Chemically Controlled Protein Assembly: Techniques and Applications

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1. Introduction

The study of biological processes has driven the efforts of modern molecular biology to unravel the microscopic capabilities of natural systems. Intrinsic to the experimental analysis of these life-governing principles is the process of testing, replicating, and visualizing the underlying biological mechanisms. As such, interacting with the nanoscale machinery of life becomes an increasingly apparent challenge.1 The range of this pursuit spans from DNA to RNA to proteins. While the controlled assembly of nucleic acid structures is widely studied,2,3 there are a smaller number of studies on the development of methods that investigate and exploit protein assembly and protein—protein interactions.4,5 These ubiquitous natural phenomena form a central founda-

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we review the assembly of proteins under the control of small molecule chemical signals, the dramatic strides made in the refinement of synthetical CID tools, and the divergent directions this work has taken as the concept of dimerization has expanded to a larger notion of chemically induced proximity (CIP) and how CIP has been used as both an investigative and a therapeutic tool. In addition, the potential for CID to be used to direct the assembly of supramolecular protein structures will also be reviewed.

2. Chemically Induced Dimerization and Chemically Induced Proximity—Developing the Systems

Chemically induced dimerization is the controlled dimerization of a pair of proteins, via any one of a number of classes of dimerizers. The dimerizer acts to bring the two proteins together, and the induced dimerization can be used to increase the effective molarity of a protein at a certain cellular substructure, thus causing chemically induced proximity (CIP) of the two previously dispersed proteins (Figure 2). The increased effective concentration of the proteins may be used to activate or control a biological event. In Figure 2, the induced proximity of proteins A and B is mediated by a different protein, which has been fused to the proteins of interest. This is a common method for causing the dimerization of proteins. The result of dimerization is flexible, as it is directed by the domain(s) fused to the protein, which is being used to effect the dimerization: the result, for example, can be association of two dispersed cytoplasmic proteins or recruitment of a freely diffusible protein to the location held by another, such as a membrane surface, a cellular compartment, or a DNA/RNA binding site.

Although this review focuses on small molecule induced dimerization, it is important to briefly mention other species which can induce dimerization, including protein-based dimers. Physiologically, induced dimerization is particularly critical in transmembrane receptor signal transduction. A broad class of hormone receptors function by ligand-induced association; through the binding of a single hormone molecule to two copies of a receptor, they are brought together and thereby activated. Human growth hormone and granulocyte macrophage colony-stimulating factor are two examples. An added layer of complexity has been unearthed for the hormone erythropoietin and its receptor, for which hormone binding conformationally reorganizes a preassembled receptor to initiate signaling. The human interferon-γ has also been shown to induce receptor dimerization in solution and on cell surfaces. Nucleic acids are also used as inducers of dimerization in nature and have been exploited in the formation of nanostructures in vitro.
Combining a DNA binding domain (DBD) and a protein binding domain, the activation domain (AD), within one species has led to the creation of molecules which can be used to cause the dimerization of DNA and protein complexes. These species have been used as artificial transcriptional activators to control gene expression. The DBD recognizes and binds a particular DNA sequence, allowing specific gene activation, while the AD interacts with one or more components of the natural transcriptional machinery. A number of DBDs have been used for this purpose, including polyamides, peptides, and triplex forming oligonucleotides and peptide nucleic acids. While there is good understanding of the characteristics required for designing DBDs, research continues toward discovering suitable ADs. Induced dimerization of proteins has also been used to control gene expression and is reviewed in section 3.2.

Figure 2. Example of chemically induced proximity (CIP). In the absence of the dimerizer, the effective molarity of protein B is low, while in the presence of dimerizer, protein B is recruited to the cellular location of protein A, thus controlling or initiating a biological stimulus.

Figure 3. Initial demonstration of the CID concept. On the left, two FKBP monomers bind the bivalent drug FK1012. On the right, FKBP-TCR fusion proteins are dimerized by FK1012, initiating intracellular signaling. Figure reprinted from ref 25 with permission. Copyright 1993 AAAS.
2.1. Initial Report of Small Molecule Induced Dimerization

The concept of chemically induced dimerization initiated by a small molecule, rather than a hormone, and its first application was introduced in a landmark paper in 1993 by Schreiber, Crabtree, and co-workers. They demonstrated a means by which a synthetic molecule could reproduce the ability of natural systems to use proximity as an activation switch. A bivalent derivative of the tight-binding immunosuppressive drug FK506 was shown to reversibly dimerize its protein target, FK506 Binding Protein (FKBP, Figure 3). Most importantly, the bivalent ligand, FK1012, functioning as a dimerizer, could be used to drive biological function. By fusing FKBP to the proximity regulated T-cell receptor, they produced a system by which binding of FK1012 activated the endogenous signal transduction cascade (Figure 3).

2.2. Expanding the Chemically Induced Dimerization Toolkit

The 15 years since this original work has seen a myriad of investigations that have elucidated the basic principles governing CID systems and their utility. FKBP has proven to be a particularly flexible agent for inducing proximity and has been used for a number of investigative and therapeutic applications as well as mutated FKBPs and modified FK1012 ligands. In one example from Clackson et al., a FK1012 analogue was synthesized with a “bump” preventing binding to the wild type FKBP by steric interference. They then made an FKBP mutant (F36V) with a compensatory “hole” which allowed binding of the modified ligand with low nanomolar affinity. After synthesizing a dimerizer based on the new ligand (AP103), they showed that the system is functional both in vitro and in vivo.

A fascinating side effect of these FKBP-remodeling studies was the coincidental identification of an FKBP mutant (FM) that forms a stable dimer \( K_d = 30 \, \mu M \) in the absence of any ligand. Moreover, synthetic nonimmunosuppressive FKBP-binding ligands were able to fully reverse self-dimerization of FM, thus creating a ligand switching system for protein aggregate disassembly (Figure 4). This phenomenon was cleverly exploited as a trigger for pharmacologically regulated secretion. Critical to this method was the observation that fusion proteins with multiple copies of FM will form not simple dimers but higher-order aggregates. Thus, by linking four copies of FM to secreted peptide hormones such as human growth hormone or insulin, the translated fusion proteins will form large aggregates retained in the endoplasmic reticulum. Addition of the FM-binding ligand disaggregated the proteins and initiated hormone secretion. In another example of ligand remodeling, Koide et al. have prepared a library of cell-permeable heterodimeric small molecules using olefin metathesis. A representative ligand library was screened for molecules which were cell-permeable, and a number were shown to induce dimerization in intact cells.

2.3. Heterodimeric Dimerization

Homodimeric dimerizers have their most elegant or economical application in switching systems that dimerize a single fusion protein, such as the original construct described by Schreiber and co-workers. Such symmetric dimerizers can be used to dimerize nonequivalent fusion proteins as well. If two fusion proteins (X–A and X–B, where X represents the dimerization domain) are present in equal mixtures, and complex formation is governed by random assortment, 50% of the ligand-induced dimers should be heterodimeric (A–XX–B) with 25% of each homodimer also formed (A–XX–A and B–XX–B). Depending on the degree of amplification available in a cellular context, this degree of activation has typically been shown to be sufficient, provided that the homodimeric species do not produce a dominant-negative effect. Reliance on these probabilities is unsatisfying, and the ability to specifically produce only AB pairs is clearly the more precise and elegant route. In
principle, such a system could be ligand directed, generated by a bivalent ligand with two distinct protein binding targets or protein directed by engineering complementary mutations into the adjacent surface of the dimerized protein to promote heterodimeric pairs.

