A C-terminal domain of the membrane copper pump Ctr1 exchanges copper(I) with the copper chaperone Atx1

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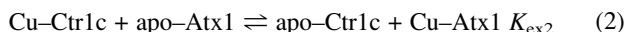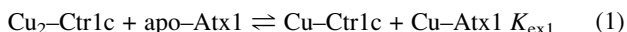E-mail: agw@unimelb.edu.au*

A cloned C-terminal domain of the yeast high-affinity copper uptake pump Ctr1 exchanges Cu(I) rapidly with the yeast copper chaperone Atx1: $10^{-2} < K_{ex} < 10^{+2}$.

Proposed pathways for distribution of copper in the yeast *S. cerevisiae* are illustrated in Fig. 1.[1,2] Ctr1[3,4] is one of two known high affinity transporters which appear to donate Cu^I to delivery proteins (chaperones) for shipment to specific organelles and copper proteins. Ctr1 homologues are proposed as the primary avenue for copper uptake in mammalian cells.[5] The yeast Ctr1 protein (406 amino acids) features three separate structural domains which are proposed to mediate different aspects of copper transport.[4,6] In particular, the cytoplasmic C-terminal sequence 280–406 (Ctr1c) is hydrophilic and includes two Cys-X-Cys motifs. These possible copper-binding sites may mediate transfer of copper from Ctr1 to the chaperones (Fig. 1).

The present Communication reports cloning, expression and isolation of Ctr1c.[7] The protein expressed with no detectable bound metal (apo–Ctr1c) but incubation with Cu^I reagents allowed binding of two equivalents of copper (Cu_2–Ctr1c) which were EPR-silent.[8] Significantly, the chaperone Cu–Atx1 (which can bind one Cu^I ion as a $Cu^I(S$-Cys$)_2$ centre; Fig. 1)[9] acted as a source of copper: incubation of apo–Ctr1c (36 μM) with 8 equivalents of Cu^I–Atx1 (292 μM) transferred 1.7 equivalents of copper to Ctr1c. This exchange may be described by the following equilibria:-

$$Cu_2\text{–Ctr1c} + \text{apo–Atx1} \rightleftharpoons Cu\text{–Ctr1c} + Cu\text{–Atx1} \quad K_{ex1} \quad (1)$$

$$Cu\text{–Ctr1c} + \text{apo–Atx1} \rightleftharpoons \text{apo–Ctr1c} + Cu\text{–Atx1} \quad K_{ex2} \quad (2)$$

$$Cu_2\text{–Ctr1c} + 2\,\text{apo–Atx1} \rightleftharpoons \text{apo–Ctr1c} + 2\,Cu\text{–Atx1} \quad K_{ex} \quad (3)$$

where

$$K_{ex} = K_{ex1}K_{ex2} = X/Y^2 \quad (4)$$

with

$$X = \{\{[\text{Ctr1c}]_{tot}/[Cu_2\text{–Ctr1c}]\} - 1\} \quad (5)$$

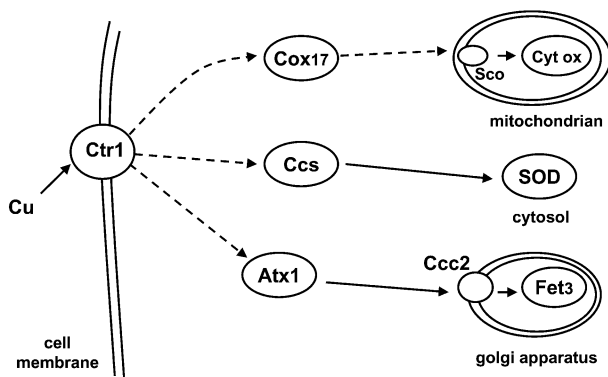**Fig. 1** Model of copper transport pathways in yeast.

