Copper Binding before Polypeptide Folding Speeds Up Formation of Active (Holo) Pseudomonas aeruginosa Azurin †

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Copper Binding before Polypeptide Folding Speeds Up Formation of Active (Holo) *Pseudomonas aeruginosa* Azurin†

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ABSTRACT: Cofactors often stabilize the native state of the proteins; however, their effects on folding dynamics remain poorly understood. To uncover the role of one cofactor, we have examined the folding kinetics of *Pseudomonas aeruginosa* azurin, a small blue-copper protein with a copper cofactor uniquely coordinated to five protein residues. Copper removal produces apo-azurin which adopts a folded structure identical to that of the holo-form. The folding and unfolding kinetics for apo-azurin follow two-state behavior. The extrapolated folding time in water, $\tau \sim 7$ ms, is in good agreement with the topology-based prediction. Copper uptake by folded apo-azurin, to govern active (holo) protein, is slow ($\tau \sim 14$ min, 50:1 copper-to-protein ratio). In contrast, the formation of active (holo) azurin is much faster when copper is allowed to interact with the unfolded polypeptide. Refolding in the presence of 10:1, 50:1, and 100:1 copper:protein ratios yields identical time-trajectories: active azurin forms in two kinetic phases with folding times, extrapolated to water, of $\tau = 10 \pm 2$ ms (major phase) and $\tau = 190 \pm 30$ ms (minor phase), respectively. Correlating copper-binding studies, with a small peptide derived from the metal-binding region of azurin, support that initial cofactor binding is fast ($\tau \sim 3.7$ ms) and thus not rate-limiting. Taken together, introducing copper prior to protein folding does not speed up the polypeptide-folding rate; nevertheless, it results in much faster (> 4000-fold) formation of *active* (i.e., holo) azurin. Living systems depend on efficient formation of functional biomolecules; attachment of cofactors prior to polypeptide folding appears to be one method to achieve this.

A considerable body of work has demonstrated that proteins fold with widely differing kinetics and with mechanisms of varying complexity. A key finding is that while many large proteins populate folding intermediates, smaller proteins often fold directly to the native state without kinetic intermediates (*1*−*3*). For such small proteins, parameters such as sequence, size, stability, and topology may to various extents affect the protein-folding rates. A recent study identified a statistically significant correlation between the topology and folding speed for a large set of small, unrelated proteins (*4*). It was shown that proteins with mainly local interactions (such as α-helices) have rapid folding transitions, whereas proteins with more complex topologies (such as β-sheets) usually fold more slowly.

Many proteins require the binding of cofactors to perform their biological activity, and these molecules fold in a cellular environment where their cognate cofactors are present. It has been demonstrated in vitro that many proteins retain the interactions with the cofactors after polypeptide unfolding (*5*−*9*). Therefore, it is possible that cofactors bind to their corresponding polypeptides before folding in vivo. Cofactors most often stabilize the native states of the proteins they interact with (*5*, *6*, *10*, *11*). However, the manner in which cofactors affect the folding pathways remains poorly understood, since kinetic-folding studies are frequently conducted in the absence of potentially complicating ligands. Only a few studies targeting the effect of cofactors on folding have been reported to date. For example, calcium ions have been shown to stabilize RNase HI and staphylococcal nuclease A proteins by a mechanism based on decreasing the unfolding speed (*11*, *12*). In contrast, α-lactalbumin was concluded to refold more quickly in the presence of metals, but the metals had no effect on the unfolding speed (*13*).

To directly address the role of a metal cofactor in protein folding, we have examined the consequences of cofactor binding on the folding pathway of *Pseudomonas aeruginosa* azurin. This is a well-characterized small (128-residue) blue-copper protein that functions as an electron carrier in bacterial respiratory chains. Azurin has a β-barrel structure arranged in a double-wound Greek-key topology (*14*−*16*). A redox-active copper [Cu(II)/Cu(I) pair] is coordinated by two histidine imidazoles (His46 and His117), one cysteine thiolate (Cys112), and two weaker axial ligands, sulfur of methionine (Met121) and the carbonyl of glycine (Gly45). The highly covalent nature of the copper−cysteine bond gives Cu(II) azurin unique spectroscopic properties: most importantly, an intense absorption at 630 nm. This feature can be used as a signature of folded holo-protein (i.e., the active form). Azurin can bind many different metals in the active site; moreover, it adopts a folded structure also without the presence of a metal cofactor. Crystal structures of apo- and holo-azurin have shown that the overall three-dimensional structure is identical with and without metal (*16*, *17*).

