Peptide and Protein Recognition by Designed Molecules

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Contents

I. Introduction

A protein in its native, folded conformation creates a solvent-exposed (exterior) surface and a solvent-excluded (interior) surface (Figure 1). Enzyme active sites are most often found at the interior of proteins. Functional group presentation at an enzyme active site is convergent; this accounts for the success of highly functionalized small molecules in inhibiting their action. Functional group presentation on the surface of a protein is primarily divergent in nature. In binding protein surfaces, designed molecules must account for the extensive solvation of the protein surface, as well as the surface topology. Tight binding therefore requires the involvement of large surface areas and multiple points of functionality.

There is growing interest in understanding and manipulating protein−protein interactions due to their central importance in many biological processes. Protein−protein interactions are known to play a critical role in the normal function of cellular/organelle structure, immune response, protein enzyme inhibitors, signal transduction, and apoptosis. Rational approaches toward the recognition of protein surfaces may provide better insights into exactly how proteins interact with one another, and is an alternative to enzyme inhibitor design as a molecule-based disease therapy. Moreover, rational protein surface recognition is a challenging test of our knowledge of molecular design.

The nature of protein−protein interfaces has been the focus of investigation for some time. Particular emphasis in the literature has been placed on structure,[1−6] energetics,[4,7] electrostatic complementarity,[8] and kinetics[9] of protein−protein interactions. A recent important breakthrough has been the identification of "hot spots" on protein surfaces.[10−13] A hot spot is a defined locale of ca. 600 Å^2 on the surface of a protein at or near the geometric center of the protein−protein interface. The residues that comprise the hot spot contribute significantly to the stability of the protein−protein complex. Mutating a hot spot residue to alanine results in a protein that will have a lower affinity for its partner. As demonstrated by Wells and co-workers, by mapping these $\Delta\Delta G$ values to the known structure of the protein, one can generate a map of the protein where mutants that contribute most to the loss of affinity are near each other on the protein surface. These residues are not necessarily contiguous in the primary sequence of the protein. Surrounding the hot spot is an area of residues that contribute slightly less to the stability of the complex. This outer area has been compared to an O-ring that excludes solvent from the protein−protein interface, stabilizing the complex.[12]

The significance of this solvent expulsion at the protein−protein interface is of special note.[12] A survey of amino acids appearing on hot spots showed a predominance of Trp, Tyr, and Arg residues. The surface area of the hot spot (generally 600 Å^2) may be the size that is critical to make a water-excluded seal around the energetically favorable interactions. This expulsion of water would result in a lower dielectric at the interface, increasing the energetic contributions of hydrogen-bonding and electrostatic interactions. But what about the hydrophobic residues at protein−protein interfaces? The argument has been made that solvation of hydrophobic residues is important for the unfolding of proteins. The authors of the survey suggest that this is similarly true for protein interfaces. They also suggest that interacting with the residues important to the expulsion of solvent may be a strategy to inhibiting protein−protein interactions.

The generality of the hot spot model of protein−protein interactions remains an open question. The systems best characterized by the alanine screening technique are protein−protein interactions between peptide hormones and their hormone receptors or antibodies with their respective antigen. While these are valid protein−protein interactions, they do not represent the complete collection of protein interface

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topologies.[1] One could imagine that a localized hot spot would be difficult to identify in a coiled coil interaction, for example.

The main goals in designing synthetic molecules that disrupt protein−protein interactions through specific recognition take two complementary threads. One is the recognition of protein surfaces in ways that agonize a biological response, mediate some protein dimerization, or stabilize the native oligomerization state of the protein.[14,15] The second objective in targeting a protein surface would be to selectively bind half of a dimeric interaction in a way that would sterically block association with the natural protein partner. The focus of this review is to introduce current strategies for binding protein surfaces with emphasis on the use of designed molecules. The progress made to this point has in large part been based on the recognition of oligopeptides by complementary, designed molecules. The principles of molecular recognition learned from these model systems are laying the foundation for progress in the field of protein surface recognition.

II. Current Strategies

A. Chemical Genetics

Schreiber has recently suggested that an important goal in bioorganic chemistry is "to identify a small

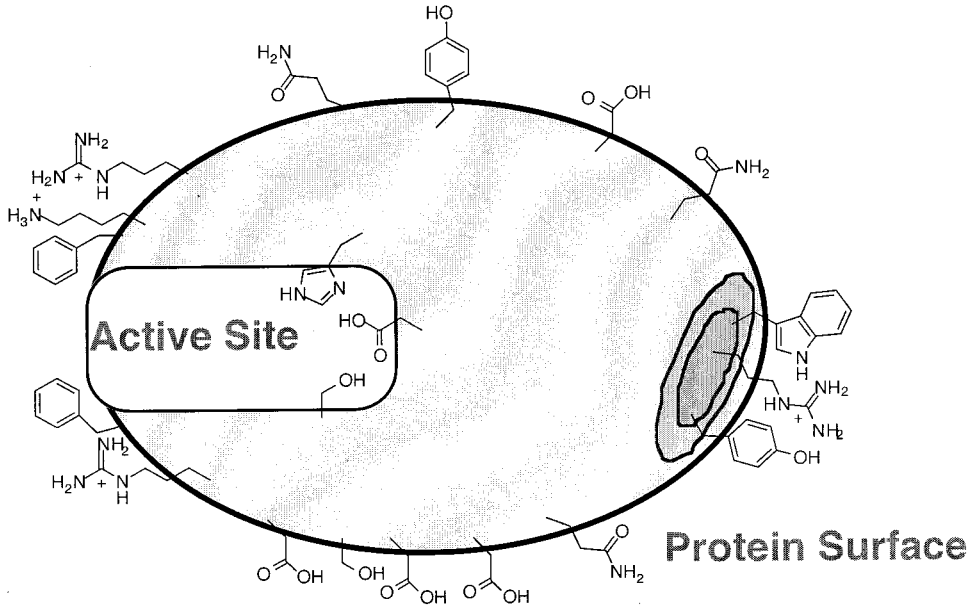

Figure 1. Schematic representation of a protein (enzyme) interior, exterior, and hot spot.

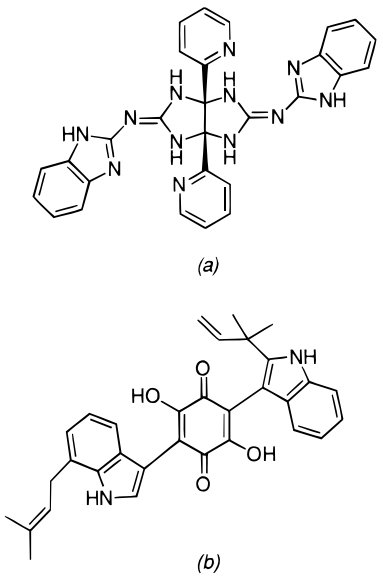

Figure 2. (a) SB 247464, a small molecule mimetic of G-CSF discovered in a library screen assay. (b) L-783,281, a small molecule mimetic of insulin.

molecule partner for every gene product".[16] Natural products and natural-product-like molecules are important scaffolds, often designed by nature for interaction with protein targets (gene products). The goal of using natural product or totally novel scaffolds to interact with proteins within living systems to cause a change in cellular phenotype represents a sizable challenge but one that holds tremendous promise.

Examples of small molecules that bind protein surfaces in a fashion similar to that of natural proteins have been identified from screening synthetic libraries or natural product isolates for their ability to activate cell surface receptors. Many cell signaling pathways begin with a cytokine/cell surface receptor interaction that causes dimerization of the receptor and initiates a phosphorylation cascade of specific cellular proteins toward the nucleus. These receptor tyrosine kinase (RTK) pathways have been the focus of much research over the past several years.[17]

A high-throughput, cell-based assay for the detection of compounds that activate granulocyte colony stimulating factor (G-CSF) receptor found that SB 247464 was an active compound (Figure 2).[18] The assay was based on a STAT inducible reporter gene (luciferase) that is responsive to G-CSF activation. The active compound exhibited a biphasic dose response curve which is a property of cytokine dimerizers of cell surface receptors. Its efficacy was 30% of the natural cytokine. Experiments using murine−human G-CSF receptor chimeras indicated that SB 247464 was selective for the murine extracellular domain, and bound a region that was distinct from the G-CSF binding domain. The authors noted that the 2-fold symmetry in this molecule suggests an ability to dimerize G-CSF receptor in a way similar to that of G-CSF itself. This was the first example of a synthetic small molecule which was able to dimerize cell surface receptors. However, its exact mechanism and site of interaction on the cell surface receptor remain to be determined.

