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Short communication

A dimerization interface mediated by functionally critical residues creates interfacial disulfide bonds and copper sites in CueP

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ABSTRACT

CueP confers bacterial copper resistance in the periplasm, particularly under anaerobic conditions, through an unknown mechanism. The only available structure and limited solution data suggest that CueP forms noncovalent dimers in solution, whereas sequence conservation suggests important roles for three cysteines and two histidines as copper ligands. Here we report evidence of a dimerization equilibrium mediated by a newly identified interface of functional relevance, which occludes internal copper sites and disulfide bonds but allows for intra- and interchain disulfide bonding, an extensive disulfide relay, and interfacial copper sites. Our results suggest a role for CueP linking redox-state sensing and copper detoxification.

Copper is essential for life but toxic above trace levels, thus its intracellular level is tightly regulated [1]. Gram-negative bacteria employ the *cue* regulon and the *cus* locus to cope with copper toxicity [2,3]. The *cue* regulon confers higher resistance levels, standing as an interesting target of pathogens such as *Salmonella enterica* [4]. It includes CueR, which upon a raise in copper levels induces the expression of genes coding for a copper ATPase, a periplasmic multicopper oxidase, and a periplasmic protein known as CueP, subject of this work [5–7].

CueP's role in copper homeostasis is beginning to be unveiled. It is required for resistance particularly under low oxygen conditions [7] and delivers copper to the periplasmic Cu/Zn SodCII in low-copper environments [8]. It binds metal ions in vitro and in vivo [7–10] but the metal-binding motif(s) have so far eluded structural characterization by X-rays [9] and other methods (our unpublished results), and only its metal-free structure is available (Fig. 1B) [9]. Among putative copper-binding residues, His94, His99, Cys96, Cys104 and Cys172 locate in the conserved long loops Ala87–Val115 and Thr165–Leu178 (Fig. 1 and S1). A third loop conserved among CueP homologues is spatially close. Since loops tend to accommodate substitutions with little impact on structure [11], the high conservation of these loops suggests functional importance. The presence of three conserved cysteines is also puzzling, since periplasmic copper proteins avoid them as they are sensitive to oxidation [12].

The crystallographic unit cell of metal-free CueP (PDB ID 4GQZ) contains a noncovalent dimer of two noncovalent dimers mediated by interfaces "1" and "2" in Fig. 2. Yoon et al. proposed that the dimer mediated by "2" is the major species observed in solution by size-exclusion chromatography [9]. They further proposed a buried copper-binding site in the place of a crystallographic water molecule, involving His99 and the three cysteines. Cys96 and Cys172 form a disulfide bond in the X-ray structure, but Yoon et al. proposed that bonds Cys96–Cys104 and Cys104–Cys172 could also exist depending on the conditions [13,14]. We report here that CueP's oligomerization equilibria extend beyond noncovalent dimerization, including disulfide-mediated binding through a newly identified interface ("3") that involves the conserved loops, interferes with copper binding, and includes residues critical for CueP's function in vivo.

We produced the mature form of *S. enterica* CueP in *E. coli* [7]. Samples of metal-free or copper-incubated protein display a single band in SDS-PAGE under reducing conditions at 17 kDa, the mass expected for monomeric species (Fig. 2A). Non-reducing SDS-PAGE, instead, displays a complex pattern with two major bands at around 30–34 kDa, two bands at around 15–17 kDa and another at 68 kDa, corresponding to dimeric, monomeric and tetrameric species, respectively (Fig. 2A). The relative abundances qualitatively agree with those

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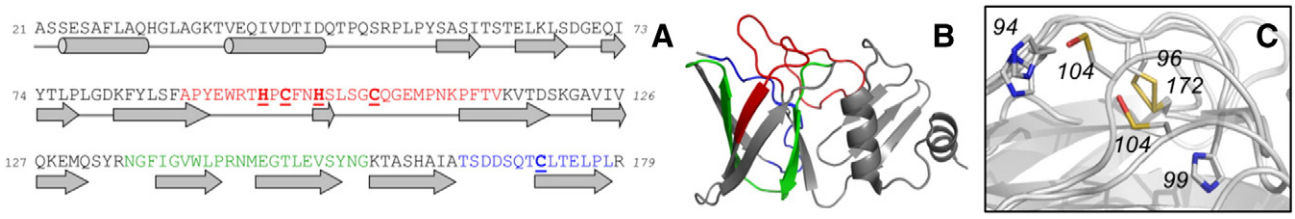

Fig. 1. (A) Amino acid sequence and secondary structures of mature CueP. The three conserved regions are shown in colors; residues discussed in the text are in underlined bold. (B) Structure of one CueP chain with the conserved segments colored as in (A) rendered from PDB ID 4GQZ. (C) The two main conformations observed for the long conserved loop, with essential histidines and cysteines shown as sticks (Cys104 is most likely oxidized to its sulfenic acid derivative in the structure according to the electron densities).

observed by Yoon et al. in the elution profile of a size-exclusion chromatography column.[3] In the presence of copper, the same species exist but with larger amounts of the two bands running close to the monomer, considerably less of the dimer, and slightly more of the tetramer. This suggests that CueP's oligomerization equilibrium includes dimers mediated by disulfide bonds, and that copper binding disturbs the formation of the covalent dimer. Substitutions of Cys104 or Cys172 by serine hamper formation of the tetramer and substantially decrease the amount of dimer (Fig. S2) indicating that both cysteines are involved in covalent dimerization. This seems incompatible with Cys172 being completely buried and compromised in a disulfide bond with Cys96 as in the X-ray structure, but could point at the existence of multiple redox species and large conformational freedom. Substitution of Cys96, which is close to the surface of the protein, does not affect oligomerization in native SDS-PAGE (Fig. S2).