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Thermodynamic stability data for *Pseudomonas aeruginosa* copper (oxidized and reduced) azurin as well as for zinc-substituted and the apo-form of the protein have been reported (*10*, *18*). Cu(II) azurin is more stable than the reduced Cu(I) form; both holo-forms are more stable than apo-azurin. Upon unfolding of holo-azurin, induced by the chemical denaturant guanidine hydrochloride (GuHCl),[1] the copper remains bound to the unfolded polypeptide in a trigonal coordination (*19*). However, the copper-bound unfolded state of Cu(II) azurin is not as stable with time, as is unfolded Cu(I) azurin, due to a slow redox reaction that takes place between the cysteine thiol and Cu(II) under aerobic conditions (*20*). EXAFS experiments revealed that one copper ligand in unfolded azurin is Cys112 (*21*); subsequent mutant studies [on His117Gly and His46Gly point-mutated azurin proteins (*22*)] confirmed that His117 is one of the other ligands in the unfolded state. The third copper ligand in unfolded azurin has not yet been probed: suggestions include the sulfur of Met121 or a water oxygen. A small peptide comprising the C-terminal part of azurin, including three native-state copper ligands (Cys112, His117, and Met121), was recently shown to specifically bind copper and acquire β-like structure (*7*). Based on these findings, this C-terminal polypeptide segment (adopting a β-hairpin in native azurin) was proposed to act as an initiation site for azurin folding in the presence of copper.

In this report, we probe the involvement of oxidized copper in the folding process of *Pseudomonas aeruginosa* azurin. The speed of active (holo) azurin formation is examined for two different reaction pathways: (1) copper added to the unfolded polypeptide at the initiation of refolding; and (2) copper added to previously folded apo-azurin. We find that the uptake of copper by folded apo-protein (path 2) is very slow. In contrast, if copper is included in the refolding buffer, i.e., presented to the unfolded protein (path 1), formation of active (holo) azurin is more than 3 orders of magnitude faster. Azurin refolding in the presence of copper occurs in two kinetic phases, suggested to be related to two denatured protein populations interacting differently with copper. Variations of copper-to-protein mixing ratios, and complementary peptide-binding studies, support that copper binding is not rate-limiting. Refolding rates for the azurin polypeptide in the presence and absence of copper are not much different, indicating that copper binding to the unfolded state does not speed up the polypeptide-folding process. Nevertheless, to function in vivo, azurin must have copper in its active site; holo-azurin is obtained much more rapidly when copper interacts with the polypeptide *before* folding takes place.

EXPERIMENTAL PROCEDURES

Materials. The azurin peptide (sequence: FCTFPGHSALMK) was purchased from Louisiana State University Core Laboratory. Purity (>98%) was verified by mass spectroscopy and HPLC. Peptide solutions (100 mM Tris-HCl, pH 7.2) were made fresh; concentrations were determined by Ellman's assay of free thiols. All chemicals were obtained from Sigma Chemical Co. GuHCl was of ultrapure grade.

[1] Abbreviations: GuHCl, guanidine hydrochloride; CD, circular dichroism.