A recent report has also described the discovery of a small molecule insulin mimetic which activates insulin receptor tyrosine kinase activity in a cell-based assay.[19] While the phenotype of cells treated with L-783,281 (Figure 2b) is similar to that of insulin, the mechanism of action is markedly different. Tryptic digests of the insulin receptor (IR) with L-783,281 suggest that it binds near the ATP binding site, changing its conformation, which is a known (and general) mechanism for IRTK activation.

This example underscores a significant difference between the design of protein surface binders and chemical genetics. The prevailing philosophy of chemical genetics is to use medium- to high-throughput screening of functional assays of a specific cellular phenotype. This is not the case for molecular design. Here, testing the ideas of molecular complementarity that have been incorporated into the molecule is equally important to the overall enterprise, and this is best served by direct binding assays. Both approaches are equally meritorious; the choice between them should be based solely on the question being asked and how best to answer it.

B. Monoclonal Antibodies

The immune system is remarkably effective at protein surface recognition.[20] The humoral immune response collects the antibodies that best bind to an antigen and then executes a process of affinity maturation to generate fully matured antibodies. Through the now common preparation of monoclonal antibodies, a discrete epitope on a target protein may be bound with high affinity. A detailed investigation of the common features found in antibody−protein complexes has been gained from X-ray analysis.[21] In all cases, large surface areas (600−950 Å^2) on the antibody are buried on contact with the protein antigen.[22] Within the interfacial region are found a high proportion of aromatic residues with an important role for charge complementarity and hydrogen-bonding contacts.[5,23]

The use of antibodies in molecular and cellular biology as diagnostics for the presence of a protein epitope is widely accepted. This represents a good example of functional protein−protein interactions. The specific recognition of protein surfaces can be used in antibody-based cancer therapy.[24] The recent approval of a monoclonal antibody (recombinant humanized anti-p185^{HER2}) to inhibit the Her2/neu homodimerization as a therapy for breast cancer has been a long awaited breakthrough for monoclonal technology. The immune system is an important model for chemists to study toward the deeper understanding of intermolecular interactions.

C. Phage Display

The chemical diversity created by randomization of peptide or protein segments expressed on the surface of phage particles is a powerful technique for identifying molecules that bind to protein surfaces.[25] A multistep approach to protein minimization has recently been reviewed by Cunningham and Wells.[26] Alanine scanning of the protein identifies residues

important for the binding interaction. Subsequent minimization to the identified binding epitope (with available structural information) and affinity optimization by phage display generate new peptides with properties similar to those of the wild-type protein. This approach has been utilized in the synthesis of minimized proteins such as the cytokines atrial natriuretic peptide (ANP)[27,28] and the Z-domain of protein A.[27] Recently, a naive phage display library has led to the identification of a mimic of the peptide hormone erythropoetin (EPO).[29]

Through the mirror image phage display technique, novel D-amino acid peptides can be identified for binding to protein surfaces. In short, the technique entails the chemical synthesis of a protein which contains all D-amino acids in place of the natural stereoisomers. The "mirror image" protein in the normal screening and selection protocols for binders identifies natural peptide sequences which, when synthesized with all D-amino acids will bind to the native protein.[30] This technique has most recently been used to identify short cyclic peptide sequences of D-amino acids which inhibit the entry of the HIV virus by binding the core coiled coil trimer of gp41.[31]

D. Designed Protein Surface Binding Agents

The remainder of this review will focus on the development of the principles of molecular design toward the recognition of peptides and proteins. The practice of molecular design relies on a full understanding of noncovalent forces. Often, many of these forces (hydrogen bonding, electrostatics, van der Waals forces, $\pi-\pi$ interactions, conformational energy, etc.) work in concert, making it difficult to isolate the contributions of any one component. Model systems can be used to illustrate the contributions of these forces in a systematic manner. The primary goal in this research is the design of molecules with predictable recognition properties. Among the greatest tests of the knowledge gained from model systems is their application to a natural target. Here the requirements for specificity and tight binding are more rigorous than in the model systems. Protein surfaces represent a challenging target for the field of molecular design. Large surface areas (500−2000 Å^2) must be covered and multiple interactions utilized to achieve sufficient binding affinity to observe an effect. Several interrelated strategies toward this goal are discussed below.

III. Oligopeptide Recognition

A. Sequence-Selective Recognition of Short Peptides

A distinction should be made between cell surface receptors and the common molecular recognition term "receptor". Host−guest chemistry traditionally called its designed molecules (the host) receptors for a given recognition target (the guest). Cell surface receptors are membrane-bound proteins involved in signal transduction cascades. Both usages are common in the literature.

Oligopeptides represent an intermediate step toward the recognition of protein surfaces for the field of molecular design. Short peptide sequences (3−20 amino acids) are themselves worthwhile targets for recognition due to their potential applications in separation, diagnostic, or biological areas. The notion of peptide recognition as a stepping stone to protein binding arises because many of the early synthetic receptors involved a small number of functional group interactions. Binding a protein surface should require large arrays of interacting functionality in a conformationally defined context. Also, these arrays may not be confined to one domain or secondary structural element of the protein. Short peptides present an additional factor to binding because they are themselves usually conformationally flexible. Work from the group of Still has focused on polypeptides as targets for molecular recognition. The major goal of his work was to enumerate the principles that govern peptide recognition by designed molecules and to use this information in the determination and computational prediction of binding affinities. In particular he has exploited an elegant combination of combinatorial, one-bead, one-compound assays with molecular designs that are well-suited to both convergent recognition and library generation.[32−35] These sequence-selective tripeptide receptors have recently been developed into effective peptide sensors (Figure 3).[34] The design is such that, in the absence of the target peptide, the fluorophore (**F**) is quenched

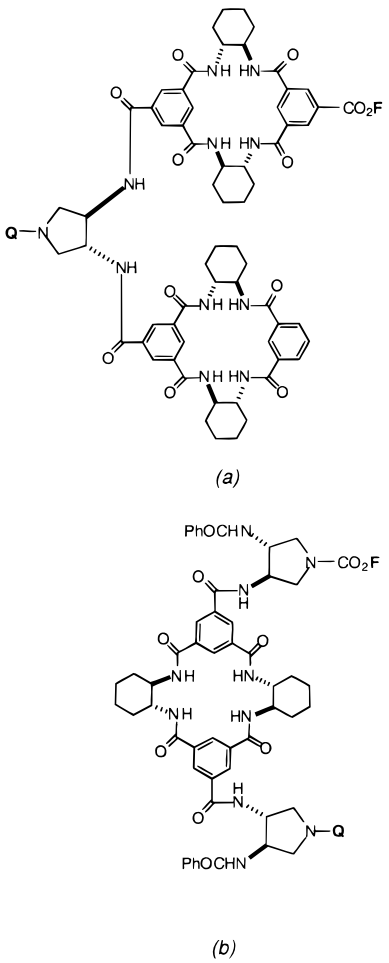

Figure 3. Fluorescent tripeptide chemosensors. **F** = $(CH_2)_2SO_2$-dansyl. **Q** = dabsyl. (a) Selective for ^{D}Pro-^{L}Val-^{L}Gln. (b) Selective for ^{L}Gln-^{D}Asn-^{L}Gln.

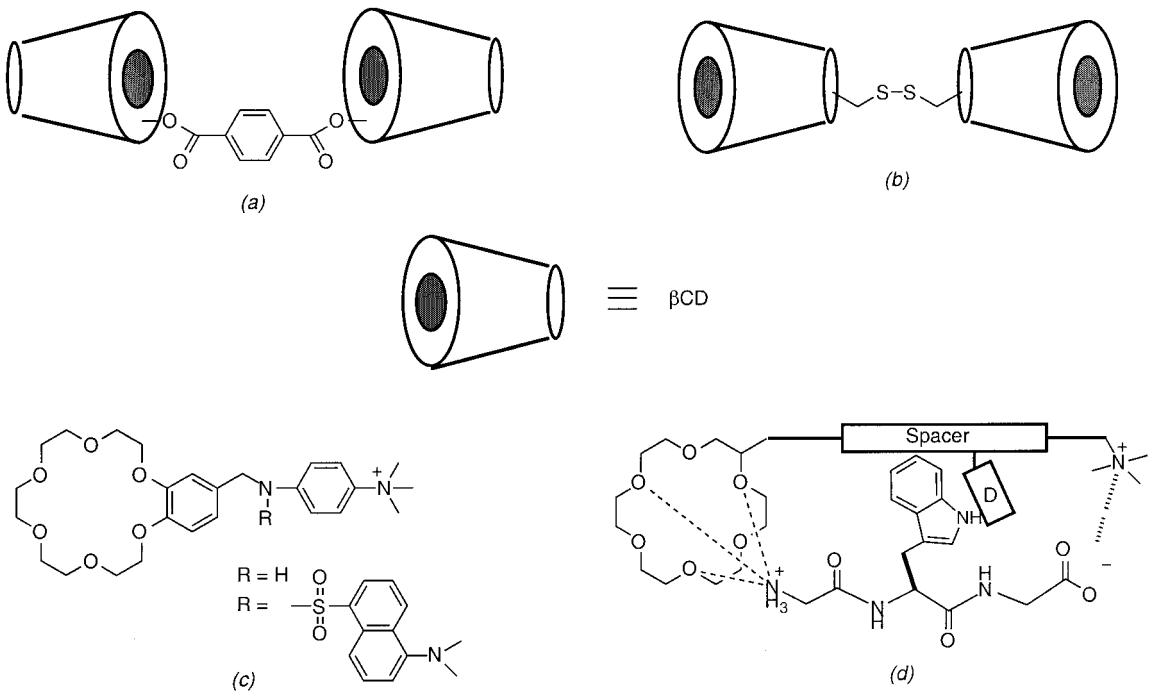

Figure 4. (a, b) β-cyclodextrin dimers (schematically) bound through the (a) secondary or (b) primary face. The dimeric molecules were used to bind Trp/Phe-containing peptides in aqueous solution. (c) Tripeptide binding molecule designed by Schneider. (d) A schematic of the interaction of the dansyl-functionalized molecule and the unprotected Gly-Trp-Gly.