Schreiber and co-workers, choosing the former route, constructed a heterodimeric dimerizer from FK506 and cyclosporin, referred to as FKCsA (Figure 5), and demonstrated three distinct modes of selective intracellular signaling. The pro-apoptotic effects of FKCsA were tested in a construct that used a membrane-localized triple-copy FKBP to recruit multiple cyclophilin-Fas fusion proteins. Dimerizer-induced localization of the Fas intracellular domain at the inner membrane surface produced a concentration dependent reduction in secreted alkaline phosphatase activity, used as a marker of cell viability. Finally, FKCsA was shown to direct nuclear localization of a GFP-cyclophilin fusion protein via dimerization with an FKBP-GAL4 fusion target.

A second method developed for generating selective heterodimers exploits the natural product rapamycin, an immunosuppressant that also binds to FKBP. Rapamycin and FK506 both exert their biological effect by an unusual mechanism: after binding to FKBP, the combination of the protein and the ligand (but neither one alone) binds to and inhibits a second cellular protein target, calcineurin, a serine-threonine phosphatase critical to T-cell receptor signaling. FRAP is a gigantic protein, 289 kD and 2549 amino acids, an undesirable size for protein engineering. However, the rapamycin binding function can be localized to a mere 90 amino acid segment, a rapamycin binding fragment (FRB, 11 kDa) that preserves the full binding affinity of the complete protein. In light of these successes, the prominent role played by the immunophilins in the CID literature becomes clear: FKBP and FRB are particularly small (and therefore unobtrusive) and bind their ligands very tightly (sub nM), ideal traits for protein engineering.

For derivatives of rapamycin to be maximally useful in therapeutic dimerizer systems, the immunosuppressive activity needed to be neutralized, a process that had already been undertaken in the remodeling of cyclosporin A and its binding target cyclophilin, among other examples. Guided by the crystal structure of rapamycin in binary complex with FKBP and FRB, Schreiber and co-workers successfully designed an orthogonal rapamycin–FRB pair. Replacement and stereochemical inversion of the C16-OMe group in a native rapamycin produced conformationally distorted analogs—“rapalogs”—that bound wild-type FRB with up to 300-fold lower affinity (Figure 6). Given the substantial conformational change in the modified rapamycin, FRB remodeling by rational design was judged impractical. Rather, an iterative genetic screen that used a three-hybrid technique—another evolving adaptation of the CID principle (section 3.5)—was applied to select for mutations of FRB that restored binding to the altered ligand (Figure 6). The critical α-helix in FRB adjacent to the modified section of rapamycin was targeted for modification, and a triple mutant capable of binding the rapalog with nanomolar affinity was identified, completing a rapamycin-based orthogonal pair suitable for in vivo applications. Further rapalogs have been developed to permit orthogonal control of protein activity.

2.4. Affinity Modulation

Chemically induced dimerization consists of two binding events—the primary event, between the monomeric protein and the dimerizer, and the secondary event, between the monomer–dimerizer complex and another monomer. This secondary binding event has been used as a means to perturb the equilibrium of the first event, a notion referred to as affinity modulation. Conceptually, the protein–protein interactions introduced via dimerization, if favorable, can serve to amplify the binding strength of a ligand, akin to the mechanism by which the FK506-FKBP complex functions to inhibit calcineurin. Conversely, unfavorable protein–protein interactions could reduce binding affinity, potentially...
desirable as a means to reduce toxicity in selective molecular contexts (Figure 7A). In the larger context, multiple-target binding has been proposed as a universal technique for engineering therapeutic selectivity by creating ligands that exert their pharmaceutical effect only in the presence of specific combinations of protein targets. Crabtree and co-workers synthesized an FK506-peptide dimer and demonstrated the ability of FKBP-target interactions to enhance the affinity of an SH2 domain binding peptide by a factor of 3 or decrease it by a factor of 6.46,49

Figure 6. Schematic showing rapamycin with the position of modification (C16) highlighted and the experimental scheme to identify a rapalog-mutated FRB pair. The rapalogs were individually screened against a library of FRB mutants with increased expression from the reporter gene being used to identify matched rapalog-FRB mutant pairs. Figure elements reprinted from ref 44 with permission. Copyright 1997 National Academy of Sciences, U.S.A.

Figure 7. Concept of affinity modulation. (A) A bivalent drug, capable of binding either to the FKBP protein (gray semicircle) or to the DHFR target protein (gray rectangle) but not to both proteins simultaneously due to destabilizing protein-protein interactions. (B) Cell-selective activity of a bivalent drug. In cells that possess an abundant, high affinity FKBP protein, the bifunctional molecule will partition preferentially to bind to FKBP, leaving DHFR uninhibited. In cells lacking FKBP or with a protein which binds weakly to FKBP ligands, the bifunctional molecule will selectively partition to inhibit DHFR and elicit a cytotoxic response. Figures reprinted from ref 50 with permission. Copyright 2003 American Chemical Society.

The ability to harness unfavorable protein-protein interactions to generate ligand selectivity has been demonstrated in vivo. Wandless and co-workers exploited differential binding of FKBP to convert MTX into a plasmodium-selective DHFR inhibitor. Alone, MTX is completely unselective, with equivalent binding affinity for both plasmodium and human DHFR. A genome search revealed that P. falciparum possesses a single FKBP homologue (pfFKBP), which is present in the parasite cytoplasm at a 50-fold lower concentration than the level of hFKBP in human cells.
Moreover, the plasmodial enzyme was observed to bind SLF, a synthetic ligand for FKBP, with 14-fold lower affinity than did hFKBP. Wandless and co-workers thus synthesized an MTX-SLF heteroligand, reasoning that these in vivo differences in the prevalence and affinity of FKBP could effectively modulate the toxicity of MTX (Figure 7B). In vivo, MTX-SLF displayed weak cytotoxicity to MES-SA uterine cancer cells, with an IC₅₀ of 25 μM. This detoxification could be reversed, lowering the IC₅₀ 68-fold, by saturating the intracellular FKBP with 5 μM FK506-M, a nontoxic, monomeric FKBP-binding ligand. In contrast, when MTX-SLF was tested against live plasmodia, coadministration of the monomeric FKBP-binding ligand. In contrast, when MTX-SLF was tested against live plasmodia, coadministration of FK506-M had no effect on the IC₅₀ of 1.5 μM, yielding a parasite-selective therapeutic index of 16.7.50

2.5. Theoretical Principles Governing Dimerization

In order to better characterize the increasing number of developing practical applications for dimerizer-based systems, theoretical models describing dimerization have been derived and refined by several groups. All basic theoretical models of induced dimerization can be crudely described via the equilibrium expression shown in Scheme 1.

Perselos and DeLisi derived one of the earliest expressions for treating a mixture of protein and dimerizer, allowing for calculation of the fraction of dimerized protein present in the solution.51 However, this expression relied on the assumption that the total concentration of dimerizer was equal to the concentration of free dimerizer. Simply put, the dimerizer concentration must be much greater than the protein concentration. For dimerizer-based systems, where the aim is to maximize the fraction of P₂D complex, this assumption does not hold.

