Abstract

Optimization of $\beta^3$-peptide structure and function with applications to a novel viral target

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This dissertation describes the development of first generation $\beta$-peptides that incorporate optimized electrostatic interactions into their 14-helical structure, and bind a novel peptidyl model of the Severe Acute Respiratory Syndrome (SARS) Coronavirus (CoV) prefusion complex. Chapter 1 presents a systematic analysis of the relationship between salt bridge composition and 14-helix structure within a family of model $\beta$-peptides in aqueous buffer. An inverse relationship between side-chain length and the extent of 14-helix structure, as judged by circular dichroism, was found. Introduction of a stabilizing salt-bridge pair within a previously reported $\beta$-peptide ligand for hDM2 led to changes in structure that were detectable by two-dimensional NMR spectroscopy. Chapter 2 describes the development of a peptidyl model of the SARS prefusion complex organized around a metal bipyridine center, and the first generation $\beta$-peptide inhibitors that bind this model. The novel human coronavirus associated with the 2003 outbreak of SARS-CoV exploits a type 1 surface glycoprotein, spike (S), to facilitate host cell entry. The S protein is essential for cell surface receptor binding and subsequent fusion with a host cell and is therefore an attractive target for potential anti-SARS therapeutics. As a first step towards developing $\beta$-peptide inhibitors of S protein-mediated cell fusion, a synthetic peptide model of the S protein prefusion complex (bDG2) was designed. In this model, three copies of residues 899-928 of the S protein, each linked to a single 5-
carboxy-2,2’-bipyridine ligand, assemble into a stable trimer upon coordination with Fe$$^{II}$$.

Fe(bDG2) is highly $$\alpha$$-helical and soluble in a variety of physiological buffers. Two 14-helical $$\beta^3$$-decapeptides ($$\beta$$LVL1 and $$\beta$$LVL2) were subsequently identified, by FRET and ELISA assays, that bind Fe(bDG2) in the nanomolar concentration range. Substitution of $$\beta^3$$-homoalanine in place of residues on two faces of $$\beta$$LVL2, suggest that these $$\beta$$-peptides bind Fe(bDG2) in a novel way. An in vivo neutralization assay recognized $$\beta$$LVL1 as 30% effective in decreasing viral infection. Fe(bDG2) is anticipated to prove useful for in vitro binding studies of SARS inhibitors, and further elaboration of the $$\beta$$LVL peptides could generate potential leads for therapeutic inhibition of SARS.
Optimization of β³-peptide Structure and Function with Applications to a Novel Viral Target

A Dissertation
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Dissertation Director: Dr. Alanna Schepartz
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Chapter 1  Relationship Between Salt-Bridge Identity and 14-Helix Stability of $\beta^3$-Peptides in Aqueous Buffer
Introduction

The importance of β-peptides

One of the most attractive goals of chemical biology research is the synthesis of unnatural polymers that structurally and functionally emulate systems in nature. Folding is an attribute common to biological molecules such as proteins and nucleic acids.\textsuperscript{1} The activity of proteinogenic, biomolecular “machines” is often intrinsically related to the three-dimensional structure of the proteins involved in the complex. New systems for molecular recognition, catalysis, and protein folding can be uncovered by the design, synthesis and detailed characterization of artificial molecules that utilize non-covalent forces to modulate folding into specific and predictable secondary structures.\textsuperscript{1,2} “Foldamers,” or structurally defined non-natural oligomers, are such artificial molecules. Examples of foldamers include artificial duplexes of tethered 2-aminotriazines (modeled after DNA)\textsuperscript{1}, peptoids (N-substituted glycine oligomers)\textsuperscript{3}, and peptides exclusively of β, γ, or ω substituted amino acids.\textsuperscript{4} Oligomers of β-amino acids (i.e. β-peptides) have shown the ability to form stable helical, pleated-sheet and tubular arrangements. β-peptides can fold into stable and diverse secondary structures with a much richer conformational energy surface when compared to their α-peptide counterparts.\textsuperscript{5} In addition, β-peptides have shown resistance to cleavage by common peptidases up to two days of exposure.\textsuperscript{6} Small β-peptides have shown helical character by NMR and circular dichroism experiments at temperatures of 80 °C and above.\textsuperscript{7} Considering the inherent stability of β-peptide secondary structure, there is a great potential for β-peptides as biomimetics, imitating protein-protein recognition and possibly creating useful drug candidates. This potential has also been harvested by controlling β-peptide tertiary
structure and the creation of large, protein-like molecules from β-amino acids with active sites and enzymatic activity is not an impossibility.

**β-Peptide Research: A Synthetic Approach**

With an extra carbon-carbon sigma bond capable of free rotation, β-amino acids were long thought to have a low tendency to form highly ordered structures when oligomerized into β-peptides. Early work by Seebach and co-workers replaced the oxygens in a chain of oligo-(R)-3-hydroxybutanones with NH groups with the hope of finding helical substructures stabilized by hydrogen bonds. What they found was an array of possible helical secondary structures, defined by the manner of substitution on the C-C bond. β3-amino acids, in which substitution is on the third carbon of the β-amino acid backbone, can be enantioselectively prepared from enantiomERICALLY pure, commercially available α-amino acids. The conversion can be achieved in two steps using the Arndt-Eistert homologation of N-protected α-amino acids followed by the Wolff rearrangement of the diazoketone reactive intermediate to give the β-amino acid derivative (Figure 1.1). Early syntheses of β3-peptides were performed in solution using (benzyloxy)carbonyl (designated as “Z”) or (tert-butoxy)carbonyl (designated as “Boc”) protecting groups and standard carbodiimide activation. However, solid-phase techniques provided rapid access to a larger and more diverse set of β-peptides. Therefore, the Arndt-Eistert homologation and Wolff rearrangement were applied in the preparation of (9H-fluoren-9-ylmethoxy)carbonyl (designated as “Fmoc”) protected β3-amino acids, which are amenable for use with the Wang (4-(benzyloxy)benzyl alcohol) resin in solid phase synthesis. A significant loss of the Fmoc protecting group was
Figure 1.1 Scheme representing the Arndt-Eistert Homologation (steps one and two, that go through a mixed anhydride intermediate) and Wolff Rearrangement (step three) to synthesize an Fmoc-protected $\beta^3$-amino acid starting from an Fmoc-protected $\alpha$-amino acid.
observed under conditions using triethylamine. This loss was found to be dependent upon the nature of the amino-acid side chain: aliphatic side chains provided good yields with only slight Fmoc deprotection, whereas acidic and basic side chains, even if protected, had extensive Fmoc cleavage. For future work, milder conditions were applied using weaker base, N-methyl morpholine (NMM). Other published methods for β³-amino acid synthesis include the addition of enolates derived from acetic acid esters to chiral sulfinimines, and the Mitsunobu reaction on derivatives of β-serine. Work in formulating more structurally diverse β-amino acids has occurred: β²,³-amino acids can be obtained by α-alkylation of urethane-protected β³-amino acid methyl esters; geminally disubstituted β-amino acids have also been prepared by a wide variety of methods.

**β-Peptide Research: Secondary Structure Determination**

The structures of β-peptides were first investigated using circular dichroism (CD), nuclear magnetic resonance (NMR) spectroscopy and X-ray analysis. Circular dichroism has been used to qualitatively detect secondary structure in α-peptides, such as α-helix, β-sheet, β-turns or random coils. Oligomers of β³-amino acids (β³-peptides) consistently portray an intense CD minimum at ca. 215 nm, a zero crossover at 208 nm and a maximum at ca. 200 nm. CD spectroscopy, however, does not provide structural information at atomic resolution, and thus is considered complementary to NMR spectroscopy. An early X-ray crystal structure of a β³-amino acid trimer showed all four carbonyl groups pointing in the same direction, enabling the carbonyl and amide protons to form a close hydrogen bonding pattern. The subunits appeared to form closed 14-membered hydrogen bonded rings with 14 atoms between the amide protons and the
oxygen of the carbonyl groups (Figure 1.2). The NMR solution structure of a β3-hexamer gave very different coupling constants between β-C protons and each of the α-C protons. This signified a lack of rotation around the dihedral angle between HN-C(β)-C(α)-CO on the NMR time scale, indicating the presence of secondary structure. An NOE of medium intensity was observed between two successive NH residues which have been known to indicate the presence of helical structure in α-peptides. In addition, there were notable NOEs between the amide proton of residue i and the H-C(β) of residues $i + 2$ and $i + 3$. There are five such interactions possible in the hexamer and all five were observed. It has since been determined, based on these initial results and supporting evidence, that β3-(L)-amino acid oligomers form a left-handed helix containing hydrogen bonds that extend across 14 atoms, between the amide proton ("$i$") and the main chain carbonyl ("$i+2$"). The idealized form of the 14-helix is composed of three L-β-amino acid residues per turn, with the side chains in the $i$ and $i+3$ positions pointing perpendicularly to the helix axis and parallel to each other in ca. 5Å distance (Figure 1.3). The helix backbone is virtually cylindrical with successive amino acid side chains projected 120° from each other on the cylinder surface.

In more recent work, Dr. Joshua Kritzer, of the Schepartz laboratory, determined the NMR solution structure of a ten residue β-peptide, β53-1, that binds hDM2 oncoprotein and inhibits hDM2 interaction with the activation domain of p53 (Figure 1.4). Previous CD, analytical ultracentrifugation experiments and NMR line widths were consistent with a monomeric, 14-helical structure for this β-peptide, and were confirmed by the NMR results. The proton resonances were unambiguously assigned using TOCSY and natural abundance $^1$H-$^{13}$C HSQC spectra, in CD$_3$OH at 10°C.
Figure 1.2 Stereoview of an overlay of 14 different structures of Seebach’s compound 2 with lowest energies based on 2D NMR spectroscopy. The residues in yellow, red, and blue are $\beta^3$-HVal, $\beta^3$-HAla, and $\beta^3$-HLeu, respectively. The side chain of the $\beta$-amino acids are omitted for clarity.
Figure 1.3  (left) Depiction of the alpha and 14-helices showing helical pitch and also handedness, all amide nitrogens in blue, all carbonyl oxygens in red.\textsuperscript{15} (right) Depicts the fourteen atoms over which the mainchain hydrogen bond forms to initiate the 14-helix.
Figure 1.4 (left) Overlay of the methanol solution structure of $\beta^{53}$-1 (in red) with the crystal structure of p53AD-derived peptide (in yellow) bound to hDM2 (gray surface). (right) Helical net diagram depicting the sequence of $\beta^{53}$-1, each $\beta$-amino acid is shown in one letter code.
ROESY experiments were then performed allowing the confirmation of 14-helical character. Three classes of medium-range ROEs characterize a 14-helical conformation: those between HN(i) and Hβ(i+2), HN(i) and Hβ(i+3), and Hα(i) and Hβ(i+3) – all 20 potential ROEs of these types were observed (Figure 1.5). The helix itself was characterized by approximately 1.61 Å rise per residue and 3.0 residues per turn for residues 1-6, with a slight unwinding to approximately 1.40 Å rise per residue and 3.3 residues per turn for residues 7-10. The unwinding distortion seen at the C-terminus appeared to be unique for β53-1, not observed for other β3-peptide structures previously studied by Seebach and co-workers.2, 16, 17 The structure revealed details of a helix-stabilizing salt bridge formed by the interplay of potential ion pairs across one face of the β-peptide and a novel “wedge into cleft” nestling of methyl groups on successive β3-homovaline residues packed against each other on another face of the β-peptide. The third face contained subtle unwinding, making the β3-homophenylalanine side chain not align with the β3-homoleucine and β3-homotryptophan side chains along this β-peptide face. The subtle distortion, possibly from the large, aromatic residues avoiding steric clashes, allowed the side chains to better mimic the recognition face of the p53AD α-helix, providing a link between structure and function in β-peptide foldamers.

**Manipulating β-peptide 14-Helix Stability in Methanol**

The helical conformations of β-peptides can be examined in terms of the main chain torsional angles, designated as ω, φ, θ, and ψ.18 The θ torsional angle, defined by the C2-C3 bond, requires a gauche conformation for folded β-peptide helices. Alkyl substituents at position C2 or C3 favor the gauche conformation and can provide a strong
Figure 1.5 Unambiguous ROEs observed for \( \beta 53-1 \) in CD\(_3\)OH.
conformational constraint (Figure 1.6).\textsuperscript{11} Gauche-type torsional angles are even more strongly promoted when the atoms are included in a cyclohexane or cyclopentane ring, as in ACHC, trans-2-aminocyclopentanecarboxylic acid and derivatives. The ring size determines the precise C\textsuperscript{2}-C\textsuperscript{3} torsional preference which influences the type of β-peptide helix that is formed.\textsuperscript{18}

A disulfide bridge was used to fix 14-helical structure in a β-hexapeptide using two β\textsuperscript{3}-HCys residues in the \(i\) and \(i+3\) positions.\textsuperscript{19} While 2D-NMR and CD data in methanol indicated that this molecule forms a left-handed helix with 14-membered hydrogen-bonded rings across the backbone, the conformational restraints imposed by the disulfide bond slightly twisted the helix and offset the other side chains from each other. This study did, however, provide evidence for the use of conformational constraints to facilitate 14-helix stability.

