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Discrete Roles of Copper Ions in Chemical Unfolding of Human Ceruloplasmin†

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ABSTRACT: Human ceruloplasmin (CP) is a multicopper oxidase essential for normal iron homeostasis. The protein has six β-barrel domains with one type 1 copper in each of domains 2, 4, and 6; the remaining copper ions form a catalytic trinuclear cluster, one type 2 and two type 3 coppers, at the interface between domains 1 and 6. We have characterized urea-induced unfolding of holo- and apo-forms of CP by far-UV circular dichroism, intrinsic fluorescence, 8-anilinonaphthalene-1-sulfonic acid binding, visible absorption, copper content, and oxidase activity probes (pH 7, 23 °C). We find that holo-CP unfolds in a complex reaction with at least one intermediate. The formation of the intermediate correlates with decreased secondary structure, exposure of aromatics, loss of two coppers, and reduced oxidase activity; this step is reversible, indicating that the trinuclear cluster remains intact. Further additions of urea trigger complete protein unfolding and loss of all coppers. Attempts to refold this species result in an inactive apoprotein with molten-globule characteristics. The apo-form of CP also unfolds in a multistep reaction, albeit the intermediate appears at a slightly lower urea concentration. Again, correct refolding is possible from the intermediate but not the unfolded state. Our study demonstrates that in vitro equilibrium unfolding of CP involves intermediates and that the copper ions are removed in stages. When the catalytic site is finally destroyed, refolding is not possible at neutral pH. This implies a mechanistic role for the trinuclear metal cluster as a nucleation point, aligning domains 1 and 6, during CP folding in vivo.

Ceruloplasmin (CP; EC 1.16.3.1)[1] is a circulating copper protein found in vertebrate plasma, which belongs to the family of multicopper oxidases together with ascorbate oxidase and laccases (*1*−*4*). In humans, CP accounts for ∼95% of the plasma copper and plays an important role in iron metabolism due to its ability to oxidize Fe^{2+} to Fe^{3+}, which allows for subsequent incorporation of Fe^{3+} into apotransferrin (*5*−*8*). CP is also believed to function as an antioxidant agent to scavenge free radicals, as an amine oxidase to control levels of biogenic amines, and as a copper transporter to deliver copper to extrahepatic tissues (*8*−*10*). CP is synthesized in hepatocytes and secreted into the plasma after incorporation of six copper ions in the secretory pathway (*5*). Failure to incorporate this metal during biosynthesis results in the secretion of an unstable polypeptide that is rapidly degraded in the plasma (*11*).

CP consists of a 1046 residue polypeptide and six integral copper ions classified into five different sites (*6*, *9*). The crystal structure of CP (*6*, *9*) demonstrates that the polypeptide is folded in six β-barrel domains arranged in three pairs forming a triangular array around a pseudo 3-fold axis (Figure 1). Three copper ions are found in type 1 (T1) mononuclear centers located in domains 2, 4, and 6. Copper in domains 4 (T1B) and 6 (T1A) is coordinated by two His, one Cys, and one Met, whereas the Met is absent in the metal-binding site in domain 2 (T1C) (*6*, *9*). The high reduction potential of the T1C copper indicates that it is permanently reduced and not catalytically relevant (*12*, *13*). The absorption at 610 nm that gives CP its intense blue color is a result of the oxidized coppers T1A and T1B. The remaining three coppers form a trinuclear cluster at the interface of domains 1 and 6. This cluster consists of a mononuclear type 2 (T2) and a binuclear type 3 (T3) copper site. It is envisaged that electrons are donated by substrates

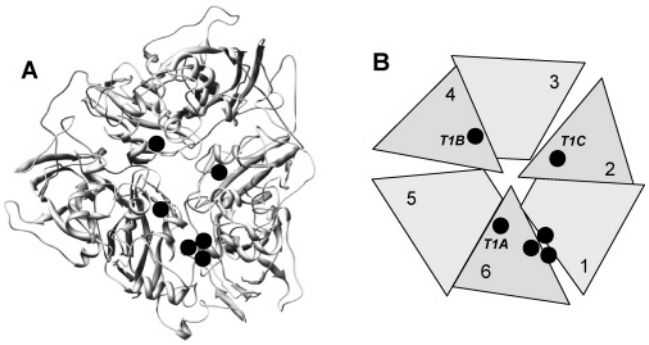

FIGURE 1: (A) Crystal structure of human CP (1kcw, pdb) with highlighted copper ions (circles) bound to the protein (UCSF Chimera). (B) Scheme of holo-CP showing the positions of the coppers (circles) in the six domains. The T1 coppers in domains 2, 4, and 6 are designated as T1C, T1B, and T1A, respectively.

