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Forum

Metalloprotein Folding

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One of the most intriguing problems in the biological sciences is the question of how to predict protein structure and function based on knowledge of a gene sequence. While significant progress has been made toward this goal during the past decade, fundamental challenges remain, such as understanding important post-translational modifications. One of the most critical post-translational modifications is the incorporation of a metal or metal cofactor into a catalytic or structural site in order to gain proper function. At the same time, some proteins function solely to recognize, transport, or sequester metals. When we consider how metals affect protein structure and function, we should not neglect their role in protein folding and stability as well as their participation in protein dynamics, as all are essential for optimal activity. Thus, workers in bioinorganic chemistry have an opportunity to be major contributors to the fields of protein folding and structure prediction.

This first *Inorganic Chemistry* Forum presents a series of modern investigations that address fundamental issues in metalloprotein folding that are essential for understanding the role of metals in biology. Metalloprotein folding in some cases is strongly coupled to metal binding. The ubiquitous zinc-finger proteins fall into this class. The zinc-finger proteins contain a zinc-binding motif consisting of four residues (Cys or His), and the binding of zinc or another transition metal is required for proper protein folding. Proteins in this family are typically multidomain biopolymers, with zinc-binding motifs joined by linker regions. The paper by Berg and co-workers ("Site Selection in Tandem Arrays of Metal-Binding Domains") explores the interesting possibility that metal binding to such multidomain proteins may occur through a number of modes. Binding may be exclusively in the canonical mode to individual zinc-binding domains, or binding may span domains, effectively incorporating the "linker" into the metal binding domain. Utilizing Co(II) as a substitute for Zn(II) in order to exploit the rich spectroscopic properties of this d^7 ion, this study shows that binding to the canonical sequence is thermodynamically preferred for zinc-binding peptides derived from the protein TFIIIA. In contrast, binding to the canonical sequence is not preferred for peptides derived from the protein TRAF despite the prediction from sequence homology. This work opens new areas in the study of multidomain binding proteins, and raises the interesting possibility of cooperative metal binding in some of the zinc-finger proteins.

Ghosh and Pecoraro in "Understanding Metalloprotein Folding Using a de Novo Design Strategy" have focused their folding efforts on de novo designed coiled coil proteins. The use of synthetic peptides allows investigators to examine a single facet of metalloprotein folding. In this case, a heptad repeat motif is utilized to prepare amphiphilic peptides that contain a single Cys residue along the hydrophobic face of an α-helix. When the peptide contains 30 amino acids, a stable three-stranded coiled coil is achieved in the absence of metal, whereas peptides containing only 23 amino acids remain unassociated until the addition of a nucleating metal. The authors describe the process of metal insertion into the prefolded system (which is thought to occur through breathing motions of the coiled coil) and contrast this process of metal complexation with a shorter peptide which requires the presence of metal to induce the desired peptide fold. Regardless of the starting peptide aggregate, the authors successfully produce a soft-metal binding site forming trigonal thiolato coordination geometry. This work demonstrates that, contrary to common belief, a preorganized metal binding site is not essential to stabilize suboptimal coordination geometries in metalloproteins. Detailed analyses of thermodynamics and kinetics of metal binding and folding of these systems have revealed how metal−ligand and

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peptide−peptide interactions together determine folded state structure and metal coordination structure.

An alternative design approach is described by Doerr and McLendon in "Design, Folding, and Activities of Metal-Assembled Coiled Coil Proteins". These authors make use of template-assembled synthetic proteins (TASP) for studying metalloprotein folding. In TASP, polypeptides are bound to a template; the template induces short-range interactions that promote and direct folding of the attached polypeptides. Metal−ligand interactions can be used as such a template, and the authors employ metal binding to bipyridyl groups covalently attached to amphiphilic peptides for this purpose. Introducing a six-coordinate metal ion then induces the formation of an octahedral complex and three-helix bundle. The range of stereochemical arrangements available to the bipyridyl groups leads to a range of possible structures, and exchange among these structures results in virtual combinatorial libraries. This work illustrates an important advantage of designed proteins, which is the facile use of chromophores and interactions not readily accessible in natural proteins. This approach also provides a means to address the challenging problem of screening and optimizing hydrophobic packing interactions. Finally, the introduced metal site provides functionality to the designed protein (an electron transfer site) and enriches the spectroscopic characterization of the system.

Azurin is a naturally occurring electron transfer protein that does not require a metal ion for folding. The fact that apoazurin has a structure very similar to that of the holoprotein, which binds a single Cu(I/II) in a blue (or type 1) copper site, raises an interesting question: Does azurin fold around a copper ion, or does copper bind after folding? In vivo, the answer is not known. In vitro, the answer is that both can happen, although formation of holoprotein occurs at quite different rates in the two cases. Wittung-Stafshede in her paper "Role of Cofactor in Folding of the Blue-Copper Protein Azurin" has shown that unfolded azurin binds copper, but this interaction does not influence polypeptide folding rates. Holoazurin formation, however, is significantly faster starting from copper-bound unfolded polypeptide than from unfolded polypeptide in the absence of copper. This difference in rates is a consequence of slow binding of copper to folded apoazurin. These studies have implications for our understanding of metal ion homeostasis in the cell. It is possible that copper binding to unfolded proteins such as azurin plays a role in intracellular copper transport.

