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Forum

Role of Cofactors in Folding of the Blue-Copper Protein Azurin

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Many proteins in living cells coordinate cofactors, such as metal ions, to attain their activity. Since the cofactors in such cases often can interact with their corresponding unfolded polypeptides in vitro, it is important to unravel how cofactors modulate protein folding. In this review, I will discuss the role of cofactors in folding of the blue-copper protein *Pseudomonas aeruginosa* azurin. In the case of both copper (Cu^{II} and Cu^{I}) and zinc (Zn^{II}), the metal can bind to unfolded azurin. The residues involved in copper (Cu^{II} and Cu^{I}) coordination in the unfolded state have been identified as Cys112, His117, and Met121. The affinities of Cu^{II}, Cu^{I}, and Zn^{II} are all higher for the folded than for the unfolded azurin polypeptide, resulting in metal stabilization of the native state as compared to the stability of apo-azurin. Cu^{II}, Zn^{II}, and several apo forms of azurin all fold in two-state kinetic reactions with roughly identical polypeptide-folding speeds. This suggests that the native-state β-barrel topology, not cofactor interactions or thermodynamic stability, determines azurin's folding barrier. Nonetheless, copper binds much more rapidly (i.e., 4 orders of magnitude) to unfolded azurin than to folded azurin. Therefore, the fastest route to functional azurin is through copper binding before polypeptide folding; this sequence of events may be the relevant biological pathway.

1. Do Cofactors Play a Role in Protein Folding?

Proteins are involved in virtually every biological process. To function, linear chains of amino acids must fold into their unique three-dimensional structures. Many small proteins fold rapidly in apparent two-state processes, whereas the folding of larger proteins often requires the involvement of intermediates and longer times.[1] Correlations of the experimental folding rates for many small single-domain proteins (without cofactors), folding by two-state mechanisms, have been studied against several parameters (e.g., thermodynamic stability, polypeptide chain length, transition-state placement, and native-state topology). Interestingly, the native-state contact order, reporting on the degree of local versus nonlocal contacts in the native state, was found to be the most statistically significant parameter.[2,3] Relative contact order (CO) is calculated as $CO = [1/(LN)]\sum\Delta Z_{i,j}$, where N is the total number of contacts, $\Delta Z_{i,j}$ is the number of residues separating contacts i and j, and L is the number of residues in the protein;[2] note that the sum $\sum$ is over all contacts N. Proteins with many short-range interactions such as α-helices (corresponding to low CO) fold faster than proteins with many long-range interactions such as β-sheet structures (corresponding to high CO). The empirical contact-order correlation was subsequently explained in terms of the topomer-search model which is a theory stipulating that the search for unfolded conformations with a grossly correct topology is rate-limiting in folding.[4] This theory suggests that the transition-state corresponds to an expanded version of the native structure and thus is similar to the "extended nucleus" description of the folding-transition state proposed earlier.[5]

More than 30% of all proteins in living cells require cofactors (e.g., metals and organic moieties) to perform their activities.[6] These proteins fold in a cellular environment where their cognate cofactors are present. As demonstrated

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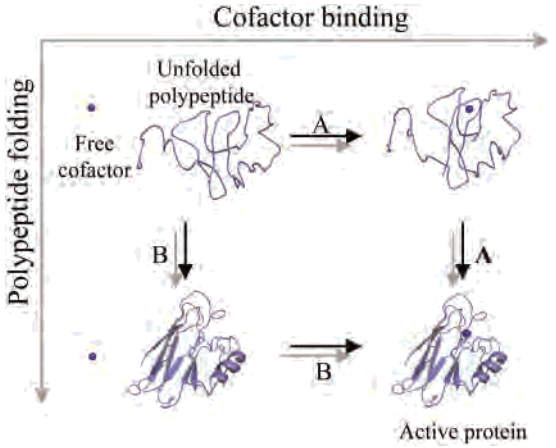

Figure 1. The two extreme mechanisms for going from free cofactor and unfolded polypeptide to a functional cofactor-binding protein. In part A, cofactor binding occurs prior to polypeptide folding. In part B, cofactor does not bind until after polypeptide folding is completed.

in vitro, many metalloproteins (e.g., cytochrome b_{562}, myoglobin, azurin, and the Cu_A domain) retain strong metalloligand binding after polypeptide unfolding.[7−10] This implies that in vivo cofactors may interact with their corresponding proteins before polypeptide folding takes place and, therefore, impact the folding reaction (Figure 1). Local and nonlocal structure in the unfolded protein may form due to coordination of a cofactor.[11] Such structural restriction may dramatically decrease the entropy of the unfolded state, limiting the conformational search for the native state.[12] The cofactor may in this way serve as a nucleation site that directs polypeptide folding. Cofactors often stabilize the native states of proteins.[7,13,14] However, the manner in which cofactors modulate polypeptide-folding pathways is not well understood.[15]

