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Unfolding pathway of CotA-laccase and the role of copper on the prevention of refolding through aggregation of the unfolded state

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ABSTRACT

Copper is a redox-active metal and the main player in electron transfer reactions occurring in multicopper oxidases. The role of copper in the unfolding pathway and refolding of the multicopper oxidase CotA laccase *in vitro* was solved using double-jump stopped-flow experiments. Unfolding of apo- and holo-CotA was described as a three-state process with accumulation of an intermediate in between the native and unfolded state. Copper stabilizes the native holo-CotA but also the intermediate state showing that copper is still bound to this state. Also, copper binds to unfolded holo-CotA in a non-native coordination promoting CotA aggregation and preventing refolding to the native structure. These results gather information on unfolding/folding pathways of multicopper oxidases and show that copper incorporation *in vivo* should be a tight controlled process as copper binding to the unfolded state under native conditions promotes protein aggregation.

1. Introduction

Laccases are the simplest members of the multicopper oxidases (MCOs) family of enzymes that contain approximately 500 amino acid residues and are composed of three Greek key β-barrel cupredoxin domains (domains 1–3) that come together to form three spectroscopically distinct types of Cu sites, i.e. type 1 (T1), type 2 (T2), and type 3 (T3) [1]. T1 Cu is characterized by an intense $S(\pi) \rightarrow Cu(d_{x^2-y^2})$ charge transfer (CT) absorption band at around 600 nm, responsible for the intense blue color of these enzymes. This is the site of substrate oxidation and a wide range of compounds, such as polyphenols, diamines and even some inorganic metals are electron donor substrates. The trinuclear center comprises two T3 and one T2 copper ions and is the site where dioxygen is reduced to water. The two T3 Cu ions show a characteristic absorption band at 330 nm. Several studies have been performed on these enzymes, due to their high potential for biotechnological industry, considering their wide range of oxidizing substrates, the use of readily available oxygen as final electron acceptor and the lack of complex cofactors such as the heme group of peroxidases [2].

The role of copper on the stability, folding and dynamics of multicopper oxidases is of particular interest [3]. Copper is the second most common element to participate in electron-transfer chains. *In vivo*, several chaperones participate in copper homeostasis acting on its uptake, efflux as well as distribution to target protein/enzymes. Also for recombinant multi-copper oxidases overexpressed in *Escherichia coli*, full copper loaded/full activity enzymes are only obtained under specific growth conditions [4]. *In vitro*, chaperones are absent and the loading of copper into client proteins as well as its effect on unfolding/folding needs to be further addressed to detail more clearly the role of this metal in protein structure–function relationships. Biophysical insight is not only important to complement *in vivo* experiments but also to potentiate the applicability of these enzymes. Despite recent advances towards understanding the role of copper on protein folding and stability [3,5] many issues remain to be clarified especially in larger copper-dependent proteins.

Azurin, a small redox-active copper protein has been widely subjected to *in vitro* folding/binding studies. The folding pathway is two-state as eventually anticipated for a small size protein (128 residues) and the single metal ion remained bound to the unfolded state contributing to the reversibility of the unfolded transition [6]. Studies on more complex systems were mostly focused on multicopper oxidases CotA, human ceruloplasmin and yeast Fet3p. Ceruloplasmin displayed a complex unfolding pathway both upon thermal and chemical perturbation. One equilibrium intermediate characterized by loss of one or two of the six copper ions of ceruloplasmin was identified [7,8]. The unfolding pathway of Fet3p upon thermal induced unfolding is also very complex with sequential or coupled domain unfolding depending on copper occupancy [9]. We have been focused on the MCO CotA, namely on protein stability and role of copper ions. CotA-laccase is a termoactive protein (T_{opt} = 75 °C), thermostable with DSC melting temperatures