Incorporation of the two backbone carbons of a β-amino acid into a small carbocycle provides substantial rigidity which leads to enhanced conformational stability.\textsuperscript{20} Gellman and co-workers first used computational methods to examine the effects of such conformationally restricted amino acids introduced to a minimized deca-β-alanine helix. Minimization and dynamics studies predicted that the 14-helical form of a decamer of \textit{trans}-2-aminocyclohexanecarboxylic acid (\textit{trans}-ACHC) residues is the most stable.\textsuperscript{20} Based on these computational predictions, they synthesized oligomers of optically active \textit{trans}-ACHC residues. A hexamer was found, by crystallography, and by the NH/ND exchange rate, to adopt 14-helical conformations. The highly folded stability of \textit{trans}-ACHC containing β-peptides in methanol prompted the question of whether
Figure 1.6  The main chain torsional angles of $\beta^3$-amino acid are such that the monosubstitution at C3 is favored by a gauche conformation which minimizes steric repulsion present in other conformers.$^{11}$
more highly functionalized monomers, such as carbohydrate derivatives, could develop stable folding patterns in aqueous solution.\textsuperscript{20}

**Manipulating β-peptide 14-helix Stability in Aqueous Buffer**

For the investigation of β-peptides under physiological conditions it became necessary to have water soluble molecules that retained 14-helical structure. Several studies have been performed to optimize β-peptide 14-helices in water.

Initially, Seebach and co-workers prepared β-peptides containing hydrophilic or polar side chains, β\textsuperscript{3}-L-Lysine or β\textsuperscript{3}-L-Serine.\textsuperscript{4} CD results indicated that β\textsuperscript{3}-L-HSer has a smaller helix-disrupting effect in water than β\textsuperscript{3}-HLys. Following this, Gellman and co-workers synthesized a series of hexa-β-peptides varying the proportions of ACHC, $R,R,R$-2,5-diaminocyclohexanecarboxylic acid (DCHC), which resembles ACHC with NH\textsubscript{3}\textsuperscript{+} group substitution, and acyclic basic and aliphatic residues.\textsuperscript{21} Decreasing 14-helicity was observed in β-peptides of increasing acyclic residue composition. It was confirmed that the use of conformationally constrained β-amino acid residues allows the creation of specific three-dimensional structure that is also extremely stable in water. Then, Seebach and co-workers designed a β-heptapeptide which utilized a salt-bridge to confer both 14-helicity and solubility in water.\textsuperscript{2} 2D-NMR spectroscopic studies showed a well-defined, left-handed, 14-helical conformation with side chains β\textsuperscript{3}-HGlut and β\textsuperscript{3}-HOrn stacked atop each other allowing salt bridges to form. This was the first high resolution structural data supporting evidence that β-peptides can form salt-bridge stabilized, unique secondary structures in aqueous solution. Independently, DeGrado and co-workers developed a β\textsuperscript{3}-peptide containing alternating β\textsuperscript{3}-HLys and β\textsuperscript{3}-HGlut residues
on two faces of a 14-helix. CD data for this molecule obtained at pH values above and below the pKa values expected for basic and acidic side chains and in the presence of increasing concentrations of sodium chloride showed the loss of virtually all 14-helical character. These experiments further supported the evidence that electrostatic interactions strongly stabilize the β-peptide 14-helix.

Recent work by Dr. Scott Hart in the Schepartz laboratory showed that alleviating the 14-helix macrodipole by strategically placed positively and negatively charged β3-amino acids along one face of the helix provides stable, water soluble, 14-helical β-peptides. The sequences of the β-peptides were chosen based on adapting a set of design principles first put forth by Seebach and co-workers. The expectations were such that the use of charged residues would ensure solubility in water, residues with positively and negatively charged side-chains positioned at i and i+3 juxtaposed on the 14-helix would allow for salt-bridging, and that the use of β3-HOrnithine rather than β3-HLys to pair with β3-HGlu would ensure less entropy loss upon salt-bridge formation. Dr. Hart’s design frees up two faces of the molecule for heterogeneous side chains, unlike previous β-peptides containing salt-bridges. Both CD and high resolution NMR measurements confirmed the significant 14-helicity of an undecapeptide containing two correctly oriented β3-HGlu/β3-HOrn salt bridges on one face, three β3-HAla residues on a second face and either β3-HVal, β3-HTyr, or a glycine equivalent on the third face (1 and 2, Figure 1.7). The presence of free, uncapped N and C termini and the locations of charged residues were determined as critical for designing well-folded 14-helices in water. In addition, they also explored the extent of 14-helical modulation by β3-amino acid choice. Several β-peptides were synthesized which contained singular substitutions of β3-amino
Figure 1.7 Helical net diagrams of β-peptide scaffolds 1 and 2 used in the host guest and original salt bridge studies. The placement of β³-HOOrn residues and β³-HGlu is such that they alleviate the β³-peptide macrodipole which points in the direction of C-terminus to N-terminus.
acids with diverse side-chain functionality in place of each of the three β³-HAla residues. Initial results indicated that γ-branched residues, such as β³-HIle or β³-HVal, had a positive effect on the 14-helicity of these β³-peptides in water.

**Modulating 14-helix structure through side chain choice**

The propensity of wider range of β³-amino acid residues to promote 14-helicity was examined through a host-guest analysis by the Schepartz laboratory, and evidence that γ-branching contributes to 14-helical structure was further explored. A “host” peptide was designed with a high water solubility, a residue facilitating UV spectrophotometric concentration determination, and available sites for substitution of “guest” side chains (2, Figure 1.7). The β³-homoalanines at the 3, 6, and 9 positions provided points for side chain substitution. The overall analysis involved comparison of CD minima near 214 nm between each of 27 variants and the host peptide. Each of the three positions on the host were substituted with each of nine different proteinogenic side chains which varied widely in functionality and included charged, aliphatic, polar and aromatic groups. In agreement with expectation, β³-homolysine was 14-helix stabilizing when located near the N-terminus and destabilizing at the C-terminus. The opposite trend was observed for β³-homoglutamate which decreased the extent of 14-helix structure by 31% when located at the N-terminus, but increased the structure by 21% when located at the C-terminus. Isopropyl and isobutyl containing side chains (β³-homovaline and β³-homoisoleucine, respectively), increased mean 14-helix structure 31-44% relative to the host peptide when substituted across any of the three positions. However, the sec-butyl side chain of β³-homoleucine was either neutral or destabilizing.
\(\beta^3\)-homothreonine contains branching at the first carbon of the side chain, much like \(\beta^3\)-HVal and \(\beta^3\)-HIle, therefore it was little surprise that this residue was stabilizing at all three positions. Each of the large aromatic side chains studied were either neutral or destabilizing to the 14-helical character. Based on the structural evidence of 14-helical unwinding in \(\beta\)-peptides that contain large, aromatic side chains, the results were not unexpected and yet still showed that bulky side chains could be well tolerated by the 14-helix.\(^{14, 24}\) Overall, when considering the contribution to 14-helix stability from electrostatic residues, the patterns resemble those observed for \(\alpha\)-helical stability in natural peptides. The extent of structure stabilization observed due to charge-macrodipole interactions is similar in magnitude to that seen for \(\alpha\)-helices.\(^{24}\)

**Ion pairs in \(\alpha\)-helices**

Ion pairs, of the type \(i, i+3\) and \(i, i+4\) contribute to the stability of solvent exposed \(\alpha\)-helices.\(^{25}\) Although \(\alpha\)-helices are generally considered unstable in aqueous environments, there are several examples of such secondary structure in natural proteins that are rendered stable by the presence of ion pairs. Troponin C, and its unusual dumbbell shape, contains a central alpha-helix “handle” that is stabilized by several oppositely charged residues juxtaposed 3 or 4 residues apart.\(^{25}\) There is a high probability, across proteins containing \(\alpha\)-helices, of finding a Glu\(^-\) four residues away from a Lys\(^+\). Tropomyosin and myosin rod coiled-coils have interhelical salt bridges that contribute to the stability of their tertiary structures. Additionally, a large number of intrahelical ion pairs of the type \(i, i+3/4\) were found in these coiled coils and were found to stabilize the helical secondary structure, as well.\(^{25}\) It was seen that the total number of
helices with ion pairs involving Glu–, Asp–, Lys+, and Arg+, in a study of 299 α-helical segments of 47 proteins by Sundaralingam and co-workers, is much higher than the number of helices with like pairs, especially in α-helices of greater length (4-6 turns).25 There was an observed preference for two adjacent, like-charged residues at each of the helix termini, most likely due to the favorable interaction with the helix dipole, much like what was later observed in the Schepartz laboratory in relation to β-peptides.

Salt bridges are found in 60% of protein structures, with one third of the charged amino acids participating found in complex salt bridges (with three or more amino acids) and 20% of the total salt bridges residing in α-helices.26 Solvent-exposed salt bridges contribute only marginally to protein stability, whereas buried salt bridges stabilize native protein structures by up to 5 kcal/mol. The strength of salt bridges is determined by the geometry and distance of the interactions, the degree of solvent exposure, and the effect of neighboring residues. Thermophilic proteins have a high number of salt bridges, suggesting a connection between salt-bridge number and increased protein stability.26 The enzyme barnase has an Asp-Arg-Asp complex salt bridge that confers 0.8 kcal/mol stability, whereas the λ repressor has a stabilizing salt bridge (1.5 kcal.mol) and hydrogen bond (0.8 kcal/mol), in an Asp-Ser-Arg triplet.26 In the case of barnase, the strengths of the two individual salt bridges is coupled, and the stabilization energy is reduced upon removing the triad to a single salt bridge.27 In another example, the GCN4 leucine zipper is stabilized by 1.7 kcal/mol by an Arg-Glu-Arg at the helix surface. Most triplet salt bridges are found to be cooperative where the free energy of the pairs present simultaneously is greater than the sum of the individual parts.26 However, it was found that a solvent exposed complex salt bridge in an 18 residue α-helical peptide, triplet Glu-
Lys-Glu, in \( i, i+4, i+8 \) arrangement, did not confer a cooperative stabilizing effect.\(^{26}\) The Glu-Lys interaction was found to be weaker than the Lys-Glu interaction, and when the triplet was present, Lys-Glu dominated so that the Lys mostly adopted the rotamer that pointed it towards the \( i+4 \) Glu, such that Lys was only interacting with one of the Glu residues at any time. The inability of a central Lys to form two interactions simultaneously, lead to the non-cooperativity of the salt-bridge.

Kennan and co-workers illustrated the effect of alterations to the electrostatic interface between helices in tuning dimeric coiled-coil stability.\(^{28}\) The contact between side chains in heptad \( e/g \) positions was manipulated to produce complexes ranging in stability from ones that are essentially unstructured to those that were not thermally denatured at temperatures as high as 90 °C. Variation in side chain length was shown to dramatically impact the stability of the heterodimeric coiled coils.\(^{28}\) The template dimer structure contained Leu residues at all but the central hydrophobic core position (occupied by Asn), helix-promoting/solubilizing Ala, Gln, and Lys residues in the solvent-exposed \( b, c, \) and \( f \) positions. The \( e/g \) residues were substituted with one of four acidic or basic amino acids, bearing side chains having one to four methylene groups between the backbone carbon and the terminal carboxylic acid (pAsp, pGlu, pHGlu, pHHGlu) or amine (pDap, pDab, pDapt, pLys).\(^{28}\) A general trend of increasing complex stability with chain length was observed. Substitutions of HHGlu, or HGlu for Glu, or Lys for Dab/Dapt gave strongly helical structures by CD spectroscopy, and melting temperatures in excess of 90 °C. The capability of tuning coiled-coil dimer stability with the side chain structure of solvent exposed salt-bridges expanded the field into the territory of stabilized protein tertiary structure by electrostatic effects.
To investigate the effect of solvent exposed salt bridges on protein stability, Kallenbach and co-workers used the GCN4 leucine zipper, a coiled coil structure consisting of two 34 amino acid $\alpha$-helices that form a dimer. GCN4 consists of four heptad repeats in which most $a$ and $d$ residues are aliphatic amino acids that comprise the duplex hydrophobic core, and the $e$ and $g$ residues interact with the opposite strand and influence both stoichiometry and stability of the coiled coil. The $b$, $c$, and $f$, sides were substituted with Ala, and simple or complex salt bridges. An Arg-Glu-Arg complex salt bridge stabilized GCN4 by 1.72 kcal/mol, with a large coupling free energy of the central Glu (0.88 kcal/mol) calculated from free energy differences between Arg-Glu-Arg and Arg-Ala-Arg, Ala-Glu-Ala, and all Ala. Electrostatics were seen to play a significant role in these stabilizing effects where substitution of only 3 of the 34 amino acids contributed much relative to the wild type. A melting temperature increase of 22 °C over the wild type $T_m$, due to the single complex salt bridge, suggested that a network of bridges could contribute significantly to the stability of thermophiles.