intramolecularly by a second chromophore (**Q**). In the presence of the bound peptide the distance between **Q** and **F** increases, and the fluorescence emission of the system is enhanced.

An important common objective is the sequence-selective binding of short peptides in aqueous (biological) solvent. Work in the Breslow lab has made use of β-cyclodextrins to bind Phe residues in aqueous solvents.[36] Dimerization of β-cyclodextrins through the secondary face was designed to allow greater cooperativity in binding the target peptide, Phe-^{D}Pro-X-Phe-^{D}Pro.[37] Association between the receptor shown in Figure 4a and cyclic ($K_1 = 2590$, $K_2 = 1120$ M^{-1}) or acyclic ($K_1 = 1100$, $K_2 = 114$ M^{-1}) versions of this sequence were evaluated in aqueous buffer at 25 °C by isothermal titration calorimetry. K_2 refers to the binding of a second equivalent of peptide to the 1:1 complex. Modeling studies showed that binding to the acyclic form should be preferred over the cyclic analogue. The lower measured affinity of the acyclic peptide was attributed to an energetic penalty of unfolding the Phe-Phe associated peptide prior to binding the bis-β-cyclodextrin. Although not cooperative, the disulfide-bound β-cyclodextrin dimer (Figure 4b) was shown to bind the sequence Trp-Trp with similar affinity ($K_a = 1200$ M^{-1}).

Incorporation of functionality to complement both side chains and the termini of an extended peptide is exquisitely described in the work of Hossain and Schneider.[38] Molecules that were complementary to the zwitterionic form of unprotected peptides were designed such that an 18-crown-6 unit could associate with the N-terminus of a tripeptide while a peralkylammonium group associated with the C-terminus (Figure 4d). NMR titrations in water ($K_a \approx 200$ M^{-1}) or methanol ($K_a \approx 10^4$ M^{-1}) demonstrated binding in this system. The incorporation of a dansyl group served two purposes. First, complementarity to aromatic side chain functionality was now incorporated into the molecule, and second, the binding could be measured by fluorescence spectroscopy. This series gave affinities of 2000 M^{-1} for a target tripeptide Gly-(Phe/Trp)-Gly in water at 25 °C. The most significant aspect of this work is the recognition of multiple functionalities in one system. A porphyrin unit has also recently been exploited as a UV sensor of amino acids and tripeptides using the same strategy.[39]

The clinically important antibiotic vancomycin binds the ^{D}Ala-^{D}Ala carboxyl-terminal sequence of bacterial cell wall components. This system has been an intensively studied natural example of short peptide binding.[40] Using this well-defined peptide−peptide interaction, Whitesides has designed oligomeric vancomycin derivatives that bind the corresponding oligomeric ^{D}Ala-^{D}Ala derivatives with high affinity (Figure 5). The binding of dimeric and trimeric ^{D}Ala-^{D}Ala substrates by dimeric and trimeric derivatives of vancomycin has been reported.[41,42] K_d values in these systems were 1.1 nM and $\sim 4 \times 10^{-17}$ M, respectively. These systems illustrate the utilization of polyvalent interactions for the design of ligands and inhibitors.[43] The macrocylcic "right-hand side" binding pocket of vancomycin has been used as the starting point for the parallel synthesis of vancomycin analogues that preferentially bind ^{D}Ala-^{D}-Lac.[44] This approach identified molecules which bound ^{D}Ala-^{D}Lac 5 times more tightly than vancomycin in aqueous solution. This is an important first step in identifying antibiotic compounds to combat vancomycin-resistant bacterial strains through specific recognition interactions to the mutated peptidoglycan strand.

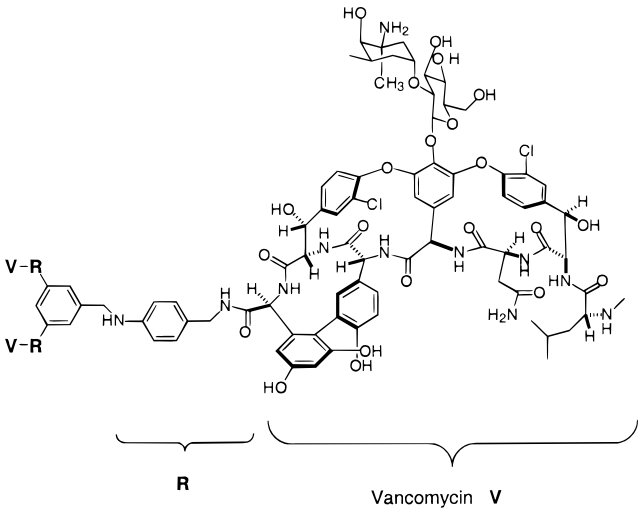

Figure 5. Trimeric vancomycin derivative for binding trimeric ^{D}Ala-^{D}Ala substrates.

B. Recognition of α-Helices and β-Sheets

Strategies for α-helix and β-sheet recognition and stabilization have been reviewed by Schneider and Kelly.[45] Monomeric α-helices may be stabilized through covalent attachment of N-terminal templates which spatially orient H-bond acceptors for the amides at the first turn of the helix. Most notable of these are the templates of Kemp[46] and Bartlett.[47] Helix nucleation is the greatest energetic cost to helix formation, and the template strategies have proven effective in stabilizing isolated α-helices in aqueous buffer. Intrastrand interactions, most often in an *i*, *i* + 4 orientation and using noncovelant forces such as ion pairing[48] and hydrophobic interactions[49] also stabilize helicity. Further, covalent linkages have also been shown to stabilize the α-helical conformation in model systems. In addition to the lactam[50,51] or disulfide-bridged examples,[52] Blackwell and Grubbs have shown that a ring-closing metathesis (RCM) reaction on allyl-protected serines or homoserines in a heptapeptide can stabilize peptide helical conformations.[53] Using water-soluble metathesis catalysts, one can imagine strategies where several cross-linked side chains could stabilize longer stretches of α-helical peptides.

Rational recognition of the side chain functionality found on protein surfaces has been studied in our group using model α-helical peptides and molecules designed for binding in competitive solvents. Our approach is based on the hydrogen-bonding complementarity between peptide carboxylate groups and synthetic receptors containing guanidinium sites. This interaction is pronounced in organic solvents due to reduced desolvation energies and the lower dielectric of the medium. Our initial concept was to bind aspartate side chains with a rigid diguanidinium molecule (Figure 6a). The spacing of the guanidinium functionality in the original system was based on the distance between side chains spaced one turn away from each other in either an *i*, *i* + 3 or *i*, *i* + 4 orientation. A marginal preference for binding the helix with an *i*, *i* + 3 orientation of aspartates was observed in 10% H_2O/90% CH_3OH.[54,55]

Increasing the number of guanidinium−carboxylate interactions in the complex has led to increased affinity, but a more interesting aspect is the importance of shape complementarity within the functional group array. When this binding motif was extended into a tetrameric system, the difference in side chain orientations relative to the helix axis was accentuated.[56] The periodicity of residues in an α-helix is 3.5 Å/turn, which puts the *i*, *i* + 3, *i* + 6, *i* + 9 orientation out of phase with the helix. The effect (which is related to the presentation of leucines in a coiled coil) creates an array of side chains that are in a left-handed superhelical orientation of their own. Residues spaced *i*, *i* + 4, *i* + 7, *i* + 11 correct for this tendency and thereby place functionality linearly along one face of the helix (Figure 7).