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around 75 °C and has a high chemical stability with an energy gap between the native and unfolded state of 10 kcal mol^{-1} and a mid-point of near 5 M of guanidine hydrochloride [10]. Copper loading is crucial to maximize activity and full loading with proper coordination geometry was only achieved when the recombinant *E. coli* used to express CotA is grown under microaerobic conditions [4]. Coordination of the T1 copper is a very delicate balance as mutational analysis of the axial ligand Met502 increases the redox potential without changing significantly the overall coordination geometry but compromises activity and results in the accumulation of a stable intermediate during chemical-induced unfolding [10]. Surprisingly, removal of a disulfide bridge from CotA did not affect neither the long-term nor the thermodynamic stability but only protein dynamics with consequences namely on copper release [11]. These studies on CotA highlight the subtle balance between structure and function mediated by copper ions in MCOs. In this study we have used double-jump stopped-flow kinetics to solve the unfolding pathway of apo- and holo CotA. Acid-induced CotA unfolding was used instead of the more classical chemical- and thermal-induced unfolding approaches. Protein unfolding at acidic pH values is driven by preferential H^+ binding to the acid unfolded state over the native state [12]. Acid-induced protein unfolding is a good alternative for unfolding studies compared to thermal and chemical induced unfolding and has been used to study other metal proteins such as superoxide dismutase [13]. Indeed, thermal unfolding usually leads to irreversible denaturation preventing the application of thermodynamic principles [14]. Furthermore, urea or guanidinium-induced unfolding is usually reversible but quantification of protein stability in the absence of denaturant relies on long extrapolation procedures which are prone to error [15]. In fact, it was found that chemically-induced unfolding of multicopper oxidases results in copper release at lower denaturant concentration than structural unfolding preventing quantification of the copper stabilizing effect [10].

2. Materials and methods

2.1. Overproduction and purification

Strain AH3517 (containing the wild-type *cotA* gene [16]) was grown in Luria–Bertani medium supplemented with ampicillin (100 mg/mL) at 30 °C. Growth was followed until OD_{600} = 0.6, at which time 0.1 mM isopropyl-β-D-thiogalactopyranoside and 0.25 mM $CuCl_2$ were added to the culture medium to obtain the holo protein before lowering the temperature to 25 °C. Incubation was continued for further 4 h when a change to microaerobic conditions was achieved [4]. Cells were harvested by centrifugation (8000xg, 10 min, 4 °C) after a further 20 h. The apo protein was obtained by using the same procedure but $CuCl_2$ was not added to the growth medium and cells were grown under aerobic conditions for the whole period of culture. Proteins were purified by using a two-step purification procedure as previously described [16,17].

2.2. Unfolding of CotA induced by acid

Equilibrium unfolding of apo- and holo CotA induced by acid was followed by fluorescence emission and by absorbance at 600 and 330 nm to probe copper ions. For the unfolding experiments the proteins in 20 mM Tris–HCl, pH 7.6, were mixed with 100 mM Britton–Robinson, pH 1.6, to a final pH of 1.8.

2.3. Stopped-flow kinetics

Kinetic experiments were carried out on an Applied Photophysics Pi-Star 180 instrument with fluorescence intensity detection. A double-jump configuration was used with a mixing ratio of 1:1 in the first and second jump. A final protein concentration of 2 μM was used in the optical cell. In the first jump, CotA in 20 mM Tris–HCl, pH 7.6, was mixed with 100 mM Britton–Robinson, pH 1.6, to induce protein unfolding at a final pH of 1.8. After fixed delay times, the second jump mixes the protein solution with 100 mM Britton–Robinson, pH 5 to induce refolding. Refolding was followed by fluorescence emission with an excitation wavelength of 296 nm, and emission detected above 320 nm using a glass filter. All kinetic traces were analyzed according to a multi-exponential fit using the Pro-Data Viewer software provided by Applied Photophysics.

2.4. Data analysis

Equilibrium unfolding induced by acid was fitted according to a two-state process using the following equation:

$$\lambda_{\max\,\mathrm{obs}} = (\lambda_{\max\,\mathrm{U}}\exp^{(-\Delta G/\mathrm{RT})} + \lambda_{\max\,\mathrm{N}})/(1+\exp^{(-\Delta G/\mathrm{RT})}) \quad (1)$$

where λ_{max} is the wavelength at the maximum of fluorescence emission ($\lambda_{max\ U}$ and $\lambda_{max\ N}$ for the unfolded and native state, respectively) and ΔG is the free energy change between the N and U state calculated from the equilibrium constant. The parameter λ_{max} can be replaced by the molar extinction coefficient (ε) at 600 and 330 nm to probe copper ions in the holo protein. When ΔG is zero half of the protein molecules are unfolded and a pH value at the mid-point of unfolding can be calculated.