In the splittings observed for each band in non-reducing conditions, the slower-migrating bands match better with the molecular weights expected for pure species, and we thus attribute the faster-migrating bands to more compact forms of the loops (indeed observed in the X-ray structure, Fig. 1C) arising from copper binding and/or formation of intramolecular disulfide bridges. Supporting this interpretation, preincubation with Cu(II) enriches the most compact form (Fig. 2A), and substitutions of Cys104 or Cys172 by serine affect the relative amounts of the compact and loose forms. Notably, substitution of Cys96 by serine has no effect on this and has a mild effect on copper binding, although it is essential for CueP's activity (see below). Copper contents add up to the complex scenario: wild type CueP purified from cells grown in media with 1 mM $CuSO_4$ contains ~1 equivalent per molecule (as reported previously [10]), whereas substitution of Cys104 or Cys172 abolishes copper binding and substitution of Cys96 results in half an equivalent (Table S1). However, in vitro titration of the apo, thiol-reduced forms of wild type and mutant variants with Cu(II) reveals that they are all capable of binding not one but two equivalents of the metal ion (Fig. S3); and moreover, extensive dialysis does not remove all the ions and rather leaves a variable amount of bound metal (1–2 ions per molecule, data not shown). These results point at complex equilibria coupling dimerization, intra- and inter-chain disulfide bonds and copper binding. Finally, we observed that substitution of any of the three cysteines by serine or any of the two histidines by arginine impairs CueP's ability to confer resistance in *Salmonella* (Fig. S4), indicating they are all critical for function and suggesting physiological relevance for the observed equilibria.

Oligomerization mediated by disulfide bonds is not consistent with interfaces "1" or "2" observed in the structure, and analysis with the EPPIC and PISA webservers [15,16] return only "2" as energetically significant. However, both detect another slightly more favorable interface across unit cells ("3" in Fig. 2) stabilized by ~10 hydrogen bonds and a predicted disulfide bond between Cys104 of both monomers (Figs. 2B and S5).[4] Interface "3" includes the conserved loops, consistent with the results presented above and supporting its biological relevance.

We then sampled dimers mediated by interfaces "2" and "3" through molecular dynamics simulations, searching for conformations compatible with intra- and inter-monomer copper-binding sites and disulfide bonds as done previously [18]. We observed a variety of such arrangements exchanging in a nanosecond timescale (Fig. 2C, D, S6, S7). For example, Cys104 can easily reach the two other cysteines from the same monomer or Cys104 from the monomer across, which could allow for disulfide shuffling or relays connecting all 6 cysteine residues of the dimer. Cys104 can also join His94, His99, Cys96 and Cys172 from the same monomer, or His94 and Cys104 from the monomer across, into arrangements reminiscent of known copper-binding sites [1,17]. Although we cannot conclude that all these motifs truly exist in solution, they support the idea of a large heterogeneity and facile mixture of species arising from interactions through interface "3".

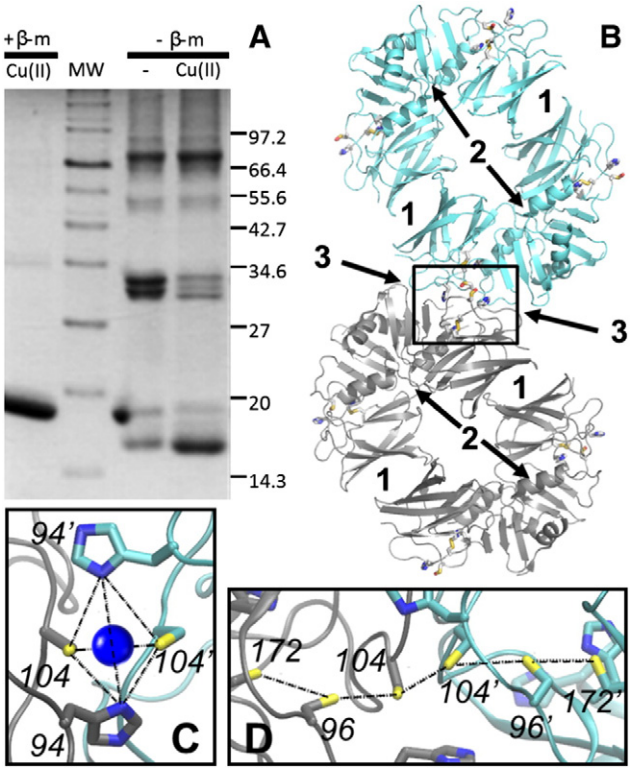

Fig. 2. (A) SDS-PAGE analysis of wild type CueP samples incubated with β-mercaptoethanol (β-m) and with excess Cu(II) before denaturation and loading in gels (first lane), or without reducing agents but with/without preincubation with copper (two rightmost lanes). (B) The dimerization interfaces discussed in the text (see also Fig. S2) mapped on two adjacent crystallographic unit cells (cyan and gray). (C and D) Simulation snapshots showing one potential interfacial copper-binding site (C, a blue sphere mimics the position of a possible copper ion) and a disulfide relay (D) at the newly identified interface (see also Figs. S6 and S7).

[3] The tetramer is not described by the authors but is visible in the elution profile they report.

[4] Residues interacting in interface "3": 50–61, 66–73, 91–95 and 100–110. This interface buries ~900 Å^2 of surface and is stabilized by −6.2 kcal/mol, against ~990 Å^2 and −4 kcal/mol for interface "2".

We propose that plastic dimerization through interface "3" could couple CueP's detoxifying activity to the systems that sense the redox state of the periplasm or with shuttles of reducing equivalents such as DsbC [14], and/or mediate interactions with periplasmic SodCII within its copper delivery pathway [10].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jinorgbio.2014.07.022.

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