Biological Relevance of Metal Binding before Protein Folding

Irina Pozdnyakova and Pernilla Wittung-Stafshede*

Department of Chemistry
Tulane University
New Orleans, Louisiana 70118

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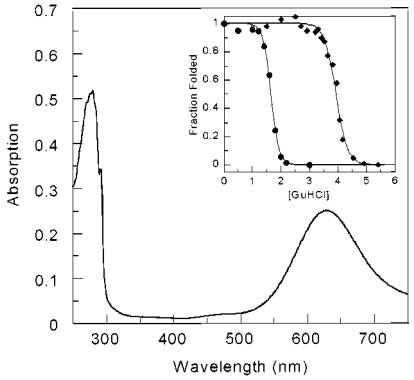
Many proteins in Nature require the binding of cofactors to perform their biological activity, and these molecules fold in a cellular environment where their cognate cofactors are present. Here we report that in the case of azurin (a copper protein mediating electron transfer), active protein is formed over 4 orders of magnitude faster if the cofactor is allowed to interact with the unfolded polypeptide as compared to binding after folding. Our discovery implies that binding of cofactors prior to polypeptide folding may be an efficient method for living systems to ensure rapid formation of functional biomolecules.

Although 30−40% of proteins in living systems bind cofactors such as metal ions to attain function, how and when cofactors become inserted into proteins are mostly unknown. It has been demonstrated in vitro that many proteins retain the interactions with the cofactors after polypeptide unfolding.[1] Therefore, it is possible that in vivo cofactors will bind to polypeptides before folding. Here we address the possibility that such behavior might result in a biological advantage. *Pseudomonas aeruginosa* azurin is a small blue-copper protein[2] with a Greek-key topology (Figure 1). The copper coordinates five protein residues in a unique geometry that gives rise to an intense absorption at 625 nm (Figure 2A). Crystal structures of apo- and holoazurin show that azurin adopts an identical (but nonfunctional) structure also without the cofactor,[2] but the holo form has higher thermodynamic stability (Figure 2A, inset).

The speed of active-azurin formation was compared for two different reaction pathways: (1) copper added to previously folded apoazurin and (2) copper added to the unfolded polypeptide at the initiation of refolding. In Figure 2B, we present the natural logarithm of the observed rate constants as a function of chemical-denaturant guanidine hydrochloride (GuHCl) concentration.[3] When copper is added (in excess) to folded apoazurin, metal incorporation is slow: time constant in water of 863 ± 5 s (∼14 min), corresponding to a second-order rate constant of ∼2.3 M^{-1} s^{-1}. (In comparison, a rate of 30 M^{-1} s^{-1} for copper binding to folded apoazurin in the presence of imdazole has been reported.[4])

Figure 1. Structure of azurin (pdb1azu) with copper and its ligands highlighted.



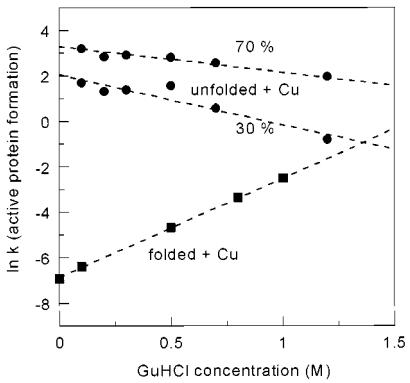


Figure 2. (A) Visible absorption of native (holo) azurin. (Inset) Equilibrium-unfolding transitions, induced by GuHCl additions, for apo (circles) and holo (diamonds) azurin. (B) Natural logarithm of pseudo-first-order rate constants for formation of active azurin, as a function of final GuHCl concentration. Folded apoazurin mixed with copper (squares); unfolded (in 3 M GuHCl) apoazurin mixed with refolding buffer containing copper (circles). The % amplitudes of the two kinetic phases found for the "unfolded + copper" case are indicated next to the data for each phase.

In sharp contrast, when copper is added to the unfolded protein, we detect rapid formation of active protein. The experimental kinetic traces are best fit to biexponential decays, with 70% of the total amplitude in a faster and 30% in a slower phase. Apoazurin folds in a single kinetic phase in the absence of copper,[5] confirming that it is the presence of copper that governs the biphasic kinetics. This suggests that copper interacts with the unfolded polypeptide in two different ways. The rate constants increase as the denaturant concentration is decreasing for both phases (negative slopes), in accord with folding being faster at more nativelike conditions. The fast and the slow fractions form

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(3) Equilibrium (far-UV CD detection) and kinetic (Applied Photophysics SX18MV stopped-flow) experiments performed at 20 °C, in 0.1 M Tris, pH 7. Formation of native azurin was probed by the absorbance increase at 625 nm upon stopped-flow mixing of copper ($CuSO_4$) and, either folded or unfolded, apoazurin; 1:50 ratio of protein to copper (10 μM final protein concentration). Linear extrapolation of data points yielded rate constants in absence of denaturant, i.e., in buffer, pH 7 (Santoro, M. M.; Bolen, D. W. *Nat. Struct. Biol.* **1988**, *6*, 825−831).

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active azurin in water with time constants of 42 ± 10 and 135 ± 20 ms, respectively.[6]

Strikingly, a functional blue-copper site can be formed 20 000-fold faster in vitro when copper is allowed to interact with the polypeptide before the structure has formed. The rigid structure of folded azurin may limit copper penetration to the active site, whereas the copper ligands are more exposed in the flexible, unfolded state. In accord, additions of small amounts of denaturant to folded apoazurin, not sufficient to globally unfold but enough to increase backbone dynamics, also increases the copper-binding speed.[7]

Only a few other studies targeting the effect of cofactors on folding have been reported. Calcium ions have been shown to stabilize RNase HI and staphylococcal nuclease A proteins by decreasing the unfolding speed.[8] In contrast, α-lactalbumin refolds more quickly in the presence of metals, but the metals have no effect on the unfolding speed.[9]

In a living eukaryotic cell, the pool of total free copper ions has been estimated to be only $\sim 10^{-18}$ M, which represents less than one atom of free copper per cell.[10] Instead, metallochaperones are suggested to be essential and act in intracellular trafficking, and delivery to target proteins, of copper in these cells (and perhaps also to some degree in prokaryotic cells). Three organelle-specific trafficking pathways for copper have been described in eukaryotes.[10] It is possible that copper delivery, believed to occur through direct physical interactions between the target and the chaperone proteins, may be facilitated more easily when the target protein is unfolded and the metal site exposed.

Translation of gene messages into proteins must be rapid for an efficient maintenance of cellular activities. Active azurin forms many orders of magnitude faster in vitro when the cofactor binds to the unfolded polypeptide instead of to the folded protein. We propose that coordination of cofactors (perhaps mediated by metallochaperone proteins) prior to polypeptide folding may be one of the methods that are used in Nature to ensure rapid formation of functional cofactor-binding biomolecules.

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(6) Formation rates for active azurin when starting from unfolded protein and copper were identical for 1:10, 1:50, and 1:100 protein-to-copper ratios, indicating that polypeptide folding, and not copper binding, is rate limiting.

(7) Note the positive slope (rate constants increase when denaturant concentration is increased) for "copper + folded apoazurin" kinetics in Figure 2B between 0 and 1 M GuHCl, denaturant conditions where the apoprotein remains fully folded (see inset, Figure 2A).

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