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Mechanistic insights into Cu(I) cluster transfer between the chaperone CopZ and its cognate Cu(I)-transporting P-type ATPase, CopA

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Running Title: Cu(I)-cluster transfer between *B. subtilis* CopZ and CopAab

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² Abbreviations used: AU, crystallographic Asymmetric Unit; DTT, dithiothreitol; Mops, 3-morpholinopropanesulfonate; SAD, Single wavelength Anomalous Dispersion.

ABSTRACT: Multinuclear Cu(I) clusters are common in nature, but little is known about their formation or transfer between proteins. CopZ and CopA from *Bacillus subtilis*, which are involved in a copper-efflux pathway, both readily accommodate multinuclear Cu(I) clusters. Using the luminescence properties of a multinuclear Cu(I)-bound form of the two N-terminal soluble domains of CopA (CopAab) we have investigated thermodynamic and kinetic properties of cluster formation and loss. We demonstrate that Cu(I)-bound forms of dimeric CopZ containing more than 1 Cu(I) per CopZ monomer can transfer Cu(I) to apo-CopAab leading to the formation of luminescent dimeric CopAab. Kinetic studies demonstrated that transfer is a first order process and that the rate determining steps for transfer from CopZ to CopAab and *vice versa* are different processes. The rate of formation of luminescent CopAab via transfer of Cu(I) from CopZ was more rapid than that observed when Cu(I) was added 'directly' from solution or in complex with a Cys variant of CopZ, indicating that transfer occurs via a transient protein-protein complex. Such a complex would likely require the interaction of at least one domain of CopAab with the CopZ dimer. Insight into how such a complex might form is provided by the high resolution crystal structure of $\text{Cu}_3(\text{CopZ})_3$, a thus far unique trimeric form of CopZ containing a trinuclear Cu(I) cluster. Modelling studies showed that one of the CopZ monomers can be substituted for either domain of CopAab to result in a hetero-trimer, thus providing a model for a 'trapped' copper exchange complex.

INTRODUCTION

Copper is involved in a wide range of biochemical processes including aerobic and anaerobic respiration, oxidative stress response, iron and oxygen transport and fruit browning. Its usefulness in nature is largely based on its ability to form tight complexes with amino acid residue side chains and to cycle between +1 and +2 oxidation states; however, these properties also make copper potentially highly toxic [1, 2]. Therefore, organisms of all types have evolved complex trafficking pathways that involve the transfer of the metal, as Cu(I), from one trafficking protein to the next, with the result that 'free' copper is kept at a very low level and the metal is delivered only where it is required [2, 3].

Amongst copper trafficking/detoxification proteins and the regulatory proteins that control their production are several that have the capacity to bind multiple Cu(I) ions in the form of Cu(I)-clusters. For example, in eukaryotes, the copper-responsive transcription factors Ace1 and Mac1 [4, 5], the Cu(I) transporter Ctr1 [6], the chaperone Cox17 [7] and the six Atx1-like domain repeats lying towards the N-terminus of the human Menkes Cu(I)-transporting P-type ATPase [8] have each been shown capable of accommodating a multinuclear Cu cluster (often a Cu_4 cluster). Our recent work on the bacterial copper trafficking proteins CopZ (an Atx1-like chaperone) and CopA (a Menkes-like P-type ATPase transporter), which are important for Cu(I)-detoxification in *Bacillus subtilis* [9], has demonstrated that these proteins have the capacity to bind multiple Cu(I) ions. CopZ can form a number of discrete Cu(I)-bound forms, including $\text{Cu}(\text{CopZ})_2$, $\text{Cu}_2(\text{CopZ})_2$ and $\text{Cu}_4(\text{CopZ})_2$ [10]. Although the physiological importance of such forms is unclear, the ease with which they form suggests that they are likely to be important under conditions of copper stress (when these proteins are required) [9]. The high resolution structure of the latter complex was recently solved, revealing a tetranuclear Cu(I) cluster at the interface of the two CopZ monomers [11]. *B. subtilis* CopA contains two soluble Atx1/CopZ-like domains towards its N-terminus (commonly referred to as the two N-terminal domains of CopA and denoted as CopAab). A soluble protein containing these domains can also accommodate multiple Cu(I) ions, leading to the formation of a highly luminescent $\text{Cu}_4(\text{CopAab})_2$ species [12, 13].

Essentially nothing is known about how such multinuclear clusters might form and undergo inter-protein transfer within the cell, but the principles of avoiding free intracellular copper that have emerged from studies of copper trafficking are likely to hold [14, 15]. The mechanism of copper transfer from Atx1-like chaperones to the N-terminal domains of Cu(I)-transporters has been studied in some detail, from which a mechanism involving the formation of a Cu(I)-mediated hetero-complex between the Atx1 protein and one of the N-terminal domains has emerged [14]. Verification of such a relay mechanism was provided by the NMR structure of such a hetero-complex between Atx1 and the first N-terminal domain of Ccc2 (termed Ccc2a) from yeast [16]. At least some Atx1-like chaperones undergo Cu(I)-mediated homo-dimerisation [10, 17], which introduces an additional step in the mechanism of transfer, where one of the monomers must dissociate to enable hetero-complex formation.

For the transfer of Cu(I) between higher order, multinuclear Cu(I) forms of CopZ and CopA, it does not seem likely that transfer occurs via the mechanism described above. For $\text{Cu}_4(\text{CopZ})_2$, for example, the ability to bind a multinuclear cluster is dependent on the CopZ dimer; dissociation into monomers would

lead to the loss of at least some Cu(I) from the protein because a single CopZ monomer cannot accommodate more than one Cu(I) ion. Secondly, dissociation of the CopZ dimer is likely to be slow because the cluster is coordinated at the monomer-monomer interface. Therefore, alternative transfer mechanisms, that do not involve release of Cu(I) or dissociation of CopZ into monomers, need to be considered.

Here, by following the luminescence of multinuclear Cu(I) forms of CopAab, we demonstrate that Cu(I) transfer can occur from multinuclear Cu(I) forms of CopZ to apo-CopAab and *vice versa*, and that this occurs at a significant rate that is consistent with a protein-protein transfer mechanism. The crystal structure of a novel trimeric form of CopZ containing a trinuclear Cu(I) cluster at the interface of the three monomers provides significant new insight into the structural flexibility of copper chaperone proteins and modelling studies, in which one of the CopZ monomers is replaced by an N-terminal Atx1-like domain of CopA, suggest how dimeric CopZ could transfer Cu(I) to an N-terminal soluble domain of CopA without first dissociating into monomers.

