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Mechanism of Cu_A assembly

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Abstract

Copper is essential for proper functioning of cytochrome *c* oxidases, and therefore for cellular respiration in eukaryotes and many bacteria. Here we show that a new periplasmic protein (PCu_AC) selectively inserts Cu(I) ions into subunit II of *Thermus thermophilus* ba_3 oxidase to generate a native Cu_A site. The purported metallochaperone Sco1 is unable to deliver copper ions; instead, it works as a thiol-disulfide reductase to maintain the correct oxidation state of the Cu_A cysteine ligands.

Cu_A is a dinuclear copper site within the soluble domain of subunit II (Cox2) of bacterial and eukaryotic cytochrome *c* oxidases (CcOs), whose function is to convey electrons from a soluble cytochrome *c* to the catalytic heme a_3-Cu_B center of CcO (refs. 1,2). The proper assembly of the Cu_A site is essential for the catalytic machinery of a functional oxidase. Several proteins have been identified as key players in the delivery of metal ions to the Cu_A site3, but the detailed molecular mechanisms and the specific roles of each protein are poorly understood4. In prokaryotes two protein families have been proposed to be involved in Cu_A site formation. The first includes proteins that are able to bind Cu(I) through methionine and histidine residues arranged in a highly conserved $H(M)X_{10}MX_{21}HXM$ motif 5 (referred to as periplasmic Cu_A chaperone (PCu_AC) hereafter). The second consists of the Sco proteins, whose mechanism of action in Cu_A assembly as thioredoxins or metallochaperones is still debated6. These proteins (PCu_AC and Sco) are often found in the same bacterial operon, and most of the identified operons that encode Sco also contain a gene for Cox2 (ref. 7). PCu_AC and Sco proteins occur together in the *Vibrio cholerae* bacterium (http://string.embl.de).

The Cu_A-containing subunit II from *Thermus thermophilus* ba_3 oxidase (*Tt* Cu_A hereafter), *T. thermophilus* PCu_AC (*Tt* PCu_AC) and *T. thermophilus* Sco1 (*Tt* Sco1), which are all located in the bacterial periplasm, were expressed as truncated soluble versions in which the transmembrane helices (and the signal peptide region in *Tt* PCu_AC) were not included, thus

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AUTHOR CONTRIBUTIONS

L.A.A. cloned and expressed *Tt* Sco1 and *Tt* Cu_A proteins and contributed to the NMR structure of *Tt* Sco1 and NMR titrations; P.G. cloned and expressed PCu_AC protein and performed redox experiments; S.W. solved the structures of PCu_AC and contributed to the NMR titrations and NMR structure of *Tt* Sco1; S.C.-B. supervised and coordinated the acquisition of NMR data and structures, and the performance of titration experiments; I.B., L.B., G.A.S. and A.J.V. developed and directed the project and contributed to the writing. All authors were involved in the discussion of the biochemical meaning of the experiments.

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resulting in constructs of 136, 172 and 121 amino acids, respectively (Supplementary Methods and Supplementary Fig. 1 online).

Tt PCu_AC binds one equivalent of Cu(I), as also observed for the homologous protein DR1885 (ref. 5). The Cu(I) binding affinity, measured by competition experiments with DTT followed by ^1H-^{15}N HSQC, is $(2.2 \pm 0.1) \times 10^{-13}$ M (Supplementary Fig. 2, Supplementary Data and Supplementary Methods online). Upon addition of Cu(I) up to a 1:1 metal/protein ratio, the most affected region includes residues His46, Met61, Met83 and His85 and the neighboring residues (Supplementary Fig. 2), thus defining the copper binding ligands (similar to the Met_3His ligand set found for DR1885 in homologous positions).

Sco proteins, in contrast to the *Tt* PCu_AC protein family, are able to bind both Cu(I) and Cu (II) ions8–10. Cu(I) binding followed by NMR confirmed the involvement of metal ligands conserved in all Sco1 proteins (Cys47, Cys51 and His137) (Supplementary Fig. 2 and Supplementary Data). Apo-*Tt* Sco1 showed weaker Cu(I) binding capabilities compared with its eukaryotic homologs (a lower limit of ~10^{-10} M was estimated for the K_d of Cu(I)–*Tt* Sco1). Cu(II)–*Tt* Sco1 presents spectroscopic features resembling those of the human and *Bacillus subtilis* homologs8,10,11 (Supplementary Data and Supplementary Fig. 3 online).