Hu and co-workers, while examining the DHFR-based dimerization system described in section 2.2, initially reported that the formation of P₂D did not correspond to a simple, noncooperative binding model that would be assumed for such a complex.51 In such a model, wherein each protein binds independently to the dimerizer, complex formation should build toward a maximum when the protein/dimerizer ratio equals 2, after which the addition of dimerizer should drive the equilibrium toward the binary complex. Although the data fit the noncooperative model up to the protein/dimerizer ratio of 2, the fraction of P₂D was generally unaffected even in the presence of a 50-fold excess of dimerizer. To explain this discrepancy, the concept of affinity modulation (cooperativity or Kₛ) was introduced into the theoretical description of dimerization (Scheme 2).

Given this equilibrium expression and the mass balance eqs 1 and 2,

\[ P_{tot} = [P] + [PD] + 2[P₂D] \]  

Scheme 1. Basic Dimer Equilibrium

\[ P + D \overset{K_a}{\rightleftharpoons} PD + P \overset{K_p}{\rightleftharpoons} P₂D \]

Scheme 2. Dimer Equilibrium Accounting for Binding Cooperativity

\[ P + D \overset{K_a}{\rightleftharpoons} PD + P \overset{K_p,K_a}{\rightleftharpoons} P₂D \]

expressions for the concentration of singly and doubly bound protein as well as free monomer can be derived (eqs 3–5).

\[ [P] = \frac{(1 + K_a[D]) + \sqrt{(1 + K_a[D])^2 + 4K_a^2K_p[D]}}{2K_aK_p[D]} \]  

(3)

\[ [PD] = K_a[P][D] \]  

(4)

\[ [P₂D] = K_p^2K_a[P]^2[D] \]  

(5)

With the theoretical model governing dimerization now taking into account binding cooperativity, the role of putative protein–protein interactions at the newly formed protein interface in the stability (or instability) of the non-native protein dimer could be calculated. However, Carlson et al. began to examine the effects of another critical component of the DHFR dimerization event—the ligand itself. Given the flexible nature of the linker tethering the functional parts of the dimerizer, it can be envisioned that such molecules are subject to a number of intramolecular interactions, some of which may render the dimerizer unable to bind its protein target. Through molecular modeling, gel filtration experiments, and NMR analysis, it was found that bis-MTX adopts a primarily folded state in solution, which limits the concentration of dimerizer available for binding.32 Given this realization, the theoretical treatment of dimerization can be amended to contain the equilibrium expression between active and inactive dimerizer, as shown in Scheme 3. Given this equilibrium expression and the mass balance eqs 1 and 2, a new expression for the concentration of dimer can be derived (eq 6):

\[ [P₂D] = \frac{K_p^2K_a[P]^2[D]}{(K_{eq} + 1)} \]  

(6)

While this treatment of the theoretical basis for dimer formation has illustrated the concepts of both protein cooperativity and ligand conformational equilibria, the model still requires an initial guess for certain concentrations followed by an iterative fit of the data to generate equilibrium constants. Whitesides and co-workers sought to simplify and generalize this model by allowing for the direct calculation of parameters of interest.52 Revisiting the equilibrium expression found in Scheme 2, and approaching the expressions using the monovalent ligand dissociation constant Kₛ rather than Kₚ, a new expression can be written as found in Scheme 4.

In this equilibrium expression, the apparent dissociation constants can be described in terms of the monovalent ligand dissociation constant as shown in eqs 7 and 8.

\[ D_{tot} = [D] + [PD] + [P₂D] \]  

Scheme 3. Dimer Equilibrium with Cooperativity and Ligand Behavior

\[ P + D \overset{K_a}{\rightleftharpoons} PD + P \overset{K_p,K_a}{\rightleftharpoons} P₂D \]

Scheme 4. Dimer Equilibrium Based on Apparent Dissociation Constants

\[ P + D \overset{K_a'}{\rightleftharpoons} PD + P \overset{K_p'}{\rightleftharpoons} P₂D \]
in this context, is a measure of all stabilizing or destabilizing procedures. An additional consideration is that cooperativity, cooperativity without the use of approximations or data fitting of evaluating the ligand dissociation constant and protein ligand conformational behavior, it remains an exact method one can estimate the cooperativity, $K_d$. Based on this equation, the maximum $D_p$ is dependent on $P_{tot}$, $K_d$, $K_c$, and $D_{tot}$. Given the values of $D_{p,max}$, $P_{tot}$, and $K_d$, one can estimate the cooperativity, $K_c$.

Although this model does not take into consideration ligand conformational behavior, it remains an exact method of evaluating the ligand dissociation constant and protein cooperativity without the use of approximations or data fitting procedures. An additional consideration is that cooperativity, in this context, is a measure of all stabilizing or destabilizing interactions, which can range from protein–protein or protein–ligand effects to entropic or solvation contributions. Overall, the further expansion of a theoretical understanding of protein dimerization and oligomerization will lead toward general and practical applications to chemical dimerizer design.

3. Exploiting the Chemically Induced Dimerization Toolkit

The inducible dimerization systems described above have been exploited as investigative tools for the selective activation of various cellular processes at the levels of signal transduction, gene transcriptional, and protein post-translational modification; they all rely on the ability of dimers to switchably colocalize their fusion protein partners. By CID-selective activation/localization, the function of known proteins is dissected; the biophysics and cellular roles are thereby elucidated. It has also been used to discern the identity of novel proteins when they are captured via their interaction with a dimerizer-like ligand and identified by the biological readout they induce.

3.1. Selective Activation of Transduction/Cascade Pathways

In early work, Schreiber and co-workers employed their novel FK1012 switching system as a means both to analyze the dynamics of Fas signaling in vitro and also to probe its role in T-cell biology in vivo. They also showed that Fas dimerization and subsequent signaling transduction was involved in apoptosis in human keratinocytes. However, this approach of inducing apoptosis through Fas signaling can depend on the developmental stage of the cell. Alternatively, activation of caspases via small molecule control results in activation of the apoptotic machinery and the resulting cell death. Induced aggregation of caspase-1 or caspase-3 fusion proteins by addition of a FK1012 analogue has been shown to cause apoptosis in target cells. While caspase-1 shows autotoxicity in the absence of the chemical dimerizer, capase-3 shows no such autotoxicity. The apoptosis was also shown to bypass the Bcl-xL checkpoint. Further, the dimerization and activation of caspase-3 has been shown to cause conditional cell ablation in transgenic mice. The transgenic mice had fusion caspase-3 proteins expressed in their liver, and injection of the dimerizer resulted in activation of caspase-3 and dose-dependent hepatoocyte ablation. One week after the application of the dimerizer, liver regeneration was measured and normal architecture was observed. In the absence of the dimerizer, no liver injury was detected. The production of a FKB-caspase-9 fusion protein and its subsequent selective activation with an FK1012 analog has illuminated the downstream events in the caspase-9 signaling cascade.

Fusion proteins and their induced dimerization have also been used to investigate the necessity of protein dimerization in other cellular cascade events, including the mitogen-activated protein kinase cascade activation of further downstream kinases. The role of oligomerization in signal transduction has been shown by the dimerization dependent activation of the Ras-GTP regulated protein kinase, Raf. In two separate studies, employing both the FK1012-FKBP and coumermycin—GyrB systems, dimerization has been shown to activate Raf kinase in vitro. Dimerization has also been shown to be important for the activation of G-protein coupled receptors, transmembrane proteins which are responsible for the activation of cellular signal transduction pathways. The importance of dimerization on the phosphorylation activity of the dsRNA-dependent protein kinase, PKR, has also been illustrated through the use of a CID system (Figure 8). In the natural system, PKR dimerizes through binding to ds-RNA, which leads to phosphorylation of the eukaryotic translation initiation factor elf2α and subsequent downregulation of protein expression (Figure 8-1). Removal of the N-terminal RNA binding domain resulted in inactive protein (Figure 8-2). Replacement of the RNA binding domain with GyrB and treatment of the cells with coumer-
mycin resulted in increased detected levels of phosphorylation and a concomitant decrease in protein expression (in this case, luciferase activity was decreased by 90% compared to that of cells not treated with coumermycin), suggesting dimerization is necessary for activation of the kinase domain (Figure 8-3).