Protein Expression and Purification. Pseudomonas aeruginosa apo-azurin was expressed in *Escherichia coli* strain RV308 as previously described (*10*, *22*, *23*). In brief, cells were grown in LB medium containing 100 μM ampicillin. Protein expression was induced (at $OD_{600} = 0.6$) by addition of isopropyl-β-D-thiogalactoside (0.5 mM). The cell paste was resuspended in sucrose buffer (20% sucrose, 0.3 M Tris-HCl, pH 8.1) and subjected to osmotic shock (0.5 mM $MgCl_2$). Contaminating proteins were precipitated from the periplasmic preparation by decreasing the pH to 4.1 (50 mM ammonium acetate), yielding azurin-containing supernatant. Zn- and apo-azurin fractions (pI = 4.6 and 5.2, respectively) were separated on an SP Sepharose HP (Pharmacia) ion-exchange column with a pH gradient from 4.5 to 9.0 (50 mM ammonium acetate). Following PEG dialysis, gel filtration on a Superdex 75 (Pharmacia) column yielded 95−98% pure apo-azurin. Copper was never added during cell harvest and purification. Protein purity was determined by gel-electrophoresis and copper-reconstitution experiments.

Equilibrium Studies. GuHCl-induced equilibrium unfolding of apo-azurin was monitored by far-UV circular dichroism (OLIS Inc. instrument; 200−300 nm, 1 mm cell) and tryotophan fluorescence (Varian Eclipse; excitation 285 nm, emission 308 nm, 5 nm band-pass). All experiments were performed in 100 mM Tris-HCl buffer (pH 7.2, 20 °C). There was no protein-concentration dependence for the unfolding transition (in the range 5−100 μM), the two spectroscopic methods yielded identical results, and the reaction was fully reversible. The experimental unfolding curve was fitted (in KaleidaGraph) to the following expression derived for a two-state process:

$$Y_{obs} = \{Y_U + Y_F[\exp((\Delta G_U(H_2O) - m[\text{denaturant}])/RT)]\}/\{1 + [\exp((\Delta G_U(H_2O) - m[\text{denaturant}])/RT)]\}$$

where Y_{obs}, Y_U, and Y_F are the observed spectroscopic signal, denatured-protein baseline, and folded-protein baseline, respectively. From the fit, $\Delta G_U(H_2O)$, the free energy of unfolding in aqueous solution, and m, the dependence of the free energy on denaturant concentration (*24*, *25*), were determined.

Kinetic Studies. Time-resolved folding and copper-binding [Cu(II), supplied as $CuSO_4$] measurements were made on an Applied Photophysics DX.18MV stopped-flow reaction analyzer (with two monochromators) in its fluorescence (excitation at 285 nm; emission at 308 nm; 8 nm band-pass, 0.2 cm path) or absorption (at 630 nm for protein; at 340 nm for peptide) mode. Buffer in all experiments was 100 mM Tris-HCl, pH 7.2, 20 °C. Tris-HCl was selected, since high concentrations of Cu^{2+} are not soluble in phosphate buffer. For each experimental condition, 5−8 transients were averaged.

Refolding and unfolding of apo-azurin were monitored by tryptophan fluorescence. For unfolding, five parts of denaturant solution were mixed with 1 part of protein solution (10 μM final protein concentration). Refolding was initiated from 2.5 M GuHCl by 1:5 mixing with buffer solutions including appropriate GuHCl concentrations. No missing amplitude was observed within the instrument dead time (6−8 ms for 1:5 mixing).

Kinetics of copper uptake by folded apo-azurin were studied by monitoring the appearance of 630 nm absorption upon 1:1 mixing of apo-azurin with buffer containing $CuSO_4$ in 10:1 and 50:1 copper-to-protein ratios (10 μM final protein concentration). Similar experiments were also performed in which low concentrations of GuHCl were included in both syringes (0.1, 0.5, 0.8, 1.0 M GuHCl). No absorption increase was observed within the instrument dead time (2 ms for 1:1 mixing).

To investigate azurin refolding in the presence of copper, 1 part of unfolded apo-azurin (in 2.5 or 3 M GuHCl) was mixed with 5 parts of buffer containing $CuSO_4$ in 10-, 50-, or 100-fold excess over protein (10 μM final protein concentration) as well as appropriate GuHCl concentrations. Also in these experiments, no missing amplitude was found in the dead time. The kinetics of copper binding to the small peptide were investigated by mixing equal volumes of peptide solution (50 μM peptide) with buffer containing 15-, 20-, or 30-fold excess of $CuSO_4$ while monitoring the increase in 340 nm absorption.

