apparent that conformational reorganization coupled to the ionization of the buried group is a major determinant of these pK_a values. Specifically, the creation of charge in hydrophobic environments can trigger a shift from the fully folded state to local or partially unfolded states in which the charge can gain access to water or to an environment where the charge can be solvated. These alternative conformational states are not normally populated owing to the large free energy difference between the alternative and fully-folded native states; however, the partially unfolded states can become the new ground state under pH conditions where the internal group is charged. If the ionization of an internal group promotes the transition to a new conformational state then its pK_a should be sensitive to the global thermodynamic stability (ΔG°) of the protein because this determines the energy gap between the ground and the alternative states. This was tested by measuring the pK_a of two internal Lys residues in variants of staphylococcal nuclease with thermodynamic stabilities ranging from 8.4 to 13.8 kcal/mol. The magnitude of the shift in the pK_a of the internal Lys residues was found to be sensitive to the ΔG° of the protein confirming that the pK_a values of these Lys residues are determined by the probability of structural reorganization more than by local dielectric properties of their microenvironments. These observations imply that structure-based pK_a calculations for buried groups and other electrostatic processes in hydrophobic environments require accurate treatment of conformational reorganization, which remains an extremely challenging proposition.

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Critical Role of a Glycine Residue in an Allosteric Switch

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Thermodynamic coupling in proteins is ubiquitous but mechanistically poorly understood, particularly for systems in which the coupling occurs over large distances. In the E. coli biotin repressor the coupling between homodimerization and allosteric effector, bio-5′-AMP, is −4.0 kcal/mol. The two coupled processes occur at sites separated by 33Å. Structural and thermodynamic studies indicate that the coupling is accompanied by disorder-to-order transitions at the two distant functional sites. Perturbations to the transition at the ligand binding site via alanine substitutions alter both ligand binding and coupled dimerization. Alanine substitutions in four loops in the dimerization surface yield a range of energetic consequences for BirA dimerization. For one of these variants, BirAG142A, the free energies of dimerization and corepressor binding are consistent with complete abolition of coupling. Structural studies of the variant indicate that the loss of coupling is accompanied by disruption of the disorder-to-order transitions at both functional surfaces. In this work the structural basis of coupling between the dimerization and ligand binding surfaces was further investigated by measuring the consequences of alanine substitutions distributed in three of the dimerization loops on corepressor binding. Isothermal titration calorimetry measurements indicate that, in contrast to BirAG142A, the variants, several of which dimerize very weakly, all bind to bio-5′-AMP with energetics indistinguishable from wild-type BirA. The results indicate that a single glycine residue serves as a switch for allosteric regulation of BirA.

Enzymes and Protein Dynamics II

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Zinc Inhibits Hedgehog Autoprocessing: Linking Zinc Deficiency with Hedgehog Activation

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Zinc is an essential metal with wide-ranging biological functions while Hedgehog (Hh) signaling plays crucial roles in both development and disease. Here we describe a mechanistic interaction between zinc and Hh signaling. Hh ligand, the upstream activator of Hh signaling, originates from Hh autoprocessing, in which Hh precursor protein undergoes self-cleavage and cholesterol modification. In vitro assay showed zinc inhibits Hh autoprocessing with IC50 of 2 μM. Solution NMR revealed that zinc interacts with active site residues of Hh autoprocessing domain while ITC indicated that the binding is driven mostly by enthalpy with 1:1 stoichiometry. We further demonstrated zinc inhibition of Hh autoprocessing extends to a cellular environment through cell culture studies. In normal physiology, zinc likely acts as a negative regulator of Hh autoprocessing and inhibits the generation of Hh ligand and Hh signaling. In many diseases, zinc deficiency and elevated level of Hh ligand co-exist, including prostate cancer, lung cancer, ovarian cancer and autism. Our data suggest a novel, causal relationship between zinc deficiency and the overproduction of Hh ligand: zinc deficiency likely enhances Hh autoprocessing and the production of Hh ligand, thereby activating Hh signaling in diseases.

