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CopC Protein from *Pseudomonas syringae*: Intermolecular Transfer of Copper from Both the Copper(I) and Copper(II) Sites

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The CopC protein from *Pseudomonas syringae* pathovar *tomato* is expressed as one of four proteins encoded by the operon CopABCD that is responsible for copper resistance. It is a small soluble molecule (10.5 kDa) with a β-barrel structure and features two distinct copper binding sites, which are highly specific for Cu^{I} ($K_D \geq 10^{-13}$) and Cu^{II} ($K_D \approx 10^{-15}$). These dissociation constants were estimated via ligand competition experiments monitored by electronic spectral and fluorescence probes. The chemistries of the two copper sites are interdependent. When the Cu^{II} site is empty, the Cu^{I} ion is oxidized by air, but when both sites are occupied, the molecule is stable in air. The availability of an unoccupied site of higher affinity induces intermolecular transfer of *either* Cu^{I} or Cu^{II} while maintaining free copper ion concentrations in solution at sub-picomolar levels. This intriguing copper chemistry is consistent with the proposed role of CopC as a copper carrier in the oxidizing periplasmic space. These properties would allow it to exchange *either* Cu^{I} or Cu^{II} with its putative partners CopA, CopB, and CopD, contrasting with the role of the Cu^{I} (only) chaperones found in the reducing cytoplasm.

The CopC protein is a small soluble molecule of β-barrel topology (Figure 1) proposed to act as a copper chaperone in the oxidizing environment of the periplasmic space of *Pseudomonas syringae* pathovar *tomato*.[1−4] In contrast to cytoplasmic copper chaperones, it contains no cysteine residues (e.g., see refs 5 and 6). NMR and EXAFS structural data indicate the presence of two binding sites, separated by 30 Å.[3,7] One is specific for Cu^{I}, while the other is specific for Cu^{II}. The proposed ligand environments are Cu^{I}(His)-(Met)$_x$ ($x = 2$ or 3) and Cu^{II}(His)$_2$(Asp)(Glu)(OH$_2$). Intriguingly, the copper ion appears to exchange between the two sites upon a change of the oxidation state; i.e., the protein may function as a so-called redox switch.[7] However, it remains unclear how such an exchange occurs and what the specific affinity of each binding site is. An *Escherichia coli* homologue PcoC also binds both Cu^{I} and Cu^{II}, but an exchange has not been documented.[8,9] We have employed

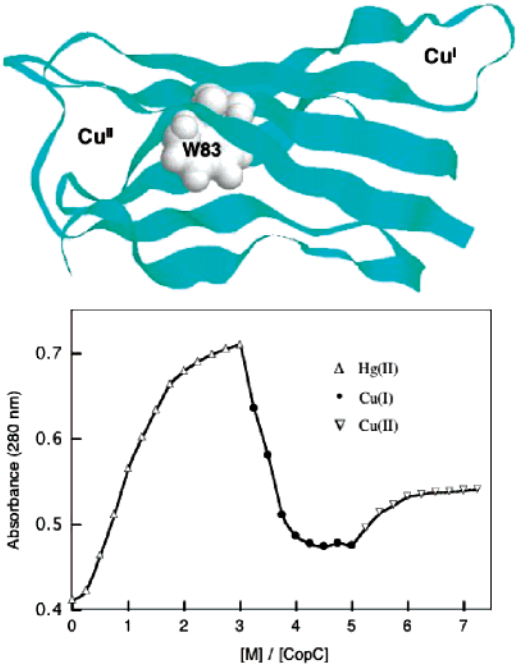

Figure 1. Upper: ribbon representation of the averaged molecular structure of apo-CopC (PDB coordinates 1M42). Residue W83 is highlighted. Lower: change in the absorbance at 280 nm upon sequential titration of apo-CopC (47 μM) in a 20 mM Mes buffer (pH 6) with $Hg^{II}Cl_2$, $Cu^{I}(MeCN)_4ClO_4$, and $Cu^{II}SO_4$. [M] = [Hg(II) + Cu(I) + Cu(II)].

