Role of Cofactors in Protein Folding

PERNILLA WITTUNG-STAFSHEDE*
Department of Chemistry, Tulane University,
New Orleans, Louisiana 70 118

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ABSTRACT

Although cofactors are essential components of many proteins to attain biological activity, the role of cofactors in protein folding is not well understood. Biophysical characterization of four types of cofactor-binding proteins (with copper, flavin moiety, iron−sulfur cluster, and heme cofactors, respectively) provides the following insights. (1) The presence of the cofactor often stabilizes the native protein. (2) The cofactor has the ability to interact specifically with the unfolded polypeptide. (3) The presence of the cofactor is sometimes essential for the polypeptide to fold. (4) Coordination of the cofactor prior to polypeptide folding can dramatically accelerate formation of the functional protein.

Introduction

With the sequencing of the human genome completed, the current focus is turned to protein structure and stability, protein−protein and protein−ligand interactions, relationships between structure and function, and how these relate to disease and drug discovery. Several diseases are related to misfolding and/or aggregation of proteins, most often with β-sheet structure, making a better understanding of protein folding highly desired. In vitro, proteins fold with widely differing kinetics and with mechanisms of varying complexity. While many large proteins (>100 residues) populate folding intermediates, smaller proteins (<100 residues) often fold directly to the native

state without kinetic intermediates.[1−3] For such small proteins, parameters such as sequence, size, thermodynamic stability, and topology may to various extents affect the protein-folding rates. In 1998, a statistically significant correlation between folding speed and native-state topology (described by the parameter relative contact order) was observed for a large number of small, unrelated proteins (lacking cofactors) folding by two-state kinetics.[4] 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. This finding was subsequently explained in terms of an extended nucleus with native-like topology in the transition state for folding.[5]

Many proteins (>30% of all proteins in living cells) require cofactors (metal ions, but also organic moieties) to perform their biological activities (such as electron transport, oxygen transport, metal transfer and storage, and catalysis). These proteins fold in a cellular environment where their cognate cofactors are present as free ions in the intracellular media or coordinated by "delivery/chaperone" proteins. It has been demonstrated in vitro that many cofactor-binding proteins have the ability to retain interactions with the cofactor after polypeptide unfolding, and also when no covalent bonds are linking the protein and the cofactor.[6−10] For example, coordination of the hemes in cytochrome b_{562} and myoglobin to the corresponding unfolded polypeptides has been observed.[6,9,11] In the case of the copper proteins azurin and the Cu_A domain,[12−14] the metals were found to remain associated with the unfolded polypeptides. Taken together, it is possible that in vivo cofactors interact with their corresponding proteins before polypeptide folding takes place and may, therefore, impact the folding reaction.

The conformation of an unfolded polypeptide (chemically denatured in vitro or after leaving the ribosome in vivo; note, however, that these unfolded states may be different) is not completely random-coil, although most secondary and tertiary structure is absent. Inherent structures preferred by various peptide sequences, and fluc-

Pernilla Wittung-Stafshede received a M.S. in chemical engineering in 1992 from Chalmers Institute of Technology (Gothenburg, Sweden). Thereafter, she conducted graduate work on peptide nucleic acids under the guidance of Bengt Norden (at Chalmers) and obtained a Ph.D. in physical chemistry in 1996. During 1997−1998, she was a Postdoctoral Scholar at Caltech, working with Harry B. Gray on laser-triggered protein folding. She joined the faculty at Tulane in 1999 and is currently an Assistant Professor of Chemistry and an Alfred P. Sloan Research Fellow. Most research projects in her laboratory focus on biophysical characterization of cofactor-binding and oligomeric proteins.

* E-mail: pernilla@tulane.edu.