All kinetic traces were fit to monophasic or biphasic decay equations using a nonlinear least-squares algorithm supplied by Applied Photophysics (see text). The pseudo-first-order rate constants derived for copper binding to folded apo-azurin and the refolding rate constants in the presence of copper were fit to functions linearly relating ln k and GuHCl concentration values (from which the rate constants in absence of denaturant were extracted). The unfolding and refolding rate constants for apo-azurin at different denaturant concentrations were fit in KaleidaGraph assuming standard linear dependences of ln k_F and ln k_U on the GuHCl concentration (*26*):

$$\ln k = \ln[k_F(H_2O)\exp(m_F[\mathrm{GuHCl}]/RT) + k_U(H_2O)\exp(m_U[\mathrm{GuHCl}]/RT)]$$

In this equation, m_U is the slope of the unfolding branch and m_F is the slope of the folding branch. $k_F(H_2O)$ and $k_U(H_2O)$ are the folding and unfolding rate constants in aqueous solution (i.e., in the absence of GuHCl).

RESULTS

Folding Dynamics of Apo-azurin. GuHCl-induced equilibrium-unfolding of *Pseudomonas aeruginosa* apo-azurin is a reversible, two-state process in Tris-HCl buffer (Figure 1, inset), as has also been reported previously using phosphate buffer (*27*). In living systems, the azurin polypeptide coordinates one copper ion. In addition to being essential for function (electron-transfer processes), the copper ion also stabilizes the protein structure. The equilibrium-unfolding midpoint for apo-azurin occurs at 1.7 M GuHCl (Figure 1, inset) whereas it is not observed until 4.0 M GuHCl for oxidized holo (copper) azurin (*20*, *27*). In terms of energetics, the free energy of unfolding in water is 29 ± 2.3 kJ/mol for apo-azurin and 52 ± 4.7 kJ/mol for holo-azurin (oxidized), respectively.

Folded apo-azurin shows strong tryptophan (Trp48) emission at 308 nm; upon polypeptide unfolding, the intensity decreases and the emission maximum shifts to higher wavelengths (~350 nm) (*10*, *22*). Folding and unfolding kinetics for apo-azurin were therefore probed by monitoring changes in tryptophan emission. The reactions all exhibit monoexponential decay curves, and a graph of ln k values versus GuHCl concentration results in a V-shaped profile (Figure 1). From the fit, the folding rate constant in the absence of denaturant, $k_F(H_2O)$, for apo-azurin was determined to be 134 ± 20 s^{-1} (i.e., ln k_F is 4.9).

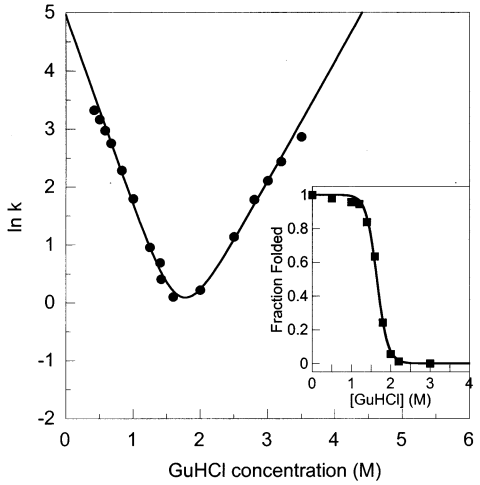

FIGURE 1: Natural logarithm of folding and unfolding rate constants for apo-azurin as a function of GuHCl concentration (20 °C; pH 7.2). Inset: Equilibrium unfolding curve for apo-azurin. Solid lines represent two-state fits (see Experimental Procedures).