Small, globular protein ubiquitin contains a pair of oppositely charged residues, Lys and Glu, located on the surface with the spatial orientation characteristic of a salt bridge. The strength of this Lys11 to Glu34 salt bridge is approximately 0.86 kcal/mol. If the orientation of the salt bridge is reversed, Glu11 to Lys34, the salt bridge is of a similar strength, however the global stability of the protein is disrupted by 0.53 kcal/mol. The overall contribution of the salt-bridge orientation is thought to be attributed to the charge-charge interactions between the salt-bridge residues and the rest
of the ionizable groups in their surrounding environment. So, while the surface salt bridges are stabilizing, it is strongly context dependent, with nearby charge-charge interactions being the largest determinant.\textsuperscript{30}

In an attempt to create putative salt bridges on the surface of a protein, Matthews and co-workers designed six mutants of T4 lysozyme, introducing new charged side chains close to one or more existing charged groups of the opposite sign on the protein surface.\textsuperscript{31} In each of the cases, the introduced electrostatic interactions occurred within consecutive turns of an $\alpha$-helix. Overall, when compared with control proteins in which the charged partner was removed from the mutants, contributions to stability from each of the engineered salt bridges was very small.\textsuperscript{31} It had previously been shown that the His31-Asp70 salt bridge of T4 lysozyme stabilizes the protein by 3-5 kcal/mol\textsuperscript{32}, so three additional double mutants were created so as to introduce His-Asp charge pairs on the protein surface. Each of these destabilized the protein, and presumably introduced strain into the protein fold.\textsuperscript{31} Pairs of oppositely charged residues on the surface of proteins generally have the freedom to adopt different conformations and often do not form structurally localized salt bridges. Such residues tend to remain mobile, interact weakly, if at all, and do not contribute significantly to protein stability.\textsuperscript{31} In fact, it was proposed that in proteins that bear strong salt bridges the folding of the protein must provide requisite driving energy to hold the interacting partners into the correct rigid alignment.

Recent work in the Schepartz laboratory elucidated the high-resolution X-ray crystal structure of a highly thermostable $\beta$-peptide octamer bundle that self-assembled in aqueous solution.\textsuperscript{8} This represents the first non-natural oligomer that is verified experimentally to look and act much like a true protein. Part of the design of the $\beta$-
peptide monomer units was to include a face of self-complementary charges to favor homo-oligomerization, therefore using salt-bridging to hold together the different helices into the oligomer. Packing between the helices involved both parallel and antiparallel interactions. The antiparallel interaction positions the salt bridge faces, of alternating $\beta^3$-HOrn and $\beta^3$-HAsp, of each helix in position to make complementary electrostatic interactions across the interface.$^8$ Additional electrostatic contacts, through terminal $\beta^3$-HOrn and $\beta^3$-HGlu on a face of aromatic residues, form electrostatic contacts with the $\beta^3$-HOrn and $\beta^3$-HAsp of a neighboring helix in a parallel fashion. Therefore, much of the packing in the octameric $\beta$-bundle is thought to be initiated by salt-bridging interactions. This novel structure has paved the way towards future development of functional “$\beta$-proteins,” and it is worth noting the large role the salt-bridging has played in $\beta$-protein structural assembly.

**Design of $\beta$-peptides for systematic study of side-chain identity of salt-bridging residues**

Six $\beta$-dodecamers were designed, each derivatives of previously mentioned $\beta$-peptide 2 (Figure 1.7). In each case, either (S)-2,4-Homodiaminobutyric acid ($\beta^3$-HDab) or $\beta^3$-HLys replace $\beta^3$-HOrnithine and $\beta^3$-HAsp replaces $\beta^3$-HGlu.$^{33}$ All six $\beta^3$-dodecamers contain helix-promoting$^{23, 24, 34}$ aliphatic $\beta^3$-HVal residues at positions 2, 5, and 8 along one face of the putative 14-helix and $\beta^3$-HAla residues at positions 3, 6, and 9 along a second face (Figure 1.8). Each molecule also contains a $\beta^3$-HTyr residue to simplify spectrophotometric concentration determination. The $\beta^3$-peptides were synthesized using standard Fmoc solid-phase methods,$^{10, 19, 35}$ purified using reverse phase HPLC, and their sequences confirmed using MALDI-TOF mass spectrometry.
Figure 1.8  Helical net diagrams depicting the designs of the β³-peptides in the salt-bridge study.
six molecules were monomeric at 80 µM as determined by analytical ultracentrifugation. One of the most 14-helix stabilizing salt-bridges was applied to β53-1, the structurally well-characterized14 β-peptide that binds oncoprotein hDM2. It was hypothesized that the substitution would lead to differences in structure observable by NMR.

**Results**

**Experimental Goals**

The primary objective of this systematic study was to identify the charged side chain β³-amino acid partners that best stabilize the β³-peptide 14-helix in aqueous buffer as judged by CD spectroscopy. By examining the 14-helical character of a panel of six β-peptides whose only difference is the length at which the charged entity is projected from the helical backbone, general trends can be interpreted and can inform future 14-helical β-peptide design. Introduction of a 14-helix stabilizing salt bridge into a previously reported hDM2 ligand was thought to lead to changes in 14-helicity detectable by 2D-NMR spectroscopy.

**Circular Dichroism Spectroscopy as a tool for probing 14-helical character**

CD spectroscopy, at neutral pH and extreme pH PBC buffer, was used to monitor the extent of 14-helix structure in each β-peptide at 25 °C. While CD data on β-peptides must be interpreted carefully,36 it is reasonable to assume that, for β³-peptides in particular, changes in intensity of the 14-helical signature correlate to changes in 14-helical population.18, 36-38 The CD spectra of all six molecules (Figure 1.9) are consistent with a 14-helix structure, with ellipticity minima between 211 and 214 nm, ellipticity
Figure 1.9 CD spectra of the β-peptides of the salt bridge study at 80 µM in PBC buffer at pH 7.
maxima between 195 and 198 nm, and a crossover between negative and positive ellipticity between 200 and 202 nm. The maximal values of negative ellipticity range from -11,500 deg cm$^2$ dmol$^{-1}$ to -19,500 deg cm$^2$ dmol$^{-1}$, representing a change of greater than 40%. The CD data suggest that the level of 14-helix structure among the six molecules is, from greatest to least: 2DabD > 2OD > 2DabE > 2KD > 2 > 2KE (Figure 1.9). CD spectra taken at extreme buffer pH showed similar overall trends and a highly disrupted 14-helicity, evidenced by the higher values of maximum negative ellipticity (Figure 1.10, Table 1.1).

The 14-helix stabilities of 2DabD, the β-peptide with the most 14-helical character of the series, and 2DabE, a β-peptide with intermediate 14-helical character and only one notable difference from 2DabD, were examined further by monitoring their CD spectra as a function of NaCl concentration at pH 7 in PBC buffer (Figure 1.11). In both cases the dependence of mean residue ellipticity (MRE) at 214 nm is approximately sigmoidal with a midpoint at 0.5 M NaCl; the plateaus observed at low salt suggest the formation of a stable conformation under these conditions. The CD spectrum of 2, 2KE, 2OD and 2KD at 98 °C in 1X PBC buffer, pH 7.0 is in accordance with the literature$^7$ since these β$^3$-peptides show no evidence of cooperative unfolding over the temperature range of the assay. All four molecules still exhibit 14-helical character, although diminished, at 98 °C. (Figure 1.12).

Substitution of better 14-helix stabilizing salt bridge into a β-peptide of known function

Based on the information provided by the CD spectra of 2 and 2OD, substitution of β$^3$-HAsp for both β$^3$-HGlu residues in β53-1 (well characterized ligand of hDM2) was
Figure 1.10. CD spectra of the β-peptides of the salt bridge study at 80 μM in PBC buffer at pH 2 (left) and pH 12 (right).
Figure 1.11 MRE$_{214}$ of 100 µM 2DabE and 2DabD vs [NaCl]$^{0.5}$
Figure 1.12  Change in minimum MRE as a result of temperature change from 4 °C to 98 °C for 2, 2KE, 2OD and 2KD of the salt bridge study.
Table 1.1  Minimum MRE (deg cm$^2$ dmol$^{-1}$ residue$^{-1}$) for β-peptides studied herein at 80 μM and 25 °C

<table>
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<th>$-\theta_{\text{min}}$ (pH 7)</th>
<th>$-\theta_{\text{min}}$ (pH 2)</th>
<th>$-\theta_{\text{min}}$ (pH 12)</th>
<th>%Δ (pH 2/7)</th>
<th>%Δ (pH 12/7)</th>
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<td>8710</td>
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hypothesized to lead to differences in structure observable by NMR. CD and a fluorescence polarization binding study of β53-1D were performed at 25 °C, in PBC buffer, and 1X PBS, respectively, for direct comparison between the two otherwise identical molecules. The CD spectrum of β53-1D gave a minimum MRE at 212 nm of -9564 deg cm² dmol⁻¹, only slightly lower, and blue-shifted, from the minimum of -8450 deg cm² dmol⁻¹ at 215 nm for β53-1 (Figure 1.13). Direct binding analysis of N-terminally fluorescein-labeled β53-1D to hDM2 gave a calculated dissociation constant of 526.6 ± 115.4 nM (Figure 1.13). As expected, the ROESY spectrum of β53-1D at 10 °C in CD₃OH showed multiple (ten of thirteen possible) long-range ROEs characteristic of 14-helic conformation. There were five of seven possible C₄H(i) → C₇H(i+3) ROEs and five of six possible C₉H(i) → C₇H(i+3) ROEs (Figure 1.14). Additional backbone ROEs may have been present but were obscured by resonance overlap, as was true for β53-1.37 No backbone ROEs inconsistent with the 14-helix were observed.

Two dimensional NMR spectra reveal subtle changes

Interestingly, aliphatic ¹³C-HSQC and TOCSY spectra revealed that the vicinal protons in the γ position of β₃-HOrn at position 1 were clearly resolved in the NMR spectrum of β53-1D but not in β53-1 (Figure 1.15). Additionally, the ¹³C-HSQC spectrum shows the same γ protons of β₃-HOrn residue 7 of β53-1D had better resolution as compared with those same protons on β53-1. The differences between both of these β-peptides were also seen in the ROESY spectrum. In the case of β53-1D, six long-range ROEs were observed between protons on β₃-HOrn and those on proximal β₃-HAsp residues (Figure 1.16). Overall, although the CD spectra of β53-1 and β53-1D are nearly
Figure 1.13  top: helical net diagrams depicting $\beta53$-1 and $\beta53$-1D. Bottom: red $\beta53$-1 and green $\beta53$-1D; left: CD spectrum in PBC buffer, pH7; right: fluorescence polarization direct binding assay with fluorescein labeled $\beta53$-1 and $\beta53$-1D binding to hDM2.
Figure 1.14  Backbone ROEs observed in the ROESY spectrum of β53-1D; C$_{\alpha}$H(i) → C$_{\beta}$H(i+3) ROEs are in red, C$_{N}$H(i) → C$_{\beta}$H(i+3) ROEs are in blue.
Figure 1.15  Differences in the 2D-NMR spectra of $\beta53$-1D (left) and $\beta53$-1 (right). Regions of the $^{13}$C-HSQC spectrum that are differentiated in $\beta53$-1D and non-differentiated in $\beta53$-1.
Figure 1.16 Differences in the 2D-NMR spectra of $\beta_{53-1}D$ (left) and $\beta_{53-1}$ (right).

Regions of the ROESY spectrum that are present for $\beta_{53-1}D$ and not for $\beta_{53-1}$. 
identical, the NMR data implies a subtle increase in the order of the salt-bridge side-chains in $\beta^{53-1D}$ when compared with $\beta^{53-1}$.

**Discussion**

*Shorter side-chain length on charged residues leads to greater helical stability*

Circular dichroism (CD) is a technique that uses circularly-polarized light to lend insight into the secondary structure of a compound. The components of circularly-polarized light will travel through an optically active medium (e.g. a $\beta$-peptide) with different velocities due to the different indices of refraction for right and left circularly polarized light. After passing through an optically active sample, the right and left handed components of the light will be changed: the electronic component, which travels perpendicularly from the direction of propagation of the light, will trace out an ellipse since the magnitudes of the two components (right and left) are no longer equal. Therefore, the mean residue ellipticity (MRE) is measured during CD spectroscopy. Circular dichroism is a powerful tool for the detection of secondary structure in $\alpha$-peptides. Natural peptides, oligomers of $\alpha$-amino acids, display particular CD spectral patterns depending on their secondary structure. The same can be said for $\beta^3$-peptides. Several trends emerge when the relative stabilities of the six $\beta$-peptides of this study are compared. First, molecules containing $\beta^3$-HAsp display higher levels of 14-helix structure than otherwise identical molecules containing $\beta^3$-HOrn (compare 2DabD vs 2DabE, 2OD vs 2, and 2KD vs 2KE). Second, molecules containing $\beta^3$-HDab display higher levels of 14-helix structure than otherwise identical molecules containing $\beta^3$-HOrn (compare 2DabD vs 2OD, and 2DabE vs 2). Finally, molecules containing $\beta^3$-HOrn
display higher levels of 14-helix structure than otherwise identical molecules containing β3-HLys (compare 2OD vs 2KD, and 2 vs 2KE). These trends suggest that the level of 14-helix structure in β-peptides relative to 2 correlates inversely with side chain length: shorter side chains improve helicity. Interestingly, solvent exposed salt bridges often contribute minimally to protein stability, and glutamate, not aspartate, is the preferred partner for intra-α-helical salt bridges in proteins of known structure. The pH dependence of the CD spectra of each β-peptide studied supports the hypothesis that electrostatic interactions stabilize the 14-helical conformation.