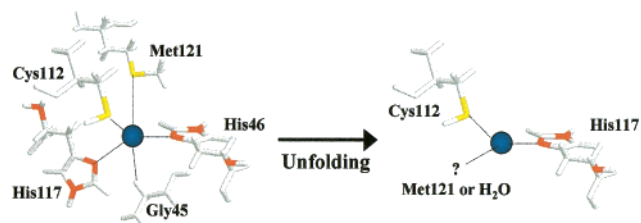**Table 1. Properties of the Cofactor-Binding Proteins Discussed in the Text**

protein	native topology	cofactor	binding motif
azurin	β	copper ion	Cys, His, His, Met, Gly
flavodoxin	α/β	flavin mononucleotide	Trp and Tyr π-stacking
ferredoxin	β	iron−sulfur clusters	Cys residues
cytochrome c_{553}	α	iron−porphyrin	His and Met
cytochrome f	β	iron−porphyrin	His and N-terminus

A

Azurin   Flavodoxin   Ferredoxin

B

Cytochrome c_{553} Cytochrome f
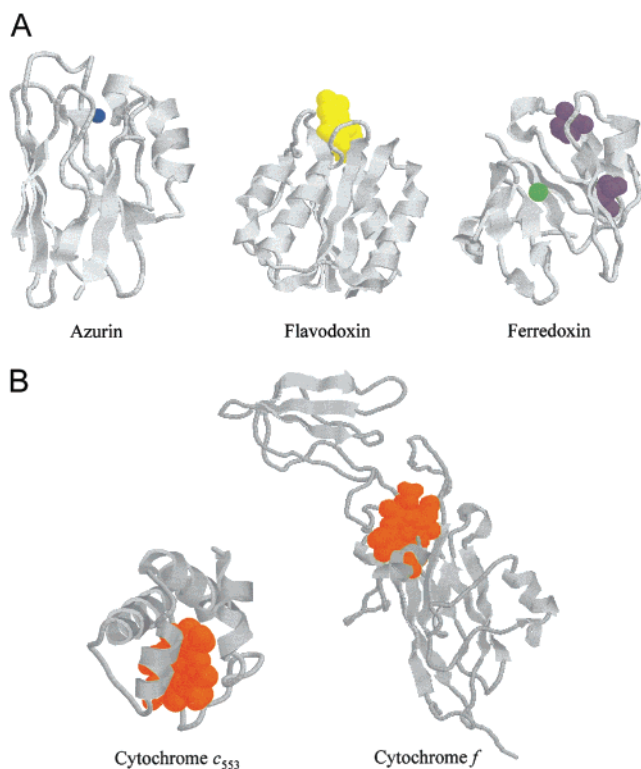
FIGURE 1. Cartoon drawings of the native states, with cofactors shown in space-filling representations, of the five proteins discussed in the text (1azu.pdb, f2x.pdb, 1dvh.pdb, 1cfm.pdb; ferredoxin was modeled using 1xer.pdb as template).

Met121
Cys112
His46
His117
Gly45
Unfolding
Cys112
His117
?
Met121 or H$_2$O

FIGURE 2. The five residues (Gly45, His46, Cys112, His117, and Met121) that form the copper-binding site in folded azurin (left) and the residues (His117, Cys112, and unknown ligand) proposed to be involved in copper coordination in the unfolded state (right).

tuating tertiary organization, may be present (extent may depend on solution conditions).[15] Local and nonlocal structure in the unfolded protein may also exist due to coordination of a cofactor. Such structural restriction may dramatically decrease the entropy of the unfolded state and therefore limit the conformational search for the native state.[15] The cofactor may in this way serve as a nucleation site that initiates, directs, and even accelerates polypeptide folding. It has been shown that cofactors often stabilize the native states of the proteins they interact with,[6,7,16,17] but the manner in which cofactors affect the folding pathway remains poorly understood. Most kinetic-folding studies to date have been conducted in the absence of potentially complicating ligands.

Some years ago, my research group began to address the roles of cofactors in protein-folding reactions using biophysical, biochemical, and molecular biology techniques. In this Account, I review our findings on four groups of cofactor-binding proteins (Table 1, Figure 1) in the context of three broad questions. First, do the cofactors bind to the unfolded polypeptides, and if so, how? Second, does the presence of the cofactors (in the unfolded state) affect the polypeptide-folding rates? Third,

what are the folding/unfolding mechanisms for these proteins, and how do the cofactors participate? The insights provided by our investigations aim toward establishing general roles for cofactors in protein-folding reactions.