EXPERIMENTAL

Transfer experiments

Wild-type CopZ, C13S/C16S CopZ and CopAab were purified as previously described [10, 12]. Prior to the addition of Cu(I), protein samples were treated with 5 mM DTT² and excess reductant was removed by passage down a G25 Sephadex column (PD10, GE Healthcare) in an anaerobic glovebox (Faircrest), in which the oxygen concentration was kept below 2 ppm. Additions of Cu(I) were made anaerobically using a solution of CuCl in 1 M NaCl and 100 mM HCl [8, 10] or in 1 M NaCl and 10 mM HCl [17]; identical results were obtained in each case. Luminescence emission spectra were recorded using a Perkin-Elmer LS55 fluorimeter at 25 °C with excitation at 295 nm and excitation and emission slit widths of 10 nm, and a 390 nm cut-off band pass filter was employed. Luminescence kinetic data were fitted using the program DynaFit (BioKin, CA, USA) [18].

Crystallisation and crystal structure determination

A solution of CopZ in 10 mM Mops buffer at pH 7.0 containing a dimeric form of the protein binding a single Cu(I) ion [10, 19], Cu(CopZ)₂, was concentrated anaerobically to 58 mg/ml. Hanging drop vapour diffusion crystallization experiments were carried out at 277 K using a Belle Technology anaerobic chamber with an oxygen concentration of no more than 0.2 ppm. Clusters of rod shaped crystals grew within 4 days in experiments containing 0.2 M ammonium acetate, 0.1 M sodium acetate trihydrate pH 4.6 and 30% (w/v) PEG 4000 as precipitant. Crystals were harvested under anaerobic conditions at 289 K by transfer into fresh crystallization solution supplemented with 25% (v/v) ethylene glycol and then rapidly frozen in liquid nitrogen. The crystals belonged to space group *P*6₃ (*a* = *b* = 64.0 Å, *c* = 27.3 Å) with a monomer of CopZ in the asymmetric unit and a solvent content of 42% (v/v). The presence of copper was verified by measurement of the X-ray fluorescence spectrum from a single crystal in the vicinity of the copper K absorption edge. A SAD X-ray diffraction dataset was subsequently collected on the high energy side of the edge ($\lambda = 1.38$ Å) at the Synchrotron Radiation Source (SRS). Images were processed using MOSFLM [20] to a resolution of 1.9 Å and scaled with SCALA [21]. This dataset was effectively untwinned ($\alpha = 0.02$). Molecular replacement was carried out with MOLREP [22] using the structure of an apo-CopZ monomer from the Cu₄(CopZ)₂ crystal structure [11] (PDB entry 2QIF) as a search model. Initial refinement was carried out using REFMAC5 [23] and COOT [24] was used for model building. Inspection of σ_A -weighted single difference Fourier maps revealed the presence of a residual peak adjacent to the sulfur atoms of the two cysteine residues of the protein. This peak at approximately 25 σ was interpreted as a copper ion and incorporated into the structural model. The presence of this single copper ion in the crystallographic asymmetric unit was verified by direct methods in SHELXD [25] using the measured anomalous difference data. The final stages of refinement were performed using SHELXL [26] to give a final structural model including a CopZ monomer, a Cu(I) ion and 20 water molecules. This model has $R_{\text{cryst}} = 19.1\%$ and $R_{\text{free}} = 26.4\%$. When analyzed for stereochemical quality using PROCHECK [27] the final structure has all residues in the most favoured or generously-allowed regions of the Ramachandran plot. Full data collection, phasing and refinement statistics are presented in Table 1. Programmes from the CCP4 programme library [28] were used throughout the structure solution and refinement process. The atomic coordinates and experimental structure factors have been deposited in the PDB with the accession code 3I9Z.

Molecular modelling and protein structure analysis

Molecular models for conceptual copper exchange heterotrimers involving two copies of CopZ and a single Atx1-like domain from CopA (either CopAa or CopAb) were generated by homology modelling using the refined crystal structure of the $\text{Cu}_3(\text{CopZ})_3$ homotrimer as a template. These models contained the trinuclear Cu(I) cluster found in the parental structure. Tightly-bound water molecules hydrogen bonded to Ser12 and to Tyr65 (see Results section) were also included. These models and the X-ray crystal structure of the homotrimer were each immersed in a water bath and subsequently energy minimized in the presence of periodic boundary conditions using GROMACS [29] with the OPLS force field. Sodium counter ions were added to each model to maintain zero overall charge. Ideal values for metal cluster geometric parameters and corresponding force constants were taken from the work of Zhang [30] and Dalosto [31]. Protein structures were superimposed using DALI [32] or SUPERPOSE from the CCP4 programme suite [28]. Analysis of subunit interfaces was performed with PROTORP [33] and NOXclass [34].

RESULTS & DISCUSSION

Thermodynamics of Cu(I)-transfer between CopZ and CopAab detected by luminescence spectroscopy

Cu(I) bound to CopZ does not give rise to a luminescence signal in the ~600 nm region of the emission spectrum. CopAab, at low Cu(I) loadings (<1 Cu(I) per protein), also does not result in a luminescence signal. However, above 1 Cu(I) per protein, CopAab does give rise to a high intensity luminescence band, which we have previously shown is due to Cu(I)-mediated protein dimerisation, resulting in the formation of a solvent-shielded multinuclear Cu(I) centre(s) [12, 13]. Luminescence intensity was observed to maximise at ~2 Cu(I) per CopAab (generating $\text{Cu}_4(\text{CopAab})_2$), before quenching upon further Cu(I)-binding. Therefore, we investigated whether Cu(I)-transfer between the proteins could be followed through the luminescence of Cu(I)-bound CopAab. Similar studies have been reported for Cu(I) transfer from CopZ to CopY of *E. hirae* [35, 36] and Cu(I) transfer from Cox17 to Sco1 and Cox11 of *S. cerevisiae* [37].

CopZ was pre-loaded with 1 Cu(I) per protein, generating $\text{Cu}_2(\text{CopZ})_2$ [10], and mixed with apo-CopAab at ratios of CopZ:CopAab of 1:1, 1.5:1 and 2:1. Luminescence intensity in the 600 nm region was not observed for any of the resulting mixtures (not shown), indicating that insufficient Cu(I) was transferred from CopZ to CopAab to generate detectable quantities of the luminescent $\text{Cu}_4(\text{CopAab})_2$ species. Even with a 10-fold excess of $\text{Cu}_2(\text{CopZ})_2$, luminescence intensity was not observed (not shown). This indicated that Cu(I) is bound significantly more tightly in the $\text{Cu}_2(\text{CopZ})_2$ complex than in $\text{Cu}_4(\text{CopAab})_2$, or that the presence of CopZ inhibits the formation of the luminescent CopAab species.