3.2. Transcriptional Control

Gene activation via dimerization has also been demonstrated using an FK506–peptide conjugate which acts as a transcriptional coactivator. The synthesized dimerizer is able to act as a bridge between a FKBP-GAL4 fusion protein and the basal transcriptional machinery and, as such, was shown to coactivate the transcription of a mammalian promoter in vitro (Figure 9). In the absence of the FKBP-GAL4 fusion protein, no activation was observed. In a similar manner, the Mapp group has synthesized molecules which replace the peptide used in the above example with a small molecule which interacts with the natural transcriptional machinery. The approach has been used to effect transcriptional up-regulation in HeLa cells.

Figure 8. Dimerization-dependent phosphorylation of PKR. PKR (protein A) contains an N-terminal domain necessary for ds-RNA binding. Dimerization of the PKR results in phosphorylation and subsequent phosphorylation of eIF2α and downregulation of the luciferase expression (situation 1). Removal of the RNA binding domain (protein B) prevents dimerization and downstream events (situation 2). Replacement of the RNA binding domain with GyrB (protein C) and addition of coumermycin results in dimerization and reduction of luciferase activity (situation 3).

Figure 9. Artificial transcriptional coactivator. The FKBP–GAL4 fusion protein binds to GAL4 sites upstream of the core promoter site. The FK506–peptide dimerizer (shown as the stick figure and ribbon) acts as a bridge between the activator and the basal transcriptional apparatus, causing transcription of the promoter. Figure reprinted from ref 64 with permission. Copyright 1997 National Academy of Sciences, U.S.A.
Crabtree and co-workers have employed CID methods to great effect in exploring the timing and dynamic response of transcriptional activation. In one case, CID switching revealed the unusual persistence of transcription postactivation; in another, the ability of transcriptional activation to induce both binary (expected) and graded (unexpected) responses was observed.

### 3.3. Post-translational Control of Protein Structure and Function

Induced dimerization has also been used to exert control over both the levels of proteins within cells and the protein structure by post-translational methods by controlling protein splicing or by “rescuing” proteins from programmed degradation. Protein splicing is a naturally occurring phenomenon in which a portion of the protein, the intein, is excised and the two flanking sequences, the exteins, are ligated. By this process, a new protein is formed from the pro-protein and—as protein function is tightly linked to its structure—provides a mechanism for the control of protein activity. This intriguing method of post-translational control has been widely studied, and a large number of inteins have been discovered and unnatural inteins have been designed.

Furthermore, it has been discovered that reconstitution of inactive N- and C-terminal halves of an intein results in splicing together of the flanking extein sequences, referred to as trans-splicing. In their initial paper, Mootz and Muir exploited the concept of trans-splicing and used induced dimerization to cause the reconstitution of the two intein halves. The principle of the approach is shown in Figure 10. In the absence of rapamycin, no splicing was observed; however, upon addition of the small molecule dimerizer, product was detected within 10 min. They have further shown the application of this system in living mammalian cells and Drosophila melanogaster, where feeding transgenic flies rapamycin resulted in the formation of full length luciferase, as determined by Western blotting. Flies fed dimethylsulfoxide (DMSO) showed similar luminescence to nontransgenic flies.

While there are many ways to deactivate proteins, from small-molecule inhibitors to gene deletions, to transcriptional/translational repression, no existing method matches the target-independent, rapidly reversible, pharmaceutically controlled characteristics that would be possible with a CID-driven system. Intriguingly, Crabtree and co-workers have described a partial solution for CID-switchable protein deactivation, a longstanding unsolved problem. This approach uses a dimerizer to maintain a fusion protein in active form; in the absence of the dimerizer, the fusion protein is proteolytically degraded (Figure 11). The switching method relies upon a destabilized variant of the rapamycin binding protein, referred to as FRB*, that is inert when dimerized by a nontoxic rapalog but, when unbound, induces proteolytic degradation of the entire fusion protein. In addition to a detailed characterization of this effect, screening experiments with eight distinct FRB* fusion proteins revealed a dynamic range of \(3.5\times100\) for CID-reversible destabilization.

Finally, rapalog-mediated recovery of a test-FRB* fusion was demonstrated in a knock-in mouse model, verifying the potential in vivo utility of this technique. It was further shown that the recruitment of natural FKBP was required to help prevent the protein degradation and that formation of the ternary complex was required to offer full stabilization. The technique has been expanded where the rapalog mediated dimerization of FRB* and FKBP is not required for the protection from degradation; simply binding of a rapalog to a FKBP analogue prevents the degradation.

Recently, Pratt et al. have combined the above two concepts of protein splicing and dimerization controlled stabilization of proteins, which would otherwise be subject to degradation by the proteasome. The system is called split ubiquitin for the rescue of function (SURF) and is shown schematically in Figure 12. The protein of interest is fused to a “degron”, which would cause the fusion protein to be degraded by the cellular proteasome. In the fusion protein,
a FRB and the C-terminal fragment of ubiquitin are placed between the protein of interest and the degron. Addition of rapamycin causes the protein to dimerize with another fusion protein consisting of FKBP and the N-terminal ubiquitin fragment. The dimerization causes the reconstitution of ubiquitin, and a protease acts on the complex, releasing the protein of interest and rescuing it from proteasomal degradation. The system has been shown to work with three different classes of proteins: proteases (caspase-3), kinases (v-Src), and translation factors (Smad 3).78

Induced dimerization has also been used to control protein levels using small molecule proteolysis targeting chimeras (PROTACs). The PROTACs minimally consist of a ligand for the target protein and a peptide (or recently a nonpeptidic79) ligand for E3 ligase (part of the SCFβ-TrCP complex). Dimerization of the target protein with the ligase causes specific ubiquitination of the target protein and hence results in protein degradation by the 26S proteasome.80 The PROTAC system has been shown to provide control of protein levels in vitro and in vivo.81,82

The Bertozzi lab demonstrated the use of the rapamycin CID system to investigate post-translational modification.7,83,84 This intriguing strategy capitalized on two aspects of biosynthetic modularity and harnessed the CID mechanism with particular elegance. Controlled cellular localization of enzymes serves as an important functional regulator, demonstrated here by the requirement that glycosyl- and sulfotransferases must reside in the Golgi to function. Second, many of the key enzymes involved in post-translational modification have functionally independent localization (Loc) and catalytic (Cat) domains. Collectively, these observations suggest a method in which glycosylation is chemically regulated by controlling the catalytic domain’s access to its proper position in the cell.