Azurin with a Copper Ion

Copper plays a key role in all living organisms, serving as a cofactor for many proteins involved in electron transfer, oxidase and oxygenase activities, and detoxification of radicals. *Pseudomonas aeruginosa* azurin is a small (128 residues) blue-copper protein that facilitates electron transfer in bacterial respiratory chains, with a β-barrel structure arranged in a double-wound Greek-key topology[18,19] (Figure 1). A redox-active copper is coordinated by two histidine imidazoles (Histidine-46 and Histidine-117), one cysteine thiolate (Cysteine-112), and two weaker axial ligands, sulfur of methionine (Methionine-121) and carbonyl of glycine (Glycine-45) (Figure 2). The highly covalent nature of the copper−cysteine bond gives azurin an intense absorption at 630 nm. In vitro, azurin can bind many different metals in the active site; moreover, 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 cofactor.[19,20]

P. aeruginosa azurin with the copper oxidized is more thermodynamically stable than the reduced copper form, and both holo forms are more stable than apo-azurin (the oxidized cofactor stabilizes the native form by 23 kJ/mol as compared to the apo-form).[16,21] Upon unfolding of holo azurin, induced by the chemical denaturant guanidine hydrochloride (GuHCl), the copper remains bound to the unfolded polypeptide.[13] EXAFS and electrochemical experiments revealed a trigonal coordination of the copper in unfolded azurin; one copper ligand was shown to be Cysteine-112.[22] Our subsequent studies on two azurin mutants revealed that Histidine-117 (but not Histidine-46) is one of the other ligands in the unfolded state.[14] The
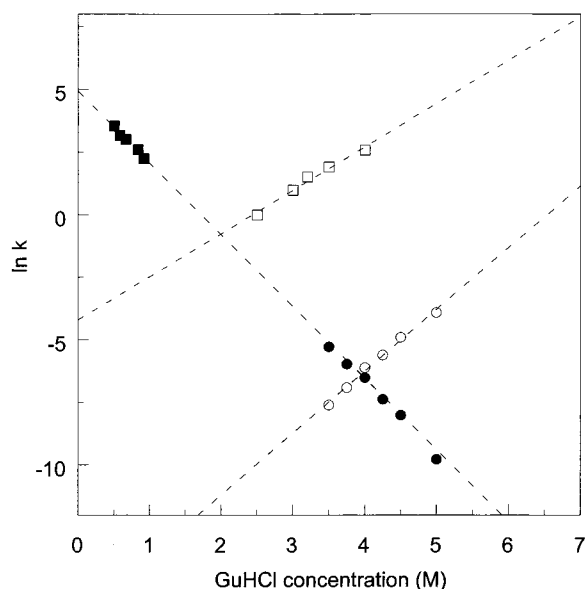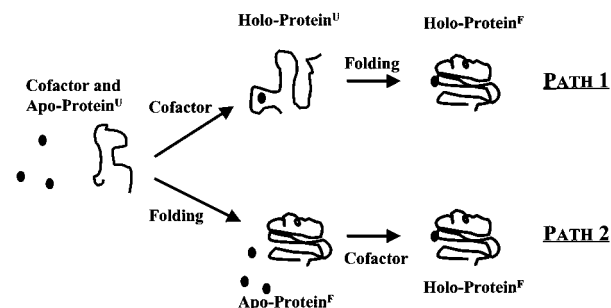
FIGURE 3. Natural logarithm of folding (solid) and unfolding (open) rate constants for holo- (circles) and apo-azurin (squares) as a function of GuHCl concentration.

FIGURE 4. Possible mechanisms in vivo for going from unfolded polypeptide and free cofactor to an active holo protein. Path 1: cofactor binding to unfolded polypeptide takes place before protein folding. Path 2: apo-protein folding precedes cofactor uptake.

third copper ligand in unfolded azurin, not yet confirmed, is believed to be the sulfur of Methionine-121 or a water oxygen (Figure 2). We recently showed that a small peptide comprising the C-terminal part of azurin, including the copper ligands Cysteine-112, Histidine-117, and Methionine-121, specifically binds copper in a 1:1 ratio. Upon copper coordination, the peptide acquires β-structure and an absorption band appears at ~340 nm.[8] The copper absorption in urea-unfolded holo-azurin agrees with that observed for the copper–peptide complex, suggesting similar metal coordinations.[14] On the basis of these findings, we proposed that the C-terminal segment, in the presence of copper, acts as a nucleation site for azurin folding.[8]