CopZ has the capacity to bind more than 1 Cu(I) per protein [10]. CopZ loaded with 1.5 Cu(I) per protein was mixed with apo-CopAab at ratios of 1:1 and 3:1. The resulting luminescence spectra, shown in Figure 1A, revealed that essentially no luminescence intensity was observed for the 1:1 CopZ:CopAab experiment, but for the 3:1 experiment, a luminescence band was observed with a wavelength maximum at ~610 nm. The intensity of the band indicated that ~12% of the CopAab sample was in a dimeric, luminescent form, and, at equilibrium, the ratio of Cu(I) bound to CopZ was a little in excess of 1:1. This is consistent with the proposal that $\text{Cu}_2(\text{CopZ})_2$ has a higher stability than $\text{Cu}_4(\text{CopAab})_2$.

CopZ loaded with 2 Cu(I) per protein (generating $\text{Cu}_4(\text{CopZ})_2$) was mixed with apo-CopAab at a 1:1 ratio and allowed to reach equilibrium. The resulting luminescence spectrum revealed significant intensity, see Figure 1B. The spectrum of $\text{Cu}_4(\text{CopAab})_2$ (that contains an identical concentration of CopAab) is shown for comparison, demonstrating that Cu(I) is fairly evenly distributed between CopZ and CopAab, with a small excess associated with CopAab. To test whether transfer can occur in both directions, $\text{Cu}_4(\text{CopAab})_2$ was mixed with apo-CopZ. The resulting luminescence spectrum was very similar to that obtained when Cu(I)-CopZ was mixed with apo-CopAab (see Figure 1A), demonstrating that Cu(I)-transfer is reversible.

Kinetics of Cu(I)-transfer between CopZ and CopAab detected by luminescence spectroscopy

Luminescence intensity was monitored as a function of time following the addition of increasing concentrations of $\text{Cu}_4(\text{CopZ})_2$ to a fixed concentration of apo-CopAab, see Figure 2A. Data for the 2:1 and 3:1 CopZ:CopAab experiments were fitted to a single exponential function and those for 4:1 and 5:1 CopZ:CopAab experiments were fitted to a double exponential function and the rate constant for the initial process taken for the purposes of comparison (the cause of the second phase is discussed below). While the

rate of the reaction was dependent on the concentration of Cu(I)-CopZ, the value of k_{obs} (corresponding to the first exponential process for the 4:1 and 5:1 experiments) did not vary significantly ($k = 0.045 \pm 0.01 \text{ s}^{-1}$ at 25 °C). The data indicated that the rate determining step of the transfer reaction is a first order process, and because the transfer reactions were not conducted under pseudo-first order conditions, we conclude that this is a true first order process. The rate-determining step could correspond, for example, to the first order dissociation of the $\text{Cu}_4(\text{CopZ})_2$ complex or the dissociation of a complex between the two proteins through which Cu(I) transfer is mediated [15, 16, 38].

To investigate this further, the kinetic characteristics of the reverse reaction, *i.e.* of Cu(I) transfer from $\text{Cu}_4(\text{CopAab})_2$ to apo-CopZ, were probed by following luminescence intensity at 600 nm as a function of time, see Figure 2B. The luminescence data resulting from loss of Cu(I)-CopAab fitted well to a double exponential function. The initial phase accounted for the majority of the amplitude and was characterised by a rate constant of $k_1 = \sim 0.024 \text{ s}^{-1}$ at 25 °C (and for the second phase, $k_2 = 0.006 \text{ s}^{-1}$). For direct comparison of the kinetics of transfer in the two directions, transfer of Cu(I) from CopZ to CopAab was measured under identical conditions, see inset Figure 2B. This fitted well to a single exponential ($k = \sim 0.053 \text{ s}^{-1}$, in reasonable agreement with the above data), consistent with a single phase of concerted Cu(I)-transfer. Importantly, the data clearly indicated that the transfer of Cu(I) from CopAab to CopZ occurred at a significantly slower rate than the that of transfer from CopZ to CopAab. Therefore, the transfer reaction kinetics is governed by a different rate-determining step for each direction.

Binding of $> \sim 2$ Cu(I) per CopAab (that is, binding of additional Cu(I) to $\text{Cu}_4(\text{CopAab})_2$) leads to a quenching of the luminescence intensity [13], and this accounts for why the intensity was observed to decrease at longer time periods for the 4:1 and 5:1 CopZ:CopAab experiments. This demonstrated that the formation of the $\text{Cu}_4(\text{CopAab})_2$ complex occurred first, and was then followed by further transfer of Cu(I). The double exponential fits of data from the 4:1 and 5:1 CopZ:CopAab experiments revealed a first order rate constant for the second, slower transfer step of $\sim 0.0015 \text{ s}^{-1}$, *i.e.* approx. one order of magnitude lower than for the initial transfer reaction.

For the 2:1 to 5:1 CopZ:CopAab transfer experiments of Figure 2A, equilibrium luminescence spectra were recorded, Figure 2C, revealing that the extent of Cu(I) transfer to CopAab was dependent on how much (excess) Cu(I)-CopZ was added. This was reflected in the relative intensity and position of the luminescence maximum at equilibrium. From a ratio of 2:1 to 5:1, the luminescence band maximum shifted from ~ 608 to 618 nm. We have shown previously that the wavelength maximum is indicative of the Cu(I)-loading of CopAab, with a longer wavelength indicative of a higher Cu(I)-loading [13]. This enabled an approximate calculation of the amount of Cu(I) transferred; for example, at a ratio of 4:1, a wavelength maximum of 614 nm indicated an average loading of ~ 2.2 Cu(I) per CopAab, with ~ 1.5 Cu(I) per protein remaining with CopZ (see *Supplementary Table S1*). As the ratio of CopZ to CopAab was increased, more Cu(I) was transferred and the average Cu(I) content of CopAab increased relative to that of CopZ. Thus, higher order forms of CopAab have a higher affinity for Cu(I) than those of CopZ. From the thermodynamic data presented here it is clear that the extent of Cu(I) transfer from CopZ to CopAab is regulated by the Cu(I)-loading level of CopZ, and, more precisely, that the transition from the monomeric to the dimeric (luminescent) form of CopAab is controlled by the level of Cu(I) bound to CopZ.