Further CD analysis of 2DabD, the β-peptide with the greatest 14-helical character, and 2DabE, β-peptide with intermediate 14-helical character and only one difference from 2DabD yielded interesting, albeit expected, results. Upon monitoring their CD spectra as a function of NaCl concentration, both 2DabD and 2DabE become significantly less 14-helical as the NaCl concentration increases from 0 to 1.5 M as judged by the MRE at 214 nm. These CD data are highly reminiscent of those reported by Cheng and DeGrado for a 15-residue β-peptide containing β3-HLys/β3-HGlu salt bridges on two 14-helical faces and a C-terminal D-Asp; this molecule also showed a sigmoidal dependence of MRE at 214 nm on NaCl concentration with a midpoint of 0.4 M NaCl.

The linear progression of decrease in 14-helical content over the course of the temperature variations provides further information about relative 14-helicities (Figure 1.12). The minimum MRE of the molecules appear to converge to some point beyond the scope of the assay (possibly the fully unfolded state). This is evidenced by the relative differences in slope (perhaps, the rate of unfolding) of the lines that fit the data.
The largest slope, greatest rate of change, is seen by the most 14-helical molecule with the lowest initial minimum MRE. In agreement with prior observation, this data indicates that both 2OD and 2KD are initially more helical than the control molecule at low temperatures and, yet, 2KD converges with 2 as temperature increases.

**Characterization of β53-1D and comparison to β53-1**

In both β53-1 and β53-1D, the CD wavelength scan at neutral pH showed much lower 14-helical content than 2 or 2OD, respectively, based on the intensity of the minimum at approximately 214 nm. This seems to indicate that the other residues, such as the large β3-HTrp and β3-HPhe of the recognition face, are affecting the unwinding of the 14-helical structure, whereas the contribution of the improved salt-bridge to the overall structure is minimal. Similarly, the $K_d$ of $^{\text{Flu}}\beta53-1D$ obtained from the fluorescence polarization direct binding assay was within error of the $K_d$ for $^{\text{Flu}}\beta53-1$ (368 ± 76 nM). Therefore, the change in salt bridge did not affect the inherent ability for $\beta53-1$ to bind its macromolecular target, even while it provided subtle improvements to 14-helicity of the total β-peptide structure. Overall, the ROESY spectrum of β53-1D closely matched that of β53-1, further supporting the conclusion that β53-1D assembles into a similar 14-helix.

**Interpretation of two dimensional NMR insights**

A portion of the aliphatic $^{13}$C-HSQC NMR spectrum (Figure 1.15) identifies the interactions between γ protons on β3-HOrn 1 and 7 and the corresponding γ carbon within β53-1D and β53-1. In the case of β53-1, the γ protons of both β3-HOrn1 and 7 were
broadened due to exchange and the exact positions of the two peaks could not be fully defined. In the case of $\beta^{53-1D}$, however, the $\gamma$ protons of $\beta^3$-HOrn1 were narrower and resolved. Also, differences, relating to the salt bridge, between $\beta^{53-1}$ and $\beta^{53-1D}$ were seen in the ROESY spectrum. These ROEs include three – those between protons of $\beta^3$-HAsp4 and $\beta^3$-Horn7 – that were not observed in the ROESY spectrum of $\beta^{53-1}$ (Figure 1.16). The ROESY spectra of $\beta^{53-1D}$ and $\beta^{53-1}$ also differed in terms of the distribution of long-range ROEs throughout the sequence. The spectrum of $\beta^{53-1D}$ showed comparably fewer unambiguous ROEs between $\beta^3$-HOrn1 or $\beta^3$-HOrn7 and $\beta^3$-HAsp4 and 10 but comparably greater number of ROEs between $\beta^3$-HOrn7 and $\beta^3$-HAsp4.

Conclusions

In summary, there is evidence that salt bridge identity exerts an influence on 14-helix stability, with shorter residues contributing to great 14-helical stability and the $\beta^3$-HDab/$\beta^3$-HAsp pair is identified as the most stabilizing of the salt bridges studied. With this information in place, we can now apply the structure-stabilizing salt-bridge effects to the design of other biologically active $\beta$-peptides, thereby further assessing the delicate connection between $\beta$-peptide structure and function.

This information has been used by other laboratories in the design of water soluble, structured $\beta$-peptides. Kaur and co-workers created a set of $\beta$-peptides using shorter side chains on salt bridging residues. A particular salt bridge in one of their designs involved a $\beta^3$-HOrn mimic and a negatively charged carboxylate mimic used to stabilize right handed 14-helical $\beta$-peptides based on L-aspartic acid monomers in aqueous solution.\textsuperscript{41} In a study by Vaz and co-workers, linear $\beta$-peptides held 14-helical
by electrostatic interactions were compared to analogous cyclic peptides. When shorter side chains were used for salt bridging residues on the linear β-peptides, the highest 14-helix propensities were seen. However, the most stable helical structure was formed with a cyclic peptide held together via a covalent bridge between β³-HOrn and β³-HGlu.

The trends seen for β³-peptides are not unlike those seen in natural proteins when closely examined. Generally, salt bridging is seen at the interior of proteins, where the charged residues are sequestered from solvents and can be held into close proximity by the fold of the protein. Since the salt bridges holding together the β³-peptides of our study are solvent exposed it is logical that the shorter side chains with charges more close to the hydrophobic backbone of the β-peptide, much like sequestered salt bridges in proteins, would contribute more to 14-helical structure. Longer side chains have a larger entropic cost when ordered into salt bridges, and also would be more available for solvation by the aqueous solution, thus leading to less salt-bridging and in effect less 14 helical structure. Additionally, longer side chains could lead to capping of the termini to ultimately alleviate the macrodipole of the β³-peptides, which could actually take away from the stabilizing complex salt bridge interaction.

**Experimental**

**General**

Fmoc-protected α-amino acids, PYBOP®, HBTu, HOBt, and Wang resin were purchased from Novabiochem (San Diego, CA). Dimethylformamide (DMF), N-methyl-2-pyrrolidone (NMP), N-methylmorpholine (NMM), trifluoroacetic acid (TFA), and piperidine were purchased from American Bioanalytical (Natick, MA). CD₃OH (99.5%
d_3) was obtained from Cambridge Isotopes (Andover, MA). All other reagents were purchased from Sigma-Aldrich. Mass spectra were acquired with Applied Biosystems Voyager-DE-Pro MALDI-TOF mass spectrometer (Foster City, CA). Reverse-phase HPLC was performed using a Varian ProStar HPLC system using VyDAC analytical (C4, 300 Å, 5 μM, 4.6 mm x 250 mm) or semi-preparative column (C8, 300 Å, 10 μM, 10 mm x 250 mm), or Waters SymmetryPrep semi-preparative column (C8, 100 Å, 7 μM, 7.8 mm x 300 mm) columns, using water/acetonitrile gradients containing 0.1% TFA. Separations were performed as indicated with flow rates of 1 mL/min (analytical), and 4 mL/min or 5 mL/min (semi-preparative). β^3^-peptides and Fmoc-β^3^-L-amino acids were detected spectrophotometrically at 214 nm and 280 nm using the Varian Prostar PDA UV/VIS detector. β-peptides were synthesized using a Symphony/Multiplex peptide synthesizer (Protein Technologies, Tuscon, AZ). Circular dichroism experiments were performed on an AVIV Model 202 or Model 212 spectrometer (Proterion Corporation, Piscataway, NJ). NMR spectroscopy was performed on an 800 MHz Varian INOVA NMR spectrometer (Varian, Palo Alto, CA).

**β-Peptide Preparation**

**β-amino acid synthesis**

Fmoc-protected β^3^-L-amino acids were prepared following methods described by Seebach.\(^4, 6, 10, 43\) The two synthetic steps required for the conversion of α to β^3^-amino acid are the Arndt-Eistert Homologation followed by the Wolf Rearrangement. Commercially available Fmoc α-amino acids were used as starting materials as the limiting reagent to the overall synthesis. The mechanism of the Arndt-Eistert Homologation begins when the carboxylate from the deprotonated acid moiety on the α-
amino acid performs a nucleophilic attack on the carbonyl carbon of the isobutylchloroformate, which in turn activates the C-terminal end of the amino acid, forming a mixed anhydride, while releasing a chloride ion. Then, diazomethane is generated by the reaction of inert Diazald with base, and attacks at the C-terminal carbonyl, releasing carbon dioxide and an iso-butyl oxide ion, forming the diazoketone. At this step, a new carbon-carbon bond has been formed, and an additional carbon has been added to the molecule. Next, the Wolf Rearrangement begins when silver trifluoroacetate drives off nitrogen gas from the diazoketone resulting in the formation of a carbene. Carbenes are highly reactive species, as the carbon has only six electrons in its shell, and a lone pair. This prompts the rearrangement to occur, by successive breaking of the bond between the alpha carbon and the carbonyl carbon, and attack of the alpha carbon on the carbene. The lone pair of the carbene forms a double bond to the carbonyl carbon to which it is already bonded. Attack by water leaves the $\beta^3$-amino acid – a methylene group separating the carbon bearing the amino acid “R” group from the carbonyl at the C-terminus of the amino acid (Figure 1.17).

The laboratory procedures involved in generating the diazoketone and the following rearrangement to get the $\beta^3$-amino acid are explained as follows. The $\alpha$-amino acid (~0.014 mol, 4.0-5.0 g) is dissolved in THF (20 mL), cooled on ice and placed in the product flask along with N-methyl morpholine (1.2 equivalents) and isobutylchloroformate (1.2 equiv.). This forms the mixed anhydride. Sodium hydroxide (15 equiv.) is then dissolved in water (9 mL), placed in the reaction vessel and heated to 75-80 $^0$C using a hot oil bath. For this reaction the mini Diazald apparatus (Sigma-
Figure 1.17. The general reaction mechanisms for the Arndt-Eistert Homologation and proposed mechanisms for formation of β3-amino acids.
Aldrich) is used, thus keeping the reaction and product flasks spatially separated. This is followed by the addition of dry ether (15 mL) and diethylene glycol ethyl ether (as a phase transfer to prevent the ether from bubbling off too quickly, 10 mL). The inert Diazald is then dissolved in ether (20-30 mL) and added to the separatory funnel which is allowed to drip slowly into the reaction vessel. This generates the diazomethane, due to Diazald’s reaction with the base in the reaction vessel. It is important that the entire procedure is performed behind a blast shield and that all glassware involved has no ground joints due to the potentially explosive nature of diazomethane. The diazomethane that forms is heated to a gas and travels across the apparatus to the condenser (which is a dry ice and acetone bath) where it drips as a liquid directly into the product flask. A trap connects the product flask across to the condenser, thus catching any diazomethane that does not make it into the product flask, and immediately placing it next to the dry ice bath. The separatory funnel is washed with ether after all Diazald has dripped through, and the reaction is continued until the drops from the condenser are clear (all ether). Any remaining diazomethane is neutralized with acetic acid, immediately forming a white precipitate in the trap. The reaction is then worked up via extraction with saturated sodium bicarbonate (2X, 50 mL) and concentrated sodium chloride (2X, 50 mL), dried over magnesium sulfate, filtered through celite and rotovapped to dryness. The final product, the diazoketone, is purified via column chromatography. The solvent system used is ethyl acetate as the polar component in hexanes with the addition of weak base N-methyl morpholine. The presence of the weak base will ensure that any remaining α-amino acid will be deprotonated, and thus will stick to the polar column ensuring the facile separation and movement of diazoketone through the column. For loading
purposes, the crude diazoketone is dissolved in chloroform. After the column is complete, monitored throughout with TLC, the purified diazoketone is rotary evaporated to dryness.

The rearrangement is performed in a single, straightforward laboratory step. The diazoketone is dissolved in THF (63 mL) and water (7 mL) and chilled to 0°C. The silver trifluoroacetate (0.11 equiv.) is dissolved in N-methyl morpholine (2.5 equiv.) and then added to the diazoketone. The entire reaction flask is covered with foil, as it is light sensitive. It is left to stir at 0°C for one hour, or overnight, followed by removal of the ice bath. It is then left to stir at room temperature for about thirty minutes, or overnight. At this point, a TLC is taken to monitor the reaction – if the reaction has not gone to completion more catalyst and base are added and the reaction is left to proceed longer. Upon completion of the reaction, the products are worked up, first by rotary evaporation to remove the THF (to eliminate the possibility that some product will be lost upon extraction due to solubility in THF), bringing it up in ethyl acetate, and then by extraction with saturated ammonium chloride (2X, 50 mL) and concentrated sodium chloride (2X, 50 mL). The crude product is dried over magnesium sulfate and rotary evaporated to dryness. The β-amino acid is then purified using column chromatography, loaded onto the column in chloroform, run through the column with the ethyl acetate in hexanes solvent system with the addition of acetic acid to ensure that the β-amino acid is protonated, thus aiding its ability in moving through the polar column. As before, the final product is rotary evaporated to dryness. Final products are analyzed for purity and identity by analytical HPLC and proton NMR.