The solution structures of apo-*Tt* PCu_AC, Cu(I)–Tt PCu_AC and apo-*Tt* Sco1 were determined using NMR-derived restraints (Supplementary Methods). The NMR spectra of apo- and Cu (I)–*Tt* PCu_AC variants show two sets of signals in a 65:35 ratio (Supplementary Fig. 3) for 23 and 24 residues, respectively, which originate from a *cis-trans* isomerization of the Gly13-Pro14 peptide bond. The two structures resulting from this isomerism in both apo- and Cu(I)–*Tt* PCu_AC were obtained (Fig. 1a–d and Supplementary Tables 1 and 2 online). The structural differences induced by this isomerization are localized in loops 1 and 8 and do not affect Cu (I) binding (Fig. 1a–d). *Tt* PCu_AC is arranged in a cupredoxin-like fold (Fig. 1a–d), except that strands μ4 and μ5 form an extended, flexible solvent-exposed μ-hairpin that is longer than the one reported for the homologous DR1885 protein5. The Cu(I) ion is coordinated in a tetrahedral arrangement to the sulfur atoms of Met61 and Met85, the Nδ 1 atom of His46 and the Nε2 atom of His83 (Fig. 1a,c). The structure of apo-*Tt* Sco1 in the reduced form adopts the thioredoxin-like fold already observed for all Sco1 homologs12–16 (Fig. 1e and Supplementary Table 3 online). Loop 8 (which includes the metal ligand His137) adopts an extended conformation in *Tt* Sco1 (Fig. 1e) resembling that observed for the human and yeast proteins, in contrast with the shorter (and less extended) loop 8 present in *Bs* Sco1. The cysteine metal ligands are solvent exposed (as in other Sco1 proteins), which is in agreement with the observation of rapid air oxidation.

The availability of the NMR resonance assignments of the apo and the metallated forms of the two possible copper donors, as well as that of the Cu_A-containing soluble fragment of the *T. thermophilus* ba_3 oxidase, allowed us to investigate, through NMR, copper uptake by the Cu_A fragment. We simultaneously monitored the occurrence of copper transfer and the formation of the correct metallated form, and also identified the copper donor protein, by detecting the resulting apo state of the protein that had transferred the copper ions. This strategy also allows the identification of possible transient intermediates.

Addition of Cu(I) to reduced ^{15}N apo-*Tt* Cu_A under anaerobic conditions gives rise to the fully metallated protein in the reduced state (Supplementary Data and Supplementary Fig. 4 online). Exposure of this species to oxygen resulted in the formation of a purple species with the characteristic electronic spectrum and ^1H NMR signals of the oxidized, mixed-valence Cu_A center (Supplementary Fig. 4)17. This indicates that the Cu_A center can be formed *in vitro* without the assistance of any protein when the cysteine residues of the Cu_A center are reduced. The affinity of both copper ions in *Tt* Cu_A is in the femtomolar range based on competition

studies with DTT (Supplementary Methods) and is thus higher than that of *Tt* PCu_AC and *Tt* Sco1. However, because Cu(I) is not freely available in the periplasmic space, a Cu(I) chaperone is needed to deliver two Cu(I) ions to the apo-Cu_A protein.

We initially explored the possible role of Sco1 as a Cu(I) or Cu(II) donor to apo-Cu_A. No evidence of copper uptake by the Cu_A protein or of metal depletion of *Tt* Sco1 was observed, which suggests that *Tt* Sco1 is not responsible for the direct delivery of Cu(I) or Cu(II) ions into apo-*Tt* Cu_A.