To investigate the efficiency of Cu(I)-transfer from CopZ to CopAab, control experiments were conducted in which luminescence increases at 600 nm were monitored following the addition of either Cu(I) directly (as an inorganic salt), or in combination with a variant of CopZ lacking the Cu(I)-binding motif cysteine residues (C13S/C16S CopZ), see *Supplementary Figure S1*. The data showed that Cu(I) binding to CopAab 'directly' from solution, or as a complex with C13S/C16S CopZ, occurred with a different kinetic profile over a longer time period. This indicated that transfer between CopZ and CopAab occurs rapidly (relative to direct additions of Cu(I)) and that transfer between CopZ and CopAab does not occur via dissociation of Cu(I) from $\text{Cu}_4(\text{CopZ})_2$ into solution followed by binding to CopAab. Furthermore, it is clear that the binding motif cysteines are important for the rate of transfer (but, importantly, not the final form of Cu(I)-bound CopAab). This indicates strongly that transfer occurs via a Cu(I)-mediated protein-protein interaction. The rate determining step in the transfer could be, therefore, the formation/dissociation of a transient Cu(I)-mediated complex between CopZ and CopAab. The nature of such a complex would be expected to be different for the two directions of transfer because CopAab undergoes significant rearrangement upon Cu(I)-mediated dimerisation [12] and so is distinct from the apo-bound form.

A CopZ trimer with a trinuclear Cu(I) cluster, $\text{Cu}_3(\text{CopZ})_3$, is observed in crystal

Our attempts to crystallize a mononuclear Cu(I)-bound form of the CopZ dimer ($\text{Cu}(\text{CopZ})_2$) under anaerobic conditions yielded crystals in space group $P6_3$ containing a copper ion and a CopZ monomer in the crystallographic asymmetric unit. The presence of a single copper ion was verified by direct methods using the anomalous differences measured from the same crystal and later through calculation of anomalous difference Fourier maps phased on the refined crystal structure (see *Supplementary Figure S2*). The arrangement of the protein and its cysteine ligands around the crystallographic 3-fold axis generates a molecular trimer in the crystal lattice in which each copper ion is trigonally coordinated by the sulfur atoms of cysteine residues Cys13 and Cys16 from the metal-binding M(S/T)CXHC sequence motif of the CopZ monomer and the sulfur of Cys16 from a neighbouring CopZ monomer in the crystallographic trimer, see *Figure 3*. This is the first high resolution structure of a trimeric form of any copper chaperone. Our assignment of the metal as a cuprous ion resulted from the trigonal coordination observed in the crystal structure together with the $1s \rightarrow 4p$ pre-edge feature observed at around 8985 eV in the X-ray fluorescence spectrum of the crystal (see *Supplementary Figure S3*). This feature is characteristic of complexes containing digonally and trigonally-coordinated Cu(I) ions [39] whilst Cu(II) ions exhibit a corresponding feature appearing 3-4 eV higher in energy. The trimeric $\text{Cu}_3(\text{CopZ})_3$ complex therefore has stoichiometry 1:1 rather than 1:2 as for the as-prepared solution phase protein. This result may be compared to our previous attempts to crystallize the dinuclear Cu(I) form of CopZ, $\text{Cu}_2(\text{CopZ})_2$, which produced crystals of a tetranuclear copper-bound dimer, $\text{Cu}_4(\text{CopZ})_2$ [11]. It is clear that the solution-phase forms of the protein which have been characterized [10] do not represent the full set of states which are stable in crystal.

The refined crystal structure contains all 69 amino acid residues from the primary sequence of CopZ (*Figure 3*) and has an R-factor of 19.1% ($R_{\text{free}} = 26.4\%$) for data $F > 4 \sigma(F)$ in the resolution range 10 – 1.9 Å (full data collection and refinement statistics can be found in *Table 1*). CopZ displays the ferredoxin-like fold observed in previous solution-phase structure determinations [40] and the structure of the monomer is very similar to that found in the high resolution crystal structure of a tetranuclear Cu(I)-bound CopZ dimer complex, $\text{Cu}_4(\text{CopZ})_2$ [11] (PDB entry 2QIF), with an RMSD based on α -carbon atoms of 0.62 Å.

The trigonal coordination of each copper ion in the cluster is afforded by the sulfur atoms of residues Cys13 and Cys16 from each CopZ monomer (distances 2.24 Å and 2.31 Å, respectively) and the sulfur atom of Cys16 of a neighbouring monomer (coordination distance 2.21 Å). These distances are consistent with those observed in similar copper sites in proteins in the PDB (~2.4 Å). The three sulfur atoms and copper ion are essentially coplanar and the most compressed of the S–Cu–S angles (107.1°) involves the sulfur atoms from Cys16 residues. Trigonal coordination of Cu(I) by cysteine sulfur in the yeast copper thionein crystal structure [41] involved a similar distortion in bond angles. The distance between copper sites in the cluster is 3.13 Å, considerably longer than the interatomic distance in copper metal (2.58 Å) and so the requirements for a true metal cluster may not be fully met. The copper ions in the cluster are fully occluded from solvent, but the sulfur atoms of residues Cys16 are solvent accessible on the near face of the trimer. Solvent access to the copper cluster via the remote face of the trimer is blocked by residues Ser12 arranged around the molecular 3-fold axis.

Two water molecules (W1 and W2) are buried in each CopZ monomer adjacent to the bound copper ion. Their low temperature factors indicate a restricted mobility. Tyr65 plays a central role in stabilizing these solvent sites, forming hydrogen bonds with each. W1 also forms further hydrogen bonds with the main chain amide nitrogen atoms of residues Ser12 and Cys13, whilst W2 forms hydrogen bonds with the main chain carbonyl oxygen of Cys16 and the sidechain of Gln63. These interactions appear critical to the conformation of the polypeptide spanning the copper binding sequence motif.

Intersubunit contacts

A limited number of hydrogen bonding interactions stabilise the subunit interfaces in the trimer, and a number of these involve residues of the metal-binding motif. Firstly, His15 forms a hydrogen bond between the N δ 1 atom of its imidazole group and the carbonyl oxygen in the main chain of Gln63. Secondly, the hydroxyl groups of each of the three copies of Ser12 in the trimer form hydrogen bonds with a single water molecule located on the crystallographic 3-fold axis, see *Figure 4*. This water molecule thus mediates a network of intersubunit interactions. Two further specific hydrogen bonding interactions occur between the sidechain of Lys18 and mainchain of Asp62 and between the sidechains of Gln14 and Asp66. Therefore, each subunit interface involves three hydrogen bonds with the donor group supplied by a residue in the range 14-18 (CopZ numbering). In each case, the corresponding hydrogen bond acceptor is found in a residue in the range 62-66. Interestingly, alignment of the sequences of a number of CopZ orthologs from a variety of microorganisms reveals conservative amino acid substitutions in these regions that may lead to

a conservation of at least a subset of the trimer interface interactions seen with *B. subtilis* CopZ (Figure 5). These include copper chaperones from other Gram-positive bacteria, such as the well-studied CopZ from *Enterococcus hirae* [35, 36, 42] but also from representative β -, δ - and ϵ -proteobacteria as well as the actinomycete *Streptomyces coelicolor*.