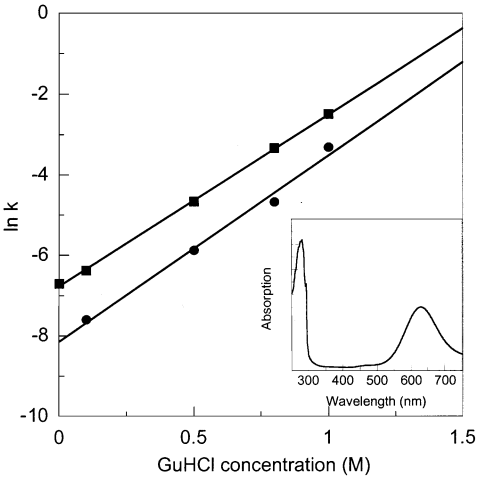

FIGURE 2: Natural logarithm of copper-uptake rate constants for folded apo-azurin mixed with excess copper (10:1 ratio, circles; 50:1 copper-to-protein ratio, squares) as a function of GuHCl concentration (20 °C; pH 7.2). Inset: Absorption spectrum of native (holo) azurin.

Copper Uptake by Folded Apo-azurin. Native (holo) azurin has a strong blue color due to ligand-to-metal charge transfer between the cysteine sulfur ligand (Cys112) and the oxidized copper ion (Figure 2, inset). The pseudo-first-order rate constants for copper uptake by folded apo-azurin were determined from the appearance of this 630 nm absorption upon mixing apo-protein with copper (1:10 and 1:50 protein-to-copper ratios). The experiments were performed in the presence of 0.0, 0.1, 0.5, 0.8, and 1.0 M GuHCl; all are conditions at which the native conformation of apo-azurin is favored (cf. Figure 1, inset). The kinetic traces were successfully fitted by single-exponential functions, and the natural logarithm of the copper-uptake rate constants was found to depend linearly on the denaturant concentration (Figure 2). As can be seen, the rates are faster for the higher

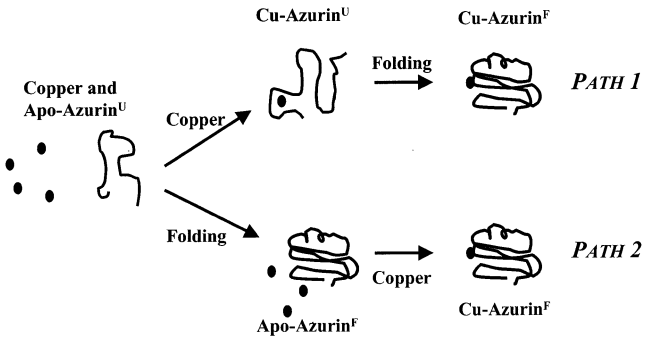

FIGURE 3: Possible mechanisms for going from unfolded polypeptide and free copper to the active holo-protein. Path 1: Copper binding to unfolded polypeptide takes place before protein folding. Path 2: Apo-protein folding precedes copper uptake.

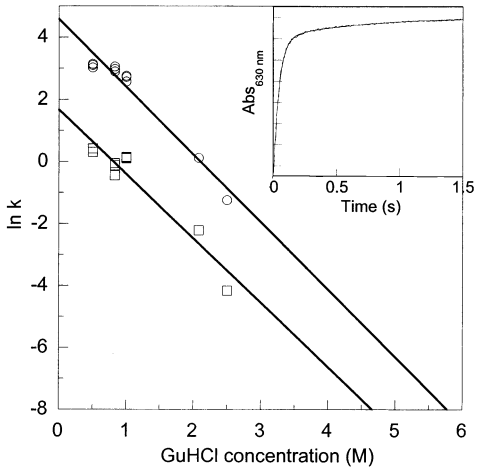

FIGURE 4: Natural logarithm of pseudo-first-order rate constants for formation of holo-azurin in the presence of excess copper as a function of GuHCl concentration (20 °C; pH 7.2). The fast phase (circles) corresponds to ~85% of the total amplitude, the slower phase (squares) to the remaining ~15%. At 0.5, 0.8, and 1.0 M GuHCl, rate constants are overlaid for 10:1, 50:1, and 100:1 copper-to-protein ratios. Inset: Example of a kinetic trace, and the corresponding two-exponential fit overlaid, observed upon mixing unfolded apo-azurin with copper-containing refolding buffer (10 μM azurin, 100 μM copper, and 0.5 M GuHCl as final concentrations).

copper-to-protein ratio, and additions of small amounts of GuHCl speed up copper incorporation. The copper-uptake time in water is 57 and 14 min for 1:10 and 1:50 protein-to-copper ratios, respectively. The second-order rate constant for copper incorporation into folded azurin that can be derived from these experiments is $2.6 \pm 0.3\ M^{-1}\ s^{-1}$.