Our results using a tetraaspartate peptide and tetracationic molecules (Figure 6b) have shown that

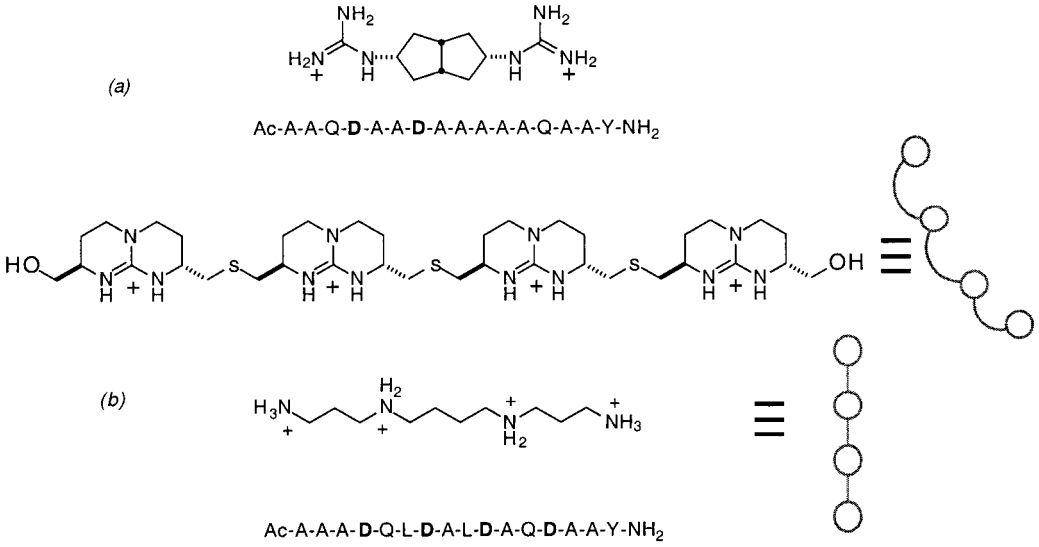

Figure 6. (a) Designed diguanidinium derivative for binding diaspartate peptides in an α-helical conformation. The *i*, *i* + 3 diaspartate peptide is also shown. (b) Tetraguanidinium and tetraammonium (spermine) structures and schematic representation of their functional group orientation with respect to the tetraaspartate (*i*, *i* + 3*n*) peptide. All cations were used as their chloride salts.

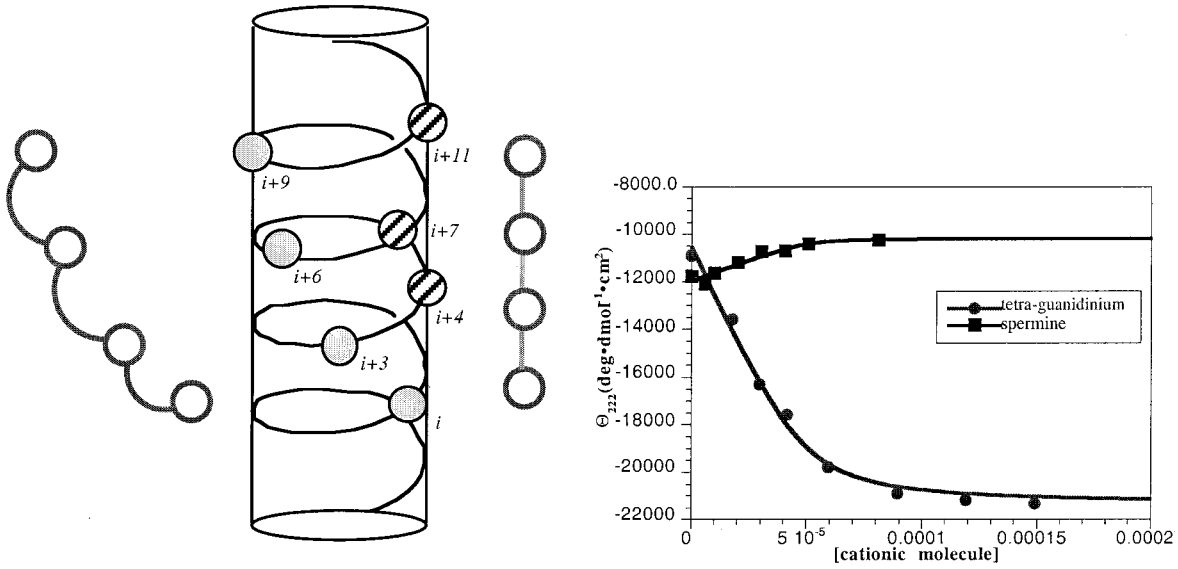

Figure 7. (Left) Comparison of side chain functional group presentation of i, $i + 3$, $i + 6$, $i + 9$ and i, $i + 4$, $i + 7$, $i + 11$ groups of an α-helix. The suggested "complementary" shapes that go along with the respective presentations are also shown. (Right) CD binding titration of tetraaspartate peptide with cationic binding molecules. The change in θ_{222} for the tetraguanidinium molecules indicates increased helicity with binding.

spermine (represented as presenting functionality in a linear fashion) is unable to bind the polypeptide in an α-helical conformation, whereas the tetraguanidinium receptor can, due to the superhelical arrangement of guanidinium functional groups ($K_a = (2-3) \times 10^5\ M^{-1}$ in 10% H_2O/90% CH_3OH). This is a result of the spacings of positive charges, and their presentation toward the amino acid side chains in the α-helical conformation. We have used CD spectroscopy as a measurement of helical induction that results from intermolecular interactions between the polypeptide and designed receptors. As seen in Figure 7, binding of the tetraguanidinium results in sizable changes in θ_{222} as a function of guanidinium concentration. On the contrary, binding of spermine results in an apparent destabilization of helicity. However, Sasaki et al. have shown that spermine can bind and stabilize a tetraglumatate peptide sequence when the spacing of the acidic residues is in an i, $i + 4$, $i + 7$, $i + 11$ orientation.[57] This suggests that orientation of functionality on an α-helical peptide should be considered in the design of binding molecules.

In their review, Schneider and Kelly articulate two strategies for nucleating β-sheet folding, through strand or turn mimetics. Molecular scaffolds such as the oligourea of Nowick[58,59] and the dibenzofuran of Kelly have proven to be very successful model systems.[60,61] These systems have been used to illustrate intermolecular β-sheet association and subsequent self-assembly. In particular, a recent version of the urea-based β-turn model of Nowick incorporates an oxalamide to mimic the H-bonding pattern along the bottom edge of a β-sheet (Figure 8).[59] A stable, antiparallel, dimeric species was observed as evidenced by intermolecular transfer ROE cross-peaks in heterodimeric examples. The most significant breakthrough here is the identification of a discrete β-sheet dimerization event which can be monitored. This permits increased understanding of the molecular and atomic details of β-sheet association.

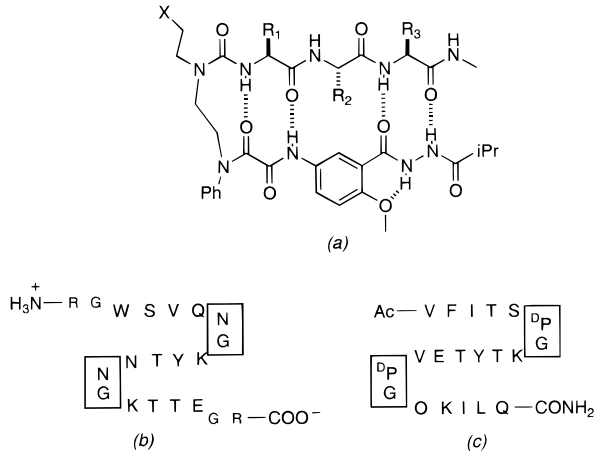

Figure 8. (a) General structure of Nowick's model β-sheet dimerizing molecule designed β-sheet structures exhibiting a cooperative fold based on a dipeptide turn motif. (b) The Serrano Asn-Gly motif. (c) The Gellman ^{D}Pro-Gly motif.

Protein association through β-sheet structures is common in nature; the model studies should afford access into designed molecules which associate with natural protein targets through β-sheet domains.