Here, I will review our biophysical efforts to learn more about roles of cofactors in protein-folding reactions in the case of the copper-binding protein *Pseudomonas aeruginosa* azurin. Although copper is an essential transition metal that acts as a cofactor in many proteins in vivo, free copper ions are toxic, and incorrect copper−protein interactions can result in disease.[16] In order to better understand metal−protein interactions in azurin, we perform in vitro equilibrium and kinetic folding experiments using chemical denaturants, such as urea and GuHCl, to perturb the structure (most often at pH 7, 20 °C). The conformational state of azurin is probed by a combination of spectroscopic methods, with each method reporting on a different aspect of the protein structure. For example, visible absorption reports on the Cu^{II} environment, tryptophan fluorescence reports on the integrity of the protein core via tryptophan-48,[17] and far-UV circular dichroism (CD) reports on polypeptide secondary-structure changes. If an unfolding process is "two-state", which means that only folded and unfolded species are populated, different spectroscopic methods should yield the same result.[18] To probe folding kinetics, we use stopped-flow mixing to achieve rapid denaturant-jumps (instrument dead time 2−3 ms) that are followed by spectroscopic detection. The logarithms of the observed rate constants (k_{obs}; folding-rate constants at low denaturant concentrations; unfolding-rate constants at high denaturant concentrations) are often plotted as a function of denaturant concentration in a so-called Chevron plot. If the data points in this appear V-shaped, that is one indication of two-state kinetic behavior.[18]

With azurin as our model system, we have addressed the following questions: Does the cofactor bind to the unfolded polypeptide, and if so, what are the ligands? How tight do various metals bind to the folded and unfolded polypeptide, and what is the effect on azurin's overall stability? Does the presence of metal in the unfolded state affect the polypeptide folding speed and mechanism? What may be a biologically relevant pathway for formation of active (holo) azurin? Our observations on azurin may have implications for β-sheet formation and metal−protein interactions in a broad perspective.

2. What Are the Basic Characteristics of Azurin?

Pseudomonas aeruginosa azurin is a small (128 residues) blue-copper protein (also called cupredoxin) that is believed to facilitate electron transfer in denitrification/respiration chains.[19] Its redox partners, cytochrome c_{551} and nitrite reductase, have been identified from in vitro experiments, but their relevance as physiological partners has not been established in vivo.[20] It was recently proposed that the physiological function of azurin in *P. aeruginosa* involves electron transfer directly related to the cellular response to oxidative stress.[20] Azurin has one α-helix and eight β-strands that fold into a β-barrel structure arranged in a double-wound Greek key topology[19,21] (Figure 2). Proteins with the β-barrel motif belong to the large family of sandwichlike proteins. The structures of these proteins are characterized by two β-sheets packed against each other (like a sandwich).[22] In *P. aeruginosa* azurin, a redox-active copper (Cu^{II}/Cu^{I}) is coordinated by two histidine imidazoles (histidine-46 and histidine-117), one cysteine thiolate (cysteine-112), and two

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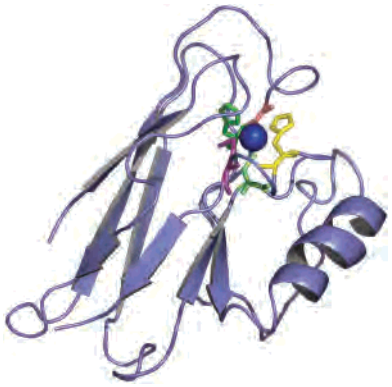

Figure 2. Three-dimensional representation of azurin (1azu.pdb) with copper in blue and its five ligands, forming the trigonal bipyramidal coordination geometry, shown in stick representation (prepared using PyMOL).

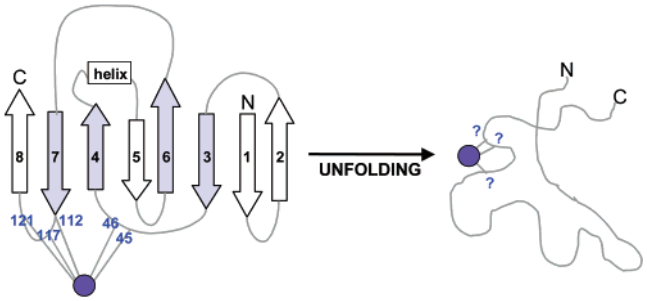

Figure 3. Native copper-azurin (left) retains the copper upon unfolding (right) in a trigonal coordination. In this schematic representation of azurin's secondary structure (left), β-strands are labeled from N- to C-termini, and the copper ligands in folded azurin are labeled (Gly45, His46, His117, Cys112, and Met121). Strands 4-5-6-3 form the Greek Key motif; the strands that form an interlocked pair, a substructure recently found highly conserved among β-sandwich proteins,[22] are shaded in blue (strands 7-4-6-3).