To address the role of the copper in the folding process, we compared folding and unfolding reactions for holo- and apo-azurin. In Figure 3, the natural logarithm of folding and unfolding rate constants for holo- (oxidized copper) and apo-azurin are presented as a function of denaturant concentration. To calculate the folding speed for holo-azurin at each denaturant concentration, we combined experimental unfolding rate constants and equilibrium constants.[23] The derived first-order rate constants are thus for azurin refolding with copper present in the unfolded state. Strikingly, the folding arm of the Chevron plot appears almost identical for the two proteins (folding time in water is ~10 ms for both apo and holo), whereas the unfolding arms differ dramatically.[23] Holo-azurin unfolds much slower than apo-azurin at all conditions (e.g., 10 000-fold difference in the unfolding rate constant at 3.5 M GuHCl). Thus, the mechanism by which copper increases azurin thermodynamic stability is solely based on slower unfolding.

We also probed the involvement of copper in the folding process of azurin by examining the speed of active (holo) azurin formation by considering two different reaction pathways:[24] (1) copper added to the unfolded polypeptide at the initiation of refolding and (2) copper added to previously folded apo-azurin (Figure 4). We found that the uptake of copper by folded apo-protein (path 2) is very slow (minutes). In sharp contrast, if copper is included in the refolding buffer, i.e., presented to the unfolded protein (path 1), formation of active (holo) azurin is 4000-fold faster (milliseconds).[24] Copper binding was shown to occur before the rate-limiting step, in accord with rapid binding to the unfolded polypeptide. The refolding rates for the azurin polypeptide in the presence and in the absence of copper do not differ significantly, indicating that copper binding to the unfolded state does not speed up (or slow down) the polypeptide-folding process. Nevertheless, to function in vivo, azurin must have copper in its active site. The active blue-copper site in azurin is formed more than 3 orders of magnitude faster when copper is allowed to interact with the polypeptide before the structure has formed. The rigidity of folded azurin may limit copper penetration to the active site, whereas the copper ligands are more exposed in the flexible, unfolded state.[24]

Flavodoxin with Organic Flavin Moiety

The flavodoxin-like motif is shared by nine superfamilies of proteins that exhibit little sequence similarity and comprise a broad range of proteins (like catalases, chemotactic proteins, lipases, esterases, and flavodoxins) with different functions. Still, they are all characterized by a folding motif consisting of one five-stranded parallel β-sheet and four α-helices (Figure 1). Flavodoxin aids photosynthetic electron transport and contains a redox-active flavin–mononucleotide (FMN) cofactor.[25-27]

The affinity of FMN to folded *Desulfovibrio desulfuricans* flavodoxin (148 residues) is high; the dissociation constant for oxidized FMN is ~0.1 nM.[28] Residues in two peptide loops form the major portion of the FMN-binding site, which consists of a combination of aromatic-stacking interactions, an apolar environment, and electrostatic interactions. In particular, the iso-alloxazine ring of the FMN is sandwiched between two aromatic residues (Tryptophan-60 and Tyrosine-98), allowing for considerable π-orbital overlap. Upon FMN removal, the apo-

protein adopts a structure identical to that of the holo form, except for more flexibility in the FMN-binding loop regions.

Equilibrium unfolding studies of holo (oxidized FMN) and apo *D. desulfuricans* flavodoxin (ATCC strain 27774) showed that the reactions are reversible, but unfolding curves differ when two spectroscopic probes are used.[25] We concluded that a native-like intermediate, with altered local structure near the tryptophan but retaining all native secondary structure, is present on the equilibrium unfolding pathway regardless of FMN presence. Equilibrium unfolding intermediates have also been observed with *Azotobacter vinelandii* apo-flavodoxin and the sequence-unrelated (apo) proteins CheY and cutinase that share the flavodoxin-like fold.[29] In contrast to the large effect on protein stability enforced by the presence of copper described above, the presence of FMN confers very little change in *D. desulfuricans* flavodoxin stability (<2 kJ/mol).[25] The affinity of FMN to the unfolded polypeptide must, therefore, be almost as high as that to the folded flavodoxin. Support for this prediction is given by the finding that FMN does not dissociate from the unfolded polypeptide.[25]

