Copper-Triggered β-Hairpin Formation: Initiation Site for Azurin Folding?

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Despite the importance of β-sheet structures as regular secondary structure elements in proteins, the principles underlying their formation and stability are not well understood.[1] The β-hairpin is the simplest form of an antiparallel β-sheet and is defined as a turn region flanked by two strands with a defined backbone hydrogen-bonding pattern. In contrast to α-helices, which have been extensively studied using synthetic and natural peptides,[2] small β-hairpins are normally not thermodynamically stable.[3] The smallest peptide found to adopt a β-hairpin structure in solution is a nonamer derived from the protein tendamistat;[3b,e] a more stable β-hairpin was found with a 16-residue peptide that included three amino acid residues that formed a stabilizing hydrophobic core.[1a,3bc,4] Here we report that a stable β-hairpin structure is formed in a 13-residue peptide, derived from the blue-copper protein azurin, when copper ligation is used as the stabilizing factor.

Azurin is a 128-residue protein with a β-barrel structure that coordinates a redox-active copper.[5] The copper ion is coordinated by two histidines (His117 and His46; *Pseudomonas aeruginosa* azurin numbering) and one cysteine (Cys112) in a trigonal plane and two axial (Met121 and the carbonyl of Gly45) ligands. Upon azurin unfolding, induced by a chemical denaturant, the copper remains bound to the polypeptide[6] coordinated in a trigonal arrangement by one cysteine, one histidine, and a third, unknown ligand.[6c] The data suggest that at least two native ligands, Cys112 and His117, are involved in copper binding in unfolded azurin. These residues, and axial ligand Met121, are within a short stretch of the polypeptide that adopts a β-hairpin conformation (connecting β-strands 7 and 8) in native azurin.

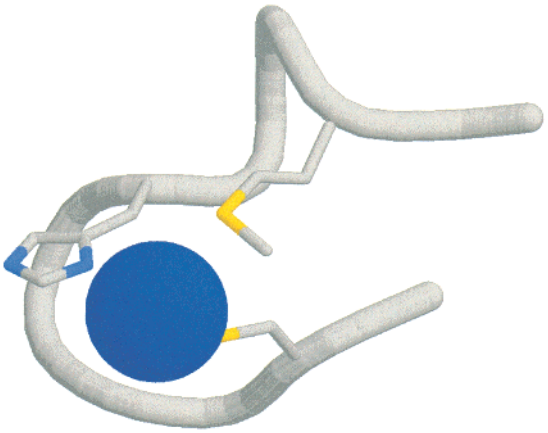

Figure 1. Residues 111−123 (FCTFPGHSALMKG) in *Pseudomonas aeruginosa* azurin (1AZU). Copper ligands (Cys112, His117, and Met121) and copper are highlighted.

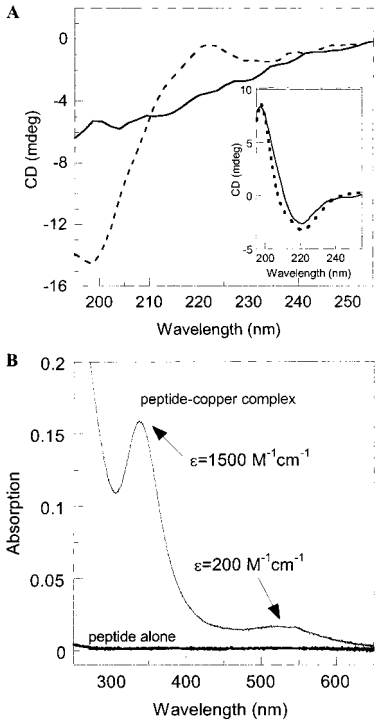

Figure 2. (A) Far-UV CD of peptide with (solid line) and without (dashed line) copper. (Inset) Difference CD spectrum: peptide−Cu complex minus peptide alone (solid line). A scaled spectrum of native azurin is also shown (dashed line). (B) Visible absorption upon adding copper to the peptide.

Any structural preference in the unfolded state of a protein will restrict the ensemble of conformations available to the peptide chain, and may function as an initiation site for folding. To test if ligation of copper to unfolded azurin induces local structure that acts as the nucleation point for folding, we studied a 13-residue peptide (FCTFPGHSALMKG) that corresponds to residues 111−123 in *Pseudomonas aeruginosa* azurin (Figure 1). The peptide includes the His117, Cys112, and Met121 copper ligands of native azurin. Nonrandom structure adopted by this peptide in isolation may also exist in the denatured state of full-length azurin.

As is expected for a short peptide, we find its conformation in aqueous solution to be that of random coil, deduced from its far-UV circular dichroism (CD) spectrum (Figure 2A).[7] However, upon addition of Cu^{2+}, the far-UV CD signal from the peptide

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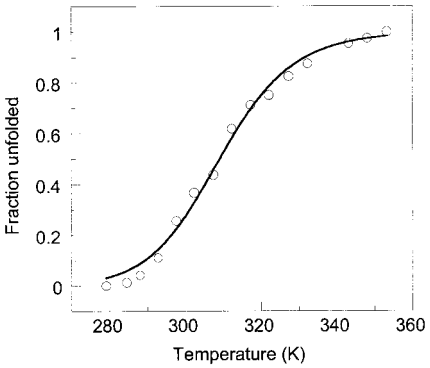