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Probing Factor XIII Substrate Specificity for Fibrinogen αC (233-425) Using Maldi-Tof Mass Spectrometry and 2D 15N-1H HSQC NMR Spectroscopy

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In blood coagulation, the transglutaminase FXIII introduces covalent γ-glutamyl-ε-lysinyl crosslinks into the fibrin clot network. The C-terminal portion of the Fibrinogen Aα chain contains the αC region (221-610) that has a flexible αC-connector segment (221-391) and a globular αC domain (392-610). Reactive glutamine (Q) residues from the αC-connector can be covalently cross-linked by activated FXIII (FXIIIa) to reactive lysines (K) located in the C-terminal portion of the αC domain on another fibrin molecule. Studies have shown that αC based crosslinks help generate a clot that has more lateral aggregation, is stronger, and is more resistant to fibrinolysis. Fibrinogen αC (233-425) contains three reactive glutamines (Q237, Q328, Q366) that participate in cross-linking but in-depth kinetic information is not available on individual residues. A combination of mass spectrometry and NMR-based methods is being used to probe for FXIIIa substrate specificity for fibrinogen αC region (233-425). In the MALDI-TOF mass spectrometry assay, FXIIIa-catalyzed cross-linking between reactive glutamines on αC (233-425) and the lysine-mimic glycine ethylester (GEE) are monitored following chymotrypsin and Glu-C protease proteolytic digests. MALDI-TOF MS runs reveal that FXIIIa cross-links GEE to all three reactive glutamines Q237, Q328, and Q366. Q237 is cross-linked first followed by Q328 and Q366. Complementary 2D 15N-1H HSQC experiments show the incorporation of 15NH4Cl or 15N -GEE into three separate glutamines on αC (233-425) in the presence of FXIIIa.
Gel electrophoresis studies further reveal that the lysine mimic dansylcadaverine and the Q-containing peptide Dansyl-Ahx-α2-antiplasmin(1-15) can each be crosslinked into αC (233-425).
The new knowledge gained about Fibrinogen αC (233-425) and substrate reactivity may later be used to design therapeutic Aα fibrinogens that could influence fibrin clot character.

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Ligand Binding to Anion Binding Exosites as Prothrombin is Converted to Active Thrombin

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The blood coagulant thrombin is originally expressed as the zymogen prothrombin (ProT). Prothrombin contains the F1.2 (Gla + Kringle 1 + Kringle 2) domain and the inactive serine protease prethrombin-2 (PT2). Kringle 2 of F1.2 protects an immature anion binding exosite on ProT called pro-ABE II. An immature, but unprotected, pro-ABE I site also exists on ProT. As part of the zymogen activation process, the prothrombinase complex can cleave ProT after R271 and release F1.2 from PT2. Subsequent cleavage of PT2 at R320 helps to generate active thrombin. Both anion binding exosites become fully mature upon production of thrombin. In the current work, solution NMR studies have been used to characterize the binding of GpIbα (269-286, 15N-labeled L275, 15N-labeled D277) to (pro)-ABE II and the binding of PAR3 (44-56, 15N-labeled F47, 15N-labeled D54) to (pro)-ABE I. The residues that are 15N-labeled have previously been shown by X-ray crystallography to make extensive contact with the thrombin surface. This observation is further supported by 1D proton NMR line broadening studies on the ligand-thrombin complexes. 15N-1H HSQC NMR titrations demonstrate that GpIbα (269-286, 15N-labeled L275, 15N-labeled D277) does not bind to the pro-ABE II site on human ProT. Ligand binding affinity to ABE II then increases as ProT is converted to PT2 and then thrombin. By contrast, HSQC NMR titrations reveal that the pro-ABE I site on ProT can already bind PAR3 (44-56, 15N-labeled F47, 15N-labeled D54). More selective therapeutics may take advantage of this immature ABE I site. Binding affinity for PAR3 (44-56) further increases as active thrombin is produced. After the two mature exosites are generated, thrombin is well poised to regulate several coagulation and anti-coagulation events.