D. desulfuricans flavodoxin from ATCC strain 27774 displays 75.3% amino acid sequence similarity to the corresponding protein from ATCC strain 29577.[28] Equilibrium unfolding experiments with the latter protein show, again, that the holo protein is only somewhat more stable than the apo form (by ~6 kJ/mol) and that the FMN remains coordinated to the unfolded polypeptide (*Protein Sci.*, in press). The folding and unfolding kinetics for *D. desulfuricans* (strain 29577) holo-flavodoxin exhibits two-state behavior. The extrapolated folding time for holo-flavodoxin in water is ~280 μs. In contrast, the folding and unfolding kinetics for the apo form are not two-state. Two kinetic phases (with rates that differ ~20-fold) are observed throughout the denaturant range, suggesting the presence of a kinetic intermediate (*Protein Sci.*, in press). Notably, the folding pathway of CheY, another protein with the flavodoxin-like fold, was shown to include a kinetic intermediate.[30] Both refolding phases for apo-flavodoxin are orders of magnitudes slower than the refolding of holo-flavodoxin. We conclude that cofactor interactions with the unfolded protein alter the kinetic pathway and speed up folding of the flavodoxin polypeptide.

Ferredoxins with (Non-Heme) Iron Centers

The interconversion of iron−sulfur proteins between apo and holo forms, and the interconversion of iron−sulfur clusters of high and low nuclearity (i.e., between [4Fe-4S] and [2Fe-2S] forms), are effective mechanisms for organisms to deal with oxidative stress and changes in intracellular iron concentrations. Iron−sulfur proteins that are dependent on cluster disassembly and conversion include novel transcriptional and translational regulators.[31] In addition, a number of [4Fe-4S] proteins undergo reversible degradation to [2Fe-2S] forms in the presence of O₂,
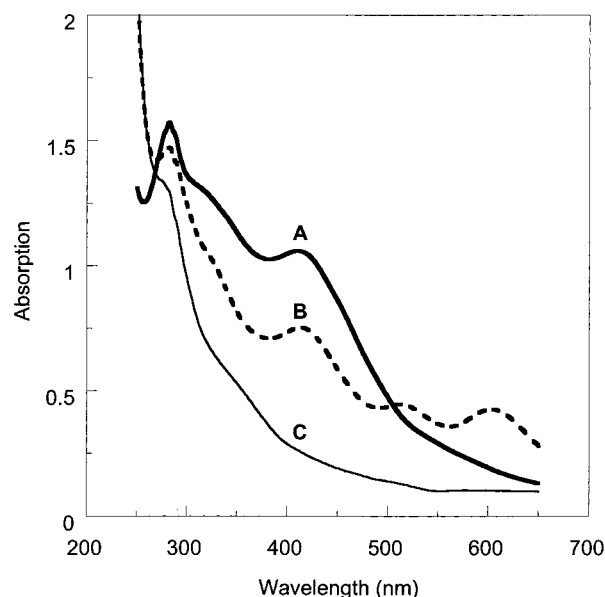
FIGURE 5. Visible absorption of folded ferredoxin (trace A), intermediate ferredoxin, i.e., protein unfolded for a few minutes (trace B), and fully denatured ferredoxin, i.e., unfolded protein incubated for >1 h (trace C).

possibly as a protective mechanism.[32] The mechanisms for hydrolysis of model [4Fe-4S] clusters, and [4Fe-4S]-containing proteins, have been characterized,[33] whereas interconversions of [3Fe-4S] clusters in proteins have been much less studied.

A good system for [3Fe-4S] cluster studies is the family of dicluster, seven-iron ferredoxins from archaea belonging to the order *Sulfolobales*.[34] These thermostable organisms live at low pH and temperatures around 70−80 °C. Their ferredoxins are small, monomeric proteins with mostly β-sheet structure and one [3Fe-4S]$^{+/0}$ and one [4Fe-4S]$^{2+/+}$ center. *Acidianus ambivalens* ferredoxin is one such protein (containing the two iron centers and a zinc cofactor; Figure 1), which we found to be highly resistant to both heat (T_m = 122 °C; pH 7) and chemical perturbation.[10] Lowering the pH dramatically decreased *A. ambivalens* ferredoxin stability ($T_m \approx 64$ °C at pH 2.5), suggesting that electrostatic interactions contribute favorably to the high stability at neutral pH. In accord, analysis of the 3D molecular model of the protein showed that there are several possible ion pairs on the surface and, in addition, the two iron−sulfur clusters and the zinc all coordinate deprotonated side chains.[35]