Recently two β-hairpin turn strategies have exhibited cooperativity in antiparallel β-sheet formation (Figure 8).[62,63] Short peptides with either Asn-Gly or ^{D}Pro-Xxx motifs to induce β-hairpins in 20-mer polypeptides were designed. Stable structures were developed using side chain rotamer modeling and carefully positioned salt bridges. Structural data, primarily from 2D NMR and CD spectroscopy (T_m and urea denaturation experiments), confirmed the designed structures. The stabilized β-sheet polypeptides consisted of three strands linked by two of the respective dipeptide β-hairpins.[64] A series of controls were available by positioning ^{L}Pro in the place of ^{D}-Pro at the first and second hairpins as well as at both in the same sequence. The authors note that the ^{L}-Pro-^{L}Pro derivative lacks structure as determined by

Table 1. Examples of Interface Peptide Inhibition of Protein−Protein Interactions

proteins	interface	techniques	refs
		Viral/Bacterial Proteins	
HIV-1 gp41	α-helix	CD, ELISA	74,77
HIV p24 (homodimer)	α-helix	ultracentrifugation, Immunoblot	111
HIV RT (homodimer)	W repeat	RT assay, fluorescence, size exclusion	112, 113
HIV protease (homodimer)	β-sheet	protease assay, size exclusion, protein cross-linking, fluorescence	114−116
HSV ribonucleotide reductase (homodimer)		reductase assay	117−119
L. casei thymidyalte synthase (homodimer)	β-sheet	TS Assay, fluorescence, NMR	120
*Eco*R1	α-helix	size exclusion, CD, cleavage assay	121
		Transcription Factors	
human estrogen receptor (hER) (homodimer)	SH2 domain	DNA gel-shift assay, size exclusion, immunoblot	122
bHLH TF E47 (homodimer)	α-helix/β-sheet	DNA gel-shift assay, size exclusion, CD	68, 123
Jun (homodimer)	α-helix	DNA gel-shift assay, size exclusion, cytotoxicity assay	124, 125
		Cellular Proteins	
Ras-Raf	β-sheet	fluorescence polarization	126
p185neu (homodimer) HER2/neu	α-helix	immunoprecipitation/autoradiography	127
Bcl-x_L-Bak	α-helix	fluorescence, NMR	128
APC (homodimer)	α-helix	native gel electrophoresis, CD, Western blot	129
guanyl cyclase (homodimer)	α-helix	Western blot, size exclusion, yeast two-hybrid analysis	130
p53-MDM2	α-helix	X-ray crystallography	131
Cdk4-$p16^{INK4a}$	α-helix	pRb phosphorylation assay	132
Src-SH2 domain	SH2 domain	X-ray crystallography	133
$p21^{Cip1/Waf1}$-cyclinE/Cdk^2	β-sheet	kinase assay, immunoblot, CD	134
$p21^{Cip1/Waf1}$-PCNA	β-sheet		
protein kinase CK2 α-β subunits		CK2α activity, ultracentrifugation, Western blot, SPR	135
poly-Pro/SH3/WW		fluorescence, PAGE	136

CD, while ^{D}Pro-^{L}Pro and ^{L}Pro-^{D}Pro show intermediate structure relative to that of the parent ^{D}Pro-^{D}-Pro sequence.

These models should prove amenable to further investigations on the nature of β-sheet formation in natural systems and more refined measurement of β-sheet propensities of amino acids and protein folding. A future extension of this work will be in the design of molecules that bind β-sheets in a well-defined manner, as has been done in the α-helical model peptide systems.

IV. Protein Surface Recognition

A. Interface Peptides

The philosophy behind the "interface peptide" strategy is straightforward; if a particular protein is involved in protein−protein interactions, then it can be inhibited by shorter peptides with the same sequence as key regions at the interface. Table 1 lists some examples of this strategy from the recent literature. This subject has also been reviewed by Zutshi and Chmielewski with emphasis on the inhibition of dimerization of viral proteins.[65] Peptide−protein interactions from a general perspective have also been reviewed.[66] The use of peptide libraries to identify sequences that inhibit protein−protein interactions is a powerful technique aimed at answering the same questions.[67] The power of interface peptides is their ability to suggest even smaller nonpeptidic structures that can inhibit the same protein−protein interactions. Specific examples in the application of this approach will be described.

The interface peptide approach has been extended from inhibition of enzyme dimers to transcription factors. Ghosh and Chmielewski have demonstrated that helix II from the basic helix−loop−helix (bHLH) transcription factor E47 was effective at inhibiting homodimerization and subsequent DNA binding of the full-length E47 protein.[68] The system was characterized by a gel mobility shift assay, size exclusion chromatography, protein cross-linking experiments, and CD spectroscopy. In particular, the CD spectroscopy results showed a transition from an α-helix to a β-sheet of E47 in the presence of the helix II peptide, which itself was shown to have β-sheet character. When helix II is added to native E47, the associated heterocomplex assumes a β-sheet structure. The effectiveness of the inhibition was attributed to the inherent β-sheet nature of the helix II sequence, which effectively sequesters the full-length protein by allowing it to assume a sheet structure.

Important modifications of the interface peptide approach are also under investigation. Retro−inverso[69] or partially modified retro−inverso peptides[70] start with active polypeptides in the L-configuration and reverse the peptide sequence, incorporating D-amino acids in place of L-amino acids. This combination of changes will present amino acid side chain functionality in a way that is nearly identical to that of the natural sequence.[71,72] Examples of secondary structural stabilization techniques being used in conjunction with interface peptides are beginning to appear in the literature.[73,74]

An elegant combination of these ideas has been demonstrated by a group at Genentech.[74] The envelope glycoprotein of HIV-1 (gp41) mediates membrane fusion between the virus and target cells. gp41 is an α-helical trimeric coiled coil protein where the N-terminal portion forms a parallel trimer at the core

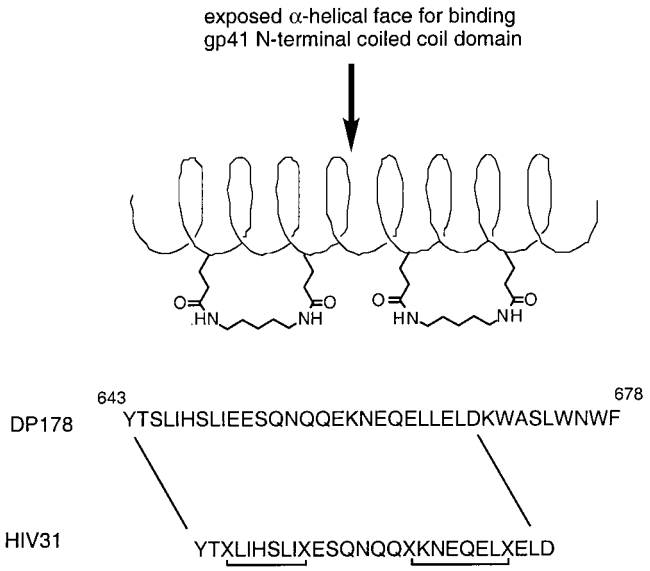

Figure 9. Interface peptides for inhibition of gp41 membrane fusion and alkylglutamine strategy for α-helix stabilization.

of the protein and the C-terminal portions arrange themselves in an antiparallel fashion around this inner core when it is in the fusogenic state. The crystal structure of the N- and C-terminal portions of gp41 has been solved, showing a six-helix bundle.[75,76] Inhibitors of the fusion could be envisioned by binding the N-terminal core of the coiled coil trimer before the C-terminal peptide binds to form the fusogenic gp41.

Earlier work had shown that the C-terminal portion of gp41 was a potent inhibitor of viral membrane fusion.[77] Fragments of the C-terminal peptide of gp41 (residues 643−678) were designed to contain covalent linkages between *i*, *i* + 7 residues based on the work of Phalen et al. (Figure 9).[51] In brief, this approach is to covalently tether glutamate (making alkyl-linked glutamines) residues with diaminoalkyl chains. This method was shown to effectively stabilize the α-helical conformation of a model peptide.

In the gp41 work, peptides having one or two covalent tethers were shown to be significantly α-helical from 7 to 37 °C by CD. The inhibition of viral infectivity was assayed by quantifying the amount of p24 antigen (from cell lysates) found in cells treated with either free HIV virus or HIV virus particles incubated with inhibitor peptides bearing one or two cross-linked glutamines by ELISA. The results showed that certain stabilized helical peptides could block viral fusion. Peptides that were cross-linked on the face of the peptide proposed to bind the core trimer were inactive, and peptides with two cross-links were more effective than those with one. The researchers drew a direct correlation between helicity and inhibitory potency. Generalization of this conclusion would suggest that conformationally restricted interface peptides should be even more effective inhibitors of protein oligomerization than linear, unstabilized sequences.