weaker axial ligands, sulfur of methionine (methionine-121) and carbonyl of glycine (glycine-45), in a trigonal bipyramidal geometry (Figures 2 and 3). The highly covalent nature of the copper−cysteine bond gives azurin an intense absorption at 630 nm.[19] It has been suggested that the polypeptide fold defines the metal site, leading to the rather unusual Cu^{II} coordination in azurin as well as in other blue-copper proteins.[23] In general, Cu^{I} favors soft ligands (such as sulfurs) and low coordination numbers, whereas Cu^{II} prefers harder ligands and higher coordination numbers.[24] In vitro, *P. aeruginosa* azurin can bind many different metals in the active site (e.g., zinc). Crystal structures of apo- and holo-azurin [apo = without cofactor; holo = with cofactor] have shown that the overall three-dimensional structure is identical with and without a metal (copper or zinc) cofactor.[21,25] Since there is significant biophysical and structural data on folded *P. aeruginosa* azurin, and on many point-mutated variants (including the ones used in our work), azurin is a superior system for in vitro studies of the interplay between β-sheet formation and metal−protein interactions.

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Table 1. K_D Values for Metal (Zn^{II}, Cu^{I}, and Cu^{II}) Binding to Folded (Buffer, pH 7, 20 °C) and Unfolded (GuHCl, pH 7, 20 °C) Wild-Type Azurin and the Thermodynamic Stability, $\Delta G_U(H_2O)$, for Each Metal Form of Wild-Type Azurin and Various Apo Forms (Buffer, pH 7, 20 °C)[27,a]

azurin variant	$\Delta G_U(H_2O)$	K_D(folded state)	K_D(unfolded state)
Cu^{II} form	52 ± 3 kJ/mol	25 fM	0.3 nM
Cu^{I} form	40 ± 3 kJ/mol	0.033 fM	3.0 fM
Zn^{II} form	39 ± 2 kJ/mol	82 nM	5.1 ± 2 μM
apo wild type	29 ± 2 kJ/mol		
apo His46Gly	18 ± 2 kJ/mol		
apo His117Gly	19 ± 1 kJ/mol		

[a] The difference in binding affinity of each metal for the folded versus the unfolded protein, $\Delta(\Delta G)_{binding} = -RT \ln[K_D(\text{folded})/K_D(\text{unfolded})]$, corresponds to the difference in thermodynamic stability between the metal form of azurin and the stability of apo-azurin, $\Delta[\Delta G_U(H_2O)]$. (No errors are given for calculated K_D values.)

3. Does Copper Remain Bound upon Azurin Unfolding?

Early equilibrium unfolding experiments (using the chemical denaturant guanidine hydrochloride, GuHCl) of oxidized (Cu^{II}) and reduced (Cu^{I}) azurin showed that the oxidized form is more stable than the reduced form (Table 1). The unfolding curves were reversible and exhibited no protein concentration dependence. This implied that the metal remained bound to the unfolded state since, otherwise, higher protein concentrations should have resulted in unfolding curves shifted to higher GuHCl concentrations.[26] Moreover, if the metal had dissociated in the unfolded state, refolding would follow the behavior of the apo-protein, and this was not observed.[26] Different spectroscopic signals yielded identical unfolding curves, in accord with two-state equilibrium-unfolding transitions. On the basis of the difference in thermodynamic stability for the two redox forms, we predicted that the copper in unfolded azurin has a reduction potential 0.13 V higher than that in folded azurin.[26] This was based on a thermodynamic cycle connecting folded and unfolded forms in their oxidized and reduced states, respectively. Subsequent cyclic voltammetry experiments confirmed this prediction of the unfolded-state reduction potential such that folded azurin has a reduction potential of 320 mV versus the normal hydrogen electrode (NHE) whereas the reduction potential for unfolded azurin is 456 mV versus NHE (20 °C, pH 7).[23] This difference in reduction potentials can be explained by a trigonal metal-coordination in the unfolded state (see below) that favors the Cu^{I} form.

The high Cu^{II}/Cu^{I} reduction potential for unfolded azurin, which is much higher than the potential for the Cu^{II}/Cu^{I} couple in solution, confirms that the metal remains associated with the unfolded polypeptide (chemical denaturation, pH 7, 20 °C) (Figure 3). On the basis of the high potential, it was suggested that Cys112 remains a copper ligand in the unfolded-state complex.[23] Unfortunately, GuHCl-induced unfolding of oxidized copper azurin is only partly reversible in aerobic conditions since a redox reaction takes place between the thiol of Cys-112 and Cu^{II} in the unfolded state.[23] Since the zinc form of azurin is always a byproduct upon

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azurin overexpression in *E coli*, and both copper and zinc may be present in the cells where the azurin polypeptide is produced, we have also probed the role of zinc in the folding of azurin. Equilibrium-unfolding studies of zinc-loaded azurin have revealed that zinc, like copper, remains bound to the polypeptide upon unfolding.[13,27] Zinc is redox inactive, and therefore, more quantitative studies can be performed on zinc-azurin than on the copper form.