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[1] Abbreviations: CP, ceruloplasmin; CD, circular dichroism, ANS, 8-anilinonaphthalene-1-sulfonic acid; CN, cyanide.

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to T1A or T1B via outer-sphere electron transfer and then transferred to dioxygen at the trinuclear cluster (*6*, *9*, *13*−*15*). The trinuclear cluster is surrounded by four pairs of His residues equally contributed by domains 1 and 6.

Previous studies have indicated that the conformation of apo-CP has molten-globulelike properties (*16*, *17*). For more than 40 years, it has been assumed that apo-CP binds copper in an all-or-none fashion (*18*). In support, recent metabolic-labeling experiments indicated that achieving the final state of CP required occupation of all six copper-binding sites without apparent hierarchy for incorporation at any given site (*5*). On the other hand, there have also been reports that invoke the possibility of partially metallated forms of CP (*19*, *20*). On the basis of cyanide (CN)-dependent metal-removal attempts, it was suggested that the T1 coppers are more sensitive than the T2 and T3 coppers to elimination (*20*, *21*). To probe the role of the coppers in the folding mechanism of human CP, we here compare the chemically induced unfolding reactions of holo- and apo-forms using a set of biophysical tools (pH 7, 23 °C). Our study demonstrates that in vitro unfolding of both CP forms involves populated intermediates; for the holo-form, the partially folded intermediate has lost two coppers and some blue color, but the trinuclear copper cluster remains intact. It appears that this species, and thus early incorporation of the T2 and T3 copper ions, is a necessary requirement for successful in vivo biosynthesis of holo-CP.

MATERIALS AND METHODS

Chemicals and Instruments. Analytical-grade chemicals and *o*-dianisidine were obtained from Sigma-Aldrich. 2,2′-Biquinoline was obtained from Fluka. Human CP (>96% purity, $A_{610}/A_{280} = 0.046$) was obtained from Vital Products (Florida). The concentration of holo- and apo-CP was determined by ϵ_{280nm} of 200 mM^{-1} cm^{-1}. The urea concentration was determined from refractive index measurements. Absorbance, fluorescence, and far-UV circular dichroism (CD) measurements were performed on Varian Cary 50, Cary Eclipse, and Jasco J-810 spectrometers, respectively.

Copper Removal. To prepare apo-CP, 10 mg/mL of CP was dialyzed for 2 h at 23 °C against 30 mM ascorbate in 0.1 M Trizma, pH 7.2. Subsequently, dialysis continued for 20 h at 4 °C against 50 mM NaCN, 10 mM EDTA, and 10 mM ascorbate in 0.1 M Trizma, pH 7.2. The dialysis proceeded for another 24 h at 4 °C (with buffer exchange after 12 h) against 50 mM sodium phosphate buffer, pH 7.0. Alternatively, a more harsh method to prepare apo-CP involved denaturation of holo-CP in 9 M urea with subsequent dialysis against 50 mM phosphate buffer, pH 7.0, to remove the urea and dissociated coppers. The copper content to confirm 100% holo-form, and to reveal loss of copper at different urea concentrations during holo-CP unfolding, was determined according to the method developed by Felsenfeld (*22*).

Unfolding and Refolding Experiments. Samples for unfolding were prepared by suitable mixing of phosphate buffer and urea solution (9.3 M urea in 50 mM phosphate, pH 7.0) and protein (protein solution in phosphate buffer) with a final protein concentration of 2−3 μM. Samples were incubated for 1 h at 23 °C. CD was obtained from the averaged signal at 220 nm collected for 120 s at 23 °C. The same samples were used for determination of the dependence of protein fluorescence on urea concentration. Excitation was at 295 nm, and both intensity and position of emission maximum were determined from spectra obtained by averaging five consecutive scans. The same parameters characterizing 8-anilinonaphthalene-1-sulfonic acid (ANS) fluorescence were obtained from averaged emission spectra (five scans) of protein/urea samples including 200 μM ANS (excitation at 390 nm). To test for reversibility, the protein was incubated in 5.5 or 9.0 M urea, dependent on whether the first or second transition was the target, respectively. The perturbed protein was then mixed with buffer solutions containing the desired (lower) concentration of urea, and spectroscopic measurements were performed as described above. All experiments were performed at 23 °C. Each individual unfolding/refolding transition was analyzed as described in refs *23* and *24* using standard two-state equations that reveal free energies of unfolding in buffer (i.e., ΔG_U) and *m* values [i.e., a measure of cooperativity of the transition or relative exposure of the hydrophobic surface upon unfolding (*23*−*25*)].