Exponential fits used to describe the increase in amplitudes of refolding in double-jump experiments were carried out using Origin software. The fraction of each state in the unfolding pathway of the apo- and holo-CotA protein was calculated based on the amplitudes of refolding phases after different unfolding (delay) times:

$$f_N = (\text{offset phase}_1 - \text{A phase}_1)/\text{offset phase}_1 \quad (2)$$

$$f_U = \text{A phase}_2/\text{offset phase}_2 \quad (3)$$

$$f_I = 1 - f_N - f_U \quad (4)$$

with offset and A being the plateau and the amplitude values for phase 1 and 2.

3. Results

The CotA laccase unfolds at acidic pH values as shown in Fig. 1. The holo protein is more stable than the apo-form showing the stabilizing effect of copper. For the apo-CotA, 50% of the molecules are unfolded at pH 3.2 while a pH of 2.4 has to be reached to unfold 50% of the holo protein. The stabilizing effect of copper can then be addressed by inducing unfolding at low pH overcoming a disadvantage of using chemical denaturants. Copper from T1 (probed by absorbance at 600 nm) and from T3 site (absorbance at 330 nm) are released or coordinated in a different geometry as monitored by the decrease of the absorbance band upon unfolding (Fig. 1). The similarity between the pH values at the mid-point measured by fluorescence (2.4 ± 0.1) and by absorbance (2.7 ± 0.1) indicates simultaneous structural unfolding and copper release form its native geometry according to a two-state unfolding process. However, often the complexity of unfolding/refolding pathways is not revealed by equilibrium studies and kinetic measurements are required to address the existence of intermediate states.

Single-jump unfolding of apo-CotA induced through pH decrease was previously characterized as a double-exponential process [11]. Double-exponential unfolding kinetics can not result from *cys-trans* proline isomerisation in the unfolded state as the fluorescence signal is not intrinsically sensitive to this equilibrium.

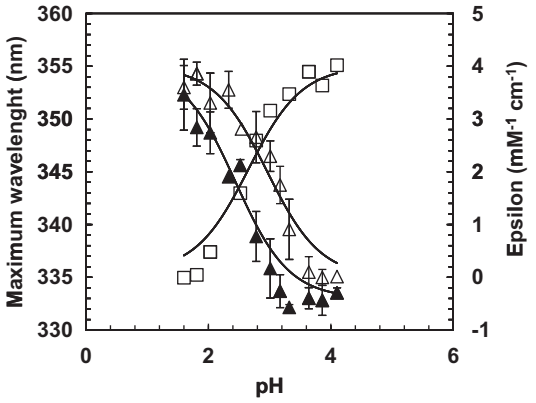

Fig. 1. Equilibrium unfolding of apo- (△) and holo-CotA (▲) followed through the fluorescence emission maximum and of holo-CotA followed by absorbance at 600 nm (□). Solid lines are the fits according to Eq. (1) shown in material and methods. The pH value at the mid-point of the unfolding transition measured by fluorescence is 3.2 ± 0.3 and 2.4 ± 0.1 for the apo- and holo-protein, respectively, and measured by absorbance at 600 (copper T1) and 330 nm (copper T3) is 2.7 ± 0.1.

Indeed, double-jump kinetics of the type measured here (unfolding jump, incubation for fixed delay times followed by a refolding jump) have been used to characterize proline isomerisation in the unfolded state [18] but they can also be used to characterize unfolding pathways especially of non two-state unfolding proteins often shared by proteins longer than 100 residues. Therefore, double-jump experiments were carried out to solve the unfolding pathway of apo-CotA. Unfolding for 20 ms leads to a single-refolding phase but unfolding for 200 ms results in a double-exponential refolding trace (see Supplementary material S1). The amplitudes of these two phases of refolding reflecting the accumulation of different unfolded states increase exponentially with the unfolding time with rate constants of 10.99 and 6.57 s^{-1} (Fig. 2A). Again, the observation of these two phases in the refolding trace can not be assigned to proline isomerisation in the unfolded state which is a much slower process (rate constant of 0.017–0.003 s^{-1} [19]). Therefore, unfolding of apo-CotA was described as a three-state process with the accumulation of an intermediate in between N and U (N ↔ I ↔ U) and the amplitudes of the refolding phases were used to calculate the amount of each species during unfolding (Fig. 2B).