Folding of Azurin in the Presence of Copper. To study the folding process for azurin in the presence of (oxidized) copper, unfolded apo-protein (in high concentration of GuHCl) was mixed with copper-containing buffer (without, or with a low concentration of, denaturant). In this way, copper is introduced to the unfolded polypeptide at the same time folding is triggered by the decrease in total denaturant concentration. There are two pathways by which folded holo-azurin can be formed (Figure 3). According to path 1, copper binding takes place prior to polypeptide folding (i.e., copper binds to the unfolded protein). In path 2, instead, apo-azurin folds first and incorporation of copper occurs in the folded state.

Azurin refolding experiments in the presence of copper were carried out under pseudo-first-order conditions (10:1, 50:1, and 100:1 copper-to-protein ratios). For all final concentrations of GuHCl studied, the kinetic traces are biexponential (inset, Figure 4). Approximately 85% of the total amplitude change at 630 nm is associated with the faster phase and 15% with the slower phase. Both the fast and slow processes are independent of copper-to-protein ratio, and the logarithms of the fast and slow rate constants both vary linearly with the denaturant concentration (Figure 4). Upon extrapolation to 0 M GuHCl, the folding rate constants in water are $98 \pm 10\ s^{-1}$ (i.e., $\ln k = 4.6$) and $5.3 \pm 0.4\ s^{-1}$ (i.e., $\ln k = 1.67$) for the fast and slow processes, respectively (Table 1). Even at final concentrations of 2.0 and 2.5 M GuHCl, where folded apo-azurin is not thermodynamically stable, formation of folded holo-azurin is observed upon mixing (Figure 4). This latter result excludes path 2 (in Figure 3) as a likely mechanism for formation of folded holo-azurin.

Copper Binding by Small Azurin Peptide. A small peptide corresponding to residues 111−123 in *Pseudomonas aeruginosa* azurin (sequence: FCTFPGHSALMK; native-state copper ligands underlined) is considered a good model of the metal-binding segment in unfolded azurin (*7*). Upon copper binding to this peptide, absorption appears at 340 nm (and, with a smaller extinction coefficient, at 530 nm), and a β-sheet peptide structure is adopted (*7*). The kinetics of copper binding to the azurin peptide (15:1, 20:1, and 30:1 copper-to-peptide ratios) were studied by monitoring the increase in absorption at 340 nm upon stopped-flow mixing in the absence of GuHCl. Two kinetic phases (with 60% of the total amplitude change found in the faster process, and 40% in the slower process) are observed (Figure 5). None of the processes are dependent on the copper-to-peptide ratio, suggesting that initial metal interaction occurs before 340 nm absorption. The appearance of this absorption band is closely related to the geometry and distance of the interaction between the cysteine sulfur (and perhaps other peptide side chains to smaller extents) and the copper ion; to form, it may require peptide conformational changes. The biphasic behavior can be explained by the peptide binding to copper in two different ways (i.e., gaining 340 nm absorbance in parallel paths), or by the presence of a populated intermediate (perhaps with one or two, of the three possible, copper ligands coordinated) on the reaction pathway. Regardless of mechanism, these experiments show that copper can interact with residues in the azurin peptide on very short time scales [the pseudo-first-order rate constant is $270\ s^{-1}$ (i.e., ln k of 5.6) for the first/fast phase; see Table 1].

Table 1: Kinetic Data for Formation of Apo- and Holo-Forms of Azurin (10 μM Final Protein Concentration, Tris-HCl Buffer, pH 7.2, 20 °C) through the Various Pathways Discussed in the Text

start point	end point	1/k	detection
unfolded apo-azurin	folded apo-azurin	7 ms	Em_{308nm}
folded apo-azurin + copper	folded holo-azurin	57 min[a] 14 min[b]	Abs_{630nm}
unfolded apo-azurin + copper	folded holo-azurin	10 ms (85%)[c] 190 ms (15%)[c]	Abs_{630nm}
azurin peptide + copper	peptide−copper complex	3.7 ms (60%)[d] 160 ms (40%)[d]	Abs_{340nm}

[a] 10:1 copper-to-peptide/protein ratio. [b] 50:1 copper-to-protein ratio. [c] Identical rates found for 10:1, 50:1, and 100:1 copper-to-protein ratios. [d] Identical rates found for 15:1, 20:1, and 30:1 copper-to-peptide ratios.