CP Oxidase Activity Assay. The oxidase activity of CP was performed by using *o*-dianisidine (an aromatic diamine) as a substrate in accordance with the procedure by Schosinsky et al. (*26*).

Analysis of Far-UV CD Spectra. Far-UV CD spectra (190−260 nm) were recorded at 23 °C; all spectra were averages of 10 scans. Several algorithms available in DICHROWEB (*27*, *28*) were used to analyze the data. The goodness of the fits was based on the NRMSD parameter (*29*), and the best score was provided by CDSSTR (*30*).

RESULTS

Preparing Holo- and Apo-Forms of CP. Our holo-CP stocks were highly pure but sometimes (depending on storage time) contained a fraction of partially metallated protein. The oxidized T1 coppers can be identified from the 610 nm absorption. The T2 and T3 copper sites form the catalytic cluster and thus are probed indirectly via CP oxidase activity. The total number of copper ions in CP can be quantified using the 2,2′-biquinoline assay (*22*). Before each experiment, 100% holo-CP (i.e., the protein coordinating six coppers) was prepared via addition of additional copper ions in the presence of ascorbate followed by extensive dialysis to remove the excess copper (*5*). On the basis of 610 nm absorption and copper quantification, this treatment resulted in a homogeneous sample of 100% holo-CP. Different approaches to preparation of apo-CP have been described (*19*−*21*): CN dialysis under reducing conditions and high-urea treatment. Our experiments revealed that the CN dialysis method is "best" as it does not perturb the protein structure as much as urea treatment (see below). Thus, for our unfolding studies, we prepared apo-CP via CN dialysis. Metal analysis of the protein after this treatment confirmed that all copper ions had been removed.

In Figure 2, we show the far-UV CD (secondary structure) and fluorescence (tryptophan environment; CP has 19 Trp) spectra of apo- and holo-forms of CP. Secondary structure analysis of the CD data for holo-CP agree with the reported crystal structure; the signal is dominated by β-sheets, turns, and random coils, with minimal or no α-helices. Analysis of the spectra for apo-CP (prepared via CN dialysis) suggests

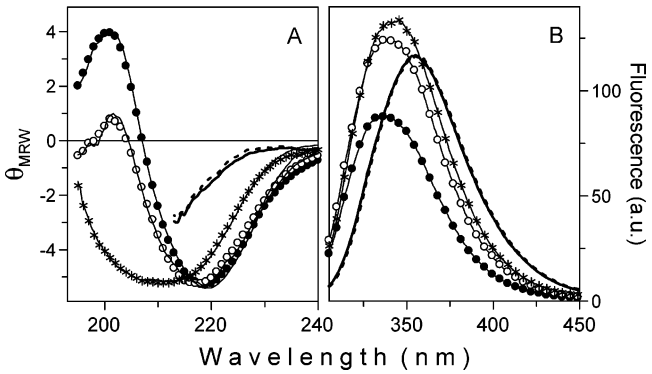

FIGURE 2: (A) Far-UV CD spectra of holo (●), apo(CN) (○), and apo(urea) (*) forms of CP. According to the CDSSTR algorithm, holo-CP contains 5% α-helix, 37% *β*-strands, 23% turns, and 33% unordered secondary structures. Corresponding contents in apo-(CN)-CP are 3% α-helix, 33% *β*-strands, 22% turns, and 41% unordered secondary structures. (B) Tryptophan emission (excitation at 295 nm) of holo (●), apo(CN) (○), and apo(urea) (*) forms of CP. For comparison, CD and fluorescence signals of fully unfolded apo- (dotted curves) and holo-CP (solid curves) (i.e., in 9 M urea) are also shown.

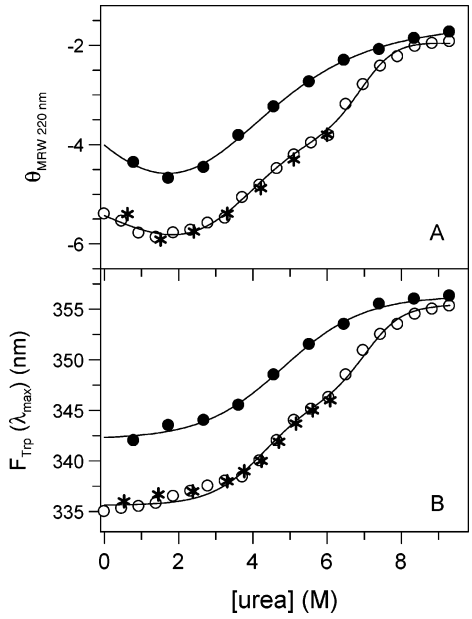

FIGURE 3: Isothermal denaturation of holo-CP followed by (A) CD at 220 nm and (B) tryptophan fluorescence (excitation at 295 nm). Unfolding of native-CP (○), refolding of CP from 5.5 M urea (*), and refolding of CP from 9.0 M urea (●). The continuous lines are fits to the experimental values (Table 1).

