pooled, buffer-exchanged against 20 mM Mops–NaOH (pH 7.0) and 50 mM NaCl, concentrated, and stored at 4°C until further use within 48 hours.

Samples with PcuC variants were transferred to an anaerobic chamber (Coy Laboratory); loaded with different amounts of CuI [tetrakis(acetonitrile)copper(I) hexafluorophosphate, Sigma-Aldrich, anaerobically dissolved in dimethyl sulfoxide], Cu2+ (CuCl2 in H2O), or a mixture of both; and incubated overnight at 5°C. Cu-loaded PcuC samples were desalted in the anaerobic chamber by gel filtration over PD MidiTrap G-25 columns (GE Healthcare), their concentration was measured, and protein samples were directly used for the experiments.

**Purification of the ScoI–Cu2+–CoxB complex**

Reduced apo–CoxB was mixed with equimolar amounts of freshly purified ScoI–Cu2+ in 20 mM Mops–NaOH (pH 7.0) and 50 mM NaCl (final protein concentrations, 20 to 30 µM). The pink ScoI–Cu2+–CoxB complex formed within seconds after mixing and was loaded onto a Superdex 75 (16/60) column equilibrated with 20 mM Mops–NaOH (pH 7.0) and 50 mM NaCl. Fractions containing ScoI–Cu2+–CoxB were pooled, concentrated with ultrafiltration (10 kDa cutoff), and immediately used for further experiments.

**Determination of protein concentrations**

Protein concentrations were measured via the specific absorbance at 280 nm using the following extinction coefficients: ScoIΔSS (28,085 M−1 cm−1), apo–CoxB (27,960 M−1 cm−1), ScoI·Cu2+·CoxB (26,950 M−1 cm−1), ScoIΔSS·Cu2+ (10,555 M−1 cm−1), apo–ScoI (10,430 M−1 cm−1), ScoI·Cu2+ (10,430 M−1 cm−1), ScoI·Cu2+·CoxB complex (38,390 M−1 cm−1), apo– and holo–PcuC, PcuC ΔC (8480 M−1 cm−1), and TEV-protease (32,290 M−1 cm−1).

**Crystallization of ScoIΔSS, ScoI·Cu2+, and CoxB·CuA**

Crystallization experiments were carried out with the sitting-drop vapor diffusion method. ScoIΔSS [0.15 µl, 54 mg/ml in 10 mM tris-HCl (pH 8.0)] was mixed with 0.15 µl of precipitant [25% (w/v) polyethylene glycol (PEG) (PEG) 1500, 0.1 M succinic acid/sodium phosphate/glycine (SPG) buffer (pH 4.0)], and crystals (space group P 21 2 1) grew within several weeks at 20°C. ScoI·Cu2+ [0.1 to 0.15 µl, 47 mg/ml in 10 mM tris-HCl (pH 8.0)] was mixed with 0.15 to 0.2 µl of 20% (w/v) PEG 6000 and 0.1 M Na-citrate (pH 5.0) at 20°C. Crystals (space group P 21 2 1) grew within several weeks at 20°C. Crystals of CoxB·CuA were obtained as a side product of our attempts to crystallize the ScoI–Cu2+–CoxB complex. ScoI·Cu2+–CoxB [0.1 to 0.15 µl, 25.9 mg/ml in 5 mM Mops–NaOH (pH 7.0)] was mixed with 0.15 µl of precipitant [25% PEG 6000, 0.1 M succinic acid/sodium phosphate/glycine buffer (pH 4.0)], and crystals (space group P 21 2 1) grew within several days at 20°C. Crystals of ScoI·Cu2+·CoxB grew in the same crystals (space group P 21 2 1) grew within several days at 20°C. Crystals of ScoI·Cu2+·CoxB grew in the same
(pH 7.0) was mixed with 0.15 to 0.2 μl of 10% (w/v) PEG 1000, 10% (w/v) PEG 8000, 0.2 M potassium bromide, and 0.1 M tris-HCl (pH 7.5). Crystals of PcuC·Cu$_3$ (space group P 1 2, 1) grew within ~10 days at 4°C.

**Crystallographic data collection and structure determination**

All crystals were cryoprepared in precipitant solution and supplemented with 15 to 20% glycerol and additional 4 to 5% of the corresponding PEG. Crystals were flash-frozen in liquid nitrogen. All x-ray diffraction data were recorded at the SLS beamline X06sa (Swiss Light Source, Paul Scherrer Institute, Switzerland).