4. What are the Metal Ligands in Unfolded Azurin?

Extended X-ray absorption fine structure (EXAFS) experiments in high GuHCl concentrations established that copper (Cu^{I}) in unfolded azurin is coordinated in a trigonal geometry to one thiolate (most likely Cys-112), one imidazole (perhaps one of the native state ligands His-46 or His-117), and a third, unknown ligand[28] (Figure 3). The nature of the third ligand could not be determined in these experiments; it could be a chloride from GuHCl, water, or another sulfur (perhaps Met-121). To elucidate if one of the two native-state histidine ligands, His-117 or His-46, is involved in copper coordination in unfolded azurin, we prepared two single-site (histidine-to-glycine) azurin variants: His117Gly and His46Gly azurin.[29] Equilibrium-unfolding experiments of His46Gly azurin loaded with copper demonstrated that copper remained bound to the protein in high urea concentrations where the protein's secondary structure is lost. In contrast, for copper-loaded His117Gly azurin, copper did not stay coordinated upon polypeptide unfolding.[29] This result strongly indicated that His-117 is the histidine coordinating copper in unfolded azurin, which is in accord with the proximity of His-117 to Cys-112 in azurin's primary sequence.

Of the five native-state copper ligands in azurin, three (Cys-112, His-117, and Met-121) are situated in a loop region toward the C-terminus of azurin's polypeptide (Figure 3). Since two of these ligands were confirmed copper ligands in the unfolded state (Cys-112 and His-117), we suspected that Met-121 may be the third, unknown ligand. To address the role of Met-121 in copper binding in unfolded azurin, we prepared the single-site mutant Met121Ala azurin, in which the methionine had been replaced by an alanine.[27] We found that copper (Cu^{II}) dissociated from Met121Ala azurin early on in the polypeptide-unfolding reaction (GuHCl-induced equilibrium unfolding), suggesting that Met-121 is important for copper retention in the unfolded copper form of wild-type azurin. Notably, the equilibrium-unfolding behavior of the zinc form of Met121Ala azurin was identical to that of wild-type azurin loaded with zinc, implying that zinc may not include Met-121 as an unfolded-state ligand.[27] This finding correlates with the crystal structure of zinc-substituted wild-type azurin, which shows that Met121 is not a zinc ligand in the native state of zinc azurin.[30]

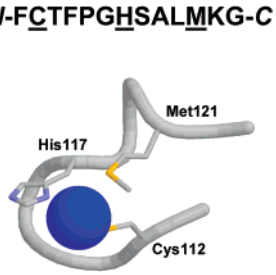

Figure 4. The top part shows the primary structure of the 13-residue model peptide (residues 111−123 in azurin) with the three residues identified as copper ligands underlined. The bottom part shows the secondary structure of the region of azurin that corresponds to the model peptide (based on 1azu.pdb) with copper ligands in stick representation.

Complementary evidence for the copper and zinc ligands in unfolded azurin comes from model peptide studies. We prepared a 13-residue peptide corresponding to the loop region in azurin (residues 111−123) that contains Cys-112, His-117, and Met-121 (Figure 4). This peptide was found to bind copper strongly, in a 1:1 stoichiometry.[11] Upon copper (Cu^{II}) binding to the peptide, visible absorption bands appeared at 330 and 530 nm, and β-like secondary structure formed. The presence of absorption at these wavelengths suggests thiolate(S) to Cu^{II} ligand-to-metal charge-transfer transitions.[31] Copper (Cu^{II}) binding to the peptide was also observed using isothermal titration calorimetry (ITC) although no thermodynamic parameters could be derived due to oxidation side reactions between Cu^{II} and the peptide cysteine.[27] Likewise, zinc also binds to the peptide in a 1:1 ratio as detected by ITC experiments; however, no secondary structure was induced when this complex formed. Metal titrations using ITC to a set of point-mutated peptides, in which Cys-112, His-117, and Met-121 were replaced by glycine one by one, confirmed that all three ligands are required for copper binding, whereas only Cys-112 and His-117 are needed for zinc binding.[27]

5. How Do Metal Affinities for Folded and Unfolded Azurin Affect Overall Stability?

Apo, Cu^{I}, Cu^{II}, and Zn^{II} forms of azurin all unfold in apparent two-state equilibrium-unfolding reactions (chemical denaturation, pH 7, 20 °C).[26,32,33] Since, in the cases of both copper (Cu^{I} and Cu^{II}) and zinc, the metal stays bound upon unfolding, the net effect on azurin's stability (as compared to the stability of apo-azurin) corresponds to the difference in metal affinity for the folded and unfolded polypeptide. The metals greatly stabilize native azurin; zinc, Cu^{I}, and Cu^{II} forms of azurin have thermodynamic stabilities of 39, 40, and 52 kJ/mol, respectively, whereas the stability of apo-azurin is 29 kJ/mol (pH 7, 20 °C).[26,32,33]