that the *β*-sheet content decreases with a subsequent increase in the random coil structure. Analogously, positions of maxima of tryptophan fluorescence of holo- (335 nm) and apo- (340 nm) forms indicate perturbation of the tryptophan environments in the apo-form of CP. Thus, apo-CP is structurally distinct from holo-CP: It appears to have lost some native structural elements. For comparison, CD and fluorescence spectra for fully unfolded forms are also shown in Figure 2.

Unfolding of Holo-CP. Unfolding of holo-CP was induced by urea additions at pH 7 and 23 °C. In Figure 3, we show the far-UV CD and fluorescence signals as a function of urea concentration. It is apparent that the process is not a simple two-state process but involves at least two steps. There appears to be a first transition with a midpoint of ∼4.2 M

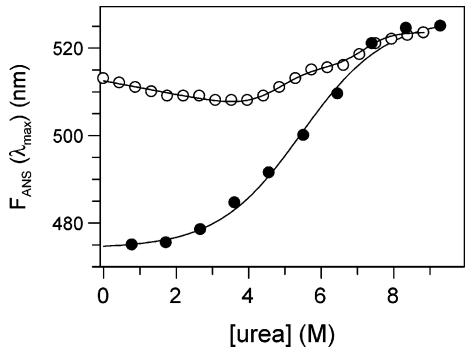

FIGURE 4: Isothermal denaturation of holo-CP followed by ANS fluorescence (excitation at 390 nm). Unfolding of native-CP (○) and refolding of CP from 9.0 M urea (●). The continuous lines are fits to experimental values (Table 1).

urea, resulting in the loss of about 30−40% of the far-UV CD intensity and a shift of the emission maximum toward higher wavelengths (i.e., from 335 to 345 nm) although the intensity does not change much. This first process is followed by a second transition that has a midpoint of ∼7.0 M urea. In this step, the fully unfolded form of the protein is reached according to the far-UV CD and tryptophan emission maximum (now at 355 nm) and intensity changes.

The unfolding reaction of holo-CP induced by urea was also probed via ANS emission (Figure 4). ANS is a fluorophore that emits strongly around 500 nm when bound to hydrophobic surfaces and pockets; it is often used as a tool to detect intermediate species of proteins as it does not significantly bind to either folded or unfolded forms (*31*, *32*). We find that the ANS emission shifts in a biphasic way that matches the two transitions observed for holo-CP with far-UV CD and tryptophan fluorescence. First, there is a small increase in the ANS emission together with a shift of the peak toward a higher maximum wavelength: This transition has a midpoint at ∼4−5 M urea. This change is followed by a further shift toward higher wavelengths of the ANS emission maximum in a second transition with a midpoint at ∼7 M urea. This result supports the presence of an intermediate species at intermediate urea concentrations (i.e., at ∼5.5 M urea).

The fates of the copper ions during urea treatment were tested by visible absorption at 610 nm (probing the oxidized T1 sites), copper analysis (probing total copper content), and oxidase activity (probing the catalytic cluster) as a function of urea (Figure 5). We find ∼50% decrease of 610 nm absorbance (which may correspond to destruction of one of the two oxidized T1 sites) in a first phase that parallels the first CD/fluorescence-detected transition. Copper quantification revealed that in total two copper ions have been removed from the protein at 5.5 M urea. The remaining blue color disappears at higher urea concentrations (6−7 M urea) together with loss of all copper ions (Figure 5). CP's oxidase activity disappears in a three-state transition that matches the disappearance of blue absorption (Figure 5): The first process results in a loss of about 60% activity. This indicates that although the intermediate form of CP at 5.5 M urea has lost some coppers, the catalytic copper cluster is still functional.