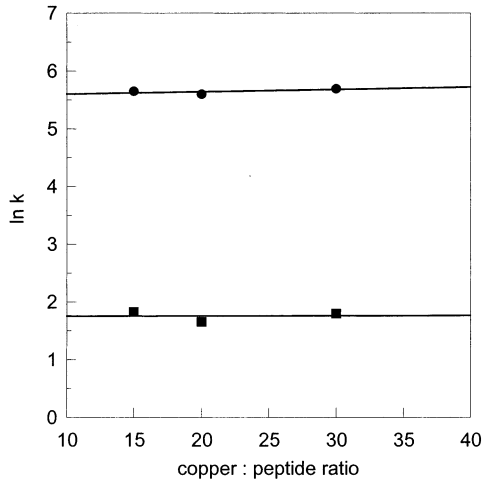

FIGURE 5: Natural logarithm of rate constants observed, by absorption appearance at 340 nm, upon mixing copper with the azurin peptide as a function of the peptide-to-copper ratio. The fast phase (circles) corresponds to ~60% of the total amplitude change, the slower phase (squares) to the remaining ~40%.

DISCUSSION

The biologically active form of azurin, as well as those of many other proteins in nature, contains a metal-ion cofactor. The copper in azurin stabilizes the folded protein significantly, although the apo-protein still adopts a nativelike structure (*10*, *20*, *27*). In this study we sought to assess whether the copper ion affects the folding pathway (and speed) of the azurin polypeptide. Copper remains bound to azurin upon polypeptide unfolding; however, this unfolded state slowly decomposes over time [most prominent in the case of Cu(II) (*20*)]. Therefore, in our kinetic experiments, we have introduced copper to the unfolded polypeptide through the refolding buffer.

Copper Binds before the Polypeptide Folds. After establishing the folding and unfolding kinetics for apo-azurin, which is an apparent two-state process with an extrapolated folding time in water of ~7 ms (Figure 1), we probed (by monitoring visible absorption accumulation at 630 nm) the speed of copper uptake by the folded apo-protein (Figure 2). This process is very slow, on the order of minutes. In sharp contrast, if azurin is refolded in the presence of copper, the expected absorption increase at 630 nm is found in two rapid kinetic phases (Table 1, Figure 4). This indicates that copper interacts with the polypeptide prior to folding, presumably in its unfolded state (Figure 3, path 1). In agreement, at final concentrations of 2 and 2.5 M GuHCl, folded apo-azurin is not favored thermodynamically; nevertheless, we still observe the two rapid phases that govern the formation of the folded holo-azurin.

Since the rate constants do not depend on the copper-to-protein ratio (for 10:1, 50:1, and 100:1 ratios), we conclude that copper binding is not rate-limiting in these refolding experiments. In accord, the kinetic mixing data for copper binding to the azurin peptide (acting as a model of the metal-coordinating segment in unfolded azurin) show that copper binding to this fragment is fast (Figure 5). When copper binds to the small peptide, an absorption band at 340 nm appears (*7*). Therefore, we searched for the absorption at 340 nm appearing transiently in the full-length protein-refolding experiments. As predicted, there is increased absorption at 340 nm within the first millisecond upon mixing unfolded apo-protein with copper (data not shown), which may thus correspond to copper binding to unfolded azurin in a coordination similar to that occurring in the small peptide.