Comparison of trinuclear and tetranuclear Cu(I) cluster complexes

In notable contrast to the situation observed in the crystal structure of the CopZ trimer, there are no direct residue-residue interactions stabilising the dimer interface in the $\text{Cu}_4(\text{CopZ})_2$ complex, the dimer being stabilised only by means of the tetranuclear cluster (see Figure 4). In order to compare the two structures, CopZ monomers taken from the trinuclear, $\text{Cu}_3(\text{CopZ})_3$, and tetranuclear, $\text{Cu}_4(\text{CopZ})_2$, structures were structurally aligned. Apart from the differences in intersubunit contacts described above, the major differences between the two are confined to the region around the M(S/T)CXHC copper-binding motif (Figure 4). These differences arise from the specific interactions employed in the binding of metal ions in the two complexes. Firstly, $\text{Cu}_4(\text{CopZ})_2$ utilises residue His15 as a direct ligand to one of its outer, trigonal Cu(I) sites, whereas the trinuclear complex features only cysteinate sulfur as ligands and His15 instead is employed to form an intersubunit contact via a hydrogen bond involving the carbonyl oxygen in the main chain of Gln63 and the N δ 1 atom of its imidazole. Secondly, Ser12 acts as part of the second coordination shell to one of the inner, diagonal Cu(I) sites in the Cu_4 cluster, whereas in the $\text{Cu}_3(\text{CopZ})_3$ complex the trigonal coordination of the copper ions renders this interaction superfluous and the hydroxyl groups from each of the three Ser12 residues instead form hydrogen bonds with a single water molecule, as described above. Also observed are differences in the positioning of some other residues around the copper binding site. In the tetranuclear cluster, the tyrosyl hydroxyl group of Tyr65 forms a long hydrogen bond with the sulfur of Cys16. This interaction is lost and the residue becomes more buried within the CopZ monomer in the structure of the $\text{Cu}_3(\text{CopZ})_3$ complex. We note that Tyr65 in CopZ is replaced by a histidine residue in the atypical Atx1-like copper chaperone from *Synechocystis* PCC 6803 and by lysines in the eukaryotic chaperones from this family, such as Hah1 from *H. sapiens* and Atx1 from yeast.

Modelling of putative copper transfer complexes

The high resolution structure of a complex involving three CopZ monomers not only demonstrated an extraordinary flexibility in Cu(I) coordination and monomer-monomer interactions, it also raised the possibility that a transient trimeric heterocomplex between CopZ and CopA could occur as part of a novel Cu(I)-transfer mechanism. Molecular modelling was used to test this hypothesis. Two models were created for the hybrid complexes, with one of the CopZ monomers in the trimer replaced by a CopA N-terminal ferredoxin-like domain (CopAa and CopAb). Note that the short linking peptide between the two copper-binding domains in CopA would preclude the formation of a $\text{Cu}_3(\text{CopZ})(\text{CopAa})(\text{CopAb})$ complex formed from a single CopA molecule. No such restriction is apparent on the formation of such a complex involving identical or different CopA domains from separate CopA molecules. These models were prepared by selecting a subunit from the crystal structure of the $\text{Cu}_3(\text{CopZ})_3$ trimer and using homology modelling to create a model for the CopA N-terminal domains. This procedure was followed rather than simply employing the available NMR structure of apo-CopAab [12] because the structure of the apo-protein does not reveal changes that are presumed to be induced by the binding of Cu(I) in the region of the MXCXXC metal-binding sequence motif (we note that the structure of Cu(I)-bound b-domain is available [43], but that of Cu(I)-bound wild-type a-domain is not); furthermore, the high sequence identities between CopZ and the a- and b-domains of CopA (30.4 and 27.5%, respectively) suggest close structural similarity and that homology modelling is an appropriate alternative.

No steric clashes occur in either of the models and all of the coordinating interactions mediating the trinuclear copper cluster were present. As a control, energy minimisation was performed for a $\text{Cu}_3(\text{CopZ})_3$ molecular model, revealing that all of the intersubunit interactions that presumably contribute to the stabilisation of the subunit interfaces of the CopZ homotrimer were present after minimization. Discounting the water-mediated hydrogen bonds involving Ser12, a total of nine intersubunit hydrogen bonds were found in the CopZ homotrimer. In both the CopAa and b domains, the structural equivalents of Gln14 and His15 in CopZ are alanines and so cannot participate in similar hydrogen bonding interactions. As a consequence, the total number of intersubunit hydrogen bonds in the two heterotrimer models was six and five, respectively (Figure 6).

The specific interactions between molecular subunits at the interfaces in the trimeric complexes are limited. The total surface area lost on formation of the three modelled complexes from individual subunits ranged from 702 to 783 Å². Of this, around 60% was contributed by non-polar and neutral atoms. The

usual indicators of permanency for protein complexes (e.g. [44]) cannot be used for situations such as this where a major driver for stability is presumably the formation of specific Cu(I) ion to cysteine thiolate coordinate bonds. However, the relatively low value of the solvent accessible area lost on complex formation and the low number of inter-subunit hydrogen bonds are at least consistent with a tentative classification of the modelled complexes as transient.

Mechanistic implications

The role of the N-terminal soluble domains of Cu(I)-transporting P-type ATPases remain unclear. It is known that they are not required for Cu(I) transport itself [45] and it has been proposed that they may fulfil a regulatory function by influencing transport efficiency [46, 47]. In the Menkes and Wilson proteins, the soluble domains are important for the correct localisation of the protein [48, 49]. Since the loading of CopZ is presumably controlled by levels of cellular Cu(I), an interesting possibility is that the dimerisation of CopAab (and, therefore, of CopA itself), which may be linked to the activity of the transporter, is gated by CopZ and only occurs when cytoplasmic levels of Cu(I) reach a particular level. The current experiments were conducted in the absence of low molecular weight thiols, which are known to influence the form of CopZ and CopAab at higher Cu(I) loadings, but we note that CopAab dimerisation was still observed in the presence of excess cysteine (a major low molecular weight thiol compound in *B. subtilis*) [12]. Furthermore, the recently discovered low molecular mass thiol of Bacilli, which has been named bacillithiol [50], is present at relatively low concentrations compared to, for example, glutathione in *E. coli* or in eukaryotic cells.