The binding site of aromatic diamine substrates such as *o*-dianisidine used here is located in domain 4 near T1B (*6*); thus, if this is the perturbed oxidized T1 site in the

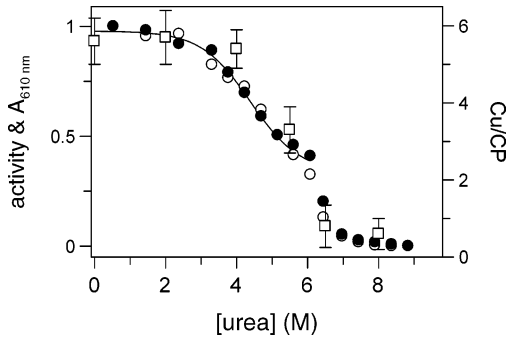

FIGURE 5: Isothermal denaturation of holo-CP followed by oxidase activity (○) monitored by reaction of CP with *o*-dianisidine and by visible absorbance at 610 nm (●). The total copper content in CP at different urea concentrations are also shown. The amount of copper (□) was quantified after dialysis of samples at each specified urea concentration, to remove any dissociated copper ions. The continuous lines are fits to experimental values (Table 1).

intermediate, one may expect less activity. However, also, removal of T1A may affect electron transfer from T1B to the catalytic cluster. On the basis of our data, we cannot distinguish which oxidized T1 site is perturbed. The T1C copper is spectrally invisible as it is permanently reduced (*13*). It is probable that this copper ion, missing one protein ligand as compared to the other T1 sites, will dissociate most readily of all copper cofactors in CP. Thus, it appears reasonable that this copper is one of the two metals removed in the intermediate. We note that the tryptophan fluorescence in folded holo-CP is quenched by the metals; in accord with several metals remaining in the intermediate, the emission stays quenched until ~7 M urea has been added (data not shown).

Next, refolding of holo-CP from the intermediate (i.e., 5.5 M urea) and fully unfolded (i.e., 9 M urea) states was tested. We find that nativelike far-UV CD and Trp fluorescence signals appear upon refolding from the intermediate species (Figure 3). Also, the blue color and oxidase activity return to native-state levels when refolded from the 5.5 M urea state. This suggests that albeit two coppers are gone in the intermediate, these can rebind upon protein refolding.

In contrast, refolding from the unfolded state (9 M urea) results in a protein species distinctly different from the native state. This form has an intermediate-sized far-UV CD signal (and different shape), only partial restoration of the native-like tryptophan emission, no blue color, no copper bound, no oxidase activity, and is found to interact strongly with ANS (Figures 3 and 4). For this species, the ANS emission is increased 3-fold as compared to that for the native-state and 2-fold as compared to that for the intermediate and unfolded states; moreover, the emission maximum is largely red-shifted to 480 nm. Taken together, these properties are indicative of a molten-globulelike species (*33*−*38*). Refolding into the molten-globule species occurs in a broad transition with a midpoint of 4.0−4.5 M urea. Repeated unfolding reveals the same urea-unfolding curve as the one obtained in the refolding experiments from 9 M urea, in accord with reversibility (data not shown). On the basis of the spectroscopic properties, the molten-globulelike CP form is not identical to the apo-form that is prepared via CN dialysis (see below).

The parameters obtained from fits to the different holo-CP unfolding curves are reported in Table 1. It is clear that

Table 1: Parameters Describing the Conformational Transitions of Holo- and Apo-CP as Monitored by Far-UV CD (CD_{220} nm), Tryptophan (F_{Trp}), and ANS (F_{ANS}) Fluorescence Maxima Placement, Blue Copper Absorbance (A_{610} nm), and Oxidase Activity[a]

detection	urea change (M)	ΔG_U (kJ/mol)	*m* (kJ/mol, M)	$[urea]_{1/2}$ (M)
		holo-form		
CD_{220nm}	0 → 5.5	15.3 ± 0.7	3.7 ± 0.2	4.1
CD_{220nm}	5.5 → 9	39.5 ± 3.0	5.7 ± 0.4	6.9
CD_{220nm}	5.5 → 0	−15.2 ± 1.1	3.6 ± 0.3	4.3
CD_{220nm}	9 → 0	−8.6 ± 0.9	1.9 ± 0.2	4.6
F_{Trp}	0 → 5.5	15.2 ± 1.3	3.5 ± 0.2	4.3
F_{Trp}	5.5 → 9	37.4 ± 2.0	5.3 ± 0.4	7.0
F_{Trp}	5.5 → 0	−17.1 ± 1.5	3.8 ± 0.3	4.5
F_{Trp}	9 → 0	−10.9 ± 1.0	2.3 ± 0.2	4.2
F_{ANS}	0 → 5.5			5.0
F_{ANS}	5.5 → 9			7.1
F_{ANS}	9 → 0	−11.7 ± 1.2	2.1 ± 0.2	5.4
activity	0 → 5.5	15.6 ± 1.9	3.6 ± 0.4	4.4
A_{610nm}	0 → 5.5	17.2 ± 1.2	4.1 ± 0.3	4.2
		apo-form		
CD_{220nm}	0 → 5.5	14.4 ± 1.1	3.1 ± 0.3	3.8
CD_{220nm}	5.5 → 9	36.5 ± 3.9	4.8 ± 0.5	7.6
CD_{220nm}	5.5 → 0	−13.2 ± 1.1	3.6 ± 0.3	3.7
F_{Trp}	0 → 5.5	14.6 ± 0.7	4.2 ± 0.2	3.5
F_{Trp}	5.5 → 9	28.8 ± 2.0	4.5 ± 0.3	6.4
F_{Trp}	5.5 → 0	−12.5 ± 3.7	3.7 ± 0.7	3.4
F_{ANS}	0 → 5.5	13.6 ± 1.5	2.9 ± 0.3	4.6