*Analytical data for Fmoc-β³-HDab (Fmoc-L-β-(γ-Boc)-2,4-homodiaminobutyric acid)*
White powder (0.166 g, 20%), $[\alpha]_D = -4.2$ (c=1, CHCl$_3$), IR (CHCl$_3$): 3683.7w, 3623.6w, 3019.8m, 2926.4w, 2400.3w, 1710.6w, 1514.0w, 1478.2w, 1425.5w, 1215.5s, 1045.5w, 929.0w, 795.0s, 669.4s. $^1$H-NMR (400 MHz, CD$_3$COCD$_3$) 1.59 (tBu, 9H, s), 1.87-1.98 (CH$_2$, 2H, d), 2.77-2.80 (CH$_2$, 2H, t), 3.18-3.21 (CH$_2$, 1H, t), 3.46 (CH$_2$, 1H, s), 4.25-4.26 (CH, 1H, d), 4.41-4.44 (CH, 1H, t), 4.49-4.58 (CH$_2$, 2H, m), 6.14 (NH, 1H, s), 6.68-6.70 (NH, 1H, d), 7.50-8.06 (CH, 8H, m). $^{13}$C-NMR (400 MHz, CD$_3$COCD$_3$, rotamers expressed in italics) 29.05, 29.68-30.83 (CH$_3$, 3C, m), 36.18 (CH$_2$, 1C, s), 38.35 (CH$_2$, 1C, s), 40.23 (CH$_2$, 1C, s), 47.36 (CH, 1C, s), 48.52 (CH, 1C, s), 67.21 (CH$_2$, 1C, s), 78.93 (C, 1C, s), 121.19 (CH, 1C, s), 126.54 (CH, 1C, s), 128.34 (CH, 1C, s), 128.89 (CH, 1C, s), 142.51 (C, 1C, s), 145.56 (C, 1C, s), 173.00 (CO, C, s), 206.39-206.98 (CO, C, s), 210.37 (CO, C, s). ESI-MS: 477.3 (M + Na) (expected mass 454.5).

$\beta$-peptide synthesis (automated method)

$\beta$-peptides were synthesized on a 25 µmole scale using standard Fmoc chemistry and Wang resin loaded with $\beta^3$-homotyrosine(OtBu) (2, 2KE, 2OD, 2KD, 2DabD and 2DabE) or $\beta^3$-homoaspartate(OtBu) ($\beta^{53-1}$D), as described. $^{23}$ $\beta$-peptides 2, 2KE, 2OD, 2KD and $\beta^{53-1}$D were synthesized using automated solid phase methods as described previously. $^{37}$ One cycle of peptide elongation consisted of the following steps. The loaded resin was first washed with N-methyl-2-pyrrolidone (NMP) (1 x 20 min, 3 x 3 min for initial resin swell, 3 x 30 sec between all other elongation steps) and the terminal Fmoc protecting group removed with piperidine in DMF (1 x 2 min, 2 x 8 min, 20% v/v). Note that following the addition of the sixth residue an additional deprotection step, piperidine (2% v/v), 1,8-diazo-bicyclo[5.4.0]-undec-7-ene (DBU, 2% v/v) (1 x 8 min) is included, as reported previously. $^{13, 35}$ The deprotected resin was then washed with NMP
(6 x 30 sec) and treated with a cocktail (30 min) containing the appropriate β²-amino acid (3 equiv.), PyBOP® (3 equiv.) or HBTu (2.5 equiv.), HOBt (3 equiv.), and diisopropylethylamine (DIEA, 8 equiv.). After coupling, the resin was washed once with NMP (1 x 30 sec), and unreacted amino groups acetylated upon treatment with acetic anhydride (6% v/v) and N-methyl morpholine (NMM, 6% v/v) in NMP (1 x 8 min) and the capped resin washed with NMP (6 x 30 sec). These steps were repeated until the β-peptide sequence was complete. Once the final Fmoc protecting group was removed, the resin was washed with NMP (8 x 30 sec) and methylene chloride (8 x 30 sec) dried 20 min under N₂, and then treated for 90 min with a cleavage cocktail composed of water (2% v/v) and tri-isopropylsilane (TIPS, 2% v/v) in trifluoroacetic acid (TFA). The cleaved resin was washed once with the cleavage cocktail (1 x 30 sec) and the cleaved β-peptide was collected, concentrated by rotary evaporation, washed with acetonitrile, and reconstituted in 1:1 H₂O/CH₃CN. The final product was lyophilized to dryness.

β-peptide synthesis (manual method)

The procedure for the manual synthesis of β-peptides 2DabD and 2 DabE using a glass peptide synthesis apparatus with fritted glass at the top and bottom and a sidearm for addition of reagents (Ace Glass, Vineland, NJ) follows closely the procedure described for automated peptide synthesis, with the following differences: (1) Removal of Fmoc protecting groups during the first six cycles was accomplished using piperidine in DMF (1 x 1 min, 2 x 8 min, 5 mL of 20% v/v). Removal of the Fmoc protecting groups during the remaining cycles was achieved by piperidine in DMF (1 x 1 min, 2 x 8 min, 5 mL of 20% v/v) followed by piperidine (2% v/v), DBU (2% v/v) in NMP (1 x 8 min, 5 mL). (2) After the deprotection and NMP washing steps, three equivalents of the
appropriate β³-amino acid were reconstituted by a cocktail of HBTu (2.5 equiv.), HOBt (3 equiv.), and diisopropylethyamine (DIEA, 8 equiv.) in a glass vial followed by immediate addition to the resin and a 30 min coupling step. (3) Unreacted amino groups were treated with Ac₂O:DIEA:NMP (1:1:6, total 5.33 mL; 1 x 8 min). Once the final Fmoc protecting group was removed, the resin was washed with NMP (9 x 4 mL), dichloromethane (9 x 4 mL), and methanol (9 x 4 mL) and allowed to dry overnight under N₂. Cleavage of the β-peptide from the Wang resin occurred by shaking the resin 2h at RT in the presence of water (2% v/v), and TIPS (2% v/v) in TFA (10 mL). The free β-peptide was then blown through the RV frit by N₂ and collected, washed with the cleavage cocktail, rotary evaporated, washed with acetonitrile, and reconstituted in water/acetonitrile (1:1). The final product was lyophilized to dryness.

β-peptide purification and analysis

The success of each β-peptide synthesis was assessed initially by HPLC and MALDI-TOF analysis of the crude reaction mixture. β-peptides were then purified to homogeneity by reverse-phase HPLC. The identities and purities of purified β-peptides were assessed by analytical HPLC and mass spectrometry (Table 1.2). Following purification, β-peptides were lyophilized, kept at -20°C, and reconstituted in PBC buffer (1 mM each phosphoric, boric, and citric acids, adjusted to pH 7.0 with NaOH) immediately prior to use.

Circular Dichroism Spectroscopy

CD spectra were acquired using AVIV Model 215 or 202 spectrometers at room temperature in PBC buffer using a 2mm pathlength CD cell. Spectra represent the average of three scans (100 ms time constant, 2 nm bandwidth) and were background-
Table 1.2  Mass spectrometry data for β-peptides used in the salt bridge study.

<table>
<thead>
<tr>
<th>β³-peptide</th>
<th>[M + H] (calc)</th>
<th>[M + H] (obs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1333.4</td>
<td>1332.4</td>
</tr>
<tr>
<td>2KE</td>
<td>1361.4</td>
<td>1358.7</td>
</tr>
<tr>
<td>2OD</td>
<td>1305.4</td>
<td>1304.0</td>
</tr>
<tr>
<td>2KD</td>
<td>1333.4</td>
<td>1331.9</td>
</tr>
<tr>
<td>β53-1D</td>
<td>1361.7</td>
<td>1360.6</td>
</tr>
<tr>
<td>2DabD</td>
<td>1277.4</td>
<td>1277.1</td>
</tr>
<tr>
<td>2DabE</td>
<td>1305.4</td>
<td>1306.3</td>
</tr>
</tbody>
</table>
corrected and smoothed over 3 data points. Each β-peptide was analyzed at a concentration of 80 µM, however for 2, 2KE and 2OD, CD experiments were repeated at a concentration of 40 µM, and showed no change in minimum mean residue ellipticity (MRE). For the salt titration study, CD spectra were obtained at 25 °C using a 1 cm pathlength CD cell. Spectra represent one scan taken over 2 seconds with a 30 min stir averaging time. For 2, 2KE, 2OD and 2KD, CD data was obtained for each of the molecules in 1X PBC buffer (pH 7.0) over the temperature range of 4 °C to 98 °C with a full wavelength scan roughly every 10 °C.

Preparation of fluorescein-labeled β53-1D variant

β53-1D was prepared with an N-terminal fluorescein label by synthesizing the β53-1D β³-peptide sequence on-resin as described above. Fluorescein-5EX-succinimidyl ester (5 g, from Molecular Probes) was added in 1.5 mL NMP and allowed to mix with bubbling N₂ for 4-6 hours. The labeled product was isolated by HPLC purification and identity verified by MALDI-TOF mass spectrometry.

Fluorescence Polarization Direct Binding Assays

The fluorescence polarization experiment was performed at 25 °C in 384-well plates (MJ Research, Waltham, MA). For direct binding measurements, serial dilutions of hDM2 (obtained as a gift from Dr. Joshua Kritzer) were made in PBS buffer, pH 7.2 and an aliquot of fluorescently labeled peptide (Fluβ53-1D or Fluβ53-1) was added to a final concentration of 25 nM, to a total volume of 12 µM. The binding reaction was incubated for 30 min at RT. Thirty minutes was a sufficient length of time for the binding reaction to reach equilibrium, as judged by an absence of change in observed polarization values after 1 h and 90 min. The equilibrium dissociation constant of the β-
peptide/hDM2 complex (LP) may be determined by fitting the fluorescence polarization (FP) data to the equation: 

\[ F = F_L + \frac{(F_{LP} - F_L)([L]_T + [P]_T + K_d - ([L]_T + [P]_T + K_d)^2 - 4[L]_T[P]_T)^0.5}{([L]_T + [P]_T + K_d - ([L]_T + [P]_T + K_d)^2 - 4[L]_T[P]_T)^0.5}, \]

where \( K_d \) = the equilibrium dissociation constant of the LP complex; \( F_L \) = fluorescence polarization of free ligand L; \( F_{LP} \) = the observed fluorescence polarization of the LP complex; \( [L]_T \) = total concentration of ligand L; and \( [P]_T \) = total concentration of protein P.

**NMR Spectroscopy of β53-1D**

*Sample Preparation and Data Acquisition*

**β53-1D** (~4.4 mg) was dissolved in CD$_3$OH (~430 µL) for NMR analysis. All data were obtained by, processed and, in part, interpreted by Dr. Mike Hodsdon. The data were acquired at 10 °C on an 800 MHz Varian Inova NMR spectrometer (Varian, Palo Alto, CA) with a 5 mm triple resonance (HCN) probe equipped with triple axis (XYZ) pulsed magnetic field gradients. All pulse sequences were part of the Varian Biopack user library. Homonuclear two-dimensional (2D) NMR spectra were acquired with spectral widths of 8000 Hz in both dimensions, a 3 s recycle delay between successive scans, and acquisition times of 0.256 and 0.032 s along F2 and F1, respectively. For the z-filtered\(^39\) 2D TOCSY\(^40\) NMR experiment, isotropic mixing was applied for 100 ms using an ~11 kHz DIPSI-2\(^44\) subsequence. Similarly, spin-locking during the 2D ROESY\(^45\) NMR experiment was achieved using a 5 kHz continuous radiofrequency field applied during the 300 ms ROE mixing period. The solvent resonance was suppressed in both experiments using WET\(^46,47\) subsequences containing 5 ms selective sinc pulses. Acquisition required a total of 15 and 52 hours due to signal averaging of 32 and 128 scans for the TOCSY and ROESY experiments, respectively.
Assignments were aided by the collection of natural abundance $^1$H, $^{13}$C-HSQC NMR spectra with a 2 second recycle delay between scans, and acquisition times of 0.205 and 0.022 seconds for the F2 and F1 dimensions, respectively. Total acquisition time required for each HSQC (one for aliphatic-region carbons, one for aromatic-region carbons) was 20.3 hours due to signal averaging of 64 scans. All spectra were processed using NMRpipe and analyzed using the Sparky software package. Unambiguously-identified ROEs observed between sequentially non-adjacent residues are shown as solid arrows in the following diagram. There were additional potential ROEs corresponding to 14-helical structure that may have been present but were obscured by resonance overlap (Figure 1.14).