Data were indexed and scaled with XDS (59) and refined with Refmac (60), and the models were built with Coot (61). Ramachandran plot: 4W9Z: Pref. 93.1% Allowed 6.1% Outliers 0.8%; 4WBR: Pref. 97.3% Allowed 2.1% Outliers 0.7%; 4WBJ: Pref. 96.3% Allowed 2.4% Outliers 1.4%.

**EPR spectroscopy**

For cw EPR measurements, proteins were concentrated to 100 to 400 μM and supplemented with 20% (v/v) glycerol as a cryoprotectant. Samples of 60 μl were filled into 3-mm quartz tubes and flash-frozen in liquid nitrogen. Samples containing Cu$^{1+}$ were kept anaerobically, and the quartz tubes were sealed under vacuum after freezing.

Cw EPR spectra were recorded in independent duplicates on an ElexSys E500 spectrometer (Bruker BioSpin, Rheinstetten, Germany) at X-band (~9.4 GHz) using an SHQ resonator and an ESR900 helium flow cryostat (Oxford Instruments, Oxfordshire, UK). All spectra were baseline-corrected by a first-order polynomial, the magnetic field offset was corrected using diphenyl-1-picylhydrazyl (Sigma-Aldrich, Buchs, Switzerland) as a reference, and the magnetic field axis was rescaled to display all spectra at a common microwave frequency of 9.5 GHz. The spectra were recorded at 40 K using microwave powers of 8 to 32 μW (nonsaturating conditions). The magnetic field modulation amplitude was set to 0.3 mT with a frequency of 100 kHz for lock-in amplification with a conversion time and time constant of 81.92 ms. EPR spectra were analyzed using MATLAB and EasySpin (62) and scaled to correct for differences in protein concentration in the samples. EPR spectra of mixtures were decomposed by least-squares fitting of weighting factors of individual experimental spectra such that the weighted sum corresponded to the experimental spectrum of the respective mixture.

Pulsed EPR distance measurements between Cu$^{1+}$ ions were carried out by ultra-wideband four-pulse DEER (46, 47) at a 9.3 GHz and a temperature of 10 K using a homebuilt X/Q band pulse EPR spectrometer (63) equipped with an MS-3 resonator (Bruker BioSpin, Rheinstetten, Germany) and 40-μl sample in 3-mm quartz tubes (as above). Observer pulses were all rectangular 12-ns pulses applied 50 MHz below the spectral maximum with an initial interpulse delay of 200 ns and incremented in eight steps of 8 ns to average out nuclear modulations. The pump pulse was a hyperbolic secant pulse of order 6 with a length of 64 ns and an excitation bandwidth of 450 MHz, was applied with an offset of +125 MHz, and included nonlinear compensation for resonator and microwave amplifier profiles (63). The pump pulse was stepped by 8 ns, and the repetition rate of the pulse sequence was 100 Hz. The data were analyzed using DeerAnalysis 2018 (64).

**NMR spectra of PcuC variants**

NMR samples consisted of 180 μl of 100 μM PcuC (13C-methyl) methionine in 20 mM MOPS (pH 7.0) (25°C), 10% NaCl, and 180 μl of 100 μM PcuC·Cu$^1$·Cu$^2$ in 3-mm NMR tubes (Norell, S-3-HT-7). Labeled protein was produced by adding l-methionine-(methyl-13C) (1 g/liter) (Sigma-Aldrich, 299146) to LB medium at the time point of induction (65). Depending on the sample, 0 to 3 meq of CuCl$_2$ or tetrakis(acetonitrile)copper(II) hexafluorophosphate were added under anaerobic conditions and incubated overnight at 5°C. All copper-containing samples were desalted over a disposable PD-10 column before the measurement to remove unbound copper. Only the PcuC·AC and the peptide samples used for the titration with CuCl$_2$ were measured without previous desalting step. For obtaining stable preparations of Cu$^{1+}$ complexes, 50 mM Na-dithionite was added after desalting. None of the substances were deuterated.