Denaturant addition to ferredoxin promotes conversion of the native brownish protein to a transient intermediate (black/purple) form with absorption features at 520 and 610 nm[10] (Figure 5). On the basis of comparisons with model complexes and the resemblance to beef heart aconitase at high pH,[36,37] this intermediate was suggested to incorporate linear [3Fe-4S] clusters.[10,35] Formation of the linear clusters occurred in parallel with the disappearance of the polypeptide's secondary structure, but the new clusters remained bound to the unfolded polypeptide.[10,35] A subsequent slower phase correlated with the conversion of the black/purple species into one lacking

color, presumably the unfolded protein from which the irons had dissociated.[10] In a wide range of conditions (pH 2.5−10, various denaturants and temperatures), the unfolding path for *A. ambivalens* ferredoxin involves the transient state in which the polypeptide coordinates rearranged, linear [3Fe-4S] iron clusters.[35]

Chemical and thermal unfolding processes (ultimately leading to cluster dissociation and degradation) are irreversible. It was shown for a [2Fe-2S] ferredoxin[38] that enzymatic cluster assembly and insertion drive the polypeptide to fold. Thus, without inserted iron−sulfur clusters, ferredoxins appear incapable of adopting their native structures. In vivo, therefore, cluster insertion may precede folding of the ferredoxin polypeptide. Desulfoferrodoxin, another non-heme iron protein with two different iron centers, was also found to unfold before the metals dissociated.[39] Only as long as the metals remained coordinated to the unfolded polypeptide was refolding of the protein possible.

Cytochromes with Heme Groups

C-type (characterized by covalent attachment of an iron−porphyrin at two cysteine residues) cytochromes are proteins implicated in electron-transfer processes in both eukaryotic and prokaryotic organisms.[40] Although the transfer of electrons is always achieved by passing from the oxidized to the reduced form of the iron in the heme, there is nevertheless a great diversity in the sequence and three-dimensional structure of these proteins (which are classified into four types[40]). The type I cytochromes *c* comprise the largest group and include mitochondrial cytochrome *c* and bacterial cytochrome c_2 among others. They are usually small, soluble proteins with helical structures. The single heme is covalently attached near the N-terminus, with histidine as the fifth ligand and methionine as the sixth ligand to the iron.[40] Studies on horse and yeast cytochrome *c* proteins have shown that non-native heme ligations in the unfolded state (by histidine, methionine, or the N-terminus) complicate and slow down the folding process at neutral pH.[41−43]

Cytochrome c_{553} from the sulfate-reducing *Desulfovibrio vulgaris* bacteria[44] (79 residues) retains the essential structural characteristics present in all type I cytochromes (Figure 1). In contrast to horse, yeast, and tuna cytochrome *c*, the heme iron in unfolded cytochrome c_{553} was found to be in a high-spin state at neutral pH (i.e., lacking a sixth ligand).[45] The kinetic traces for unfolding and refolding at pH 7 of oxidized and reduced cytochrome c_{553} were best fit by monoexponential decay equations, implying a two-state mechanism.[46] The folding time in water for oxidized cytochrome c_{553} is estimated to be less than 200 μs, and the reduced protein folds even faster (Figure 6). The folding speed for oxidized cytochrome c_{553} (pH 7) is 100-fold faster than that for cytochrome *c* at low pH[47] and at least 1000-fold faster than for other cytochrome *c* proteins at neutral pH.[48,49] Clearly, non-native heme ligations during, or prior to, folding can dramatically affect the folding landscape for c-type heme proteins.
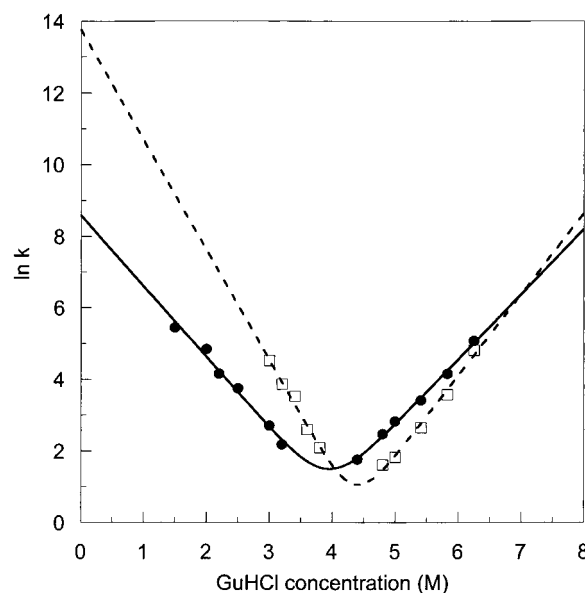
FIGURE 6. Natural logarithm of folding- and unfolding-rate constants for oxidized (solid circles) and reduced (open squares) cytochrome c_{553} as a function of GuHCl concentration. Solid and dashed lines are two-state fits.