Genomic studies have suggested that *Tt* PCu_AC homologs could be involved in copper transport in the periplasmic space of bacteria5,18, and we decided to test this previously unexplored hypothesis. When apo-*Tt* Cu_A was added to a solution of ^{15}N Cu(I)–*Tt* PCu_AC, a new set of resonances corresponding to apo-*Tt* PCu_AC was observed (Fig. 2a). A step-wise titration led to the progressive disappearance of the signals corresponding to Cu(I)–*Tt* PCu_AC, with the concomitant increase of signals from the apo form (Fig. 2a). This process was complete at a *Tt* Cu_A/*Tt* PCu_AC ratio of 1:2 (Fig. 2a). The complementary experiment to characterize the metallated species was performed by adding two equivalents of unlabeled Cu(I)–*Tt* PCu_AC to ^{15}N apo-*Tt* Cu_A, which led to the typical ^{1}H-^{15}N HSQC pattern of native $Cu(I)_2$–*Tt* Cu_A (Fig. 2b). A step-wise titration disclosed the formation of an intermediate species when less than one equivalent of Cu(I)–*Tt* PCu_AC was added to the Cu_A domain (Fig. 2b,c). This species then converted into the dimetallated Cu(I)2–*Tt* Cu_A upon further addition of the donor protein (Fig. 2c). This intermediate species displayed resonances with chemical shifts differing from those of both the apo- and the fully metallated species (Fig. 2b), and it did not show any line broadening with respect to the other forms of the Cu_A domain. This behavior, together with the observation that no intermediate was identified by monitoring the copper release from Cu(I)–*Tt* PCu_AC to apo-*Tt* Cu_A, allowed us to rule out the detection of a possible complex between the two proteins, and suggested that this intermediate corresponds to a singly metallated Cu_A species. We conclude that *Tt* PCu_AC is capable of transferring two Cu(I) ions to the reduced apo-*Tt* Cu_A site sequentially, thereby eliciting the formation of the binuclear Cu_A center in the reduced state (Fig. 2c). Aerobic oxidation of this mixture leads to the mixed-valence, oxidized Cu_A center, as revealed by its characteristic electronic spectrum (Supplementary Fig. 4).

Sco1 is able to bind Cu(II) ions. The possible direct transfer of Cu(II) ions was explored by following the titration of a sample of ^{15}N-labeled Cu(II)–*Tt* Sco1 with unlabeled apo-*Tt* Cu_A. There was no evidence of the formation of apo-*Tt* Sco1 in the ^{1}H-^{15}N HSQC spectra, thus revealing the inability of this protein to transfer Cu(II) ions to the Cu_A domain. When 2 equivalents of unlabeled Cu(I)–*Tt* PCu_AC were added to the mixture of ^{15}N-labeled Cu(II)–*Tt* Sco1 and ^{15}N-labeled apo-*Tt* Cu_A, the reduced $Cu(I)_2$–*Tt* Cu_A center was formed (Supplementary Fig. 5 online). This result indicates that (i) the presence of Cu(II)–*Tt* Sco1 does not prevent Cu(I)–*Tt* PCu_AC from transferring Cu(I), and (ii) as the final product is the reduced $Cu(I)_2$–*Tt* Cu_A center, the mechanism cannot involve the concerted action of both Cu(II)–*Tt* Sco1 and Cu(I)–*Tt* PCu_AC, which would give rise to a mixed-valence, oxidized Cu_A site.

Sco1, which has already been shown to be essential for copper upload into the Cu_A site in *B. subtilis*19, is characterized by a thioredoxin-like fold. We therefore investigated the possible thiol-disulfide oxidoreductase activity of *Tt* Sco1 during copper uptake. Oxidized apo-*Tt* Cu_A, in which the two cysteine ligands had been aerobically oxidized to form a disulphide bond (ox-apo-*Tt* Cu_A hereafter), was not able to bind copper ions when Cu(I)–*Tt* PCu_AC was added. However, when ^{15}N-labeled ox-apo-*Tt* Cu_A was titrated with a 2:1 mixture of Cu(I)–*Tt* PCu_AC and reduced apo-*Tt* Sco1, the backbone resonances of the native $Cu(I)_2$–*Tt* Cu_A appeared (Fig. 3a). Exposure of the reaction sample to atmospheric oxygen allowed the development of the typical UV-vis spectrum of the oxidized, mixed-valence Cu_A center, which