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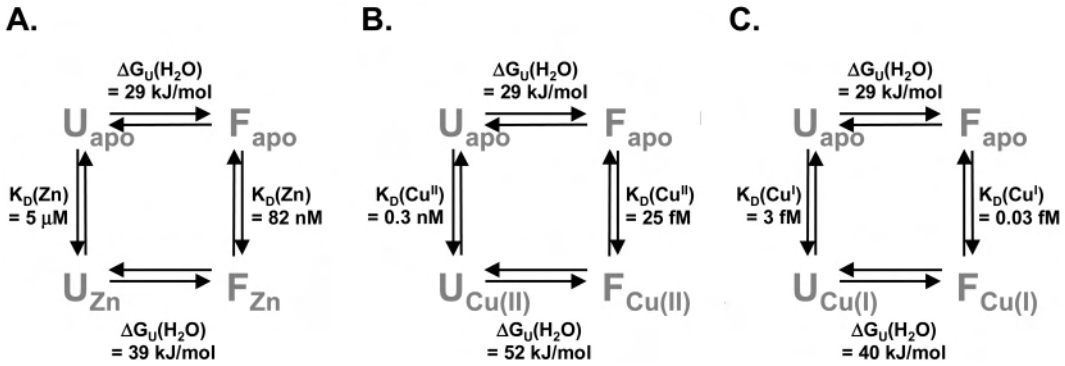

Figure 5. Thermodynamic cycles connecting folding of apo- and holo-forms of azurin, $\Delta G_U(H_2O)$, with cofactor binding (metal−protein dissociation constants, K_D) to the unfolded and folded states, respectively (pH 7, 20 °C): (A) zinc, (B) Cu^{II}, (C) Cu^{I}.

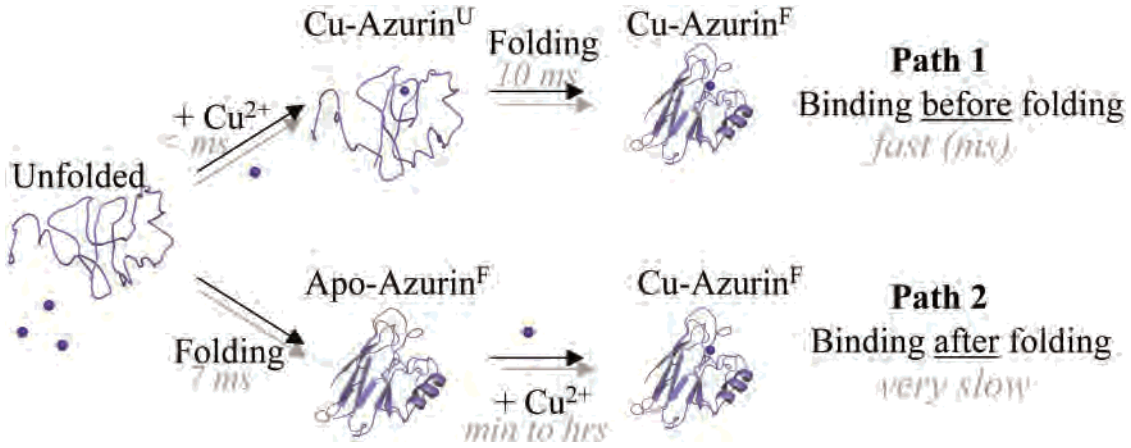

Figure 6. Time scales for formation of active azurin via pathway 1 (cofactor binding before polypeptide folding) and pathway 2 (polypeptide folding prior to cofactor binding) (pH 7, 20 °C). For each step, the approximate time is given. Upon comparison, pathway 1 is 4 orders of magnitude faster than pathway 2 (milliseconds versus minutes−hours).

A thermodynamic cycle can be constructed that links the free energy of zinc-binding to unfolded and folded wild-type apo-azurins, with the free energy of unfolding of apo- and zinc-bound polypeptides (Figure 5A). We determined the affinity of zinc for unfolded wild-type azurin to be 5 μM (pH 7, 20 °C) in ITC experiments[27] (Table 1), which results in an affinity (K_D) of zinc for folded wild-type apo-azurin of 82 nM (pH 7, 20 °C). In the case of Cu^{II} binding to apo-azurin, we could not estimate accurate K_D values from the ITC isotherms due to oxidation side reactions although metal binding could be confirmed. However, another group, using an innovative approach, reported a K_D of 25 fM for Cu^{II} binding to folded apo-azurin.[34] This value together with the stability of apo and Cu^{II} forms of azurin allowed us to estimate an affinity of copper for the unfolded polypeptide of 0.3 nM (pH 7, 20 °C)[27] (Figure 5B, Table 1). Remarkably, the metal affinity for the unfolded polypeptide is about 17 000-fold higher for Cu^{II} than for zinc, which may in part be explained by stabilization from the Met121 coordination with respect to copper, but not in the case of zinc.