References


Chapter 2  $\beta$-peptide Inhibitors of a Novel Model of the SARS-CoV Prefusion Complex
Introduction

**Severe Acute Respiratory Syndrome (SARS): an emerging infectious disease**

Severe acute respiratory syndrome (SARS) is an atypical pneumonia that first surfaced in the Guangdong Province of China in the Fall of 2002. This life-threatening, infectious disease eventually spread to approximately 30 countries and infected over 8000 people with a 10% mortality rate.\(^1\) The causative agent was identified as a novel coronavirus (SARS-CoV) that is neither the result of a mutation nor recombination of any previously studied coronaviruses isolated from humans or animals, therefore it was assigned to a new, distinct group, group IV, within the genus.\(^2\)\(^3\) Much speculation as to the origins of the coronavirus, particularly the likely explanation of animal-to-human transference, has lead to numerous theories. Soon after the first viral strains were discovered, six civet cats, a raccoon dog and a badger from a southern Chinese animal market all tested positive for the SARS virus. The Asian palm civet, a cat-like mammal and the supposed animal origin of SARS, is native to south-east Asia and southern China and is frequently considered a sought after delicacy in the Chinese live animal markets. At first, it was thought that improper handling of the virus-carrying civets lead to transmission of the coronavirus across species, however current research by Prof. Daniel Janies at Ohio State University dispels the assumption that civets were the animal reservoir stating phylogenetic evidence that several species of Chinese bats harbor a strain of SARS that best describes the virus before its transmission to human hosts.\(^4\)

In general, the initial symptoms of SARS are flu-like, including fever, myalgia, sore throat, shortness of breath and coughing. Most symptoms appear within 2-3 days of exposure with 10-20% of cases eventually requiring mechanical ventilation. The
development of diagnostic kits is ongoing with the identification of the SARS coronavirus genome.

SARS-CoV is an enveloped, positive strand RNA virus with a genome of 29 kb. There are at least three structural proteins contained in the viral envelope: the membrane (M) protein, most abundant, a triple-spanning integral membrane protein, the envelope (E) protein which is involved in viral particle assembly, and the spike (S) type I integral membrane glycoprotein responsible for entry of the virus into host cells. The S protein decorates the surface of SARS-CoV, as it contains a large extracellular domain, a short transmembrane domain and a small cytoplasmic carboxyl-terminus. A cryo-electron microscopy image of the S protein at 16-Å resolution revealed a large globular surface epitope held to the viral membrane through an elongated stalk region (Figure 2.1)

**Class I fusion mechanisms: How SARS-CoV fits in among its classmates**

The S glycoprotein behaves as a Class I fusion protein. In general, fusion proteins mediate the amalgamation of two lipid bilayers. First, the fusion protein will bridge both membranes, in this case the viral and the host cell, followed by a domain rearrangement that leads to positioning both membrane anchors into close proximity which, in turn, leads to the fusion of the two membranes. Various surface glycoproteins associated with viruses of the families Orthomyxoviridae, Paramyxoviridae, Retroviridae, Filoviridae and Coronaviridae fall under Class I by their similar fusion mechanisms. Notably, hemagglutinin 2 (HA2) of influenza virus, gp41 of HIV-1 virus, GP2 of Ebola and F2 of human respiratory syncytial virus are characterized by the same general structures as SARS-CoV S protein. While the S protein is not cleaved prior to viron assembly, its
Figure 2.1  Three dimensional reconstruction of SARS-CoV from cryo-electron microscopy image.\(^6\)
sequence alignment with other coronavirus S proteins (namely murine hepatitis virus (MHV)) allows for the definition of two distinct regions S1 and S2. S1 is the receptor binding fragment that forms a large, globular extracellular domain that targets the Angiotensin-converting enzyme 2 (ACE2) receptor on the cell surface.\textsuperscript{8, 9} Cleavage of the S protein into the separate S1 and S2 domains by protease Factor Xa has been identified as a post-translational processing step that triggers virus-cell membrane fusion, however this cleavage is not an absolute requirement for fusion.\textsuperscript{3, 10} The S protein mediates entry into host cells via two different pathways depending on the presence of proteases. In the absence of proteases, SARS-CoV enters the cell via a pH-dependent endosomal pathway where S protein is taken into the cell via an endosome and then is activated for fusion by cathepsin L protease, active only under the acidic conditions of the endosome. In the presence of proteases such as trypsin or elastase, SARS-CoV S protein circumvents endosomal entry and directly attaches to the ACE2 receptor on the host cell surface which leads to envelope-plasma membrane fusion and direct cell-surface entry.\textsuperscript{11} Given the flexibility of SARS-CoV, with two pathways for cell entry regardless of S protein cleavage, the ability of the S2 domain to initiate the actual fusion event remains an important target for therapeutic inhibition.

Following S1 receptor binding and cleavage (or dissociation) from S2, the S2 portion goes through a transient intermediate, called a pre-hairpin, that bridges both the viral and host cell membranes. S2 has two 4,3-hydrophobic heptad repeat regions, HR1 (or HRN, as it is N-terminal) and HR2 (or HRC as it is C-terminal) that form three helix bundles that comprise the stalk stretching between the viral and host cell membranes. With the departure of S1, a hydrophobic fusion peptide at the N-terminal region of S2,
adjacent to HR1, imbeds into the host cell membrane, while a transmembrane domain, C-terminal and adjacent to HR2, remains anchored in the viral cell membrane. This extended structure is transitory, often called the pre-hairpin intermediate. A conformational change occurs by which the three helices of HR2 loop around and pack in an antiparallel fashion against the core formed by the three helices of HR1. Hydrophobic residues at the $a$ and $d$ positions (of heptad repeat appellation $(abcdefg)_n$ per helix turn) of HR2 are used to pack against the grooves formed by the $e$ and $g$ positions of HR1. The six $\alpha$-helix coiled coil bundle formed by HR1 and HR2 brings the two lipid membranes, from viral and host cell, into close proximity with each other enabling membrane fusion and subsequent viral entry (Figure 2.2).

**Structural characterization of the SARS-CoV S fusion core**

Several structures of the S protein post-fusion core have been solved using X-ray crystallographic techniques. In 2004, two structures of various length fragments of the post-fusion core were determined and published. First, Rao and co-workers expressed, purified and determined the structure of post-fusion HR1 and HR2 consisting of residues 900-948 and 1145-1184, respectively, with a 22-amino acid long flexible linker between the HR1 and HR2 regions. This allowed for easy expression of the fusion core while permitting the natural interaction between HR1 and HR2. The structure of the resulting 6-helix bundle was determined at 2.8 Å resolution and can be viewed in Figure 2.3. Notably, HR1 was found to form a typical three $\alpha$-helix core whereas HR2 packed against the core in partial helical conformation. Simultaneously, Supekar and co-workers published a 1.6 Å resolution structure of residues 897-972 of HR1 and 1142-
Figure 2.2. Schematic representation of the different states of the S fusion protein during viral entry.\textsuperscript{12}
Figure 2.3. From structure by Xu and co-workers: (in gray) trimeric, proteolytically stable α-helical core composed of HR1 (in green) helical and extended regions of HR2 which pack against HR1 in an antiparallel fashion (pdb 1wnc).\textsuperscript{13}
The individual HR1 and HR2 peptides used in this study had previously been characterized as a proteolytically stable core determined by treatment with proteinase K followed by HPLC-MS analysis.\(^3\) HR2 was shown to pack against HR1 using a mix of helical and extended regions. Many hydrogen-bond associations between polar HR1 amino acid side chains and main-chain nitrogen and oxygen atoms of HR2 appeared to constrain the conformation of the extended parts of HR2.\(^4\) Residues 909-928 of the HR1 trimer create relatively deep surface grooves against which the hydrophobic amino acids of HR2, Ile-1161, Leu-1168, Val-1171, and Leu-1175, pack.\(^4\) A serine scanning mutagenesis study of HR2, by Nunberg and co-workers, focused on the \(a\) and \(d\) positions in the heptad repeat as these contribute most to interhelical knob-in-hole packing interactions dominating stable coiled-coil structures (Figure 2.4).\(^{15, 16}\) The polar residue, serine, is expected to disturb hydrophobic packing while retaining good \(\alpha\)-helical propensity. Previous studies incorporating alanine residues were insufficient to disrupt six-helix bundle packing.\(^{15}\) It was determined that hydrophobic residues of the \(a\) and \(d\) positions only within the short helical segment of HR2, were considered critical for membrane fusion. This highlights the distinct region of six helix bundle formation as imperative to the viral entry event.

In 2005, Duquerroy and co-workers also solved the structure of the HR1/HR2 complex to 2.2 Å resolution by X-ray crystallography. Their structural portrayal provided important insights into the stabilization of the HR1 central core and the constraints it imposes upon the HR2 coiled coil.\(^{16}\) In this structure, the HR1 \(\alpha\)-helix does not uniformly display the typical heptad repeat. The \(a\) and \(d\) positions become out of register after several turns which shifts the face of the helix that forms the hydrophobic
Figure 2.4. Helical wheel depicting the knobs-into-holes interactions in HR1-HR2 6-helix bundle formation.$^{16}$
core. This results in stutters in the 3-4-3-4-3 periodicity of the heptad repeat (a-d, d-g, then a-d again), and creates a 3-4-4-3-4 heptad repeat at two positions (Figure 2.5). This can lead to the differences in sequence assignments of a and d, which will be discussed in the later section describing the designs of a novel model of the SARS-CoV prefusion complex. Additional structural information regarding the stability of the HR1 core was elucidated. Two chloride ions were chelated by polar, buried residues Gln 902 and Asn 937 at each of the three chains of the HR1 helix bundle. The side chains of both of these residues are involved in a hydrogen bond network with the main chain amide and carbonyl groups and with the side chains of adjacent residues in addition to the main chain of HR2. In this particular structure, both ends of HR2 were capped by hydrogen bonding to other regions within HR2 (N-terminal cap) or by important interactions with HR1 (C-terminal cap). Additionally, binding of the HR2 extended conformations to HR1 occurs through a series of Asn and Gln side chains from HR1 which hydrogen bond to the HR2 main chain.

Work by Deng and co-workers identified and trapped four different \( \alpha \)-helical structures comprised of the HR regions: an antiparallel four stranded coiled coil, a parallel trimeric coiled coil, a four-helix bundle and the six-helix bundle heretofore described as the final, fusogenic form of the protein. This succession of structures provides insight into the mechanism by which the HR regions refold in the promotion of membrane fusion. The four-stranded coiled coil was actually comprised of all HR2 domains, showing a nonclassical “knobs-into-knobs” packing of Leu and Ile side chains in a labile, yet well-ordered antiparallel structure.
Figure 2.5  From Duquerroy and co-workers, ribbon diagram depicting the structure of the HR1 (in green) and HR2 (in black) polypeptides which show the heptad repeat stutters throughout the HR1 sequence.¹⁶
solution structure of the HR2 domain prefusion state was determined by Hakansson-McReynolds and co-workers that illustrates the three, parallel $\alpha$-helix bundle that HR2 forms prior to the fusion event, characterized by the knobs-into-holes packing typical of coiled-coils.\textsuperscript{1} The four stranded coiled-coil would most likely occur after this HR2 prefusion state has begun to reorganize but prior to the antiparallel packing against HR1 forming the six-helix bundle. Similar to the Hakansson-McReynolds HR2 three helix bundle, the parallel trimeric coiled coil described by Deng was comprised of the HR1 three helix core. As reported by Duquerroy and co-workers in their examination of the six-helix bundle, the HR1 three helix coiled-coil seen here shows the classical knobs-into-holes packing with some regions of unusual core packing that break the 4-3 periodicity.\textsuperscript{17} The four helix bundle observed by Deng and co-workers was actually a dimer comprised of the C-terminal half of HR1 with a dimer of HR2. The resulting four-helix bundle resembles the final six-helix bundle state, with sometimes irregular 4-3 heptad repeat of HR1 and HR2 packing in a “wedges-into-grooves” manner notably with Leu-1175 and Leu-1168 from HR2 making interhelical van der Waals contact with the grooves of HR1.\textsuperscript{17} This four helix structure had not yet been seen in viral fusion proteins and therefore is thought to be another transitory state of HR1/HR2 complexation on the road to viral entry.

**Anti-SARS-CoV Therapeutics: Small Molecules and Antibodies**

While not involved in cell entry of the virus, SARS-CoV 3CL protease is the enzyme involved in cleaving a polyprotein into the polypeptides that are essential for viral replication and transcription. The 3CL enzyme has been declared a primary target
for therapeutic intervention. Contrary to common proteases for several other viruses, 3CL has a Cys-His catalytic dyad and cleaves the replicase polyprotein in 11 conserved sites with a Leu-Gln/(Ser, Ala, Gly) sequence. Several 3Cl protease inhibitors have been prepared, including $C_2$-symmetric diols, bifunctional aryl boronic acids, keto-glutamine analogs, isatin derivatives, $\alpha,\beta$-unsaturated esters, anilide, and bezotriazoles. The latest, trifluoromethyl ketones (TFMKs), have been used as inhibitors to serine and cysteine proteases by forming covalent bonds with the hydroxyl or thiol of the active site thereby preventing further docking of the polyprotein substrates.