confirmed that the native site had formed. A 4-acetamide-4′- amleimidylstilbene-2,2′-disulfonic acid, disodium salt (AMS)-reacted SDS gel of the reaction mixture (run under nonreducing conditions, Supplementary Methods) indicated that, at a *Tt* Cu_A/*Tt* Sco1/*Tt* PCu_AC ratio of 1:1:2, *Tt* Sco1 is mostly in the oxidized state (Fig. 3b). Similarly, in a 1:1 *Tt* Cu_A/*Tt* Sco1 mixture, *Tt* Sco1 is essentially in an oxidized state, whereas *Tt* Cu_A is in a reduced state (Fig. 3b). These experiments show that *Tt* Sco1 is able to reduce the disulfide bond of ox-apo-*Tt* Cu_A protein, which demonstrates that this protein behaves as a thiol-disulfide reductase in *in vitro* Cu_A assembly.

Although it is possible that the *in vivo* pathway is more complex, the structural characterization of *Tt* PCu_AC and of its copper binding properties and its ability to selectively and sequentially deliver two Cu(I) ions to apo-*Tt* Cu_A, thus giving rise to the native $Cu(I)_2$–*Tt* Cu_A site *in vitro*, strongly supports the annotation of this protein as a periplasmic Cu(I) chaperone. Our data also indicate that *Tt* Sco1 is able to reduce the disulfide bond of the Cu_A center, thus allowing the Cu_A site to accept Cu(I) ions from *Tt* PCu_AC. The mechanism of bacterial Cu_A assembly therefore consists of a sequential insertion of two Cu(I) ions donated by a metallochaperone once the disulfide bond of the Cu_A center is reduced by a thioredoxin. In eukaryotes the assembly of the Cu_A site is different, as Scos have a larger affinity for Cu(I) and may act as both chaperones and thioredoxins12,20–22.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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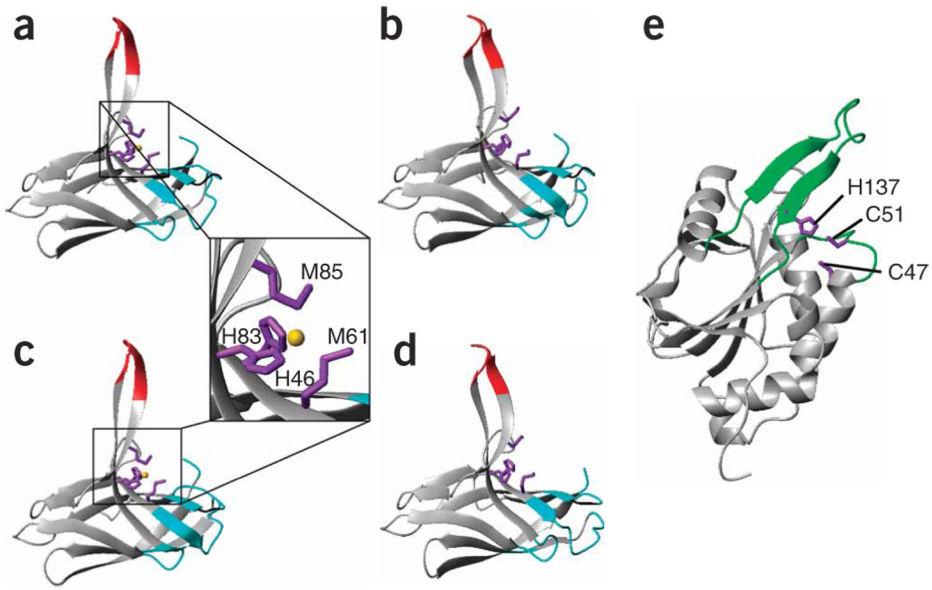

Figure 1. Solution structures of apo-*Tt* PCu$_A$C, Cu(I)–*Tt* PCu$_A$C and apo-*Tt* Sco1