In vivo, the environment is reducing, and copper is most often found as Cu^{I} although Cu^{II} may sometimes be present.[16] The Cu^{II} and Cu^{I} affinities for folded apo-azurin can be coupled through a thermodynamic cycle to the reduction potentials (E's) of the Cu^{II}/Cu^{I} pair in aqueous solution (E_{aq} = 150 mV vs NHE) and in the folded protein (E_{Az} = 320 mV vs NHE). For a closed cycle, the difference in reduction potentials (ΔE) should equal the difference in protein affinity ($\Delta[RT\ln K_D]$) according to the following: $RT \ln[K_D(Cu^{I})/K_D(Cu^{II})] = nF(E_{Az} - E_{aq})$, where F is Faraday's constant and n is number of electrons transferred, which is 1 in this case. Using the known values, the calculated a Cu^{I} affinity for the folded protein is ~0.033 fM (pH 7, 20 °C).[23] The affinity between Cu^{I} and folded apo-azurin can then be linked in another cycle to the stability of the apo and the Cu^{I} forms of azurin, to calculate a Cu^{I} affinity for unfolded azurin of ~3 fM (pH 7, 20 °C)[27] (Figure 5C, Table 1). Taken together, the data show that Cu^{I} binds stronger to both folded and unfolded azurin as compared to Cu^{II}. This is consistent with the higher reduction potentials for the Cu^{II}/Cu^{I} pair when bound to the folded and unfolded polypeptides (i.e., 320 and 456 mV vs NHE, respectively) as compared to copper in aqueous solution (i.e., 150 mV vs NHE).[23] In all cases (Figure 5), the metal affinity is higher for the folded than for the unfolded polypeptide which results in metal-mediated stabilization of the native-state of azurin.

6. Does Copper (Cu^{II}) Play a Role in Azurin's Kinetic Folding Process?

To address possible pathways for formation of active (i.e., folded protein with copper in the active site) azurin in vivo,

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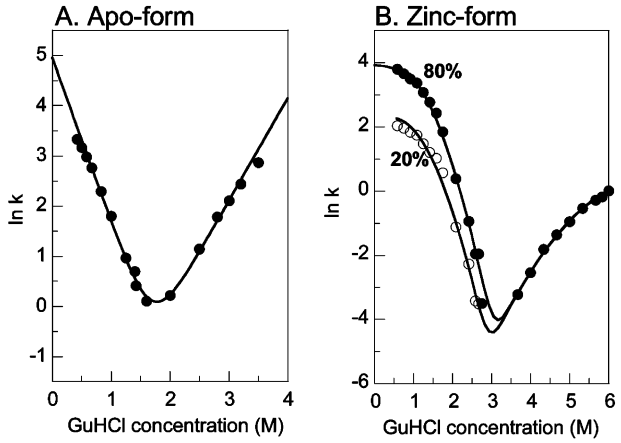

Figure 7. Semilogarithmic plots (so-called Chevron plots) of unfolding and refolding rate constants versus GuHCl concentration (pH 7, 20 °C) for the apo form of azurin (A) and zinc-substituted azurin (B; filled symbols, major phase; open symbols, minor phase). The solid curves are (in A) two-state and (in B) second-order polynomial fits.

the time scales for the two extreme scenarios (Figure 6), copper binding before polypeptide folding (pathway 1) and copper binding after polypeptide folding (pathway 2), were investigated. The folding and unfolding kinetics for apo-azurin follows two-state behavior (Figure 7A).[14,32,33,35,36] The extrapolated folding time in water, $\tau \sim 7$ ms, is in good agreement with the topology-based CO prediction. The relative contact order for folded apo-azurin is 16.7%, which corresponds to a predicted speed of 62 s^{-1} (i.e., τ of 16 ms).[32] Copper uptake by folded apo-azurin, which governs active protein through pathway 2, is very slow (i.e., $\tau \sim 14$ min depending on protein-to-copper excess). Thus, pathway 2 (Figure 6) is limited by slow copper incorporation to the folded apo-protein.[35,36]

In contrast, we found the formation of active azurin to be much faster when copper is allowed to interact with the unfolded polypeptide. Refolding experiments of azurin in the presence of 10:1, 50:1, and 100:1 copper-to-protein ratios yield identical time trajectories. Active azurin forms in two kinetic phases with folding times, extrapolated to water, of $\tau \sim 10$ ms (major phase, 85% of molecules) and $\tau \sim 200$ ms (minor phase, 15% of molecules).[35,36] Correlating copper binding studies, with the small model peptide, supported that initial cofactor binding is fast ($\tau \sim 2.5$ ms) and thus not rate-limiting. Thus, in these latter experiments, active azurin formation follows pathway 1 (Figure 6), with rapid copper uptake before polypeptide folding. Upon comparison, introducing copper prior to protein folding results in much faster (>4000-fold) formation of active (i.e., the folded holo-form) azurin.[35,36]

Since copper induces structure in the model peptide upon binding,[11] we initially speculated that the corresponding C-terminal region in full-length azurin may act as a nucleation site that, upon copper coordination, would speed up the folding of azurin. However, the kinetic experiments above (neglecting the minor slower phase) show that the folding speed for azurin's polypeptide is not affected by the presence of bound copper in the unfolded state (Figure 6). This suggests that azurin's folding transition state does not involve the copper-binding loop connecting β-strands 7 and 8.