With the ACE2 protein identified as the host receptor for SARS-CoV S1 binding, some of the development of inhibitors to SARS-CoV infection are based on blocking the receptor binding activity. ACE2 is essential in the regulation of cardiac function, yet is also found highly expressed on lung alveolar epithelial cells and enterocytes of the small intestines, both locations consistent with SARS-CoV infection pathogenesis. Given the important role of ACE2 in cardiovascular function there is a potential for therapeutic intervention to give mechanism-based toxicity. However, several ACE2 inhibitors have been studied. MLN-4760 is an active site directed inhibitor that binds the ectodomain of ACE2. A crystal structure of the receptor in the native form and with inhibitor bound shows a deep active site pocket near the bottom of a long "canyon" formed by two neighboring domains, with the open cleft that closes to wrap around the inhibitor when it is bound. From the crystal structure, it seems the negative ridges surrounding the ACE2 catalytic site would complement the largely positive residues of S1. MLN-4760 is a picomolar inhibitor of ACE2 ($IC_{50} = \sim 0.44 \text{ nM}$) with $>5000$ fold selectivity over ACE. The large conformational changes associated with binding of MLN-4760, as seen from
the two crystal structure comparisons, may disrupt the interaction between S1 and ACE2, thus changing the capacity of ACE2 to bind S1. Another small molecule inhibitor of ACE2, VE607, was identified using a phenotype-based screen in which Vero cells were infected with SARS-CoV, and was evaluated in both a cell-based plaque reduction assay as well as a pseudotype viral S protein/ACE2 mediated entry assay. The IC$_{50}$ for viral entry was 3.0 $\mu$M, while the EC$_{50}$ for plaque reduction came out to 1.6 $\mu$M.

There are many strategies currently being used to develop anti-SARS-CoV vaccines including inactivated viruses, subunit vaccines, virus-like particles (VLPs), DNA vaccines, heterologous expression systems, and vaccines derived from reverse genetics. Many antibodies have been generated against select regions of the S protein. Notably, monoclonal antibodies (MAb 201) isolated from transgenic mice with human Ig genes immunized with a recombinant ectodomain of the entire S protein neutralize infection in Vero E6 cells at nM concentrations. MAb 201 was shown to bind a soluble fraction of the S protein with a 34 nM dissociation constant measured by Biacore, and the neutralization epitope was mapped to the ACE2 receptor-binding domain of S1. Mice were protected from SARS-CoV infection by MAb 201, and clinical trials are currently planned to evaluate the safety and efficacy of such monoclonal antibodies for therapeutic use in humans. The coronavirus N protein generally is accessible from the surface of infected cells. A crystal structure of human major histocompatibility complex I (MHC-I) bound to a nine amino acid peptide derived from SARS-CoV N-protein was determined recently and the interaction it describes is thought to be a template for peptide-based vaccine design.
At the time of the SARS outbreak none of these therapeutic avenues were in place. However, a series of measures were taken to treat patients suffering with the respiratory disease. Ribavirin, a nucleoside analogue with \textit{in vitro} activity against a number of RNA and DNA viruses, was initially used in conjunction with corticosteroids. As ribavirin has no selective antiviral activity against SARS, and has been known to give adverse side effects, it is not an ideal therapy for patients infected with SARS-CoV.\textsuperscript{21} Interferons, known to be broad spectrum antiviral agents, used in the treatment of hepatitis B and C, were used, also concurrent with, corticosteroids in the treatment of SARS-CoV during the initial outbreak.\textsuperscript{21} In an uncontrolled study in Toronto, SARS patients receiving the combination therapy during an early stage of disease responded well with overall shorter times associated with resolution of lung abnormalities, better oxygen saturation level, and more rapid return of enzyme levels to normal.\textsuperscript{21} Those patients who had advanced to a late stage of the disease, however, apparently died regardless of the combination therapy, therefore indicating the importance of early treatment. Lastly, the HIV protease inhibitor, lopinavir, had been used in combination with ribavirin therapy, to good overall effect with no resulting deaths at 30 days after the onset of symptoms if taken at the onset of symptoms.\textsuperscript{21} As the SARS-CoV protease does not use the same mechanism as HIV protease, further work in this area to create more specific and potent protease inhibitors could lead to better therapies.

\textit{Anti-SARS-CoV Therapeutics: Peptide inhibitors to S mediated viral fusion}

Several peptides that encompass either the S2 HR1 or HR2 region have been examined as inhibitors to both the viral fusion event and viral infection. It is thought that
a peptide that comprises, for example, the HR2 region could bind to native HR1 in solution therefore preventing native HR2 from making the conformational change that results in viral and cell membrane fusion. Several laboratories have examined the inhibitory effects of peptides from S2. Liu and co-workers synthesized several peptides between 34 and 40 residues in length from either the HR1 or HR2 regions, and evaluated their efficacy both in vitro and in vivo. Only the CP-1 peptide (see Figure 2.8 for sequence) which comprised residues 1153-1189 of HR2, showed inhibitory activity on the SARS-CoV-induced cytopathic effect in Vero E6 cells, with an IC_{50} value of about 19 \mu M. To identify the mechanism of inhibition, biotinylated CP-1 was immobilized on streptavidin sensor chips and treated with peptides from the HR1 region. Binding of NP-1 (residues 892-931 of HR1, comprising the residues that form the deep surface grooves into which the helical portion of HR2 packs) was determined by surface plasmon resonance using BIACORE and a high binding affinity of 16.2 nM was obtained. Further characterization of the NP-1 and CP-1 interaction using sedimentation equilibrium showed that both peptides interact to form a six-helix bundle consisting of three molecules of NP-1 and three of CP-1, when mixed in equimolar concentration. The molecular mass was equivalent to a trimer of dimers. Therefore, CP-1 was identified as a first generation inhibitor with vital use in probing the HR1/HR2 interaction and that could be used as a lead in designing more potent peptide therapeutics.

Zhu and co-workers identified a synthetic peptide, HR2-38, comprising residues 1149-1186 of HR2 (see Figure 2.8 for sequence) with a strong inhibitory effect on SARS-CoV viral fusion. The IC_{50} for inhibition of cytopathic effect in cultured Vero E6 cells was 0.5-5 nM, as compared with 66.2 nM for a GST-fused HR2-38 that had been bio-
engineered and expressed. This result clearly indicates HR2-38 as a potential drug lead and Zhu and co-workers state rigorous pursuit of its clinical application among their future goals.

Yuan and co-workers showed that two specific peptides, HR1-1 and HR2-18, stood out as potential inhibitors when a series of 25 peptides encompassing the regions of HR1 and HR2 were screened using a SARS pseudotyped virus assay originally involving 293T cells co-transfected with HIV-luciferase and SARS-CoV S protein to create a pseudotyped virus that was then used to treat Vero E6 cells. Both peptides had antiviral activities, with EC\textsubscript{50}’s of 0.14 µM for HR1-1 (residues 889-926 of HR1) and 1.19 µM for HR2-18 (residues 1161-1187 of HR2). Both of these peptides were further tested in a wild-type SARS-CoV viral inhibition assay yielding EC\textsubscript{50} values of 3.68 µM for HR1-1 and 5.22 µM for HR2-18. Surprisingly, this study produced an HR1 based inhibitor in contrast to that of most other enveloped viruses, such as HIV-1 and Murine Hepatitis Virus (MHV), in which HR2 based peptides significantly outperform HR1 peptides in inhibiting viral fusion. HR1 peptides are thought to be poor inhibitors because they often self-associate into oligomers and can form aggregates in solution, especially as native HR1 forms a stable trimeric core, interfering with their ability to block the six-helix bundle formation. However, circular dichroism analysis of both HR1-1 and HR2-18 showed no strong α-helical signature, although the complex of HR1-1 with HR2-18 shows some increased α-helical characteristics and could be indicative of helix-bundle complexation.

Bosch and co-workers prepared peptides corresponding to the HR regions using a bacterial GST expression system. These were tested for their inhibitory potency in an
infection inhibition assay using Vero cells inoculated with SARS-CoV. HR2-1 (residues 1126-1189) showed concentration-dependent inhibition with an EC$_{50}$ of 43 ± 6.4 µM. Second generation inhibitors based on HR2-1 included increasing four-residue N-terminal truncations, or four and eight-residue C-terminal truncations, or C-terminal expansion by four residues.\textsuperscript{25} All peptides were tested similarly, with three particular HR2 based peptides showing lower EC$_{50}$’s than the parent molecule. One in particular, HR2-8, had an EC$_{50}$ of 17 ± 3 µM and spanned the residues 1126-1193 of HR2. On the whole, Bosch and co-workers found that the SARS-CoV six-helix bundle, as determined by biophysical characterization of the complexation of peptides spanning HR1 and HR2, has lower stability as compared to other enveloped viruses, such as MHV, with subsequent HR2 peptides having lower potency (µM EC$_{50}$) as compared with MHV inhibitors (nM EC$_{50}$).\textsuperscript{25} However, HR2-8 could serve as a lead for further development of SARS-CoV peptide inhibitors.

Using a syncytia inhibition assay, in which one line of cells displayed the ACE2 receptor on the surface and another displayed the S protein, two peptides, P8 and P9, were identified, by Lu and co-workers, as inhibitors. Both peptides were from the HR1 region, residues 540-559 for P8 and 731-753 for P9, and decreased luminescence, the reporter gene readout for syncytia formation, to 38%, and 28%, respectively.\textsuperscript{26} Lu and co-workers also assessed the ability of peptide-induced polyclonal antibodies to inhibit syncytia formation, however the antibodies tested did not have viral neutralizing activities.\textsuperscript{26}

Sainz and co-workers sought to create peptide inhibitors to SARS-CoV that are outside the HR regions. They used the Wimley and White interfacial hydrophobicity
scale to identify regions in SARS-CoV S2 that could favor association with lipid membranes, so as to create peptides analogous to these regions that could behave as inhibitors to viral fusion. Two particular peptides, SARS\_{WW-III} (residues 1028-1049) and SARS\_{WW-IV} (residues 1075-1093) coincided with the loop region that intercedes between HR1 and HR2. Both of these peptides showed dose dependent inhibition of SARS-CoV plaque formation on SARS-CoV infected Vero E6 cells. The IC\textsubscript{50} for both SARS\_{WW-III} and SARS\_{WW-IV} were \textasciitilde 2 \mu M. At \textasciitilde 30 \mu M concentration of peptides the inhibition level was great than 70\%.\textsuperscript{27}

Despite the advantageous interface between proteins and peptides, peptides have limited therapeutic value due to low cell permeability, vulnerability to proteolytic lysis, and poor pharmacokinetics, which creates a major challenge in creating peptide-based therapeutics. HR2 based peptides are generally designed to inhibit formation of the trimer-of-hairpins by binding to the HR1 region. T-20, the first peptide inhibitor to be approved by the FDA for treatment of HIV, is based on the gp41 HR2 region and has major limitations as a therapeutic due to the difficulty of peptide synthesis that results in high costs of production.\textsuperscript{28} To lower the cost of inhibitor production, and particularly to produce highly stable inhibitors with the sequence and structure complementarity of a peptide, various strategies have been invoked. Two proteins, HR121 and HR212, analogous to HR1 and HR2, were constructed as recombinant proteins that contain the sequence for HR1-HR2-HR1 or HR2-HR1-HR2, respectively, connected by linker regions. Both were tested in a pseudovirus mediated cell fusion assay that utilized HIV/SARS pseudotyped virus method.\textsuperscript{28} In this case, an HIV-luciferase vector was pseudotyped with SARS S envelope glycoprotein, co-transfected into 293T cells and the
HIV/SARS pseudovirus-containing supernatant collected. This virus was mixed with the inhibitors of interest and used to treat Huh 7 cells that contain the CD4+ receptor (the HIV receptor). Luciferase activity is measured by fluorescence and determines the degree of infection. The IC\textsubscript{50} values obtained for HR121 and HR212 were 4.13 ± 0.43 µM and 0.95 ± 0.12 µM, respectively.\textsuperscript{28} Overall, these recombinant proteins could open the doors to new therapeutics, as they were easily purified with low cost of production and had good inhibitory activity.

Yan and co-workers chose the region of HR2 from residues 1150-1185 and made structure-based substitutions to the peptide to examine the correlation between helical stability and ability to bind a region of HR1.\textsuperscript{29} These HR2 analogs were all synthetic, acetylated at the N-terminus and amidated at the C-terminus, and contained either increased hydrophobicity in the hydrophobic core (HRC1 which has an Ala to Ile mutation at position 1172), helical propensity (HRC2 and HRC4 which have a variety of residues changed to Ala), a combination of both hydrophobic and helical enhancement (HRC3 which has the Ile mutation and the various Ala mutations), the introduction of an \(i\) to \(i + 4\) lactam bridge that uses covalent constraint to stabilize \(\alpha\)-helical structure (HRC5) and the introduction of intrachain \(i\) to \(i + 3\) or \(i\) to \(i + 4\) salt bridges to stabilize \(\alpha\)-helical structure (HRC6 and HRC7).\textsuperscript{29} Biophysical techniques, namely CD spectroscopy, native polyacrylamide gel-electrophoresis (PAGE) analysis, and surface plasmon resonance analysis, were used to determine the interaction between each of the HR2 analogs to residues 902-950 from HR1. Using CD spectroscopy, analogs HRC2, 5 and 6 were all determined as forming complexes with HR1 that were as stable or slightly more stable than native HR1/HR2, with HRC5 as the strongest complex, not even completely
unfolded at $95^\circ$C. The SPR binding analysis was performed by immobilizing the HR1 sequence on BIACORE chips, flowing the HR2 analogs over the surface. The highest affinity binders to HR1 proved to be HRC2 and HRC5, which both contained enhancements in $\alpha$-helicity. Additionally, there was significant interaction of the HR2 analogs with themselves. Therefore, this suggests that HR2 is helical as a trimer and helical upon interaction with HR1.