The above approach has offered further support toward the proposed mechanism of viral membrane fusion (Figure 10). This entails a large-scale gp41 protein reorganization upon gp120 binding to CD4 and CXCR4 on the target cell. Upon binding, the C-terminal domains (the region from which the

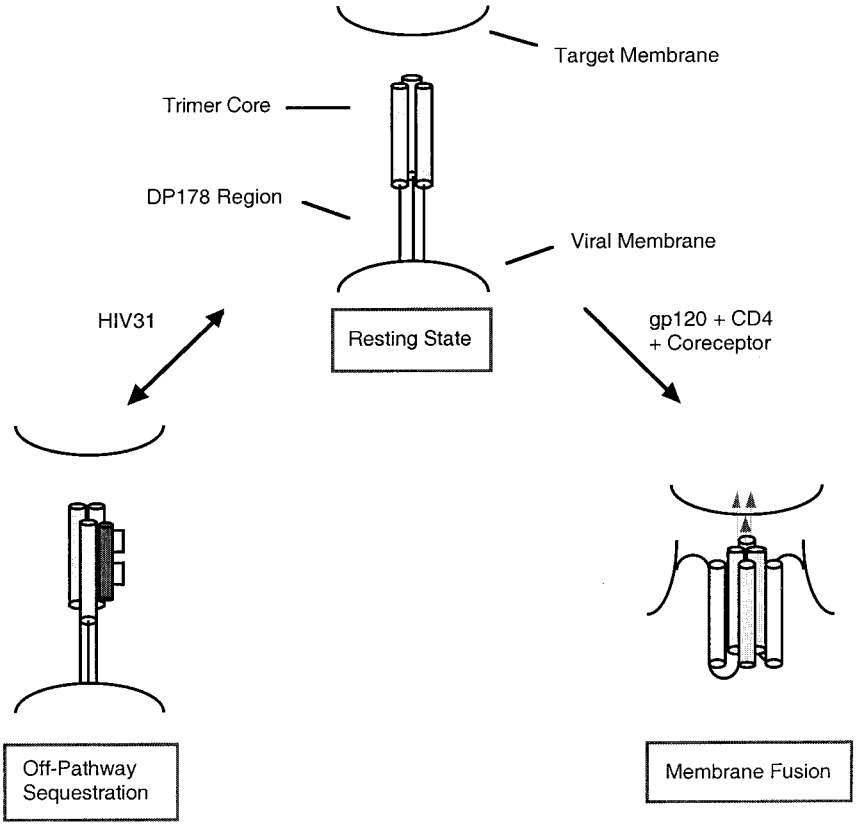

Figure 10. Proposed HIV viral membrane fusion mechanism and its inhibition by stabilized interface peptide HIV31 (adapted from ref 74).

inhibitory peptides in this study were derived) of gp41 fold over to bind the coiled coil trimer of the N-terminal fragment, thereby putting the viral membrane in close proximity to the cell membrane (Figure 10). The stabilized interface peptides were equally effective at inhibiting viral infectivity of cell culture strains of HIV-1 and primary isolates, suggesting that this process within the viral life cycle is relatively conserved across viral strains and could be a general approach to inhibit viral membrane fusion.

B. Peptidomimetics of Protein Secondary Structure

To this point, the discussion has focused on sequence-selective recognition of short peptide sequences by designed molecules, and the binding of multimeric proteins using interface peptides, a design feature taken from the interacting proteins. Combining these strategies offers an attractive route to protein surface recognition involving a secondary structure mimetic approach in which protein features are incorporated into a nonprotein framework. Currently, there is considerable effort in defining the proper molecular scaffolds that can efficiently mimic protein secondary structures.

β-Turn mimetics in the context of nucleating a β-sheet have already been discussed. However, β-turns themselves mediate a myriad of protein−protein interactions. β-Turn mimetics for the inhibition of specific protein−protein interactions have been designed from carbohydrate scaffolds. In work by Hirschmann et al., a mimetic for the cyclic peptide somatostatin (Figure 11a) has been synthesized using a β-D-glucose scaffold (Figure 11b).[78,79] Key residues in the β-turn portion were identified from deletion analogues of the hexapeptide. The shape and substitution pattern of D-glucose were found to best present the Trp, Lys, and Phe side chains. Somatostatin receptor binding assays using ^{125}I-labeled somatostatin proved the mimicry of the structure, although with reduced activity relative to that of the natural hormone ($\Delta IC_{50} \approx 10^4$).

Similarly, Nicolaou and co-workers have synthesized a number of cRGDF*V* mimetics for inhibition of integrin/ligand interactions where different orientations about the pyranose ring and glycosidic bond were evaluated.[80] These mimetics were shown to be mostly inactive in cell adhesion assays. The authors suggest that subtle requirements for the active rigid conformation may be needed for bioactivity in this system. The benefits of such carbohydrate scaffolds include an attractive balance between rigidity and diversity of functional group orientation. Also, the polyoxygenation of such compounds may aid in water solubility.

Smith and Hirschmann have also made significant inroads into the synthesis of short portions of extended β-sheet mimics. They have defined a pyrrolinone-based scaffold that mimics peptide β-sheet backbone hydrogen-bonding donors, acceptors, and side chains. This is a remarkable achievement in molecular design to reproduce all of the key recognition features of a short peptide within a low molecular weight nonpeptide analogue. The approach was applied to the design of substrate mimetics of HIV-1 protease.[81] Of particular interest is their recent report extending this motif to ligands of MHC class II proteins.[82] In this work, the bispyrrolinone scaffold was incorporated into a peptide sequence by making an amino acid derivative (Figure 12a). Basing their approach on crystal structural evidence of a bound hemagglutinin peptide fragment, they anchored the mimetic by maintaining those amino acid residues that contribute the most to the binding energy. The peptidomimetic structure was shown to have an affinity similar to that of the original peptide sequence for the MHC class II molecule (K_d = 89 nM for Figure 12b, 137 nM for Figure 12c). Methylation of the amino nitrogens in the pyrrolinone structure favors different conformations that resemble turns or helical structures.[83] An alternative scaffold for β-sheet mimicry based on a triterpene framework has been utilized in the inhibition of dimerization of

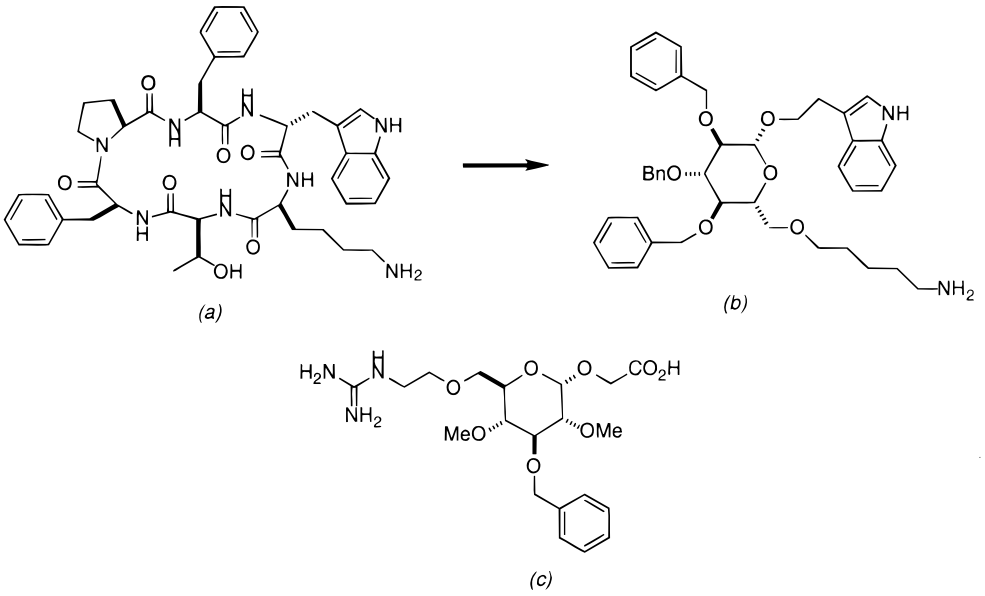

Figure 11. Carbohydrate-based peptidomimetics of β-turn molecules. (a) Somatostatin and (b) β-D-glucose-derived mimetic of somatostatin. (c) Representative mimetic of a cRGDF*V* turn.

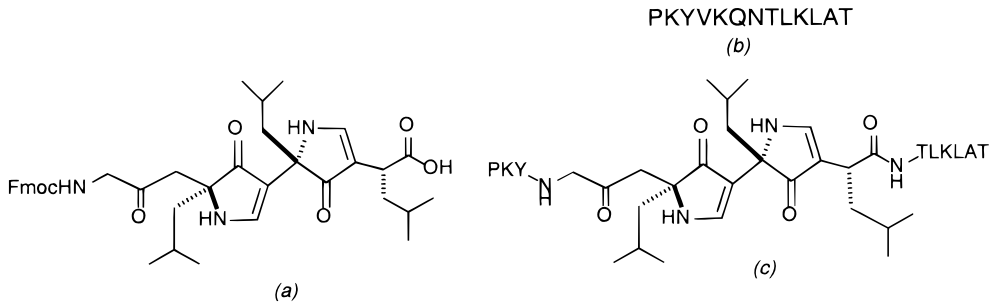

Figure 12. Pyrrolinone-based β-sheet mimic. (a) Pyrrolinone mimic amino acid. (b) The hemagglutinin MHC II antigenic peptide and (c) the mimetic of this sequence evaluated in binding assays.