7. Does Zinc Play a Role in Azurin's Kinetic Folding Process?

Like copper, zinc remains bound upon azurin unfolding, but in contrast to copper, zinc does not coordinate Met-121 in the unfolded state complex[27] (Table 1). Time-resolved folding and unfolding experiments with zinc-azurin revealed that unfolding is a single-exponential process, whereas refolding is best fit with double-exponential functions (Figure 7B). The faster phase corresponds to 80% of the amplitude change, the slower phase to the remaining 20%, at all denaturant conditions studied.[33] The biphasic behavior is not due to the presence of a fraction apo-azurin since folding kinetics is detected at denaturant concentrations where the apo-form would not fold. Instead, we determined that the biphasic behavior is due to the unfolded state of zinc-substituted azurin being heterogeneous.[33] Since refolding of azurin in the presence of copper is also biexponential, as is copper binding to the model peptide, it appears as if metals (zinc and copper) can bind to the unfolded azurin polypeptide in two distinct ways. Metal coordination in the major population of unfolded molecules may include Cys-112, His-117, and Met-121 for copper (and Cys-112 and His-117 for zinc), resulting in the faster refolding rates. In the minor population of unfolded molecules, one of these ligands may be replaced by another residue or a solvent molecule, resulting in retardation of polypeptide refolding.[33]

In contrast to apo-azurin (Figure 7A), the semilogarithmic plot of zinc-azurin folding and unfolding rate constants versus denaturant concentration exhibits pronounced curvature in both folding and unfolding arms[33] (Figure 7B). In general, such behavior can be caused by transient aggregation and burst-phase intermediates and by movement of the transition state placement. In the case of zinc-substituted azurin, we could assign the curvature to movement of the transition state as a function of denaturant concentration. Taking into account the curvature, the folding time in water is ~20 ms for the major population of zinc-substituted azurin.[33] Thus, neglecting the minor slower phase, refolding of zinc-bound azurin proceeds in a two-state reaction with roughly the same polypeptide-folding speed in water as apo- and copper-bound azurin (i.e., τ values of around 10 ms). Taken together, copper and zinc do not speed up azurin folding in water, but their presence still affects the kinetic-folding reactions, as shown by the biphasic behavior, seen with both metals, as well as the gradual movement of the transition state in the presence of zinc.[33,35] We are currently addressing these observations in detail.

8. Is Azurin's Folding Speed Dictated by Topology Instead of by the Cofactor?

As mentioned, the prediction of apo-azurin's folding speed in water based on its native state contact order is 62 s^{-1}.[32]

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Table 2. Folding Speeds in Water (20−25 °C, pH 7) for Proteins with Greek Key β-Barrel Fold[33,a]

Greek key protein	folding speed in water (s^{-1})
Zn^{II}−azurin	50 (strong curvature)
Cu^{II}−azurin	100
wild-type apo-azurin	130
His117Gly apo-azurin	70
His46Gly apo-azurin	60
apo-pseduoazurin	2 (via intermediate)
Ig domain CD2d1	18
Ig domain TWIg18′	1.5
FnIII domain TNfn3	6.2
FnIII domain TNfn10	240 (strong curvature)

[a] For zinc- and copper-substituted azurin, the faster (major) phase is listed: Ig, immunoglobulin; FnIII, fibronectin type III.

There is currently no method for including the metal−ligand interactions in the CO calculation. Thus, we cannot derive true CO values for copper- and zinc-forms of azurin. Since only five additional interactions should be added, we speculate that, upon averaging over all interactions, the CO value for holo-azurin would be similar to that of apo-azurin. Extrapolated folding rates in water for apo-azurin, two point-mutated apo-azurin forms, zinc-substituted azurin (major phase), and azurin in the presence of excess of copper (major phase) all fall within the range 50−150 s^{-1}.[33] Since the thermodynamic stability of these species ranges between 19 and 52 kJ/mol (Table 1), the native state topology, and not thermodynamic stability or the presence of cofactor interactions, is the major determinant of azurin's folding speed in water.

In Table 2, we have summarized published folding speeds in water for proteins with the Greek key β-barrel fold including all azurin variants we have studied;[33] the observed folding rates for these proteins range between 1.5 and 240 s^{-1}. Most of these proteins fold by two-state kinetic mechanisms, but sometimes an intermediate is involved (i.e., pseudoazurin). For the two most thermodynamically stable proteins (neglecting copper-azurin since a full Chevron plot has not been determined), zinc-substituted azurin and TNfn10, the Chevron plots exhibits curvature that down-tune the folding speeds in water. We propose that there is a speed limit that can be modulated by transition state movement in some cases, with respect to the formation of β-barrels with Greek key topology. This speed limit is on the order of 5−10 ms,[33] which is highly reasonable in terms of the complexity of the Greek key β-barrel fold.