As demonstrated by the cover art of this issue, the spectroscopically rich heme group has made the cytochrome *c* family a popular target for folding studies. For this reason, we have chosen three articles to represent this important area of metalloprotein folding. The folding and dynamics of proteins around hemes exhibit differences from the systems described above because the heme unit is a metal organic cofactor which can induce folding both through direct metal coordination and by hydrophobic contacts between the protein and porphyrin surface. In "Folding, Conformational Changes, and Dynamics of Cytochromes *c* Probed by NMR Spectroscopy", Bren and co-workers describe investigations of folding and dynamics of a number of proteins in the cytochrome *c* family. The readily detected hyperfine-shifted resonances in NMR spectra of paramagnetic cytochromes have been an important tool. The combination of high resolution and high information content of heme substituent resonances in paramagnetic systems has aided detection and analysis of non-native conformations of cytochromes *c*. For example, analysis of the resolved resonances of paramagnetic horse cytochrome *c* under mildly denaturing conditions allowed the detection of equilibrium unfolding intermediates. Heme resonance shift and line width analysis also revealed a novel fluxional motion of the heme axial Met in some bacterial cytochromes *c*. This surprising property is possibly linked to the high rigidity of the protein backbone surrounding the axial Met, as determined by hydrogen exchange and relaxation studies by NMR spectroscopy. Applying NMR techniques to investigate folding and dynamics of paramagnetic cytochromes *c* has thus elucidated phenomena difficult to observe using other biophysical techniques.

Protein folding contrasts with conventional chemical reactions in that the products are structurally well defined whereas the reactants are not. This presents a significant problem for understanding protein folding mechanisms. To address this problem, researchers have been making efforts to characterize denatured proteins as well as other non-native states of proteins. NMR spectroscopy has proven a useful tool in this endeavor. In "Insights into Partially Folded or Unfolded States of Metalloproteins from Nuclear Magnetic Resonance", Turano describes NMR approaches to characterize non-native states of metalloproteins. As an example, she demonstrates that high-resolution NMR data can be obtained for cytochrome *c* that is partially unfolded via interaction with SDS micelles. Proteins in the cell exist in a crowded environment, and their interactions with other molecules and membranes undoubtedly have a significant impact on their structures and folding. This work has important implications not only for the study of folding, but also for understanding physiological processes associated with protein unfolding such as membrane translocation. The extensive employment of heteronuclear methods for assignments has been important in this effort, as have studies of nuclear relaxation to probe polypeptide dynamics. In the case of paramagnetic metalloproteins, the hyperfine interaction introduces additional constraints, making metalloproteins particularly attractive subjects in these efforts.

Optical techniques have complemented NMR spectroscopy and provide a wealth of information on the dynamics of folding. In particular, phototriggers have been employed to elucidate the earliest events in cytochrome *c* folding while a variety of spectroscopic tools combined with heme metal substitution have enriched our knowledge of folding intermediates and population heterogeneity. In "Zinc-porphyrin Solvation in Folded and Unfolded States of Zn-cytochrome *c*", Gray, Winkler, and co-workers take advantage of the unique photophysical and redox properties of Zn-porphyrin to shed light on heme hydration in folded, unfolded, and partially folded states of Zn-cytochrome *c* (Zn-cyt *c*). Photoexcitation of Zn-cyt *c* creates a strongly reducing

porphyrin triplet state whose deactivation occurs via coupling to vibrational modes of the first solvent shell or by electron transfer to an exogenous redox reagent. Their measured isotope effects and lifetimes associated with folded (slow triplet decay) and unfolded (fast triplet decay) states reveal the extent of heme hydration in these conformations, and support a picture of a partially protected heme group in unfolded Zn-cyt *c* relative to a solvent-exposed model microperoxidase compound. In addition, the researchers observe variations in compact states for urea- and guanidinium-induced unfolded proteins, suggesting that folding pathways may be denaturant dependent. Here, the presence of a luminescent Zn-porphyrin covalently attached to the protein greatly facilitates the study of a range of non-native structures.

We hope that the combination of articles in this first *Inorganic Chemistry* Forum will convey to the broad inorganic community the importance and excitement of this developing interdisciplinary field. The field of metalloprotein folding will continue to offer challenges and opportunities for researchers into the future. The articles contained in this Forum provide an introduction to the field illustrating some of the major research objectives using a variety of systems and experimental approaches. Future advances in the field will rely upon new methods of interrogating the folding process both in the test tube and in the cell. Developing new methods that provide greater selectivity and sensitivity for the target protein and a broad range of time scales is essential. Clearly, a major challenge for investigators is relating processes observed in vitro with folding in vivo. For metalloproteins, this includes understanding how metal sites are assembled within the cell, and the vital role of intracellular metal tracking proteins called metallochaperones. While the studies reported herein using de novo designed peptides provide hints on folding pathways for trigonal thiolato systems that are reminiscent of copper chaperones, it is likely that folding in vivo, or under conditions mimicking the interior of the cell, provides further challenges because of system complexity and molecular crowding. Effective studies of folding in the cell thus will require development of methods that are highly sensitive, while also yielding signals specific for the target protein. Also important is the effort to understand metalloprotein and peptide conformational changes of biological importance. There has been a burst of interest in metalloproteins that undergo conformational switching in response to a change of conditions. Understanding how such conformational changes are linked to signal transduction remains an important challenge. Conformational changes in some metallopeptides, e.g., amyloid peptides, play an important role in a range of human diseases. The development of metallopeptides that undergo conformational changes in response to the presence of a metal, or to a change in metal oxidation state, may provide insight into the important problem of the roles of metals in conformational rearrangements in proteins and peptides. We believe that the reports in this special issue will encourage students and instructors to contribute to some of these emerging topics in bioinorganic chemistry.

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