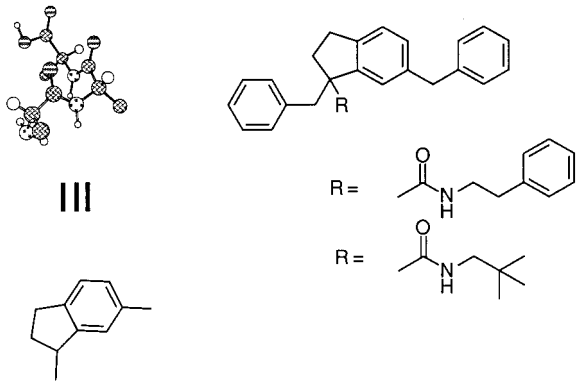

Figure 13. The indane i, $i + 1$ α-helix mimic. (Left) A schematic showing the i, $i + 1$ α-helical mimicry in the indane system. (Right) Trisubstituted indanes assayed for binding to tachykinin receptors.

HIV-1 protease.[84]

The β-amino acid structures currently being studied in the laboratories of Seebach and Gellman (among others) hold great promise.[85] Oligomerized β-amino acids (or γ-amino acids) are members of a family of new folded structures, termed "foldamers". A key to the success of these molecules is the incorporation of diverse functionality into side chains while maintaining a well-defined secondary structure in aqueous solvents. This is a complementary and novel approach to overcoming the same obstacle facing interfacial peptides, namely, conformational flexibility. Assuming that this problem can be solved, several protein−protein interactions that involve α-helices at the interface could be targeted with this strategy.

While α-helices mediate many protein−protein interactions, and are a common structural motif of protein surfaces, scaffolds that efficiently present functionality in an α-helical mimetic fashion are still lacking. Recent progress in the area of α-helix mimicry has been reviewed by Fairlie.[86] Dipeptide mimetics based on an indane scaffold are to date the best characterized nonpeptide mimics of α-helical structure (Figure 13).[87−89] These molecules do not cover enough primary sequence space to be called true α-helix mimics. A trisubstituted version of the indane scaffold having Phe and Trp mimetic side chains was shown to bind tachykinin NK_1 NK_2, and NK_3 receptors.[88] The optimal compound showed micromolar affinities for the receptors, similar to the corresponding dipeptide leads.

All of these mimetics aim at presenting functionality in a semiorganized fashion to alleviate the energetic consequences of restricting conformation upon binding by short polypeptides. The biggest challenge now is to create scaffolds that can present a diverse array of amino acid side chain mimics over an extended surface area.

C. Protein Surface Recognition by Designed Molecules

Modification of the functional groups on a protein has been utilized to allow a protein to adapt to a nonaqueous environment,[90] or for stabilization of its tertiary conformation through metal-mediated cross-linking.[91] The use of metals for the separation or modification of proteins has been proposed.[92,93] Similarly, proteins have been engineered through either cofactor reconstitution or semisynthesis to react to certain environmental factors.[94−97] This area of research is illustrated in recent work from Hamachi et al.[97] Here a semisynthetic ribonuclease S was designed to incorporate metal-chelated residues, on the basis of earlier work from Hopkins.[98,99] Synthetic ribonuclease C peptides incorporating one or two copies of the unnatural iminodiacetic acid amino acid (Ida) were synthesized. The researchers demonstrated that the activity of ribonuclease S′ was related to the concentration of Cu(II), and they proposed a mechanism where two Ida molecules cooperatively bind one Cu(II) to stabilize the ribonuclease C helical conformation and thus activate the enzyme (Figure 14). These results represent a novel example of enzyme activity influenced by the environment of the protein. Other systems can be imagined where tight, specific binding would operate a molecular switch to activate protein function only in the presence of a protein surface cofactor.

One of the rare examples of protein surface binding molecules is an antagonist of the IL-2/IL-2Rα interaction.[100] The molecule (Figure 15a) was designed to mimic a region of IL-2. Detailed investigations showed that the designed molecules bound IL-2 itself, rather than the receptor molecule (IL-2Rα). $^{15}N-^{1}H$ HSQC experiments mapped the interaction to an N-terminal loop region of IL-2 which interacts with IL-2Rα.

A second example involves the successful design of a protein A mimetic for the purification of IgG.[101] The X-ray crystal structure of the complex was crucial to the design of the mimetic. Two key residues, Phe 132 and Tyr 133, are located on the surface of a helix which binds into a shallow groove on IgG. α-Carbon distances and ζ-carbon distances were used in selecting the triazine ring as a scaffold. Additional

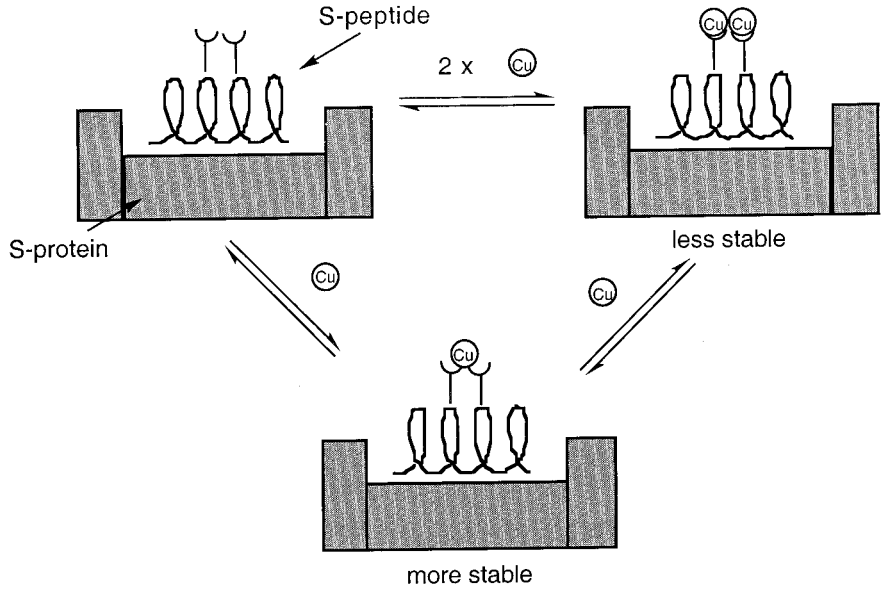

Figure 14. Schematic representation of Hamachi's Ida-derivatized S-peptide and the resulting stability of the protein as a result of the concentration of added Cu(II).

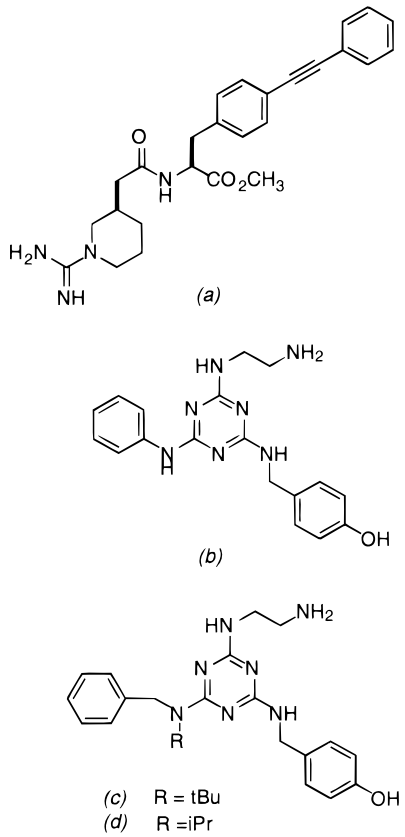

Figure 15. Small molecule protein binders. (a) An IL-2 mimetic which binds IL-2. (b−d) Designed *S. aureus* protein A mimetics for the purification of IgG.

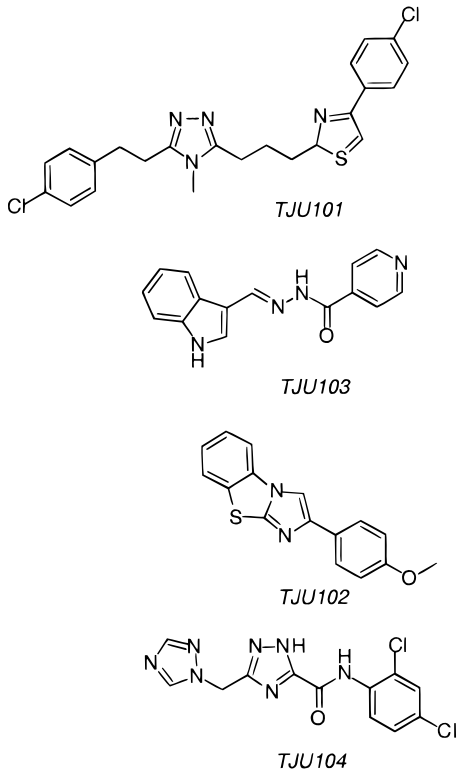

Figure 16. Four of the most potent inhibitors of CD4 MHC class II protein−protein interaction identified through the molecular modeling screening approach.

molecules based on this scaffold, but with a discontiguous Leu 136 or Phe 132 mimetic, were also designed to increase the size and volume of the interface, as established by molecular modeling. The three molecules showed similar affinities for IgG (1×10^4, 1.7×10^4, and 2.0×10^4 M^{-1}, for Figure 15b−d) as determined by an ELISA assay. These affinities are 1/1000 of the affinity of those of the natural protein, but represents a significant achievement considering the differences in size (MW 400 vs 14 000 for the protein). The originally designed mimetic was then used to functionalize an agarose column for the purification of IgG. This result is an encouraging prelude to future work that will come from the combination of computer-aided molecule design, organic synthesis, and chemical intuition.