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ion-exchange chromatography and two quantitative and complementary spectroscopic probes to initiate a study of the solution chemistry of CopC.[10]

Charge-transfer electronic transitions around 280 nm detect the presence of the bound metals copper and mercury: His → Cu^{II}; Met → Hg^{II}. Figure 1 demonstrates that apo-CopC binds two Hg^{II} ions with comparable affinity at two sites ($K_{D1} \geq K_{D2} > 10^{-6}$). Titration with either Cu^{I} or Cu^{II} displaced *only* one of the two bound Hg^{II} ions. Sequential titration with Cu^{I} followed by Cu^{II} displaced both bound Hg^{II} ions quantitatively to produce the $Cu^{I}Cu^{II}$ form of the protein (Figure 1) and to confirm the presence of two distinct binding sites specific for Cu^{I} and Cu^{II}.[10] The dissociation constant $K_D(Cu^{I})$ for Cu^{I} was estimated to be $\geq 10^{-13}$ via competition with the Cu^{I} ligand bathocuproïne disulfonate bcs ($\beta_2 = 10^{19.8}$; Table S1 and Figure S1 of the Supporting Information).[11] The dissociation constant $K_D(Cu^{II})$ for Cu^{II} was determined to be $10^{-15(1)}$ via competition with two hexadentate Cu^{II} ligands edta and cdta (Table S2 and Figure S2 of the Supporting Information).[12] These values are consistent with the proposed role of CopC as a periplasmic copper-scavenging protein.

Bound Cu^{II} but not Cu^{I} quenches the fluorescence attributed to the unique tryptophan residue W83 located between the two binding sites (Figures 1 and S2 of the Supporting Information). This provides a convenient probe for the half-loaded $Cu^{I}\subset$ and $\square Cu^{II}$ species in solution ($\subset$ is an empty Cu^{II} site, and $\square$ is an empty Cu^{I} site). $Cu^{I}\subset$ loses its fluorescence intensity rapidly ($t_{1/2} < 5$ min) in air. The intensity is recovered under anaerobic conditions by the addition of reductant ascorbate or dithionite. After the removal of unbound copper by gel filtration, the estimation of protein-bound copper by atomic absorption spectroscopy confirmed that the $Cu^{I}\subset$ and $\square Cu^{II}$ forms can be exchanged cleanly by oxidation and reduction. However, $Cu^{I}\subset$ is intrinsically unstable in air, a property apparently driven by the higher thermodynamic stability of the Cu^{II} site relative to the Cu^{I} site.