Bertozzi and co-workers thus constructed FKBP-Loc fusion proteins that resided inertly in the Golgi, and FRB-Cat fusions that were catalytically active but secreted from the cell (Figure 13). In the first demonstration, human α-1,3-fucosyltransferase VII activity was regulated to control production of sialyl Lewis X.83 Addition of rapamycin successfully dimerized the Golgi-localized FKBP-Loc domain and newly synthesized FRB-Cat, capturing the catalytic domain and producing a dose-dependent restoration of enzyme activity in the native Golgi environment. The generalizability of this technique has been verified with regulation of sulfotransferase activity by the same method, as well as the demonstration that heterologous partners—localization and catalytic domains from different enzymes—can also be effectively activated.7 Cornish, Bertozzi, and co-workers have used a similar system to describe the glyco-
slation by fucosyltransferase VII in eukaryotic cells using a “biocompatible” dimerizer. The ligand is comprised of trimethoprim (Tmp) and SLF and, as such, is capable of causing the dimerization of bacterial DHFR and FKBP. The ligand replaces the mTor-binding component of previous rapamycin analogue dimerizers with Tmp. Tmp has no endogenous substrates in eukaryotic cells, unlike the Rapamycin-like dimerizers that have been shown to cause off-target interactions and undesirable side effects in vivo. SLF is an analogue of FK506 which lacks immunosuppressive activity while retaining high affinity for FKBP. The Tmp-SLF ligand was shown to cause dimerization in a yeast-three hybrid system. Further, the system was shown to induce glycosylation in CHO cells, as measured by the presence of cell surface sialyl Lewis X.

3.4. Proximity Sensing

While the dimerized proteins are normally held together by solely noncovalent interactions between the protein and the ligand (favorable interactions may also occur between the dimerized proteins), some dimerizers have been developed which lead to the covalent irreversible cross-linking of the dimerized pair. Chemical inducers of hemovalent dimerization have been developed for cross-linking $O^6$-alkylguanine–DNA alkyltransferase fusion proteins. The mechanism of human $O^6$-alkylguanine alkyltransferase (hAGT), a DNA repair enzyme which catalyzes irreversible transfer of $O^6$-Gua alkyl groups to an internal cysteine residue, has been exploited by synthesizing $O^6$-benzylguanine–methotrexate ($O^6$-Bn-G-MTX) conjugates (Figures 1A and 14). Human AGT reacts with soluble $O^6$-Bn-G derivatives with reasonable efficiency and little selectivity, resulting in covalent attachment of the G-MTX dimerizer to a fusion protein of interest. Subsequent recruitment of DHFR fusion proteins by MTX completes the activation process. This method has been extended to covalently label a number of AGT fusion proteins, such as the human estrogen receptor (hERα) in vitro and in vivo. Fluorescent labeling of the AGT-hERα fusion protein in HeLa cells showed hERα

**Figure 13.** (A) Structure of the carbohydrate sulfotransferases showing the Loc and Cat domains containing the transmembrane and catalytic domains, respectively. (B) Induced colocalization of the Loc and Cat domains. The Loc and Cat domains were separated and fused with FKBP and FRB, respectively. Separation of the domains causes the excretion of the Cat domain fusion. Addition of rapamycin induces association of FKBP and FRB, resulting in the localization of the Cat domain in the Golgi, where it encounters the substrate and rescue of catalytic activity. Reprinted with permission from ref 7. Copyright 2004 National Academy of Sciences, U.S.A.

**Figure 14.** Dimerization using ($O^6$-Bn-G-MTX) conjugates. The hAGT fusion protein reacts with the $O^6$-Bn-G portion of the dimerizer, leading to a protein with a covalently bound MTX which can then recruit a DHFR-fusion protein.
distributed throughout the nucleoplasm but excluded from nucleoli. This approach has been used to sense the proximity of the fusion proteins.

3.5. Bioscreening

Since the first demonstration of the yeast two-hybrid system as a genetic screen for protein–protein interactions, the power of this technique has driven tremendous scientific inquiry. Among these second generation methods is the three-hybrid system, in which the fundamental protein–protein recognition event of the two-hybrid screen is replaced by a protein–ligand–protein assembly. This, of course, is the fundamental event of chemically induced dimerization. Here, it is reconfigured so that a heterodimeric bivalent ligand binds one known target and one unknown, if present, plucked from a vast genetic library to activate a switch (Figure 15). The system is based on the reconstitution of a transcriptional activator from a DNA binding domain and an activation domain by the dimerization of the two proteins via the heterodimeric ligand, a system analogous to that used for transcriptional control (section 3.2).

In their pioneering demonstration, Licitra and Liu synthesized a dexamethasone-FK506 dimeric ligand, a DNA-binding-domain-glucocorticoid receptor fusion, and showed that FKBP12 could be recovered from a library of activation-domain coupled sequences. Successful applications of the three-hybrid method have been reviewed.

An intriguing adaptation of the three-hybrid methodology is the technique of chemical complementation, described by Cornish and co-workers (Figure 16). The objective of this method is a high-throughput genetic screen for enzymatic catalysis of specific bond-making or bond-breaking reactions. In the forward direction, the heterodimerizer begins the experiment in two pieces. Each fragment has functional groups required for the reaction being studied. Enzymatically catalyzed ligation of the two free ends reconstitutes a working heterodimerizer and activates the readout. With a dexamethasone–MTX dimerizer pair, Cornish and co-workers have successfully demonstrated both versions of the chemical complementation assay. The method has also been used to identify an enzyme with 5-fold enhanced glycosynthase activity from a pool of point mutants. Further studies expanded the ability of the chemical complementation system to link bond cleavage with cell survival in yeast. The system was used to identify cellulases for the conversion of biomass to ethanol. After 8 days of selection, a cellulose variant with a 6-fold increase in $k_{cat}/K_M$ over the parent enzyme was isolated. Chemical complementation has been shown to be adaptable to different enzymatic systems, suggesting a role in the future in discovering new enzymes for important chemical transformations.

4. Application of Chemically Induced Proximity to Therapeutics

While the development of chemically induced dimerization as an investigative tool has yielded great insights into protein structure and function, the application of these tools toward pharmacological problems remains a high priority. A natural extension of the techniques explored thus far, current CID technology has focused primarily on gene expression and signal transduction. However, interesting and exciting new paths for CID-based therapies are emerging in the modification of endogenous protein–protein interactions based on small molecule induction. From a pharmaceutical standpoint, CID systems are advantageous in this regard, since the utilization of small molecules or drugs is highly desirable in many cases.

Modifications to the CID systems as described in section 2.2 are useful in the difficult problem of devising a system that is transparent to other functional machinery at a cellular level.
and organismic level. The challenges here differ for CID implementations in therapeutic versus investigational contexts. If the protein used as the CID switch is native to the organism, the risk of inhibiting its endogenous function must be considered. Reciprocally, the sequestration of the dimerizer by intrinsic cellular proteins could prevent the activation of the engineered switch. In contrast, non-native switching proteins, while potentially free of the dual interference problem, raise the risk of immunogenicity in a therapeutic context. Immunological responses to a foreign protein are clearly incompatible with the long-term viability of an introduced cellular switch.

To clarify, the discussed coumermycin-based dimerization represents an example of a system utilizing the bacterial protein GyrB, raising concerns about immunogenicity, whereas the FKBP–FRB systems are less encumbered by this, as they employ proteins of human origin. However, potential targets for an immune response still exist, such as the point mutations introduced to induce specificity for bumped dimersizers (such as the F36V mutation) or the junctions created between FKBP or FRB and the protein of interest. In any case, as the refinement and development of serum-stable, protein-based treatments advances, the opportunities for CID-based therapies will only increase.