A survey of genome sequences [51] underlined the importance of copper chaperones by revealing a large set of operons encoding CopZ-Cu-transporting ATPases in distantly-related microorganisms. The alignment of sequences of CopZ chaperones from β -, δ - and ϵ -bacteria, as well as the actinomycete *Streptomyces coelicolor* revealed that, in the majority of these proteins, the residues of *B. subtilis* CopZ that form hydrogen bonds to stabilize the trimer interfaces are conserved or conservatively varied in the other chaperones. There are obvious limitations on the extent to which such analyses can be used to make general conclusions, but the formation of trimeric complexes based on CopZ in a wide range of other bacteria cannot be precluded. However, as with the $\text{Cu}_4(\text{CopZ})_2$ structure, the physiological relevance of the trimeric structure is not clear, and there is no indication that this form of the protein is stable in solution. Nevertheless, these structures demonstrate a significant flexibility in the type of Cu(I)-mediated complexes that the protein can form. As well as interacting with CopA, CopZ may also interact directly with CsoR, the recently discovered transcriptional regulator of the *copZA* operon [52]. The flexibility demonstrated here and in our previous mechanistic and structural work [10, 11] is likely to be a key requirement for the formation of transient complexes for facilitating transfer of (multiple) Cu(I) ions.

The ability to form a trimeric complex provides a mechanism by which a multinuclear Cu(I)-bound form of dimeric CopZ can transfer Cu(I) to CopA without dissociating first into monomers, which would be kinetically unfavourable and would result in the release of Cu(I) into solution. This idea is supported by the observation that the regular trigonal coordination of copper ions in the crystal structure of $\text{Cu}_3(\text{CopZ})_3$ parallels that observed in the heterodimer structures reported for Atx1:Ccc2 [16] and Hah1:Mnk1 [PDB entry 2K1R] complexes. Such trigonal coordination makes the cluster suitably poised for ligand exchange reactions. Given the limitations of the *in silico* methodology employed, caution should be exercised, but the molecular models for heterotrimeric $\text{Cu}_3(\text{CopZ})_2(\text{CopA})$ complexes generated here appear to be entirely plausible, at least as transient complexes. We note that such a model could be further extended to include $(\text{CopZ})(\text{CopAa})(\text{CopAb})$ or $(\text{CopZ})(\text{CopAa})_2$, etc, complexes to provide a general mechanism for passing solvent-shielded copper or copper clusters between domains. Further experimentation is now necessary to provide conclusive support for this intriguing notion.

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FIGURE LEGENDS

Figure 1. Detection of Cu(I)-transfer via the luminescence of CopAab. (A) Luminescence spectra resulting from the addition of $\text{Cu}_3(\text{CopZ})_2$ to apo-CopAab at ratios of 1:1 and 3:1, as indicated. (B) Luminescence spectra resulting from additions of $\text{Cu}_4(\text{CopZ})_2$ to apo-CopAab, and $\text{Cu}_4(\text{CopAab})_2$ to apo-CopZ, as indicated. The spectrum of $\text{Cu}_4(\text{CopAab})_2$ (of equal concentration in CopAb) alone is shown for comparison. CopAab was 12.5 μM in each experiment and both proteins were in 100 mM Mops, 100 mM NaCl, pH 7.5.

Figure 2. Rates of Cu(I)-transfer probed through formation/loss of CopAab luminescence. (A) Plots of luminescence intensity at 600 nm as a function of time following the addition of CopZ containing 2 Cu(I) per protein at increasing ratios of CopZ:CopAab, as indicated. CopAab (8.5 μM), temperature 25 °C. (B) As in (A) except that apo-CopZ was added to an equal concentration of CopAab (12.5 μM) containing 2 Cu(I) per protein. Inset is the exactly equivalent reverse experiment in which CopZ containing 2 Cu(I) per protein was added to an equal concentration of apo-CopAab (12.5 μM); temperature 25 °C. (C) Equilibrium luminescence spectra following the additions of Cu(I)-CopZ to apo-CopAab in (A), as indicated. CopAab and CopZ were in 100 mM Mops 100 mM NaCl, pH 7.5. Solid lines represent fits to either single or double exponential functions, as described in the main text.

Fig. 3. The structure of $\text{Cu}_3(\text{CopZ})_3$. (A) As viewed along the three molecular 3-fold axes. Each monomer is displayed in cartoon format, with the side chains of the residues of the copper binding motif (Ser12-Cys16) shown as green sticks. The three CopZ subunits (A, B and C) are shown along with their molecular surfaces coloured green, cyan and purple. Copper ions are coloured orange. (B) A view of the Fourier map (grey) calculated for the CQHC tetrapeptide, the core of the copper-binding sequence motif of CopZ, and the Cu_3 cluster in the refined structure at 1.9 Å resolution drawn at a contour level of 1.1 σ . (C) Orthogonal views of the copper cluster. Coordination bonds are marked by dashed lines. Bond lengths are in Ångstrom. The protein subunit to which a residue belongs is given by the single letter postfix (either A, B or C) to the residue name.

Fig. 4. Interactions in polynuclear Cu(I):CopZ complexes. (A) Unique intersubunit interactions in $\text{Cu}_3(\text{CopZ})_3$. (B) The intrasubunit interactions involving residue Tyr65 in $\text{Cu}_3(\text{CopZ})_3$. (C) Interactions at the tetranuclear cluster of $\text{Cu}_4(\text{CopZ})_2$ [11]. The two subunits are coloured green and cyan. Metal-amino acid interactions are indicated by black dashed lines and residue-residue hydrogen bonds by red dashed lines.

Fig. 5. A sequence alignment of Atx1-family metallochaperones. The sequences shown are: CopZ from *Bacillus subtilis* (BsCopZ, O32221); CopZ from *Enterococcus hirae* (EhCopZ, Q47840); copper chaperone from *Ralstonia eutropha* (ReCopZ, Q0K5J5); copper chaperone from *Desulfohalobium retbaense* (DrCopZ, C1SUC1); copper ion binding protein from *Helicobacter pylori* G27 (HpCopZ); metal associated protein from *Streptomyces coelicolor* A3(2) (ScCopZ, B5ZAE1). Identical residues are indicated by a dark grey background, conservatively varied residues are boxed and shown in bold characters. Secondary structural elements in CopZ are indicated and labelled. Pairs of residues forming intersubunit contacts in the $\text{Cu}_3(\text{CopZ})_3$ trimer are indicated by matching pairs of symbols (►, ▼, ▲). In each case, the hydrogen bond donor involves the residue in the range 14-18 (CopZ numbering). The corresponding hydrogen bond acceptor is in the residue range 62-66. Note that residue Ser12 forms water-mediated contacts with the equivalent residue in the other two subunits (symbol ○).

Fig. 6. Putative copper exchange complexes. (A) Models for $\text{Cu}_3(\text{CopZ})_2(\text{CopAa})$ and (B) $\text{Cu}_3(\text{CopZ})_2(\text{CopAb})$. The a- and b-domains of CopA are coloured red and yellow, respectively. (C) Unique hydrogen bonds (shown by dashed lines) at the $(\text{CopZ})_2\text{CopAa}$ interfaces of the heterotrimer. (D) Unique subunit interface hydrogen bonds in the heterotrimer containing CopAb. The three hydrogen bonds between CopZ subunits are not shown in panels (C) and (D).