Acid-induced unfolding of holo CotA displayed a more complex pattern. Even for an unfolding time of 10 ms refolding kinetics are double-exponential (see Supplementary material S2). Longer unfolding times lead to complex refolding kinetics that can not be accurately fitted by a three or high order-exponential equation (see Supplementary material S2). Also, it is clear that after the decrease in fluorescence intensity expected for the refolding of holo CotA (due to quenching by copper) there is a quite obvious increase in fluorescence intensity for a delay time of 10 s. The amplitude of the two phases assigned to refolding (characterized by a decrease in fluorescence intensity and therefore with a positive amplitude) increase exponentially with delay time as expected for first-order steps in a three-state unfolding process (Fig. 3A). These amplitudes were used to calculate the amount of each species in the unfolding pathway according to a three-state process as we did for apo-CotA (Fig. 3B). Summarizing, both apo and holo-CotA unfold according to a three-state process with the accumulation of an intermediate in between the native and the unfolded state (N ↔ I ↔ U). The main difference is the observation of a third phase in the case of holo-CotA characterized by an increase in fluorescence intensity and therefore not assignable to refolding. The other difference is that rate constants characterizing the increase in the amplitudes of refolding are 10-fold faster for the apo protein (rate constants of phase A_1 = 10.99/A_2 = 6.57 for apo-CotA versus rate constants

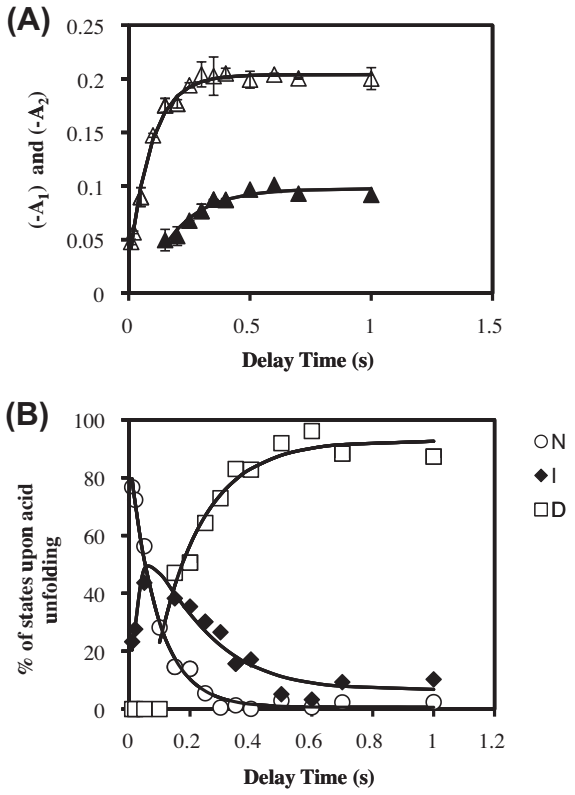

Fig. 2. Double-jump experiments for the Apo-CotA. (A) Absolute amplitudes of the phase A_1 (depicted by the symbol △ and phase A_2 (depicted by the symbol ▲) of refolding for different times of unfolding (delay time). Solid lines in (A) are the mono-exponential fits according to the equations $A_1 = -0.18136\ \exp^{(-10.991\ \text{delay}\ t)} + 0.20406$ and $A_2 = -0.14093\ \exp^{(-6.569\ \text{delay}\ t)} + 0.09754$. (B) Percentage of different states upon unfolding according to the pathway N ↔ I ↔ D. Solid lines in (B) were calculated according to Eqs. ()()()(2)–(4) shown in material and methods using the exponential equations shown above to calculate A_1 and A_2.

of phase A_1 = 1.31/A_2 = 0.68 for holo-CotA). This means that both transitions from N to I and from I to U are slower for the holo protein due to the stabilizing effect of copper as reported previously [11,20]. The third phase observed during refolding of holo-CotA is characterized by an increase in fluorescence intensity and the amplitude does not increase exponentially with the delay time (Fig. 3A). Therefore this phase can not be assigned to refolding of holo CotA. Indeed, its nature was identified using light scattering (Fig. 3C). Longer delay times of unfolding result in significantly light scattering upon refolding in the second jump. The accumulation of U during unfolding of the holo-protein leads to protein aggregation when the U state is placed under native conditions in the second-jump. The presence of copper ions in the unfolded holo-protein prevent its refolding back to the native state due to protein aggregation as summarized in the scheme below:

Apo -CotA N ↔ I ↔ U

Holo-CotA N ↔ I ↔ U →→ Agg

It is possible that further slow equilibria between different unfolded species of the holo-form participate in the aggregation mechanism as depicted in the scheme above. Light scattering still increase after unfolding times of more than 10 s (Fig. 3C) while 5 s seem to be enough to have all the molecules in the unfolded state (Fig. 3B). This point is supported by single-jump unfolding kinetics of the holo-form that was fitted with three-exponentials with one of the rate constants (0.002 s^{-1}) reflecting a very slow

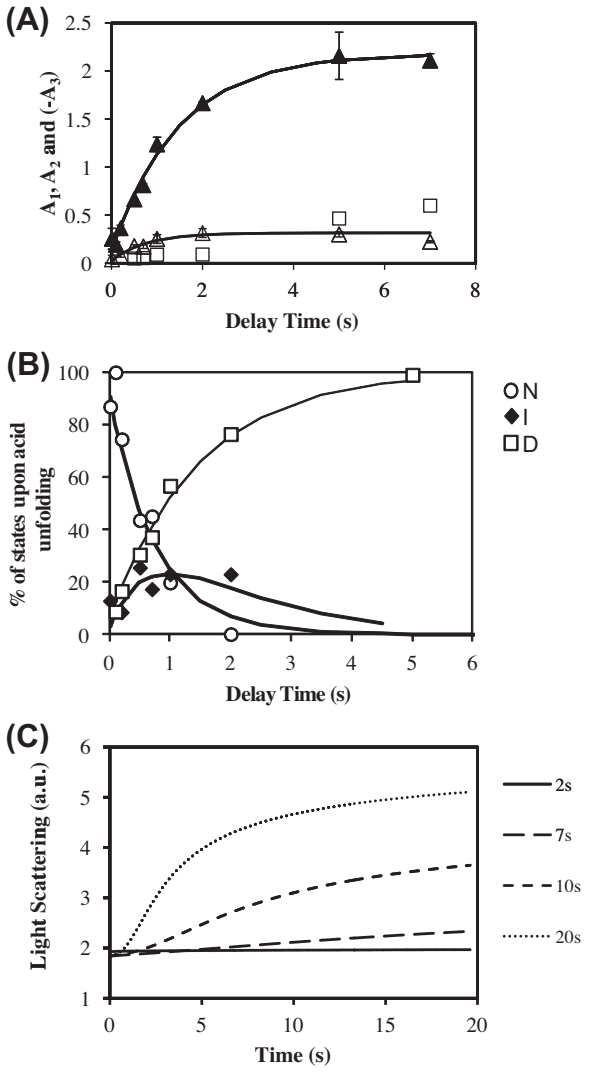

Fig. 3. Double-jump experiments for the Holo CotA. (A) Absolute amplitudes of the phase A_1 (depicted by the symbol △), phase A_2 (depicted by the symbol ▲) and phase A_3 (depicted by the symbol □) of refolding for different times of unfolding (delay time). Solid lines in (A) are the mono-exponential fits according to the equations $A_1 = -0.2891\ \exp^{(-1.3113\ \text{delay}\ t)} + 0.3165$ and $A_2 = -2.055\ \exp^{(-0.6754\ \text{delay}\ t)} + 2.1825$. (B) Percentage of different states upon unfolding according to the pathway N ↔ I ↔ D. Solid lines in (B) were calculated according to Eqs. ()()()(2)–(4) shown in material and methods using the exponential equations shown above to calculate A_1 and A_2. (C) Static light scattering upon refolding after different times of unfolding (delay times of 2, 7, 10 and 20 s).

process [11]. Anyway, binding of copper to unfolded holo-CotA promotes aggregation and prevents refolding under native conditions.