For the titration of PcuC·Cu$^{1+}$·Cu$^{2+}$ with the Scol-Cu$^{2+}$·CoxB, 50 μM of the (13C-methyl) methionine–labeled PcuC·Cu$^{1+}$·Cu$^{2+}$ in 20 mM Mops-NaOH (pH 7.0) (25°C), 50 mM NaCl, and 10% D$_2$O was titrated with 0 to 2 meq of the unlabeled Scol-Cu$^{2+}$·CoxB complex. The samples were incubated at 10°C overnight before recording NMR spectra.

All NMR measurements were carried out at 25°C (temperature calibrated with methanol-d$_4$, 99.8%) on a Bruker AVN EO 700 MHz spectrometer equipped with z-gradient coils. Gradient-selected 2D-[13C,1H] ALSOFAST-HMQC experiments (66) were used to suppress the intense signals of the protonated buffer substances. To resolve all methionine signals, a maximum $t_1$ evolution time of 121 ms was used, yielding a 13C resolution of 8.3 Hz in the indirect dimension. Each experiment was recorded in 34 min. All NMR measurements were independently repeated at least two times and proved to be fully reproducible.

**Kinetics of Scol-Cu$^{2+}$·CoxB complex formation**

CoxB (constant final concentration of 25 μM) was mixed with different amounts of Scol-Cu$^{2+}$ (37.5, 75, or 125 μM final concentration) at 25°C in 20 mM Mops-NaOH (pH 7.0) and 50 mM NaCl using a SX20 stopped-flow instrument (Applied Photophysics), and complex formation was monitored by the increase in absorbance at 520 nm. Each dataset was recorded six times and averaged. The absorbance traces were then globally fitted with DynaFit 4 according to a mechanism with reversible formation ($k_f$) and dissociation ($k_d$) of an encounter complex that irreversibly reacts to the final complex ($k_r$). Indicated errors of rate constants correspond to errors from the fits performed with DynaFit.

**Kinetics of PcuC-Cu$^{1+}$·Cu$^{2+}$–mediated Scol-Cu$^{2+}$·CoxB complex dissociation**

The rate of Scol-Cu$^{2+}$·CoxB complex dissociation by PcuC-Cu$^{1+}$·Cu$^{2+}$ was measured at 25°C in 20 mM Mops-NaOH (pH 7.0) and 50 mM NaCl after stopped-flow mixing. Identical initial concentrations of 25 μM were used for Scol-Cu$^{2+}$·CoxB and PcuC·Cu$^{1+}$·Cu$^{2+}$, and the reaction was monitored by the decrease in absorbance at 520 nm. Absorbance kinetics were measured in triplicates, averaged, and fitted with DynaFit 4 according to a second-order reaction followed by a monoexponential decay.

**Kinetics of Cu$^{2+}$ transfer from PcuC-Cu$^{1+}$·Cu$^{2+}$ to reduced Scol**

The Cu$^{2+}$ transfer from PcuC-Cu$^{1+}$·Cu$^{2+}$ to Scol (fig. S9B) was measured at 25°C in 20 mM Mops-NaOH (pH 7.0) and 50 mM NaCl in a SX20 stopped-flow instrument. Scol (25 μM) was mixed with 25 μM PcuC-Cu$^{1+}$·Cu$^{2+}$, and Cu$^{2+}$ transfer was monitored by the increase in the Scol-Cu$^{2+}$–specific absorbance at 360 nm. Three independent absorbance traces were recorded and averaged.

**Titration of reduced CoxB with Scol-Cu$^{2+}$**

The initial concentration of CoxB (reduced with TCEP and desalted) was kept constant (40 μM) and mixed with 0.5 to 5.0 meq of Scol-Cu$^{2+}$ in...
20 mM Mops-NaOH (pH 7.0) and 50 mM NaCl. After incubation at 10°C (overnight), samples were analyzed by gel filtration on a Superdex 75 10/300 column (GE Healthcare) equilibrated with the same buffer, and eluted proteins were detected via their absorbance at 280 nm. Titrations were repeated independently at least two times and proved to be reproducible within experimental error.

For absorbance titration of CoxB with Scol-Cu²⁺, the initial apo-CoxB concentration was kept constant (15 μM) and the Scol-Cu²⁺ concentration was varied between 0 and 75 μM. The absorbance signal at 520 nm was plotted against added equivalents of Scol-Cu²⁺.