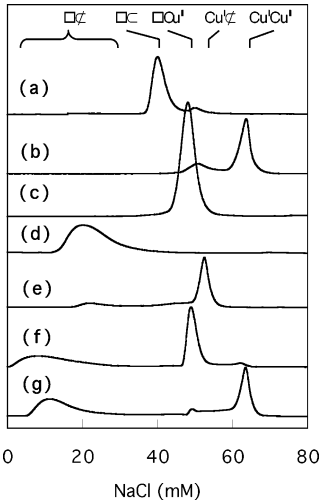

Figure 2. Elution profiles of proteins (~60 μg) on a Mono-S HR5/5 cation-exchange column in a deoxygenated buffer (10 mM KPi; pH 7.0):[14] (a) $\square\subset$ (the minor component eluting at the $\square Cu^{II}$ position is attributed to adventitious metal ions in the buffers and is removed by coelution with edta); (b) $Cu^{I}Cu^{II}$-CopC [the minor component attributable to $\square Cu^{II}$ is due to slow oxidation of the $Cu^{I}Cu^{II}$ species by air promoted by the Cu^{2+} affinity of the Mono-S resin (cf. eq 1)]; (c) 2 equiv of $\square Cu^{II}$ formed upon mixing 1 equiv of $\square\subset$ with 1 equiv of $Cu^{I}Cu^{II}$ in air (eq 1); (d) $\square\not\subset$ or a mixture of $\square\not\subset$ and $Cu^{II}SO_4$ (1:2); (e) $Cu^{I}\not\subset$ formed upon mixing $\square\not\subset$, $Cu^{II}SO_4$, and NH_2OH (1:1.2:10); (f) $\square\not\subset$ and $\square Cu^{II}$ formed upon mixing $Cu^{I}\not\subset$ with $\square\subset$ (1:1; cf. eq 3); (g) $\square\not\subset$ and $Cu^{I}Cu^{II}$ formed upon mixing $Cu^{I}\not\subset$ with $\square Cu^{II}$ (1:1) under anaerobic conditions (cf. eq 4).

In contrast, the $Cu^{I}Cu^{II}$ form is stable under the same conditions: it can be chromatographed in air (Figure 2b). However, it becomes air-sensitive in the presence of apo-protein (Figure 2c):

$$Cu^{I}Cu^{II} + \square\subset + O_2 \rightarrow 2\square Cu^{II} \qquad (1)$$

Here, intermolecular copper transfer has occurred with a change in the copper oxidation state, a process again apparently driven by the difference in the thermodynamic stabilities of the Cu^{I} and Cu^{II} sites.

Upon mutation of His1 to Phe, the affinity of the resultant variant protein H1F−CopC ($\square\not\subset$) for Cu^{II} is much diminished [$K_D(Cu^{II}) \sim 10^{-6}$]. This is consistent with His1 being a Cu^{II} ligand.[7] In fact, $\square\not\subset$ binds Cu^{II} so weakly that it cannot compete with the Mono-S cation-exchange resin and decomposed quantitatively to the apo form during chromatography (Figure 2d). In contrast to wild-type $\square Cu^{II}$, the addition of the *weak* reductant NH_2OH converted this Cu^{II} protein to the Cu^{I} form ($Cu^{I}\not\subset$; Figure 2e). Again, the reaction seems to be driven by a difference in thermodynamic stabilities between the two copper binding sites (cf. eq 1), but now the transfer is from the Cu^{II} site to the Cu^{I} site. Furthermore, in contrast to its wild-type analogue $Cu^{I}\subset$, $Cu^{I}\not\subset$ is stable: it could be isolated chromatographically in air, properties shared with the wild-type $Cu^{I}Cu^{II}$ form. $Cu^{I}\not\subset$ retained its stability in the presence of H1F apo-protein $\square\not\subset$ but reacted rapidly with O_2 in the presence of wild-type apo-protein $\square\subset$ (Figure 2f):

$$Cu^{I}\not\subset + \square\not\subset + O_2 \rightarrow \text{very slow} \qquad (2)$$

$$Cu^{I}\not\subset + \square\subset + O_2 \rightarrow \square\not\subset + \square Cu^{II} \qquad (3)$$

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(10) CopC protein was expressed and isolated essentially as reported[3] with an extra gel-filtration purification step. The protein contained <0.01 equiv of Cu. Mercury could not be detected (<0.05 equiv) after gel filtration of the $Cu^{I}Cu^{II}$ form generated under the conditions of Figure 1.

(11) A previous approach was adapted (Xiao, Z.; Loughlin, F.; George, G. N.; Howlett, G.; Wedd, A. G. *J. Am. Chem. Soc.* **2004**, *126*, 3081−3090). The total bcs/Cu^{I} ratio was maintained at 3 to ensure that all Cu^{I} not bound to the protein were present as $[Cu^{I}(bcs)_2]^{3-}$ of known stability constant ($\beta_2 = 10^{19.8}$). CopC binds Cu^{I} more weakly than bcs, but effective competition was evident from a monotonic decrease in A_{483} (characteristic of $[Cu^{I}(bcs)_2]^{3-}$) upon the addition of apo-CopC under anaerobic conditions (Figure S1 and Table S1 of the Supporting Information). However, only a minimum value of K_D can be derived because these experiments are at the limit of detection: less than 1% of protein binds Cu at equilibrium.