4. Discussion

Differential stability of apo and holo forms of CotA was addressed by acid-induced unfolding. Copper stabilizes CotA by inducing a shift in the mid-point of unfolding from a pH value of 3.2 to 2.4. Differential stability of apo- and holo-forms seems linked to conformational dynamic differences in the whole protein [21]. Double-jump stopped-flow kinetics was used to solve the acid-induced unfolding pathway of apo- and holo-CotA. Both forms unfold according to a three-state process with the accumulation of an intermediate in between the native and the unfolded state. The observation of an intermediate state in unfolding contrast with the two-state behavior of azurin, a much smaller single-copper oxidase with only 128 residues [6] compared to the 505 residues of CotA. Also, the small Cu chaperone Atox1 with 68 residues display a two-state reversible unfolding transition [5]. Copper was shown to stabilize both the native and the intermediate state of CotA. Most probably, copper is still bounded to the intermediate state causing a slower transition to the unfolded state in the case of the holo protein. An intermediate state with some copper bound was also observed in the unfolding pathway of other MCOs such as human ceruloplasmin and yeast Fet3p [3]. Copper also binds to the unfolded state promoting protein aggregation and preventing refolding of CotA under native conditions. This observation contrast to azurin where coordination of Cu(I) by the unfolded protein decreases the entropy of the unfolded state and limits conformational search for the native state [5,22]. However, coordination of Cu(II) by the unfolded azurin seems to lead to some irreversibility. As observed for holo-CotA, irreversibility of unfolding was reported in more complex systems such as MCOs human ceruloplasmin and yeast Fet3p [7–9].

A comparison between the unfolding pathway of CotA and other MCOs reveal interesting similarities. Unfolding of ceruloplasmin was also characterized by the presence of an intermediate that keeps four of six copper ions [7]. The intermediate accumulates for the apo- and holo-form albeit being less stable in the apo-form. Also, the intermediate can refold back to the native state exactly as observed for CotA. Once the unfolded state of ceruloplasmin is formed, the process becomes irreversible due to the formation of a molten globule state. This irreversibility was observed both for apo- and holo-forms, contrary to CotA where refolding of the apo- but not of the holo-form was attainable. Unfolding of ceruloplasmin was induced by urea and this may also contribute to the irreversibility of unfolding for the apo-form. For instance, unfolding of the McoA from *Aquifex aeolicus* induced by guanidinium was found irreversible due to protein aggregation [11] and chemically-induced unfolding of the blue copper protein rusticyanin leads also to aggregates [23]. Thermal unfolding of ceruloplasmin is also characterized by the accumulation of an intermediate less stable in the case of the apo-form and by irreversibility [8]. Thermal unfolding of Fet3p is also irreversible and reveals one intermediate characterized by unfolding of cupredoxin-like domain 2 [9]. Aggregation of the unfolded holo-CotA requires surely copper binding as it was not observed for the apo-form. Indeed, it has been demonstrated that many metallo-proteins retain strong metal–protein interactions after protein unfolding *in vitro*. For instance, the unfolded state of the blue copper protein rusticyanin retains copper in a non-native coordination [24] and the denaturated state of SOD retains both Cu and Zn ions although in a non-native like coordination [25]. Probably the same occurred with unfolded holo-CotA as the typical 600 and 330 nm absorbance bands disappear upon acid-induced unfolding. Protein aggregation modulated by metals has also been described in several neurodegenerative conditions. Copper was shown to induce aggregation of the amylogenic peptide β-microglobulin [26] and zinc to stabilize pathogenic aggregates of the amyloid β-protein involved in Alzheimer's disease [27].

One question that remains to be answered regarding many metal-binding proteins is when metals bind to their corresponding proteins [28]. Do they bind before, during, or after polypeptide folding? Our data shows that copper incorporation into CotA-laccase in the absence of copper chaperones should occur at a later step during protein folding as unfolded metal-bound states are prone to aggregation. Indeed, if depletion of copper from holo-CotA is carried out with EDTA, then it can be reconstituted *in vitro* with copper [4]. On contrary, expression and purification of apo-CotA followed by reconstitution *in vitro* does not allow 100% functional copper incorporation. These observations show that efficient

in vitro copper incorporation into the folded protein can be achieved but it also shows that copper incorporation is important to final subtle structural rearrangements that lead to full activity. Indeed, metal ion binding was shown to be important to fine-tune some native structures [28]. The aggregation of unfolded CotA in the presence of copper highlights the importance of sharing tight regulated pathways *in vivo* to avoid copper toxicity and to distribute copper to nascent copper-binding proteins [5].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.011.

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