Cytochrome *f* is a unique *c*-type heme protein, functioning in the cytochrome $b_6 f$ complex present in oxygenic photosynthetic organisms,[50] that is normally anchored in the membrane by a short C-terminal helix. Elimination of the membrane-anchoring helix produces a soluble protein that has no altered redox or spectral properties compared to the full-length protein.[51,52] The protein is divided into two domains, both with predominantly β-sheet structure (Figure 1). Soluble cytochrome *f* from the unicellular green algae *Chlamydomonas reinhardtii* (251 residues) has one heme covalently attached to Cysteine-21 and Cysteine-24 in the large domain. The reduction potential for the heme in *C. reinhardtii* cytochrome *f* is significantly higher than those in most other *c*-type cytochromes. This may be due to the unique heme coordination: the sixth axial iron ligand is the α-amino group of the N-terminus, a coordination not observed in any other heme protein.[50]

Chemically induced unfolding of oxidized and reduced cytochrome *f* exhibits apparent two-state behavior, despite the protein's large size and two-domain nature. Neither oxidized nor reduced unfolded cytochrome *f* can be refolded at neutral pH, but at pH 3.5 unfolding is fully reversible.[53] Reduced cytochrome *f* has much higher stability than the oxidized form (Figure 7). The heme in unfolded cytochrome *f* remains low-spin down to pH 4 but turns high-spin at pH 3.5, presumably due to protonation of the N-terminal amino group. This indicates that, as a result of the heme iron ligation, the pK_a for the α-amino group of the N-terminus is decreased from about 7.2 for a free N-terminus[54] to approximately 3.5. This observation suggests a plausible explanation, involving the cofactor, for the observed pH dependence of cytochrome *f* reversibility. At neutral pH, the presence of the interaction between the heme and the N-terminus in the
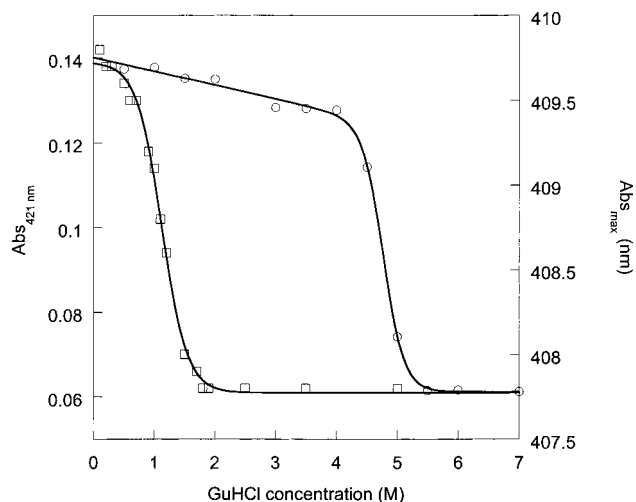
FIGURE 7. GuHCl-induced unfolding transitions at pH 7.0 for oxidized (squares) and reduced (circles) cytochrome *f*, as monitored by visible absorbance changes. The solid curves represent two-state fits.

unfolded state somehow blocks refolding (in parallel with heme misligations slowing down refolding of smaller, helical heme proteins), whereas at lower pH, the absence of this interaction allows for refolding.[53]

Topology versus Folding Speed for Proteins with Cofactors

Native-state topology has been demonstrated to be a crude determinant of folding rates for a large set of unrelated proteins (lacking cofactors) that fold by two-state mechanisms.[4] To date, it is not possible to account for cofactor interactions when calculating the contact order for a protein (only polypeptide contacts are included). Thus, the estimated contact order for a cofactor-binding protein will be that of the corresponding apo-protein. Despite this limitation, the contact order concept has been successfully used to predict the folding speed of two heme proteins: cytochrome b_{562} and cytochrome c_{553}.[11,46,55] In addition, the predicted folding speed of azurin based on its native-state topology was found to be in excellent agreement with the experimental folding rate constants for both apo and holo forms.[23] The topology-based prediction also matches the experimental folding speed for holo-flavodoxin but, interestingly, not the experimental folding speed for apo-flavodoxin (*Protein Sci.*, in press).