and

$$Y = \{\{[\text{Atx1}]_{tot}/[\text{Cu–Atx1}]\} - 1\} \quad (6)$$

A series of 28 individual exchange reactions are reported in Figs. 2 and 3.[10] The reactions involved interactions of Cu_2–Ctr1c (65 μM) with apo–Atx1 (0–360 μM) (△ in Fig. 3), of apo–Ctr1c (0–157 μM) with Cu–Atx1 (118–292 μM) (○) and also those carried out in the presence of the thiol reductant glutathione GSH (5 mM) (×). Preliminary analysis involved a Hill plot[11] of log X *versus* log Y (eqns. (4)–(6)). The experimental slope is 0.8 (Fig. 3) suggesting that transfer of the first copper ion from Cu_2–Ctr1c leads to a reduced ability to transfer the second copper ion ('negative cooperativity', *i.e.*, $K_{ex1} > K_{ex2}$). The log X intercept is log K_{ex} and Fig. 3 estimates log $K_{ex} = -0.20$ ($K_{ex} \sim 0.6$). The experimental uncertainties are large[12] and the analysis is approximate. However, K_{ex} will certainly fall in the range $10^{-2} < K_{ex} < 10^{+2}$, *i.e.*, Cu^I equilibrates between Ctr1c and Atx1.

This result contrasts with reported quantitative transfer of Cu^I from chaperone CopZ to the target repressor protein CopY in
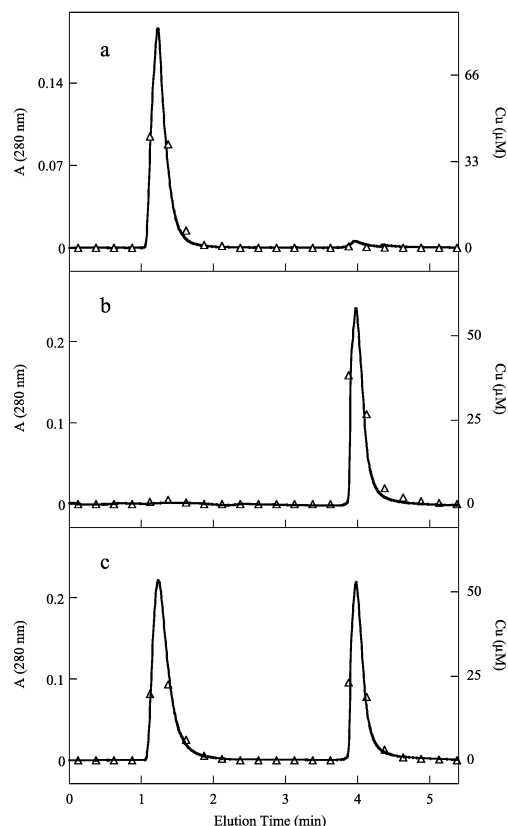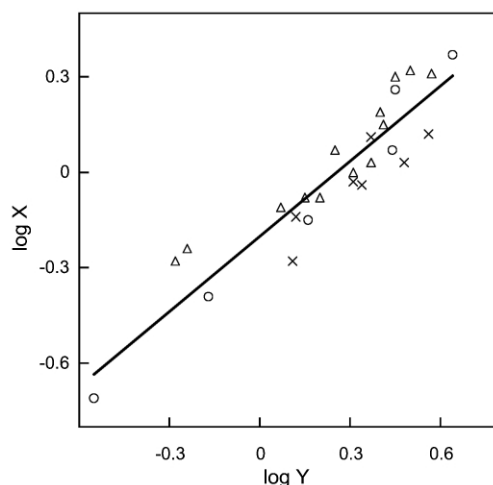

Fig. 2 Copper exchange assay between Ctr1c and Atx1. Anion exchange column elution profiles of (a) CuAtx1; (b) Cu_2Ctr1c; (c) Atx1 and Cu_2Ctr1c in molar ration 2.8:1. The copper concentration (△) in each eluted fraction was also determined by GC–AAS.

Fig. 3 Hill plot. Cu(I) donor: Ctr1c ($\triangle$) or Atx1 ($\bigcirc$). With 5 mM GSH included ($\times$).

the bacterial *Enterococcus hirae* system[13] but can be compared with $K_{ex} \sim 1.4$ for exchange of Cu^I between chaperone Atx1 and a domain of target Ccc2 of the Golgi apparatus (Fig. 1; Ccc2 is the yeast homologue of the human P-type ATPases Menkes and Wilson).[14] In addition, equilibrium is attained rapidly in the Ctr1c–Atx1 system (within 5 min of mixing) and the interaction between Ctr1c and Atx1 may be specific: the presence of physiological concentrations of the peptide thiol GSH (5 mM) does not affect the final equilibrium position (points $\times$ in Fig. 3) within experimental error, despite a reported stability constant of $\beta_2 = 10^{39}$ for binding of Cu^I by GSH.[15]

Combination of the present work with that of Huffman and O'Halloran[14] confirms that the chaperone Atx1 equilibrates Cu^I rapidly and specifically *in vitro* with domains of *both* proposed physiological partners Ctr1 and Ccc2 (Fig. 1). It strengthens the proposal that the active gradient of Cu^I in the Golgi organelle is controlled by the ATPase Ccc2.[2,14] Such a mechanism allows the organelle itself to control its copper status.