TABLES

Table 1. X-ray Data collection and refinement statistics

Data collection	
Wavelength (Å)	1.38 Å
Resolution (Å)	50 – 1.9 (2.0 – 1.9) ^a
Unique reflections	5023 (608)
R _{sym} ^b (%)	6.6 (238)
Completeness (%)	97.2 (84.2)
<I/σ(I)>	29.1 (8.6)
Multiplicity	14.1 (10.7)
R _{anom} ^c (%)	7.1 (26.3)
Anomalous completeness ^c (%)	96.7 (86.3)
Overall B-factor (Å ²)	19.7
Refinement	
Resolution (Å)	10 – 1.9
R _{cryst} ^d	19.08
R _{free} ^e	26.49
Twin fraction	0.04
Model	
Protein atoms	527
Copper ions	1
Water molecules	20
Ramachandran Analysis (%)	
Most favoured	87.1
Additional allowed	12.9
RMS deviations	
Bond length (Å)	0.011
Bond angle (°)	2.0
Average atomic B-factor (Å ²)	17.6

^a Figures in parentheses refer to data in the highest resolution bin.

^b $R_{\text{sym}} = \sum |I_i - \langle I \rangle| / \sum I_i$ where $\langle I \rangle$ is the average of symmetry equivalent reflections and the summation extends over all observations for all unique reflections.

^c $R_{\text{anom}} = \sum |\langle I+ \rangle - \langle I- \rangle| / \sum |\langle I+ \rangle + \langle I- \rangle|$. This statistic and anomalous completeness are reported to a maximum resolution of 2.0 Å.

^d $R_{\text{cryst}} = \sum ||F_o| - |F_c|| / \sum |F_o|$ where F_o and F_c are the measured and calculated structure factors

^e For R_{free} the summations extends over a randomly-selected subset (5%) of reflections excluded from all stages of refinement.

FIGURES

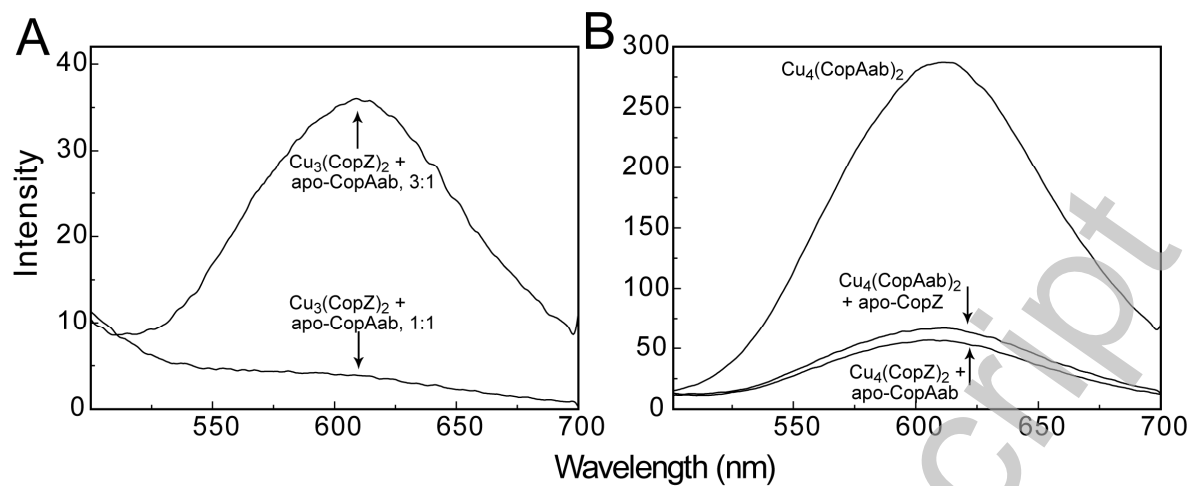


Fig. 1

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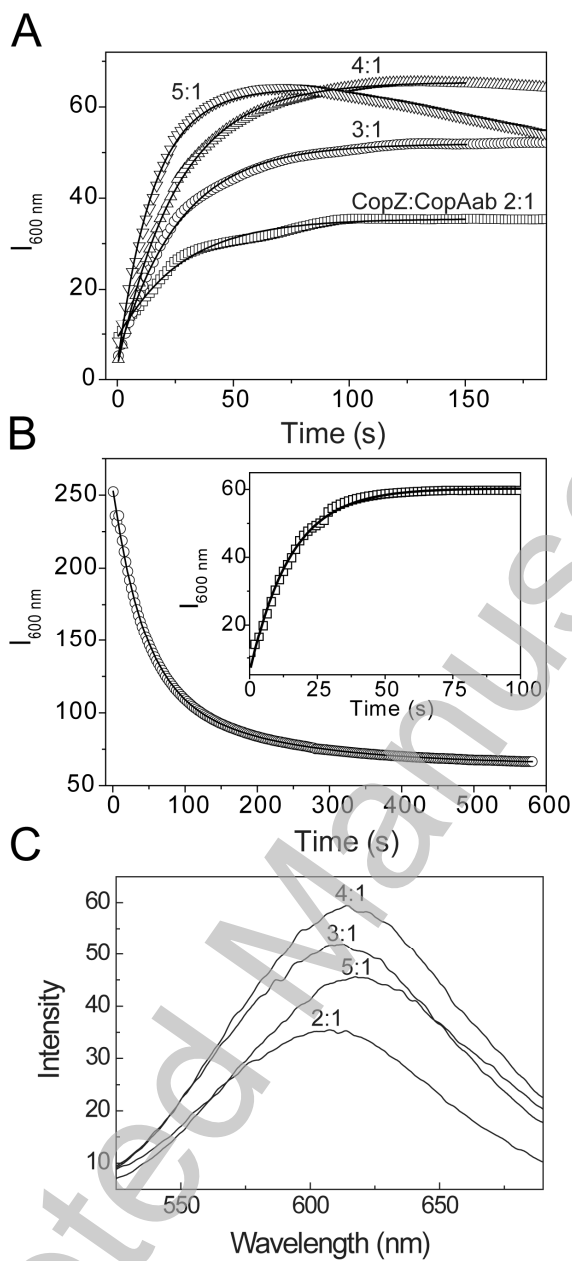