The almost perfect correlation between prediction based on native-state topology and experimental data for

cofactor-binding proteins we have studied (Table 2) suggests that cofactors, despite being coordinated to the unfolded proteins, do not significantly affect the intrinsic formation speed that is governed by each polypeptide's native-state topology. The contact order concept implies that the nature of the unfolded state should not affect the folding speed. In support, cytochrome c_{553} refolding reactions from denaturant- (random-coil structure) and methanol- (superhelical structure) induced non-native states were found to proceed with identical rates.[56]

Concluding Remarks

Research in my laboratory explores potential roles of cofactors (such as metal ions and clusters, organic and inorganic–organic moieties, etc.) in protein-folding reactions from thermodynamic as well as kinetic perspectives. Only a few other studies targeting the effect of cofactors on folding kinetics have been reported. For example (in agreement with our azurin results), calcium ions have been shown to stabilize RNase HI and staphylococcal nuclease A proteins by decreasing the unfolding speed.[17,57] In contrast (but in agreement with the flavodoxin data presented here), α-lactalbumin was shown to refold more quickly in the presence of metals, but the same metals had no effect on the unfolding speed.[58]

In living systems, functional (active) cofactor-binding proteins may form via one of two (simplified) reaction pathways (Figure 4): (1) the cofactor binds to the unfolded polypeptide prior to its folding or (2) the cofactor binds to the already folded apo-protein. Translation of gene messages into functional proteins must be rapid for an efficient response to cellular signals and for the maintenance of basic cellular activities. Thus, it appears important in vivo that the formation rates of proteins are sufficiently fast. Albeit polypeptide-folding speed is not increased, active (holo) azurin forms 3 orders of magnitude faster when the cofactor is allowed to interact with the unfolded polypeptide, instead of with the folded protein. Moreover, the presence of FMN in the unfolded state of flavodoxin speeds up folding of the polypeptide several orders of magnitude. We propose, therefore, that coordination of cofactors prior to polypeptide folding may be a relevant mechanistic pathway in Nature that has evolved to ensure rapid and efficient formation of active cofactor-binding biomolecules.

While no biological function for linear iron–sulfur clusters is known, our findings suggest they may be common intermediates after unfolding, or prior to folding,

Table 2. Experimental and Predicted (Based on Native-State Topology) Folding Rate Constants for Four Cofactor-Binding Proteins

protein	contact order prediction ln k_F	experimental rate constant ln k_F	cofactor redox state
cytochrome c_{553}	8.6	8.5	oxidized[a,b]
cytochrome b_{562}	11.5	12.0	oxidized and reduced[a]
azurin	4.1	4.9	oxidized and apo[c]
flavodoxin	8.5	8.2	oxidized[c,d]

[a] Apo form not investigated (since it does not adopt a native structure). [b] Reduced form folds faster. [c] Reduced form not investigated. [d] Apo form folds slower.

of various iron−sulfur-containing proteins. It appears reasonable to postulate that protein-mediated structural perturbations can be used in vivo to regulate iron−sulfur cluster rearrangements: such events may modulate or control various biological functions. In the same way as iron−sulfur clusters appear essential for folding of ferredoxins, heme insertion constitutes a critical step in the in vivo folding of most *c*-type cytochromes.[59] It is believed that the cytochrome polypeptide remains unfolded until heme has been covalently attached. Our in vitro results show that the subsequent folding process of the heme-linked polypeptide is strongly modulated by the presence or absence of heme ligands in the unfolded state.

To make general conclusions about the roles of cofactors in protein folding, many systems must be investigated. Nevertheless, our findings clearly show that cofactors can have significant effects on their corresponding proteins' folding and unfolding processes. Recent research has linked a number of human diseases (such as Menke's syndrome, Wilson's disease, Alzheimer's and prion diseases) to alterations in cofactor (in particular, metal) metabolism and trafficking pathways, processes all involving cofactor−protein interactions. These observations underscore the importance of understanding how cofactors interact with (folded and unfolded) proteins in living systems.

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