[a] The direction of the urea jump (i.e., unfolding or refolding) is indicated. ΔG_U, *m* values, and $[urea]_{1/2}$ were obtained from two-state fits to individual transitions. (Fit parameters for most of the ANS data were not reliable and are not shown.)

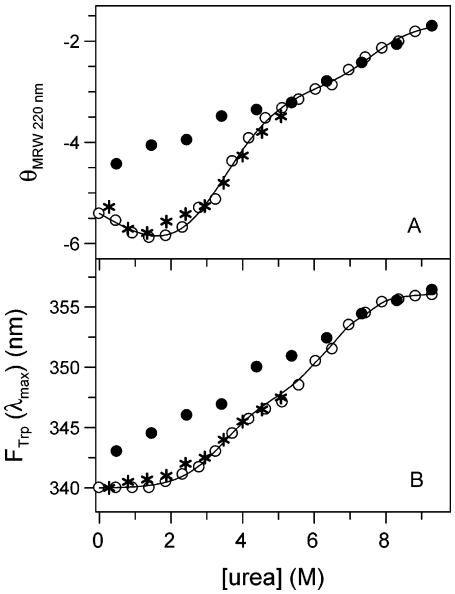

FIGURE 6: Isothermal denaturation of apo-CP followed by (A) CD at 220 nm and (B) tryptophan fluorescence (excitation at 295 nm). Unfolding of native-CP (○), refolding of CP from 5.5 M urea (*), and refolding of CP from 9.0 M urea (●). The continuous lines are fits to experimental values (Table 1).

the parameters for the first transition, from native to intermediate state, match among the five detection methods and correspond to a free-energy change of about 15 kJ/mol (pH 7, 23 °C). The *m* value, indicative of relative exposure of hydrophobic surfaces upon perturbation (*23*−*25*), is low for the protein of this size (120 kDa), supporting that only partial unfolding is involved in the transition. The second

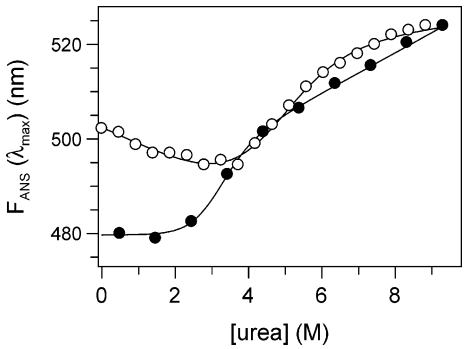

FIGURE 7: Isothermal denaturation of apo-CP followed by ANS fluorescence (excitation at 390 nm). Unfolding of native-CP (○) and refolding of CP from 9.0 M urea (●). The continuous lines are fits to experimental values (Table 1).

step, intermediate to unfolded, is irreversible, and one must use caution when analyzing the fit parameters. Nonetheless, it appears that this transition involves a higher free-energy change and more hydrophobic surface exposure than the first process. The refolding/unfolding of the molten-globule species, which is a reversible transition, corresponds to a free-energy change of about 10 kJ/mol (pH 7, 23 °C) and low cooperativity based on the low *m* value.

Unfolding Apo-CP. Urea-induced unfolding was also investigated with apo-CP prepared by CN dialysis. In Figure 6, far-UV CD and fluorescence curves as a function of urea concentration are shown. Again, unfolding appears in two phases, a first transition with a midpoint of about 3.5 M urea followed by a second transition with a midpoint of about 6−7 M urea. The intermediate species has only ~35% of the original negative far-UV CD signal; the emission maximum is shifted from 340 to 347 nm. ANS binding to apo-CP vs urea concentration was also tested; the results mimic the trends seen above for the holo-protein (Figure 7); again, a biphasic transition is observed when the ANS emission maximum is plotted vs urea concentration.