Data were fitted to a noncovalent binding equilibrium according to the following equation, where $A$ is the monitored absorbance signal, $A_0$ and $A_m$ are the absorbance values of 0 and 100% complex formation, $K_D$ is the dissociation constant, $[C]_0$ is the total concentration of apo-CoxB, and $[S]_0$ is the total concentration of Scol-Cu²⁺.

$$A = A_0 - (A_m - A_0) \cdot [C]_0 + [S]_0 + K_D - \sqrt{([C]_0 + [S]_0 + K_D)^2 - 4 \cdot [C]_0 \cdot [S]_0}$$

Fitting the data yielded a $K_D$ value of 1.54 ± 0.8 × 10⁻⁷ M, showing that the concentration of apo-CoxB was at least two orders of magnitude above the $K_D$ value, thus not allowing accurate $K_D$ determination. As we could not lower protein concentrations due to the low sensitivity of the absorbance measurements, we confined the analysis to the estimation that the $K_D$ value of the Scol-Cu²⁺·CoxB complex is below 10⁻⁷ M. Absorbance titrations were repeated independently at least two times, each confirming the 1:1 stoichiometry with the sharp kink in the titration profile (Fig. 1F).

### Analysis of Cu₄ center formation by analytical gel filtration

All samples were mixed in an anaerobic chamber and sealed in a silanized microvial (8004-HP-H/IV2μ/SZ, Infochroma) with a polypropylene screw cap (G004-HP-CB-FKSFKK10, Infochroma). Samples were incubated at 10°C overnight before gel filtration at 25°C on an AdvancedBio SEC column (PE1580-5350, Agilent Technologies, 1100 Series) equipped with a diode array detector, allowing online recording of liquid chromatography system (Agilent Technologies, 1100 Series) and washed in 0.9% NaCl solution and adjusted to a final OD₆₀₀ of 10.0. Samples of 50 μl were immediately spotted in duplicate on a filter paper soaked in freshly prepared 1% TMPD, and the reaction product indophenol blue was detected after incubation for 15 min.

### Detection of cytochrome c oxidase activity in B. diazoefficiens mutants

**B. diazoefficiens** strains 110spc4 (wild type) (67), 6611 (ΔpcuABCDE::aphII) (20), 2575 (Δscol::aphII) (19), and 3563 (ΔcoxB::aphII) (19) were grown aerobically at 30°C to late exponential growth phase in peptone–salt–yeast extract medium supplemented with 0.1% (+)-arabinose (68) and, where applicable, 50 μM CuCl₂. Spectinomycin (to wild type) or spectinomycin and kanamycin (to all other strains) were added to a final concentration of 100 μg/ml each. Cells were harvested by centrifugation and washed in 0.9% NaCl solution and adjusted to a final OD₆₀₀ of 10.0. Samples of 50 μl were immediately duplicated in a duplicate filter paper soaked in freshly prepared 1% TMPD, and the reaction product indophenol blue was detected after incubation for 15 min.