A molecular docking program (DOCK) has been used to identify nonpeptidic molecules suitable for interacting with CD4, the cell surface coreceptor of MHC class II antigen presenting proteins.[102] Inhibit-

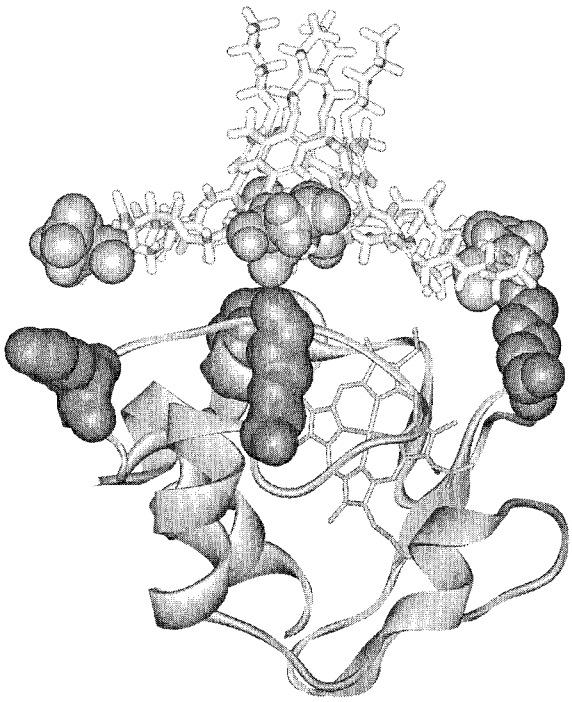

Figure 17. Proposed structure of the cytochrome *c*/antibody mimic complex.

ing this interaction would block the activation of CD4+ T cells, which is a critical process for many autoimmune diseases. The identification of a suitable binding cleft was followed by a computational screening of approximately 150 000 compounds found in the ACD database. This screening yielded 41 compounds, 8 of which showed significant activity in a cell adhesion assay. Further investigation of toxicity and efficacy showed TJU103 (Figure 16) to be the most effective compound in prolonging the median survival time of a skin allograft model. The screening approach developed in this study should be a general route for the inhibition of Ig superfamily molecular interactions.

Our approach to protein surface recognition draws inspiration from the immune system. Antibody fragment antigen binding (FAB) domains use six hypervariable loops to bind the target antigen (i.e., protein surface). We have introduced a calix[4]arene scaffold with pendant cyclic peptide units as a mimetic of antibody Fab fragments.[103] These designs provide a semirigid arrangement of several peptide loops[104−106] that mimic the antibody hypervariable loops of the complementary determining regions (Figure 18). The first synthetic mimic contained cyclic peptides with the sequences 3-amb-Gly-Asp-Gly-Asp (3-amb = 3-aminomethylbenzoic acid). This synthetic receptor was used to bind cytochrome *c* in a region of the protein rich in basic residues (Lys 17, 18, 21, 77, and 78). Natural protein partners of cytochrome *c* (cytochrome oxidase, cytochrome *c* peroxidase) bind in the same region of the protein (Figure 17). Evidence for effective complexation of cytochrome *c* came from gel permeation chromatography, ^{1}H NMR, and kinetics assays on the inhibition of ascorbate reduction of Fe^{III}−cyt *c*. In conditions of high phosphate buffer concentrations, these molecules nonetheless bind to their protein targets with K_a values in the 10^5-10^7 M^{-1} range.

The power in this approach lies in its ability to use the diversity of natural and unnatural amino acids in the cyclic peptide loops to define a wide range of antibody mimics. These mimetics are able to bind large surface areas (>600 Å^2), with a variety of surface shapes based on the amino acid sequence and nature of the central scaffold.

Binding to protein surfaces can be an alternative method for the inhibition of proteases. Enzymes can potentially be inactivated by binding near, but not *in*, the active site. The mechanism of inhibition involves a steric blocking of the approach of the substrate. Whitesides has incorporated a secondary recognition element into an active site inhibitor of human carbonic anhydrase II that utilizes hydrophobic patches near the active site cleft of the enzyme.[107] A phenylglycine was used in place of glycine to interact with a phenylalanine near the active site. An additional 10^2-10^3 M^{-1} in binding affinity was gained through this interaction.

The biological activity of some linear anionic polymer molecules has been reported.[108] These are reminiscent of heparan sulfate, which is a polysulfated polysaccharide of varying molecular weights (MW range 5000−30000). Aurintricarboxylic acid and oligophenoxyacetic acid are aromatic, polymeric carboxylic acids which have heparin-like properties in their ability to reverse basic fibroplast growth factor (bFGF) mediated autocrine cell transformation.[108] A

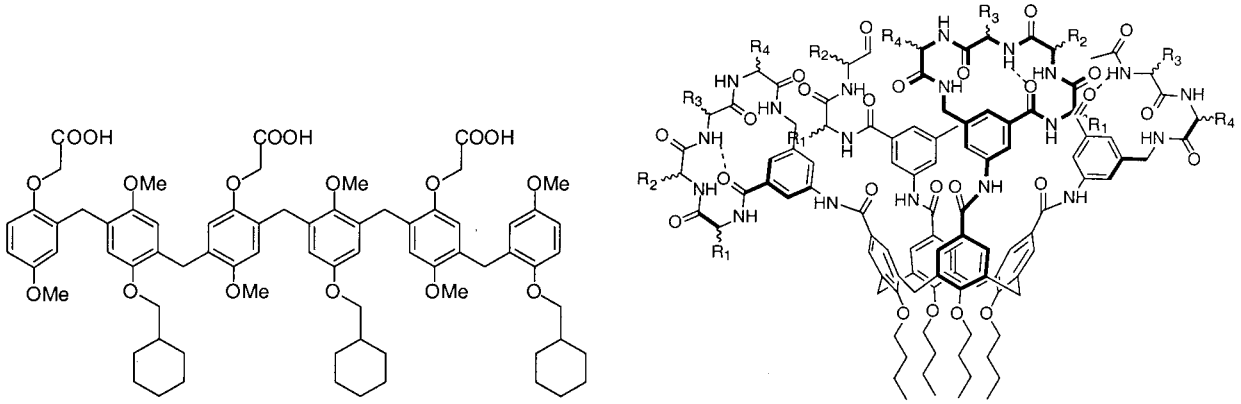

Figure 18. (Left) A designed approach to protein surface recognition using an oligophenoxyacetic acid motif. (Right) General structure of the calix[4]arene-based antibody mimics.

series of discrete diphenylmethane-based oligomers with hydrophobic and anionic functionality are potent inhibitors of human leukocyte elastase.[109] The most potent was a 6-mer oligomer (Figure 18) that presumably interacted with basic residues adjacent to the active site that were themselves surrounded by hydrophobic regions. K_i values of 17−200 nM were reported. The calix[4]arene antibody mimic motif has been used in a similar way to bind to basic residues on the surface of α-chymotrypsin.[110] In this case, a series of lysine residues are arrayed around the active site. The importance of functional group and geometric complementary in binding protein surfaces using designed molecules is demonstrated in the last two systems.

V. Perspective

The examples described above suggest great promise in the design of small molecules to bind protein surfaces. The key issues in this field are the development of better scaffolding structures for the presentation of binding functional groups in a specific orientation. This further requires more accurate mimics of larger sections of protein secondary and tertiary structure. The absence in the literature of any nonpeptide mimics of extended regions of α-helices or β-sheet structure suggests the solution is still some years away. It is highly probable that a general solution to this protein surface recognition problem, if it is to be found, will involve molecules with a large surface area. Only in this way will the large number of weak interactions be available to overcome the highly solvated character of the protein surface. Within these molecular designs the most critical feature will be a controlled balance of hydrophobic and charged regions. This balance must lead to complementarity and high-affinity interactions with the target but must not result in problems of insolubility, aggregation, or nonspecific protein binding.

VI. Acknowledgment

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VII. References

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