(**a–d**) The *trans* (**a,b**) and *cis* (**c,d**) conformations resulting from Pro14 isomerization in *Tt* PCu$_A$C are shown for each protein. The methionine and histidine residues that bind the metal are shown as purple sticks in *Tt* PCu$_A$C. The Cu(I) ion is rendered as a golden sphere. The inset highlights the metal binding site of Cu(I)–*Tt* PCu$_A$C. Residues experiencing double conformations in *Tt* PCu$_A$C as a consequence of *cis-trans* proline isomerization are shown in cyan, and those displaying fast backbone motions at 298 K are shown in red. (**e**) The metal ligands Cys47, Cys51 and His137 of apo-*Tt* Sco1 are shown as purple sticks, and loops 3 and 8 are shown in green.

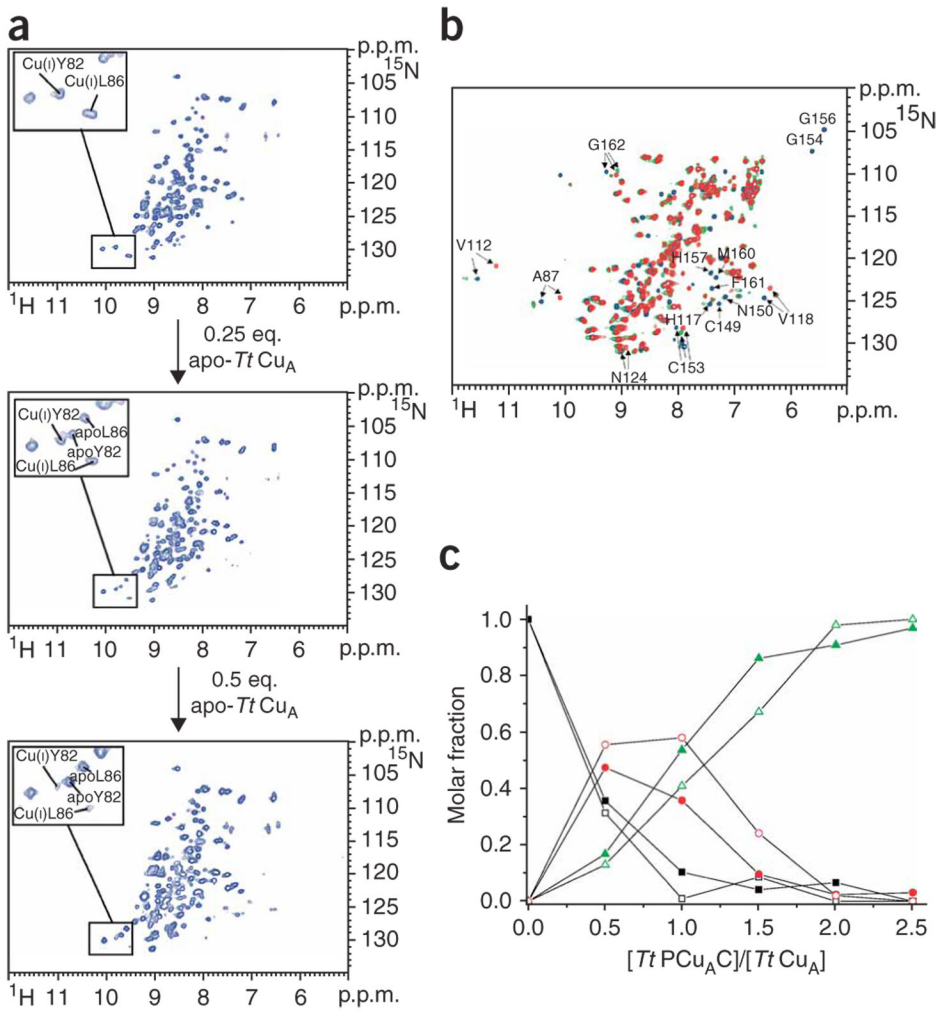

Figure 2. Cu(I) transfer reaction between Cu(I)–*Tt* PCu_AC and apo-*Tt* Cu_A

(**a**) Titration of ^{15}N-labeled Cu(I)–*Tt* PCu_AC with unlabeled apo-*Tt* Cu_A followed by ^{1}H-^{15}N HSQC experiments. In the insets, residues followed to map the metal exchange reaction are shown. (**b**) Overlay of the ^{1}H-^{15}N HSQC spectra of ^{15}N apo-*Tt* Cu_A (red), and after addition of one (green) and two (blue) equivalents of unlabeled Cu(I)–*Tt* PCu_AC. Some residues indicating the formation of $Cu(I)_2$–*Tt* Cu_A and of a partially metallated Cu_A species are labeled. (**c**) Plot showing the relative concentrations of $Cu(I)_2$–*Tt* Cu_A (green), the partially metallated Cu_A protein (red) and apo-*Tt* Cu_A (black) as a function of the *Tt* PCu_AC/*Tt* Cu_A molar ratio. The signals