Fig. 2

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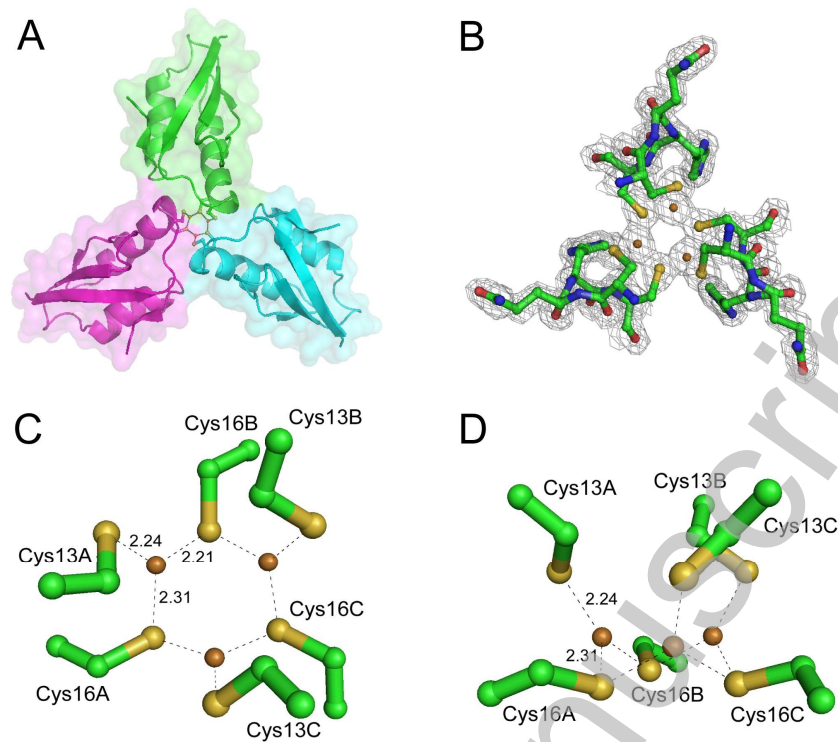


Fig. 3

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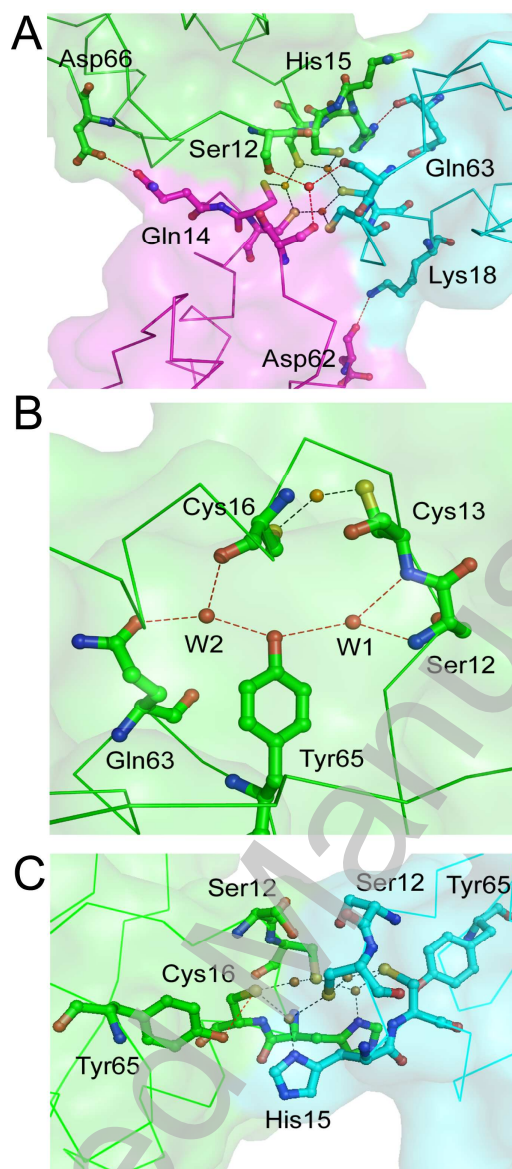


Fig. 4

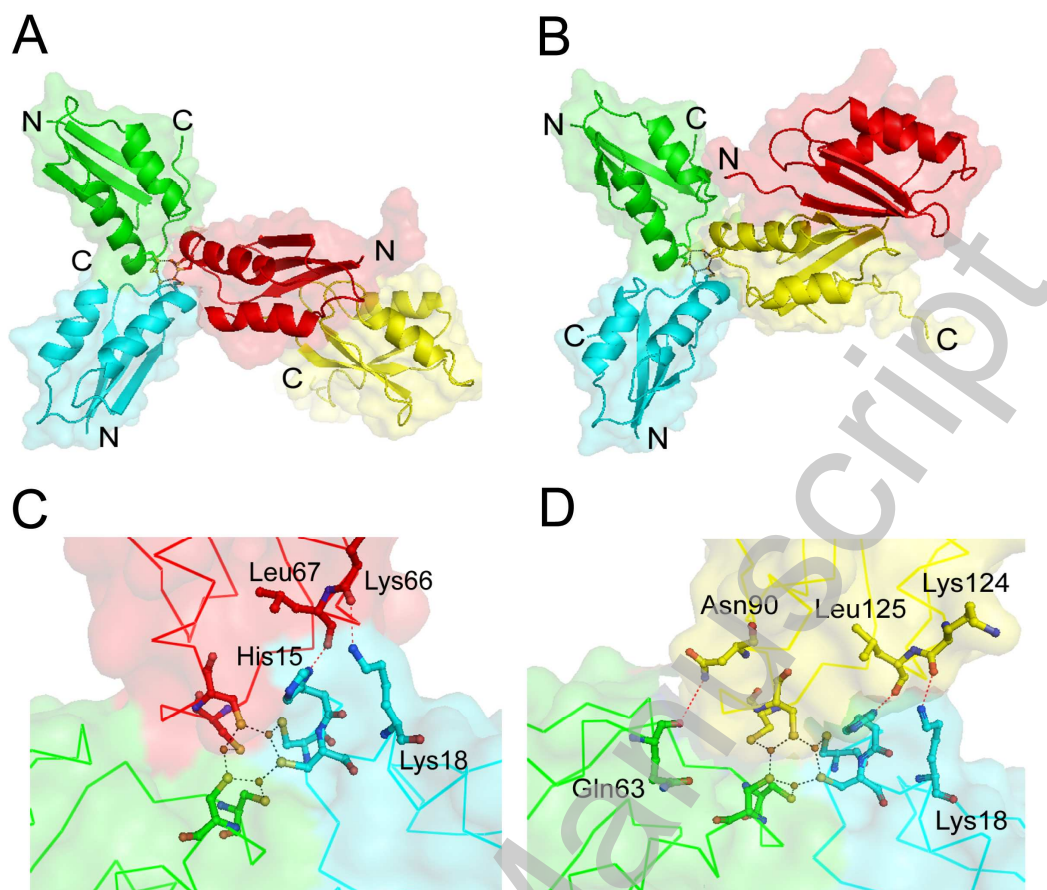


Fig. 6

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20091079

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