Figure 3. Thermal denaturation of the copper−peptide complex monitored by far-UV CD (217 nm). Both CD and absorption (340 nm) signals show identical cooperative transitions with T_m of 35 (±3) °C.

changes toward that characteristic of β-sheet, or β-hairpin, structure (Figure 2A). In parallel with secondary-structure development, absorption bands at 340 nm ($\epsilon = 1500$ $M^{-1}cm^{-1}$) and 530 nm ($\epsilon = 200$ $M^{-1}cm^{-1}$) appear (Figure 2B). The presence of absorption at these wavelengths suggest thiolate(S) → Cu(II) ligand-to-metal charge-transfer (LMCT) transitions.[8] The possibility of histidine(N) → Cu(II) charge-transfer transitions is ruled out as they appear at higher energies than 300 nm.[8] Addition of copper to a solution of L-cysteine (or L-histidine or L-methionine) alone does not result in absorption at wavelengths higher than 250 nm. It is likely that in the peptide−copper complex, copper coordinates all three native ligands in a defined geometry with good electronic overlap,[8] and therefore, visible transitions are allowed. In additional support of nativelike copper coordination is the observation of nativelike peptide structure upon copper binding (Figure 2A, inset). Titration reveals that copper coordinates to the peptide in an approximate 1:1 ratio.

A hallmark of proteins with defined native conformation is the presence of a cooperative unfolding transition. We monitored thermal denaturation of the copper-induced β-sheet structure in the peptide by far-UV CD and visible absorption. The copper−peptide complex displays a rather broad, but cooperative, unfolding transition independent of detection method (Figure 3). The midpoint of the CD-monitored transition (T_m) occurs at 308(±2) K (absorption detection gives T_m of 310 (±3) K). Our T_m is higher than that observed for the 16-residue β-hairpin,[1a,4b] but lower than that found for a 3-stranded β-sheet peptide.[1b] By using a two-state approximation, we estimated apparent thermodynamic parameters for the transition: $\Delta H(T_m) = 82$ (±5) kJ/mol and $\Delta S(T_m) = 266$ (±20) J/mol, K. Compared to data for the 16-residue β-hairpin,[1a,4b] our values are larger, in accord with a more disordered unfolded state for a shorter peptide (entropy), but more favorable native-state interactions due to the bound copper-ion (enthalpy).

The kinetics of copper-peptide complex formation was investigated in a stopped-flow mixer monitoring the appearance of the 340-nm absorption.[9] Under pseudo-first-order conditions, the formation time for the copper−peptide complex is 10−15 ms. The rate reports on interactions between copper and peptide−amino acids; however, to arrange the three ligands near the copper ion, the peptide has to adopt the β-hairpin structure. Visible-absorption development and peptide-structure formation thus must be strongly coupled processes. Laser-triggered temperature-jump experiments have shown that a small β-hairpin can fold in 6 μs.[1a] If diffusion limited, a peptide loop of 10−20 residues may form in a few μs.[10] If the azurin peptide adopts its β-hairpin structure this rapidly, the copper concentration must be very high (in the molar range) for peptide folding, and not copper-binding, to be rate limiting. For conditions with μM to mM (or less) copper concentration (such as in vivo), copper binding determines the rate of complex formation and variation in copper concentration may act as a structural switch.

Peptide fragments comprising the complete sequence of the blue-copper β-sheet protein plastocyanin have been examined.[11] All peptides displayed remarkably little propensities to adopt folded conformations in aqueous solution (copper was never added). This is in clear contrast to peptides derived from helical proteins; such peptides often display significant amount of helical preference in solution.[12] Monte Carlo simulations explained the plastocyanin results. On-lattice-trajectory calculations showed that for β-sheet proteins, such as plastocyanin and azurin, nativelike structural preferences are only required at one or two specific sites in the unfolded polypeptide chain to obtain correct folding.[13] The copper-induced β-hairpin of residues 111−123 in azurin may thus represent such a folding-initiation site (and a corresponding structure may form in unfolded plastocyanin upon copper addition).

In conclusion, a 13-residue peptide, derived from the β-barrel protein azurin, adopts a β-hairpin structure in solution upon copper ligation. Our results are important from at least three perspectives. First, in the case of the in vivo folding pathway of azurin, the region of azurin that corresponds to this peptide may act as the nucleation site that, upon copper coordination, directs (and possibly speeds up) the folding. Second, since many proteins in living systems bind metals or other ligands for specific functions, ligand−polypeptide interactions in unfolded states, generating nonrandom structures, may have importance for folding of a wide range of proteins. Finally, from a biotechnological viewpoint, our results imply that peptide-based conformational switches (sensors) using metals as triggers are within reach.

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(7) Experiments were performed in 5 mM phosphate, pH 7; peptide concentration normally 50−100 μM. Cu^{2+} was added as $CuSO_4$ (control experiments with SO_4^{2-} showed no effect). Absorption and far-UV CD were measured on Cary 50 and OLIS instruments, respectively. Thermal denaturation was monitored from 10 to 80 °C: CD (205−260 nm) or absorption (250−500 nm) monitored every 5 °C. The peptide−Cu complex did not aggregate since absorption and far-UV CD titrations showed linear dependence of complex concentration (10−400 μM), HPLC chromatography revealed similar elution times for peptide alone and peptide−Cu complex (1 mM) and, by electrospray mass spectroscopy, monomeric Cu−peptide species were detected (200 μM).

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