of residues Cys153 (solid symbols) and Gly162 (open symbols) (whose ^{1}H-^{15}N chemical shifts substantially change depending on the metallation state of *Tt* Cu_A) have been selected to evaluate the molar fractions of *Tt* Cu_A forms.

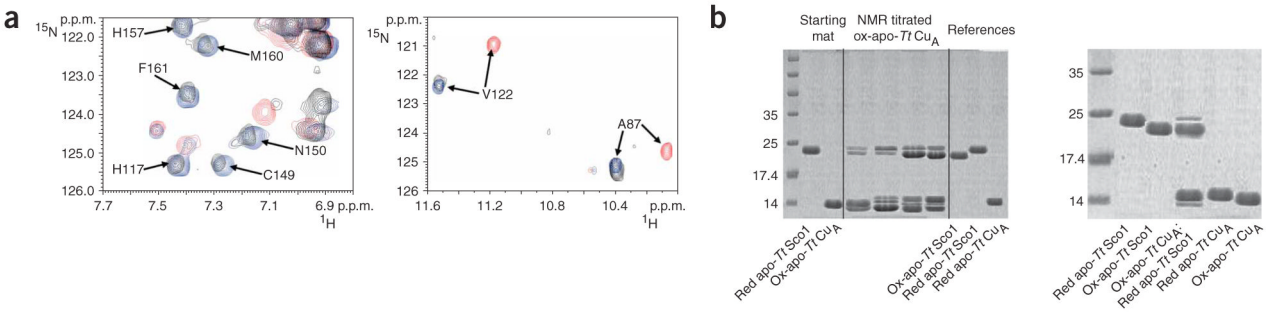

Figure 3. Cu(I) transfer from Cu(I)–*Tt* PCu$_A$C to oxidized apo-*Tt* Cu$_A$ in the presence of reduced apo-*Tt* Sco1

(**a**) ^{1}H-^{15}N HSQC overlay of oxidized apo-*Tt* Cu$_A$ (red), Cu(I)$_2$–*Tt* Cu$_A$ (blue) and the final mixture of the titration between ^{15}N-labeled oxidized apo-*Tt* Cu$_A$ and unlabeled, reduced apo-*Tt* Sco1 and Cu(I)–*Tt* PCu$_A$C in a 1:1:2 ratio, in the absence of DTT (black). (**b**) Left panel: AMS-reacted, nonreducing SDS gel of different aliquots from the NMR titration between ^{15}N-labeled oxidized apo-*Tt* Cu$_A$ and a 1:2 mixture of unlabeled, reduced apo-*Tt* Sco1 and Cu(I)–*Tt* PCu$_A$C (shown in **a**). Four steps of the NMR titration are reported. The band of *Tt* Cu$_A$ protein at 14.8 kDa is very close to that of *Tt* PCu$_A$C, which indeed has a very similar molecular weight (13.2 kDa). Starting materials and the *Tt* PCu$_A$C and *Tt* Cu$_A$ proteins in their different redox states are also reported as a reference. Right panel: AMS-reacted, nonreducing SDS gel of a protein mixture containing oxidized apo-*Tt* Cu$_A$ and reduced apo-*Tt* Sco1 in a 1:1 ratio. The proteins in their different redox states are also reported as a reference. Molecular weight markers are shown in first lanes.