Folding Pathway in the Presence of Copper. Refolding of apo-azurin is an apparent two-state process. In contrast, when copper is included in the refolding buffer, holo-azurin forms in two kinetic phases, with 85% of the amplitude in the faster phase and 15% in the slower. The faster phase (folding time of 10 ms in water) corresponds well with the refolding kinetics of apo-azurin, indicating that although copper is interacting with the protein, the speed of polypeptide folding is not perturbed for this fraction of the molecules (Table 1). Based on previous peptide studies (*7*), the transient 340 nm absorption observed immediately upon mixing in the protein-refolding experiments, and data for unfolded holo-azurin (*19*, *21*, *22*), we propose that, upon mixing, the major fraction of the copper rapidly (within milliseconds) binds to His117 and Cys112 (and perhaps also Met121) in the unfolded polypeptide. In the course of polypeptide folding, the other two native copper ligands are brought into place, resulting in the 630 nm absorption. The slower kinetic phase (folding time of 190 ms in water) may be related to a small population of unfolded molecules that initially interacts differently with copper, resulting in some retardation of the polypeptide folding speed. Perhaps the initial copper coordination here includes an additional non-native ligand, or one of the native-state ligands is absent in the initial complex.

Copper Interaction Does Not Speed Up Polypeptide Folding. As already noted above, the presence of copper in the refolding buffer does not speed up (or significantly slow) the polypeptide folding kinetics. Since copper interacts with the polypeptide in the unfolded state, we can conclude that holo- and apo-forms of azurin have very similar folding rates. Interestingly, the predicted folding time for azurin (*4*) based on its native-state topology is 16 ms (*27*). Since folding rates can vary more than 6 orders of magnitude, this folding time is in excellent agreement with the experimental results for both holo-azurin (10 ms) and apo-azurin (7 ms). This comparison between experiments and theory indicates (1) that the rate-limiting step in azurin folding does not involve the copper site and (2) that native-state topology is more important than equilibrium stability in determining the folding speed for this protein.

In a recent study, the unfolding rates for holo- and apo-azurin were compared: holo-azurin was shown to unfold 10 000-fold slower than the apo-protein (*27*). It was suggested that the mechanism by which copper increases the thermodynamic stability of azurin is solely due to a lower value for the unfolding rate constant. Our current direct experimental data on azurin refolding in the presence of copper strongly support this conclusion.

Biological Relevance for Copper Binding Prior to Folding? In living systems, active (holo) azurin may form via two different reaction pathways (Figure 3): (1) the copper binds to the unfolded polypeptide prior to its folding, or (2) the copper binds to already folded apo-azurin. Strikingly, we discovered that in vitro the active blue-copper site in azurin is formed more than 4000-fold faster when copper is allowed to interact with the polypeptide before structure has formed (Table 1). The rigid structure of folded azurin may hinder copper penetration to the active site, whereas the

copper ligands are exposed in the flexible, unfolded state. Under native conditions (0 M GuHCl), a small fraction of apo-azurin is unfolded (in 10 μM total protein, 0.1 nM will be unfolded), and the unfolding rate is 0.18 s^{-1} ($\ln k \sim -4.0$; extrapolation in Figure 1). Since the copper-uptake rates by folded apo-azurin are slower (Figure 2) than the protein-unfolding rate in the absence of denaturant, a mechanism in which the apo-protein first unfolds completely before copper becomes coordinated is consistent with the experimental data. Also in accord with this mechanism, additions of small amounts of denaturant to folded apo-azurin, resulting in faster protein-unfolding rates, increase the speed of copper uptake by the folded protein (Figure 2).

Translation of gene messages into functional proteins should be rapid for an efficient maintenance of cellular activities. Since active azurin forms many orders of magnitude faster when the cofactor is allowed to interact with the unfolded, instead of the folded, protein, we propose that coordination of cofactors prior to polypeptide folding may be one possible method in nature to ensure adequate formation of active cofactor-binding proteins. However, it must be noted that the pool of free copper (and many other metal ions as well) in living cells is very low, probably less than one copper atom per cell (*28*). This may present a kinetic problem with the proposed reaction pathway: in vivo apo-protein folding may take place faster than metal encounter with the unfolded protein. In addition, other metal ions may compete for interactions with the polypeptide. Further studies are clearly needed before conclusions with respect to in vivo mechanisms can be achieved.

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