9. What Are the Implications of Our Azurin Work?

Despite the critical role of cofactor binding proteins (metalloproteins) in fundamental processes in vivo, little is known about the molecular mechanisms of metalloprotein biosynthesis and assembly.[37] To unravel how cofactors, in particular metals, may affect and direct protein-folding reactions, here I have reviewed our in vitro work on the interplay between metal−protein interactions and polypeptide folding in azurin, a blue-copper protein (Figure 2). There are several interesting implications that can be drawn from our results.

Copper is found to bind to unfolded azurin in vitro, and the metal ligands have been identified (Figures 3 and 4). In vivo, translation of gene messages into functional proteins should be rapid for efficient maintenance of cellular activities. We discovered that although polypeptide folding speed is not increased, active azurin forms many orders of magnitude faster when the cofactor is allowed to interact with the unfolded polypeptide, instead of with the folded protein (Figure 6). We therefore propose that binding of cofactors prior to polypeptide folding may be a method to ensure adequate formation of active cofactor binding biomolecules in vivo.

We note that, in the case of copper, such ions are almost nonexistent in their free form in the cytoplasm[38] since copper's redox properties may result in oxidative damage of proteins, lipids, and nucleic acids. Instead, the cellular copper concentration is strictly controlled, and most copper ions are delivered to their destinations by copper chaperones.[39,40] Three organelle-specific trafficking pathways for copper have been described in eukaryotes: Cox17 for mitochondrial delivery, CCS for cytosolic delivery to superoxide dismutase, and ATX1 for delivery to proteins in the secretory pathway.[38] Prokaryotes, like *P. aeruginosa*, lack intracellular compartments, and thus, organelle-specific carriers of metals may not be essential. However, a homologue of ATX1 (i.e., CopZ) has been described for enteric bacteria (e.g., *Enterococcus hirae*).[38] *E. hirae*, which is the best understood prokaryotic copper homeostasis system, regulates copper uptake, availability, and export through a cop operon.[41] The cop operon is composed of four structural genes which encode for a copper-responsive repressor CopY, a copper chaperone CopZ, and two copper ATPases, CopA and CopB.[41] It is quite feasible that *P. aeruginosa* may utilize a similar copper regulator system. Currently, little is known about copper incorporation in azurin in *P. aeruginosa* although it is believed that the polypeptide is transported to the periplasm before copper insertion.[30] The periplasm is not as reducing as the cytoplasm, posing the question as to which of Cu^{I} or Cu^{II} is inserted into azurin in *P. aeruginosa*. Since the zinc form of azurin is not a byproduct in vivo,[30] either the level of copper is higher than that of zinc in *P.aeruginosa*'s periplasm, or a copper chaperone is involved. We propose that if a copper chaperone is involved in vivo, such chaperone-mediated copper delivery is easier if azurin is unfolded with the copper ligands exposed. In folded azurin, the copper site is buried 7 Å below the surface and thus shielded from solvent and other proteins.[25] Our in vitro observations of tight and specific copper binding to unfolded azurin support this idea (Table 1, Figure 5).

Since azurin in various forms, as well as other structurally similar proteins, fold with roughly the same speed (Table

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2), there appears to be a speed limit for the formation of β-barrels with Greek key topology. This implies that there may be a unifying folding transition state for proteins with this structural motif. It was recently shown that 94% of all sandwichlike proteins, such as azurin and other β-barrel proteins, contain an invariant substructure consisting of two interlocked pairs of neighboring β-strands with eight hydrophobic positions conserved.[22] In azurin, the two interlocked pairs correspond to β-strands 3, 4, 6, and 7 (Figure 3). We speculate that azurin's folding transition state, and that of other β-sandwich proteins, involves interactions between the conserved residues in these four strands. Our most recent work has revealed that four of the eight structurally conserved residues form nativelike interactions in apo-azurin's folding transition state but have only minor effects on native state stability. The other four residues do not form nativelike interactions in the folding transition state; instead, they greatly stabilize the final structure (C. J. Wilson, PWS, unpublished data). These results suggest that the structural determinants found across all β-sandwich proteins are conserved for both energetic and kinetic reasons. The curvature in the zinc-azurin Chevron plot (Figure 7B) suggests that the metal, although not changing the height of the barrier, affects its broadness and thus participates in the folding transition state. We are currently investigating the structure of azurin's folding transition state in the presence and absence of metals using both experimental and theoretical approaches.

In a wider perspective, we hope extensions of our biophysical work on azurin may aid in the curing of diseases related to protein misfolding, which often involves β-sheet aggregation (e.g., amyloid and prion-protein diseases), and cofactor metabolism, which involves incorrect cofactor−protein interactions (e.g., Menkes syndrome and Wilson's disease). Notably, Cu^{II} and Zn^{II} ions have both been shown to induce aggregation of amyloid-forming peptides.[42] Finally, *P. aeruginosa* azurin was recently shown to interact with the tumor-suppressor gene product p53 and act as an anticancer agent in cell culture studies.[43] Thus, not only is *P. aeruginosa* azurin a superior model system for in vitro studies, but it also is a putative cancer drug candidate in itself.

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