4.1. Induced Signal Transduction and Gene Expression

Over the past decades, an increase in the understanding of cellular signaling pathways and gene expression has led to an emphasis on the control of cellular machinery and the utilization of endogenous cellular defenses to combat pathogenic processes. Biomedical efforts to exploit chemical inducers of dimerization as switching systems have advanced most swiftly in this field, an arena of science with vast therapeutic promise. One major advantage of the conditionality inherent to CID systems is circumvention of the problems related to “off-target effects”, which result from the failings of nonconditional therapeutics to meet the physiologically dictated thresholds for specificity of a drug for its target and the drug target in the pathogenesis of the treated disease. A comprehensive review of this topic has been published elsewhere.95 While clinical implementation of cell-based medicine is widespread, spanning a range from the transfusion of erythrocytes to bone marrow organ transplantation, our scientific ability to fine-tune the effects of these therapies is limited. To further realize the vast therapeutic potential of genetic intervention, novel mechanisms that can regulate or fine-tune these complex biological tools will be required.

By 2001, just eight years after their initial description, a number of studies had demonstrated the utility of CID in regulating cell based therapies.96 Triggered cell proliferation at the receptor level has been used to enhance growth of genetically modified cells.97–102 In one example, skeletal myoblasts, which can be used to repair scar tissue and infarcted myocardium yet suffer from complications in reproducibly generating large enough grafts, are transfected with modified FKBP (F36V) fused to the fibroblast growth factor receptor-1 cytoplasmic domain. AP20187, a dimeric F36V ligand related to AP1903 (section 2.2), induces dimerization of the chimeric receptor and, hence, proliferation of the transfected myoblasts in a robust, reproducible manner. Additionally, after 30 days of treatment, myoblast grafts remained stable after withdrawal of the dimerizer, and differentiated normally, showcasing the reversibility of CID based systems.103

Therapeutically tailored activation of gene expression by transcription-regulating CID systems has also been demonstrated.104–107 Quintarelli et al., in an elegant combination of antitumor therapies, showed that transgenic expression of interleukins 2 and 15 by Epstein–Barr Virus-specific cytotoxic T lymphocytes (CTLs), which are tumor-specific, increased the antitumor activity of this well-studied system. The concomitant side effects of increased interleukin levels, including systemic toxicity, were mitigated by coupling this methodology with CID-inducible expression of a caspase-9 suicide gene, which afforded elimination of transgenic CTLs and interleukin production, increasing the therapeutic feasibility of the approach.108 In keeping with the superb amenability of dimerization systems to fundamental characterization, detailed analyses of the thermodynamic characteristics required for optimal CID-based activation and induction have been carried out.109,110

The competitive evaluation of dimerizer-based systems versus alternative methods for regulated gene expression has also been undertaken. In these studies, CID-based systems have shown the highest degree of regulatory selectivity and have consistently demonstrated outstanding dynamic range and sensitivity, particularly as the systems have evolved.35,111,112 The work by Xu et al. compared five popular systems for regulating induced gene expression from adenovirus (Ad) vectors—the tetracycline (Tet-on and T-REX), ecysdose, antiprogesterin, and dimerizer based systems.112 The dimerizer based system tested is an FKBP–FRB complex induced by addition of AP21967, an analog of rapamycin with decreased immunodepressive effects. The DNA binding domain (ZFHD1—a dual zinc finger domain from human transcription factor Zif 268) is fused to FKBP, while a p65-HSF1 activation domain is fused to FRB. Using a luciferase reporter assay in three different cell lines, it was found that the FKBP–FRB system maintained the lowest basal level of expression and the highest induction factor, properties paramount to the practical usage of gene expression as a therapeutic technique. Additionally, dimerizer based gene expression is independent from endogenous cellular processes and exhibits a 50-fold increase in expression at inducer levels as low as 1 nM. Lastly, the dimerizer based system benefits from the expression of binding and activation domains as individual proteins, freedom from virus-derived proteins, and the possibility that expression can be even more tightly controlled via the addition of a noninducing competitor of AP21967. Overall, the FKBP–FRB system represents a highly feasible method for the therapeutic application of targeted gene expression.

Significant progress has also been made in the engineering of dimerization based regulatory elements, optimizing the compactness and efficiency of constructs for potential therapeutic delivery.113–116 For example, the modification of rapamycin to nonimmunosuppressive agents that still efficiently bind FKBP and FRAP has found success not only in the study discussed above but also in the regulation of viral replication in antitumor therapies. Traditionally, replication-defective viral vectors are used to deliver gene-based therapies; however, use of replication-competent viral vectors represents a more efficient delivery of therapeutic genes. Unsurprisingly, there is much hesitation to use replication-competent viral vectors, since uncontrolled replication could prove disastrous to the patient. Chong et al. showed that
replication-competent vectors dependent on the presence of AP21967 improve the viral spread in tumor and surrounding tissues while allowing for temporal control of replication, thereby increasing both efficiency of gene delivery and safety to the patient.115

Growth-arresting CID therapies can also play an important clinical role: conditional CID-aggregation of the pro-apoptotic Fas effector has served as an effective safety switch in graft versus host disease (GVHD), one of the most pressing issues in transplantation medicine, in which T-cells are transduced with a gene expressing a chimeric Fas intracellular domain—FKBP fusion.125,117 While the T-cells are unaffected by the addition of the gene alone, treatment with dimerizer results in the Fas-mediated apoptosis of the cells, should GVHD occur. This technique has also shown promise in large animal models.118 Globally, steady progress has been made in validating the therapeutic viability of CID regulation in humans—defining toxicities, demonstrating efficacy in preclinical models, and monitoring for oncogenicity or other long-term consequences.119–122 For example, work by Richard et al. showed that although CID dependent association of a modified murine thrombopoietin receptor allows for in vivo selection of genetically modified hematopoietic cells in mice and dogs with little toxicity, similar work in a nonhuman primate model yielded limited expansion of genetically modified red blood cells.123 In contrast, more recent work by Nagasawa et al. employing the same methods have demonstrated the use of dimerizer-based affinity modulation as a means to inhibit the formation of the amyloid Aβ fibrils associated with Alzheimer’s disease. Small molecule inhibitors of amyloid aggregation face stern challenges common to all efforts to inhibit protein–protein interactions: they are mediated by large, often flat, surfaces that offer very little purchase for a drug to take hold.126 Given this problem of scale, a small molecule that does bind selectively to a protein-surface target may yet be unable to disrupt aggregation. Beginning with the knowledge that the dye congo red (CR) is able to bind amyloid with reasonable affinity and even prevent Aβ aggregation at high concentrations (IC50 = 2 μM), they reasoned that a SLF-CR hybrid would recruit FKBP to the surface of Aβ monomers and small aggregates, where it would serve as a chaperone to prevent formation of amyloid fibrils (Figure 17).

This particular mode of inhibition has found success in blocking Aβ aggregation, paramount to the development of Alzheimer’s disease, by utilizing a heterobifunctional dimerizer capable of binding within the aggregating Aβ fibril and concurrently recruiting the cellular chaperone FKBP, physically blocking further Aβ interactions.125 Gestwicki et al. have demonstrated that SLF-CR is capable of inhibiting Aβ aggregation in neuronal tissue culture, with an IC50 approximately 4-fold lower than that of CR alone. Seeking to optimize the potency of this effect, the authors speculated that the local dynamics of the FKBP chaperone at the Aβ surface could influence its efficacy, and they synthesized SLF-CR conjugates with variable linker length (Figure 18). The most potent compound, SLF-Benz-CR, was 40-fold more potent in the presence of FKBP than CR alone in Aβ aggregation assays, with an IC50 of 50 nM.