(12) Cu^{II} binding quenches the fluorescence of CopC linearly until 1 equiv of Cu^{II} is bound (Figure S2 of the Supporting Information). The quenching is extended linearly to higher ratios of Cu^{II} in the presence of the ligands edta or cdta (inset to Figure S2 of the Supporting Information). On the other hand, titration of these ligands into stable Cu^{II}-CopC solutions led to linear restoration of the fluorescence intensity. These experiments demonstrated an effective competition for Cu^{II} between apo-CopC and the ligands, allowing reliable estimation of $K_D(Cu^{II})$ (Table S2 of the Supporting Information).

Again, intermolecular copper transfer has occurred with a change of the copper oxidation state (cf. eq 1). The absence of an unoccupied high-affinity Cu^{II} site is required to protect the bound Cu^{I} from oxidation by air.

Ion-exchange chromatography provided further strong evidence of the interdependence of these two copper sites. Upon mixing of Cu^{I}⊄ and □Cu^{II} in an equimolar ratio under anaerobic conditions, Cu^{I}⊄ transferred its Cu^{I} ion to □Cu^{II} (Figure 2g):

$$Cu^{I}⊄ + □Cu^{II} \rightarrow □⊄ + Cu^{I}Cu^{II} \quad (4)$$

Intermolecular copper transfer of Cu^{I} has occurred but without oxidation to Cu^{II} (cf. eqs 1 and 3). Clearly, the presence of bound Cu^{II} in the wild-type protein has increased the binding affinity for Cu^{I}, and that increase was estimated to be at least 2 orders of magnitude.[13] The structural differences between proteins □⊄ and □Cu^{II} at the Cu^{II} site (30 Å removed from the Cu^{I} site) have triggered a modulation favoring Cu^{I} binding.

When reaction 4 was carried out under aerobic conditions, the Cu^{I} ion was protected against oxidation by O_2. The yield of $Cu^{I}Cu^{II}$ protein isolated from the Mono-S column dropped marginally (∼5%) with a concordant increase of detected Cu^{II}⊂. The presence of the latter is due solely to the Mono-S resin-promoted slow oxidation by air of both $Cu^{I}Cu^{II}$ (Figure 2b) and Cu^{I}⊄ (Figure 2e). Consequently, *direct* intermolecular contact must be involved for the copper transfer of eq 4.

In summary, CopC features two distinct but interdependent binding sites with high specific affinities for Cu^{I} and Cu^{II}. When both sites are occupied, the molecule is stable in air. The availability of an unoccupied site of higher affinity induces intermolecular transfer of *either* Cu^{I} or Cu^{II} while buffering free copper ion concentrations at sub-*pico*molar levels.

This unique copper chemistry is consistent with the role of CopC as a Cu carrier in the oxidizing periplasm.[4,7] Its properties would allow it to exchange *either* Cu^{I} or Cu^{II} with its proposed partners CopA, CopB, and CopD, contrasting with the role of the Cu^{I} chaperones found in the reducing cytoplasm.[5,6]

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Supporting Information Available: Tables S1−S3 and Figures S1 and S2 are given. This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) *K* for reaction 4 was assessed from the relative integrated peak area of each component of the elution profile (part g) of the figure.

(14) The identity of each protein component isolated from the elution profiles of Figure 2 was confirmed by ESI−MS and the copper content (Table S3 of the Supporting Information). apo-H1F □⊄ showed little affinity for the Mono-S resin at pH 7, eluting as a broad band of variable position close to the start of the NaCl gradient. That position depended markedly on the ionic strength and sample volume.