Whereas refolding of the intermediate species (from 5.5 M urea) results in an apo-protein that is identical to that of the starting form, refolding from 9 M urea again results in the molten-globulelike species that has distinct CD/fluorescence and ANS-binding properties (Figures 6 and 7). As in the case of the fully unfolded holo-protein, refolding of the apo-protein from 9 M urea occurs in a broad transition with a midpoint of ~4.3 M urea. Fits to the different apo-protein unfolding curves are listed in Table 1.

DISCUSSION

The majority of cuproenzymes, including blood-clotting factors, tyrosinase, lysyl oxidase, and CP, are synthesized within the secretory pathway (*39*); copper gains access to this compartment via copper-translocating ATPases present in the *trans*-Golgi membrane (*39*−*41*). Copper incorporation into polypeptides inside this compartment is not clear; it has been proposed that copper ions and proteins meet in vesicular environments before secretion (*5*, *8*). As a first step toward elucidating the interplay between copper uptake and polypeptide folding of cuproproteins made in the secretory pathway, we have characterized the in vitro chemical unfolding reactions of purified apo- and holo-forms of human CP.

Both forms of CP are found to unfold in multistep equilibrium reactions, involving at least one on-pathway intermediate and one misfolded off-pathway species. The

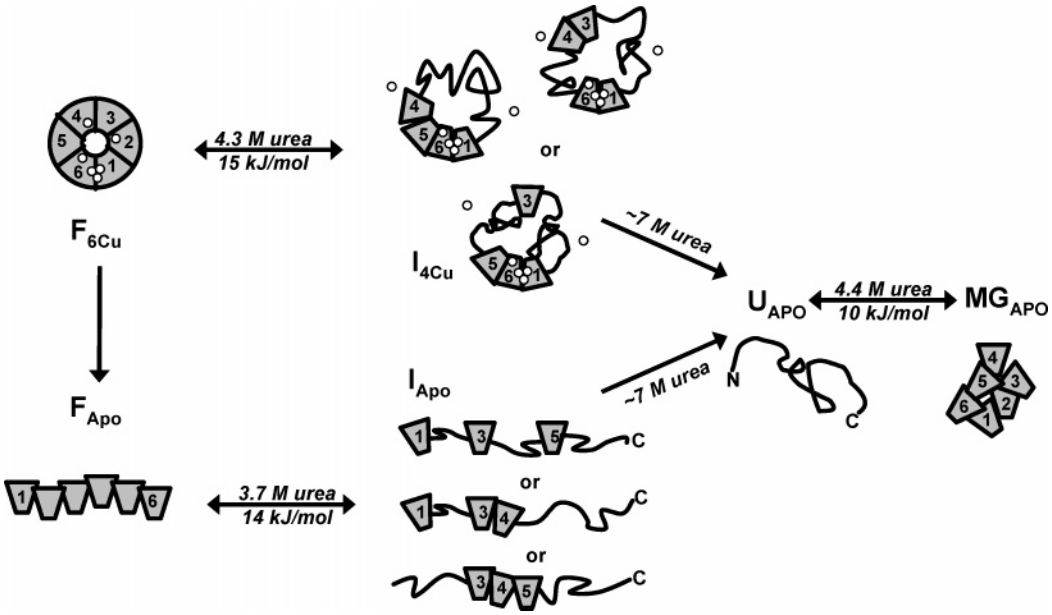

FIGURE 8: Unfolding/refolding pathways of holo- and apo-CP based on the present work. Folded holo-CP containing six bound coppers (circles), F_{6Cu}, undergoes a reversible transition up to ~5.5 M urea, with a midpoint at ~4.3 M, losing two copper ions and about half of the oxidase activity and blue absorption. This intermediate form of holo-CP is termed I_{4Cu}. This intermediate may have T1C and T1B coppers removed (see text) and two domains unfolded, perhaps involving those harboring the destroyed copper sites; the catalytic cluster and therefore domains 1 and 6 are likely intact in the intermediate. Three possible structures of I_{4Cu} are shown. A further increase in urea concentration leads to full CP unfolding accompanied by dissociation of all coppers, U_{Apo}. Analogously, F_{Apo} (prepared via CN dialysis) undergoes a reversible urea-induced transition with a midpoint at ~3.7 M (forming I_{Apo}; three possible structures are shown), followed by an irreversible transition to U_{Apo} completed at 9.3 M urea. The final unfolded state of apo-CP is similar to that obtained when starting from holo-CP. U_{Apo} undergoes a reversible off-pathway transition to a molten globulelike state, MG_{Apo}, with a midpoint at 4.4 M urea. Double-headed arrows indicate reversibility; single-headed arrows depict transitions that only go in one direction. Midpoints and stabilities are from Table 1.