### Detection of cytochrome c oxidase activity in B. diazoefficiens mutants under copper starvation

Strains 110spc4 (wild type), 3563 (ΔcoxB::aphII) (19), 6611 (ΔpcuABCDE::aphII) (20), 6611-33 (ΔpcuABCDE::aphII + pcuABCD) (6611-34 (ΔpcuABCDE::aphII + pcuABD) [both (20)], 6611-74 (ΔpcuABCDE::aphII + pcuAB) [ΔC-term]DE, 6611-715 (ΔpcuABCDE::aphII + pcuAB) [ΔC-term]DE, 6611-76 (ΔpcuABCDE::aphII + pcuABC), and 6611-1696 (ΔpcuABCDE::aphII + pcuAB) [ΔC-term]DE) were constructed according to standard protocols (20) and grown at 30°C in V3S minimal medium (9.9 mM K₂HPO₄, 10.1 mM NaH₂PO₄, 10.0 mM NH₄NO₃, 3.3 mM MgSO₄, 340 μM CaCl₂, 59 μM MnSO₄, 49 μM H₂BO₃, 7 μM ZnSO₄, 1 μM NaMoO₄, 5 μM KI, 5 μM NaSeO₃, 9 μM FeCl₃, 9 μM FeCl₂, 160 μM CuSO₄, 105 mM CoCl₂, and 105 mM NiCl₂) supplemented with 25 mM Na₂-succinate and appropriate antibiotics to mid-exponential growth phase, harvested, washed twice with sterile 0.9% NaCl, and transferred into fresh V3S with CuSO₄ to a starting OD₆₀₀ of 0.05. Strains were grown in the presence of Cu²⁺ ions in the buffer used for BCS titration experiments [20 mM Mops-NaOH (pH 7.0) and 50 mM NaCl] were removed by filtering the buffer through a 5-ml HisTrap agarose column (GE Healthcare) that had been extensively washed with 250 mM EDTA and deionized water. Apo-PcuC was then transferred into this buffer (PD-10 desalting column), and the absence of Cu ions in the apo-PcuC preparation was verified by ESI mass spectrometry. A stock solution of Cu²⁺(BCS)₂ was prepared by mixing tetrakis(acetonitrile) copper(I) hexafluorophosphate with 2 meq BCS acid (B1125, Sigma-Aldrich). The mixture was centrifuged, and the concentration of Cu²⁺(BCS)₂ in the supernatant was determined via its specific absorbance at 483 nm (13,000 M⁻¹ cm⁻¹). Titration experiments were performed under anaerobic conditions at a constant initial Cu²⁺(BCS)₂ concentration of 15 μM, mixed with 0 to 4 meq of apo-PcuC, apo-PcuC ΔC, apo-PcuC ΔH, apo-PcuC ΔHΔM, or apo-Scol. The samples were sealed and incubated for 1 hour or overnight at 25°C. Then, the absorbance at 483 nm of each sample was recorded and plotted against the molar equivalents of the respective protein added to Cu²⁺(BCS)₂.

For the reverse experiments, PcuC variants were loaded with 1 or 2 meq of Cu²⁺ as described above. Then, full-length PcuC or the PcuCΔC variants (constant concentration of 15 μM each) were mixed with 0 to 5 meq of BCS stock under anaerobic conditions and incubated overnight at 25°C. The recorded absorbance values at 483 nm were plotted against the molar BCS/protein ratio. Two independent sets of experiments confirmed reproducibility.

### Analysis of Cu¹⁺ binding to PcuC or Scol with the Cu¹⁺-specific indicator dye BCS

Potential contaminations with Cu²⁺ ions in the buffer used for BCS titration experiments [20 mM Mops-NaOH (pH 7.0) and 50 mM NaCl] were removed by filtering the buffer through a 5-ml HisTrap agarose column (GE Healthcare) that had been extensively washed with 250 mM EDTA and deionized water. Apo-PcuC was then transferred into this buffer (PD-10 desalting column), and the absence of Cu ions in the apo-PcuC preparation was verified by ESI mass spectrometry. A stock solution of Cu¹⁺(BCS)₂ was prepared by mixing tetrakis(acetonitrile) copper(I) hexafluorophosphate with 2 meq BCS acid (B1125, Sigma-Aldrich). The mixture was centrifuged, and the concentration of Cu¹⁺(BCS)₂ in the supernatant was determined via its specific absorbance at 483 nm (13,000 M⁻¹ cm⁻¹). Titration experiments were performed under anaerobic conditions at a constant initial Cu¹⁺(BCS)₂ concentration of 15 μM, mixed with 0 to 4 meq of apo-PcuC, apo-PcuC ΔC, apo-PcuC ΔH, apo-PcuC ΔHΔM, or apo-Scol. The samples were sealed and incubated for 1 hour or overnight at 25°C. Then, the absorbance at 483 nm of each sample was recorded and plotted against the molar equivalents of the respective protein added to Cu¹⁺(BCS)₂.

For the reverse experiments, PcuC variants were loaded with 1 or 2 meq of Cu¹⁺ as described above. Then, full-length PcuC or the PcuCΔC variants (constant concentration of 15 μM each) were mixed with 0 to 5 meq of BCS stock under anaerobic conditions and incubated overnight at 25°C. The recorded absorbance values at 483 nm were plotted against the molar BCS/protein ratio. Two independent sets of experiments confirmed reproducibility.
of spectinomycin (100 μg/ml) for 48 hours at 30°C to OD<sub>600</sub> of 0.8. Bacteria were harvested, washed with copper-free V3S, centrifuged, and adjusted to OD<sub>600</sub> of 7.0. Identical amounts (15 μl) of each suspension were immediately spotted onto a filter paper soaked in 1% TMDP, and indophenol blue formation was recorded after 10 to 15 min.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/7/eaaw8478/DC1

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