4.2. Physical Inhibition of Protein Interactions

Protein association plays a pivotal role in the pathological functions of many diseases. From combating viral capsid assembly to inhibiting infectious agent signaling derived from a transient protein association, the search for modulators of protein interactions is an important and relevant field of pharmaceutical research. However, the development of such small molecule inhibitors of protein association has proven elusive. Not only do the relatively large surface areas influencing protein association disperse binding energy, but these same topologies are flexible, allowing for accommodation of small molecules and lack of inhibition.125 A lean but rapidly expanding field of CID research includes circumventing this issue by utilizing induced protein dimerization as a tool for increasing the steric bulk of the underlying small molecule dimerizer. Whereas the small molecule retains its favorable pharmacological properties, the self-assembled protein complex serves as the effective pharmacophore.
A similar method of therapeutically relevant protein association lies within the “bait and trap” capabilities of bifunctional ligands in concert with ubiquitous endogenous proteins. One such protein, human serum amyloid P component (SAP), is a member of the pentraxin family of innate immune system proteins and is one of the most abundant human serum proteins. Naturally existing as a pentamer, crystallographic studies show the association of two SAP pentamers into a dimeric, face-to-face complex via a network of noncovalent -stacking interactions. The cholera toxin B-pentamer (CTB) adopts a similar pentameric conformation that binds with high affinity to cell membrane carbohydrates. Inspired by the similarity between the two structures, Liu and co-workers developed a heterobifunctional inducer of dimerization exploiting the carbohydrate binding domain of CTB and the proline binding pocket of SAP linked via an ethylene spacer. Dynamic light scattering data indicated the stable formation of a ternary SAP/ligand/CTB complex, and further competition experiments noted the increased stability of the complex relative to monovalent binding, with low micromolar IC₅₀ values at physiological SAP concentrations.

Expanding upon this work, Soloman et al. developed a similar system in which the baited protein was the Shiga toxin (Stx) pentamer. A bifunctional ligand was constructed featuring binding domains recognized by the proteins of interest (Figure 19). The trisaccharide moiety binds to Stx, whereas the cyclic pyruvate moiety binds to SAP. Gel permeation chromatography, in conjunction with dynamic light scattering data, indicated the formation of a ternary SAP/ligand/CTB complex, and in vitro binding experiments determined that, in the presence of 0.17 µM 1, SAP inhibits Stx with an IC₅₀ of 21 nM. Furthermore, compound 1 inhibited Vero cell death via a lethal dose of Stx with an IC₅₀ on the order of 15 µg/mL. However, compound 1 was shown to be inactive against a human SAP transgenic mouse model due to a high degree of clearance. Currently, studies are underway to alleviate these issues.

5. Protein Nanostructural Assembly

To this point, we have considered chemically induced dimerization as a means to control the assembly of small-scale functional complexes: initiators of transcription or signal transduction, inhibitors of protein aggregation, or localized active enzymes. The ability, however, to precisely connect patterned proteins is reminiscent of another important biological role for protein assemblies: structure. From the nanoscopic to macroscopic scale—from viral capsids to vertebrate muscle—proteins play a crucial role in biological structures. Moreover, many natural protein assemblies operate on a scale which is at present poorly imitated by synthetic approaches—too large for synthetic organic chemistry, too small for techniques of microfabrication. This 1–100 nanometer niche, occupied so successfully by biomaterials, offers a fascinating range of scientific possibilities: from advanced protein therapeutics, to proverbial nanobots, to next-generation electronic devices.

Toward this end, efforts are underway to devise techniques and unearth principles regulating the assembly of a variety of biomaterials. Nucleic acids have been a principle building material for these efforts, as sequence recognition can both encode and construct robust structural junctions. The greater structural and functional diversity of proteins offer a potentially rich opportunity for protein-based materials. In contrast to DNA or RNA, simple and elegant mechanisms for directing their assembly are lacking. In this important niche, chemically induced dimerization may play a role.

Early efforts to engineer synthetic protein assemblies have principally focused on purely protein-mediated connectivity—the association of polypeptide building blocks with intrinsic affinity, such as multimeric proteins, β-strands, coiled coils, and zinc fingers. By fusing naturally multimeric proteins, self-assembling building blocks that produce filaments (linear heterodimers) and cages (polyhedral vertices) have been designed. A second principal strategy in successful designs of these materials has been the reorganization of bundled protein domains so that they contain intermolecularly self-complementary structures, such as coiled coils with staggered ends, or helix bundles reorganized and extended for a domain-swapping interaction. These building blocks typically form large filaments, as the primary protein strands bundle into larger structures. Particularly intricate design efforts have been conducted and delineate several
strategies to functionalize these filaments.\textsuperscript{143--145} For example, recent work by Rele et al. has featured the development of D-periodic collagen-mimetic microfibers, in which tripeptide assembly is guided into an ordered nanostructure via electrostatic bias.\textsuperscript{146} These methods are limited by the fact that the assembly of self-complementary elements generally occurs spontaneously in situ as the protein is expressed. In addition, once assembled, there is no general means to remodel or disassemble the structures.

A potential enhancement to the method of spontaneous protein self-assembly is the addition of ligand control. A few examples have appeared in the literature outlining these methods that rely on the association of multivalent ligands with multivalent proteins. If chemically induced dimerization represents the association of a bivalent ligand with monovalent binding proteins, a self-limited event, then the transformation of those binding proteins into bivalent (or multivalent) molecules creates the potential for expansion into larger scale structures.

Dotan, Freeman, and co-workers designed and observed diamond-like protein crystals constructed by the assembly of tetravalent lectins (carbohydrate binding proteins) and bivalent mannose derivatives.\textsuperscript{147} Careful addition of the bismannopyranoside at 2:1 stoichiometry noncovalently cross-linked concanavilin A into a tetrahedral lattice, producing crystalline protein precipitates (Figure 20). This scaffold offers intriguing potential for further engineering of functional, three-dimensional protein networks. A disadvantage to the technique, however, is the inability to precisely regulate the degree of lattice assembly, as is the lack of a means to form soluble nanostructures.

Ringler and Schulz explored a more regulated route to ligand-mediated nanostructure assembly in their work with biotinylated building blocks.\textsuperscript{148} They produced protein nanostructures based on the ligand-directed assembly of a biotin tagged tetrameric aldolase and streptavidin (Figure 21). Biotin and bis-biotin mediated connections between the subunits enabled these constructs to be directly assembled into two-dimensional lattices and varied cruciform discrete nanoassemblies. These spatially ordered, ligand-assembled constructs offer prospects for more elegant assemblies of nanoscale protein biomaterials. Primary limitations to this approach include the restriction to rectangular arrays, the unknown stability of the streptavidin-aldolase building blocks, the difficulty in forming homogeneous complexes, and the complex process required to prepare these structures.