Structural studies of the Ctr1c domain are in progress, as are experiments to test its interaction with the other chaperones Ccs and Cox17 (Fig. 1). These are proposed to bind Cu^I as binuclear or polynuclear cysteine thiolate-bridged centres.[16,17] It remains to be seen whether similar centres are present in Ctr1c which binds two Cu^I ions and features two Cys-X-Cys motifs.

Notes and references

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7 The gene encoding the 126-residue C-terminal fragment (280–406 plus a first methionine residue incorporated as the initiating amino acid) of Ctr1, Ctr1c, was cloned into pET11a (Novagen). The resultant plasmid, pET11a-CTR1c, was transformed into *E. coli* BL21(DE3) CodonPlus and protein expression induced with isopropyl-β-*o*-thiogalactopyranoside. A lysis extract in Tris-HCl (20 mM; pH 8; 10 mM β-mercaptoethanol) was eluted from an anion-exchange column with a NaCl gradient. Ctr1c-containing fractions were combined and proteins precipitated with $(NH_4)_2SO_4$ at 40% saturation. The precipitate was dissolved and the soluble component applied to a Superdex-75 FPLC column. About 10 mg of pure protein were obtained per litre of culture. Protein identity was confirmed by N-terminal sequencing (M N R C K I A M L) and by electrospray ionisation mass spectrometry. The observed mass of 14017 Da is consistent with that of the calculated value (14018.5 Da). An additional peak at 14033 is consistent with oxidation of a methionine residue by dioxygen. The protein eluted from an analytical gel-filtration column at the expected volume but migrated anomalously at *ca.* 27 kDa on reducing SDS-PAGE gels.

8 In an anaerobic glove box, concentrated Ctr1c stocks were treated with excess dithiothreitol (DTT) and then transferred into a metal insertion buffer (Tris/Mes; 20 mM; pH 8). The protein was loaded with either $[Cu^I(MeCN)_4]BF_4$ in MeCN or $Cu^{II}(NO_3)_2$ in the presence of reductant NH_2OH or GSH. Unbound metal and reagents were removed by gel filtration. Concentrations of protein stocks were determined by absorbance at 280 nm. Quantitative drying of apo–Ctr1c samples in the volatile buffer NH_4HCO_3 provided $\varepsilon = 7660$ M^{-1} cm^{-1} and similar treatment of apo–Atx1 provided $\varepsilon = 4880$ M^{-1} cm^{-1} at 280 nm, within experimental error of that reported previously.[14] Copper concentrations were determined by graphite cuvette atomic absorption spectroscopy.

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10 The protocols follow those of refs. 13 and 14. Copper exchange experiments were conducted anaerobically in Tris/Mes buffer (20 mM; pH 8) by incubating a fixed concentration of Cu_2Ctr1c or CuAtx1 with apo–Atx1 or apo–Ctr1c of varying concentration. The total reaction volume remained constant. After incubation for *ca.* 30 min, the mixture was separated on a 1 mL UNO-Q column (Bio-Rad) in Tris-HCl buffer (20 mM; pH 8). Atx1 proteins eluted in the column flow-through whereas Ctr1c proteins eluted readily with 0.3 M NaCl (see Fig. 2). The eluted fractions were analysed for copper content by GC–AAS and for protein content by Bradford assay for estimation of the equilibrium concentrations of eqns. (5) and (6). The routine recovery rates for both Cu and Atx1 were 100 (±10) %. Ctr1c recovery was more difficult to estimate but was always > 85%. The Bradford assay is less reliable as, for unknown reasons, the Beer's Law range for Ctr1c is very narrow and the developed colour is not stable.

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12 The Cu and total protein concentrations were each determined with < 10% uncertainty. However, this leads to an uncertainty of at least 40% in (X/Y) (eqns. (5) and (6)). In addition, the mixture may deviate from its original equilibrium position during chromatographic separation, considering that Cu^I exchanges rapidly between the two proteins. These latter comments also apply to the work reported previously.[13,14]

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