proposed folding/unfolding landscape for holo- and apo-forms of CP (i.e., F_{6Cu} and F_{apo}) in urea (pH 7, 23 °C), and tentative structural aspects of the intermediates, are summarized in Figure 8. First, the activity data suggest that the holo-intermediate (i.e., I_{4Cu}) has an intact trinuclear copper cluster; thus, the interface between domains 1 and 6 where this site is located is likely intact; therefore, domains 1 and 6 remain folded. Because the holo-intermediate has lost about 30−40% of its secondary structure (which equals two out of six domains) and two coppers, one being an oxidized T1 copper (based on the blue color change), it is possible that domains 2 (containing silent T1C) and 4 (containing T1B) are unfolded in I_{4Cu}. Notably, the disulfide bonds in domains 2 and 4 link different β-strands as compared to the disulfide bonds in domains 1, 3, and 5 (*6*, *9*); this may account for differential domain stability. Earlier work (*19*) gives support that T1B, and not T1A, is the oxidized T1 copper removed in the unfolding intermediate. It was reported that upon loss of two coppers via CN dialysis, CP lost half of its blue color and most of its oxidase activity (substrate binding near T1B) disappeared whereas most of the dismutase activity (substrate binding near T1A) remained (*19*). That a few domains are unfolded and a few remain folded is in agreement with the ANS data, which do not indicate that the whole protein is partially folded [if so, we would observe a dramatic increase in ANS emission and a red shift of its maximum (*25*)]. Further additions of urea result in complete polypeptide unfolding and loss of all coppers (i.e., U_{apo}).

The apo-form of CP (i.e., F_{apo}) has been proposed to adopt a folded but extended form in which the interface between domains 1 and 6 is absent (*21*); the domains may be depicted as beads on a string with domain 1 spatially distant from domain 6. Conversion of apo-CP to the intermediate state (i.e., I_{apo}) again appears to involve selective unfolding of some of the domains based on the spectroscopic signals. Further perturbation of the apo-intermediate results in a fully unfolded state with all domains unfolded (i.e., U_{apo}). Refolding of U_{apo} (obtained from complete unfolding of both holo- and apo-forms of CP) occurs, reversibly, into an off-pathway copper-lacking molten globule (i.e., MG_{apo}). This form may have several domains folded, as it has a significant secondary structure content, but the arrangement of the domains in three-dimensional space, and interdomain interactions, may be incorrect, prohibiting copper incorporation and proper folding.

Several biological implications can be drawn from our findings. First, it is clear that, in contrast to small proteins where bound cofactors often have large effects on protein stability (*42*−*49*), the coppers in CP contribute minimally to the overall protein stability. The transition midpoints are found at roughly the same urea concentrations for both apo- and holo-forms (Table 1). Furthermore, because apo-CP may have a more extended conformation, it appears that the individual domains, and not the domain−domain interactions, govern most of the protein's stability. Second, our experiments suggest a mechanistic role for the trinuclear copper cluster in proper alignment of domains 1 and 6; only when this interface/metal site is in place is correct CP folding possible in our experiments. In agreement, several demetalation studies have demonstrated that if all six coppers are removed from CP, reconstitution is not possible at neutral and oxidizing conditions (*19*, *21*). Reconstitution of apo-CP has only been achieved in some special conditions, that is, in the presence of ascorbate at pH 5.6 and only partially via Cu(I)-glutathione in the presence of Mg-ATP at pH 7 (*5*, *17*, *50*). Third, our findings provide mechanistic insight into the recently proposed CP biosynthesis pathway (*5*). It was demonstrated via metabolic labeling and gel shift experiments that copper incorporation into CP is an all-or-none process, although the possibility of unstable, "hidden" intermediates was mentioned (*5*). We propose that the intermediate detected here with an intact trinuclear copper site is such a hidden intermediate not resolved by the methods used in ref *5*. This intermediate may form for several of the copper-site mutants studied in ref *5*, but their low stability causes them to return to the apo-form if the remaining T1 copper ions are not incorporated (due to mutations of copper ligands).

Finally, our study implies that the CP polypeptide can adopt a molten-globule form that is not on the path to the native state. Thus, timely copper incorporation, before apoprotein misfolding occurs, may be crucial for successful in vivo biosynthesis of CP. Notably, we have earlier shown that for *Pseudomonas aeruginosa* azurin, copper binding readily occurs in the unfolded state (*51*, *52*). Additional studies using strategic CP mutants and solvent conditions mimicking the biological environment in the secretory pathway will be of importance from a fundamental point of view, as it may apply to other cuproproteins made in the secretory pathway, and also from a medical aspect, as it may facilitate new approaches to incorporate copper into copper-lacking CP variants found in affected patients.

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