Another quickly evolving area of nanostructural assembly includes the development of nanotube structures for a wide range of uses in the broad spectrum of nanoarchitecture. Aside from carbon and inorganic nanotubes, which lack the functionality of higher-order protein structures, current research in the area focuses on naturally occurring polymeric proteins such as viral capsids, actin, tubulin, amyloid protein,
and flagella. Limitations with this methodology include a lack of control over in vivo assembly, as well as difficulties in accessing the entire nanotubule topology. In order to work around such problems, Ballister and co-workers have established a method of developing self-assembled, tailorable nanotubes from the toroidal hexameric protein Hcp1 from Pseudomonas aeruginosa. Systematic cysteine replacements at strategic locations along the faces of the toroidal components allow for covalent disulfide bonding between the subunits of the tubular assembly (Figure 22). Whereas size exclusion chromatography indicated the presence of high molecular weight protein complexes in solution, repeating the experiment in an excess of reducing agent (5 mM dithiothreitol) resulted in an absence of nanotube formation. Although there is a lack of a traditional multivalent ligand in this particular system, the engineered disulfide bonds serve as the chemical switch paramount to any CID system. In addition to the controlled assembly of Hcp1 nanotubes, chain termination and tube sealing was achieved using the same methodology, with cysteine mutations at the internal face of the Hcp1 toroid. This excellent level of structural control affords the self-assembly of an Hcp1 nanocapsule, with an internal environment isolated from that of the bulk solvent.

One aspect of protein nanostructure assembly given considerable attention is the increased kinetic and thermodynamic stability of multivalent complexes. An ex-

Figure 22. Protein nanotube architecture. The surface plots depict the topology of the designed nanotubes. The TEM image displays the capped nanotube. Figure elements reprinted from ref 149 with permission. Copyright 2008 National Academy of Sciences, U.S.A.
ample of this stability is described by the synthesis of a trivalent hapten that causes the aggregation of rat anti-2,4-dinitrophenol (DNP) IgG (IgG_{DNP}) into bicyclic trimers. When three 2,4-DNP moieties are tethered to a central amine via a polyethylene glycol spacer, the new compound effects the formation of trimeric IgG_{DNP}s (characterized by dynamic light scattering, analytical centrifugation, and size exclusion HPLC) with the stoichiometry IgG_3/ligand_2. Moreover, further experimentation with a competitive inhibitor indicates the relative stability of the complex is 225-fold greater than that of a singly bound species. This increase of stability is a hallmark of multivalent protein structures and represents an advantage in biotechnological applications.

Despite recent work advancing the development of self-assembled nanostructures, the problems of structure polydispersity and incomplete assembly still remain. From a practical therapeutic standpoint, the ideal nanostructure is both assembled and disassembled in a controlled manner. Whereas previous work has focused on assembly and the formation of stable complexes, controlled disassembly has often been overlooked. Previous work shows the ability of a bivalent methotrexate dimerizer to induce the dimerization of E. coli dihydrofolate reductase (DHFR), which is further stabilized by protein–protein interactions at the DHFR–DHFR interface. Further work by Wagner and co-workers has focused on the use of fusion proteins of DHFR (Figures 23 and 24, DHFR_2) to form stable, self-assembled protein nanorings where polydispersity is tunable based on the nature of the amino acid linker between the two proteins. Light scattering analysis, in conjunction with size exclusion chromatography and TEM data, confirms the formation of toroidal species in a small window of sizes. Advantageous to this method is the pharmaceutically reversible assembly of the complex, since an excess of an inhibitor of DHFR will effect dissolution of the ring. Current limitations include the ability to exert a level of control over protein integration; however, recent work indicates the possibility of dimer interface modulation as a candidate for the development of a biomolecular language.

Revisiting multivalency, further exploration from the perspective of protein rather than ligand modification has recently yielded single chain antibody (scFv)–DHFR_2 fusions which, when assembled into nanorings, have been used to produce divalent antibodies (Figure 25). The antibody chosen binds the T-cell antigen, CD3ε, of the human T-cell receptor. The nanoring containing two copies of the single

Figure 23. Protein nanoring structure: upper limb, schematic of protein nanoring structure and assembly; lower limb, TEM of DHFR nanorings. Adapted from ref 155 with permission. Copyright 2006 American Chemical Society.
chain antibody was shown to have a comparable dissociation constant to that of the parent monoclonal antibody. Confocal laser microscopy was employed to show that the antibody nanorings interacted with CD3+ HPB-MLT cells in a similar manner to that of the parental antibody. Moreover, the complexes were found to undergo controlled disassembly via the addition of the DHFR competitive inhibitor trimethoprim. The formation of larger rings in the work described in the previous paragraph leads to the vision of rings with higher valencies and avidities. Indeed, further work by Wagner and co-workers has shown that antibody nanoring sizes mimicking cellular receptor cluster topography (i.e., octomeric in nature) have led to increased avidity of fused antibodies to their targets (unpublished data). Additionally, modified dimerizers capable of delivering fluorophores, radionuclides, and drugs to targeted tissues would expand the use of biotherapeutic applications of the nanorings.

Concerning the development of mixed protein nanostructures and a technique for implementing control over nanostructure self-assembly, recent work has shown it possible to form enzymatically active nanorings using DHFR-histidine triad nucleotide binding 1 (Hint1) fusion protein (Figure 24).157 Human Hint1 (hHint1) forms a stable homodimer and acts as a phosphoramidase and acyl-adenylate hydrolase. By incorporating hHint1 into the rings, functionally active, protein-based nanorings may be produced. The length of the linker between the DHFR and hHint1 was shown to have profound effects on the size of the rings formed. When the linker consisted of Gly-Leu-Glu, a range of rings containing between 2 and 12 monomers was observed by size exclusion chromatography. The molecular weights of these rings ranged from 130 to 740 kDa. The largest rings (>11 monomers) were shown to have the greatest specific activity while the smaller rings were found to have activities greater than or comparable to the wild type hHint1.

In spite of this early progress, significant hurdles remain to be surmounted for protein—ligand nanoengineering to emerge from its nascent state. The ability to construct a greater geometric variety of structures is a high priority, as is the ability to exploit the ligand-reversibility of the assembly process in diverse environments. Tunable protein architectures, in which conformational assembly information can be encoded in the primary structure, akin to the methods used for DNA and RNA, would represent another significant advance.

6. Conclusions

The field of chemically induced dimerization, while young, is rapidly expanding into its own niche in the current spectrum of biotechnological applications. The use of CID systems as investigational tools has led to the elucidation of the role of protein interactions in a number of biological events, including signal transduction cascades and transcriptional control. A number of studies have also shown that it can be exploited to control the levels and post-translational structural modifications of proteins. In the future, an expanding number of ligand—protein dimerization systems which can be orthogonally activated will allow the ability for the study of increasingly complex systems.

Whereas current application of CID technology to therapeutics is currently limited to controlled gene expression, signal transduction, and protein oligomerization, the recent developments in the area of protein interaction disruption utilizing endogenous cellular protein are an exciting area indeed. Continued research in this area could focus on any number of diseases requiring specific protein—protein interactions. With advances in lead-based drug design and molecular docking algorithms, the discovery of new protein targets and relevant binding domains is ever-increasing. The marriage of these phenomena holds great promise for the future of pharmaceutical development, since the use of small molecules to effect changes in protein interactions has long eluded researchers.

Similar to therapeutic applications of CID, nanostructural assembly is just beginning to see refinement in technique and application. Advancements in the understanding of the increased kinetic and thermodynamic stability of multivalent complexes have been encouraging. Perhaps most importantly, recent developments in control over protein assembly hold great promise for future endeavors in this field. Molecular recognition, while hinted at in some of the cases discussed...
above, still has yet to come into its own. With the goal of forming a self-assembled, switchable, and multifunctional system of limited polydispersity, further research concerning the formation of a biomolecular language is required in order to effect the level of control necessary for such an endeavor. With such a language in place, it would be possible to use proteins as self-assembled building blocks, incorporating functionality as the researcher sees fit. Armed with such a wide array of highly refined tools, the possibilities for the application of chemical induction of protein association seem limitless.

7. References

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