Due to the success of T20 (enfuvirtide), an HR2-derived peptide, as a therapeutic against HIV-1, and the similarities of viral entry between the two viruses, Veiga and co-workers examined its potential as an inhibitor against SARS-CoV. Biophysical considerations treated the S protein-derived peptides with T20 with the addition of ANS. Fluorescence probe 1-anilino-8-naphthalene sulfonate (ANS) can be used to determine the exposure of hydrophobic regions that are normally hidden when bound by a ligand. High ANS fluorescence indicates that more hydrophobic regions of the protein or complex of interest are exposed. Additionally, the tryptophan residues of T20 provide the peptide with intrinsic fluorescence that the SARS S-based peptides do not have, therefore the measurement of fluorescence intensity of the complex can also determine the ability of T20 to bind S. The determined dissociation constant, 9.1 $\mu$M (from a $1.1 \pm 0.3 \times 10^5$ M$^{-1}$ association constant) showed moderate, yet significant, interaction between T20 and a SARS-CoV HR1-derived peptide, however it was deemed that the association was too weak for T20 to be considered a viable therapeutic for SARS-CoV.

**Metal-Assembled Triple Helix Bundles**
The use of metals to assemble peptides into parallel helix bundles of defined stoichiometry was originally designed to probe the interactions involved in protein folding. Early work by Lieberman and Sasaki demonstrated the formation of a three-α-helix bundle protein using a tris-bipyridine metal complex as a template.\textsuperscript{31} In general, designing a protein that will fold into a topologically predetermined structure is thought to follow specific rules, which lead to the concept of template-assembled synthetic proteins (TASP), first introduced by Mutter and co-workers.\textsuperscript{32} The TASP approach is as follows: the assembly of peptide building blocks reduces the conformational space accessible to the individual pieces which, when assembled, show higher thermodynamic stability than the original building blocks. The oligomeric state of the assembly is unambiguous because the individual peptide building blocks are fixed to a template which defines them.\textsuperscript{32} Short-range interactions induced by the template promote proper folding and discourage intermolecular aggregation. A metal binding site, such as an engineered 2,2′-bipyridine ligand, can be used as a template for proper assembly formation and protein folding. Amphiphilic peptides of a particular patterned sequence appended with a bipyridine at the N-terminus have been shown to form tertiary coiled coil structures upon treatment with a six-coordinate transition metal such as iron(II), cobalt(II), or nickel(II).\textsuperscript{32}

One of the first to utilize this principle, independent of Lieberman and Sasaki, was Ghadiri and co-workers with the creation of a triple-helix bundle protein comprised of three strands of 15 residue peptides appended with a 5-carboxy-2,2′-bipyridine residue at the N-termini.\textsuperscript{33} In general, the amphiphilic peptide appended with bipyridine is originally in solution in the form of a random coil, when metal ion complexation of the
bipyridine sequesters three peptide chains, creating a system bias towards trimeric assembly. The binding energy of the metal ion complexation (favorable enthalpy) is such that it drives the formation of this intermediate but also counteracts the loss of entropy in bringing the peptides into close proximity.\(^{33}\) The newly formed intermediate complex contains a greater effective concentration of hydrophobic residues. Therefore, the assembly undergoes a hydrophobic collapse that results in the formation of the triple-helical bundle (Figure 2.6).

The use of 2,2’-bipyridine ligands is advantageous as they react readily with a variety of metals, are well-studied, have high thermodynamic and kinetic stabilities and can serve as spectroscopic probes.\(^{32}\) For example, a fluorophore such as dansyl or Lucifer yellow, that has a fluorescence emission maximum (~530-550 nm) overlapping the Fe\(^{2+}\) tris-bipyridyl absorbance maximum (544 nm) will have its fluorescence quenched when brought into close proximity to an Fe(II)-assembled protein complex.\(^{34}\) Tris-bipyridyl-metal complexes are chiral and can form either left-handed \(\Lambda\) or right-handed \(\Delta\) isomers, so there are two diastereomeric triple-helical structures possible. Additionally, because the 2,2’-bipyridine molecules are asymmetric, as the peptide of interest is only attached at one of the pyridine rings, there are both facial and meridional isomers possible leaving a total of 8 possible isomers (as there are three mer for every one fac, see Figure 2.7).\(^{35}\) Fe(II) is an exchange-labile metal whereas ruthenium(II) is exchange inert, where the separation of diastereomers is possible at room temperature. In general, when examining bipy-containing \(\alpha\)-helical trimers, left-handed \(\Lambda\) isomers predominate, most likely induced by the left-handed-coiled supercoil formed by the peptides involved.\(^{36}\)
Figure 2.6. Proposed model for metal assembly of a trimeric coiled coil showing the tethering of three 2,2’-bipyridine ligands covalently linked to amphiphilic peptides by a six-coordinate metal ion.\(^{32}\)
Figure 2.7  Stereochemical outcome, showing each of the possible isomers, for an asymmetric bipyridine coordinating a metal ion.\textsuperscript{35}
In general, the sequence for the peptide involved in these three helix bundles is a coiled coil. A coiled coil consists of two to six amphiphilic α-helices associated into a left-handed superhelix. In general, each α-helix has a heptad repeat of residues, labeled \( a-g \). The side chains of \( a \) and \( d \) are generally hydrophobic, and are often \( IaLd \) for dimers, \( lald \) for trimers and \( LalD \) for tetramers.\(^{32} \) These residues mediate the hydrophobic collapse as the helices interact. Often, oppositely charged hydrophilic residues are in the positions \( e \) and \( g \), as they are complimentary between helices, and yet solvent exposed. Ogihara and co-workers designed a 29-residue peptide that folded into a coiled coil parallel three-helix bundle based on a \( VaLd \) sequence that promotes a trimeric core, with no metal coordination template.\(^{37} \)

Gochin and co-workers synthesized an N-terminally bipyridine appended peptide, P\(_{20} \), that contains Leu at both the \( a \) and \( d \) positions.\(^{38} \) Leu in these positions would actually favor either dimer or antiparallel helical arrangements while disfavoring the parallel trimer coiled-coil. This was purposefully included to ensure that the sequence did not trimerize prior to treatment with a six-coordinate metal ion, providing a measure of trimer-forming ability based on just metal complexation. Metal-ion binding was found to be coupled to helix-bundle formation, as shown by both CD spectroscopy and \(^1\)H NMR of the Ni(II) and Co(II) complexed species.\(^{38} \) Paramagnetic ions were used so that the shift and relaxation properties could be exploited to examine the peptide complex in solution. One of the Leu residues appeared to contribute to some conformational variation seen in the trimeric structure at the N-terminus, yielding spectra demonstrating two conformations for the bundle.\(^{38} \) Therefore, for a defined trimeric structure, it appears
that both the amino acid sequence and the metal-bipyridine complexation play a role, although ultimately, the stoichiometry is determined by the metal-bipyridine template.

Using metal-assembled Triple Helix Bundles to model the viral prefusion intermediate

Several methods have been applied in creating viable models for the viral prefusion intermediate of Class I viral envelope proteins for use in vitro. For example, gp41 of HIV-1 contains many of the same elements as the SARS-CoV S protein, it is membrane bound and the exposure of the HR1 three helix bundle, the target for many fusion-based inhibitors, is only transitory. Therefore, for effective in vitro assessment of inhibitor binding affinities and specificity, in addition to compound screening libraries for possible therapeutics, the necessity for a tractable, representative peptidyl model of the HR1 viral fusion core has emerged. Early work by Eckert and co-workers in Peter Kim’s laboratory demonstrated grafting the gp41 residues of interest onto a sequence bearing the GCN4 trimeric heptad repeat or an isoleucine zipper sequence to access the coiled coil and control the trimeric state.39, 40 This approach, and other related methods such as “5-helix” which is a covalent attachment of the heptad repeat helices much like HR121 and 212 described above, stabilizes the viral protein sequence as part of a larger soluble protein complex.

Gochin and co-workers synthesized a sequence from the HR1 coiled-coil domain of gp41 and used metal-bipyridine complexation to stabilize the sequence into a triple-helix coiled coil. 5-carboxy-2,2’-bipyrine was attached to the N-terminus of a 31 residue peptide containing residues 565-584 of HIV-1 gp41, with the first 11 residues from a sequence designed to stabilize three helix bundle formation.41 This molecule was named
env2.1 and contained the residues of the major grooves into which the HR2 portion of gp41 binds after the conformational change during viral fusion. Treatment with Fe\(^{II}\) lead to a magenta colored Fe\(^{II}\)-bipyridyl complex (Fe-env2.1) with an absorbance maximum of 545 nm. This overlapped well with the dansyl fluorophore, previously mentioned, which absorbs at 340 nm and emits at 542 nm. A C-peptide, with a sequence from the HR2 region of gp41, appended with the dansyl fluorophore, bound Fe-env2.1 well with a dissociation constant of 0.40 ± 0.09 µM.\(^{41}\) The binding event was monitored by the decrease in fluorescence intensity upon increase in Fe-env2.1. FRET quenching occurred as the fluorophore was brought into the close proximity of the Fe\(^{II}\)-bipyridine complex, which was a result of the C-peptide sequence binding the HR1 derived sequence of env2.1. Additionally, competition experiments were performed, by which the concentration of dansylated C-peptide and Fe-env2.1 were held constant and unlabeled C-peptide was titrated into the mixture. The IC\(_{50}\) was much higher than the \(K_d\), 273 ± 35 µM, and it was determined that the dansyl-labeled peptide needed to be at concentrations higher than the \(K_d\) in order for the fluorescence to be detected.\(^{41}\)

Gochin and co-workers improved the assay and analyzed the effects of changing the stability and sequence of the C-peptide on binding, in 2006.\(^{42}\) A systematic comparison of Co(II), Ni(II) and Fe(II) coordinated trimers of env2.1 determined that Fe(II) has the most pronounced stabilizing effect, as Fe(II) binding to bidentate ligands is cooperative. They created five different C-peptide ligands: an 18-residue wild type sequence from HR2 (C18-WT), the C-peptide with 5 residues changed to salt bridging Glu and Lys in the \(i\) and \(i + 4\) positions (C18-SB), \(\alpha\)-amino-isobutyric acid substitution that could reduce the entropic penalty associated with binding, creating a constrained,
pre-structured, α-helical peptide (C18-Aib), the C-peptide with a slightly shorter and scrambled sequence (C16-Scr) and the same shorter C-peptide with all alanine residues in place of the hydrophobic residues (C16-Ala). Each of the C-peptides was appended with a Lucifer yellow (LY) fluorophore, chosen based on its environmental insensitivity, high quantum yield and superior overlap with the Fe(II)-bipyridine absorption band. Therefore, less fluorophore-labeled peptide is necessary for binding experiments, eliminating the problems associated with dansyl-labeled peptides present in higher concentrations than the $K_d$. The highest affinity ligand was C18-Aib, with an average $K_d$ of 2.3 µM, just slightly above the wild type C18 (with an average $K_d$ of 4.7 µM), whereas C16-Ala had the worst affinity, with a dissociation constant greater than 100 µM.

Fluorescence recovery upon addition of a specific concentration of unlabeled peptide to the mixture of Fe-env2.0 (related to 2.1, but lacking the 11 residue helix-promoting region) and fluorophore labeled peptide was utilized to rank order the ability of the peptides to behave as inhibitors. The same trends were seen as with the direct binding FRET quenching assay, however the observed fluorescence recovery was 10-20 percentage points below the expected value. While light scattering, bimolecular quenching and possible inhibitor peptide aggregation could all contribute to the difference, there is also the issue of stoichiometry of binding that could impact the fluorescence recovery obtained. There are three possible binding sites for a C-peptide, labeled or unlabeled, per Fe-env2.1 complex. With the concentration of LY-labeled peptide kept low, most likely only one site will be occupied, therefore since the concentration of competing, unlabeled peptide is much higher, it could bind to the unoccupied sites on Fe-env2.1 before competing away the labeled-peptide bound in the
third site. Since so much more competing agent is needed to visualize a fluorescent effect, and much of the competing agent binding would not be monitored as it is binding an empty site and not displacing an LY-peptide, the IC$_{50}$ would come out artificially high.

Gochin and co-workers took the assay a step further for use in screening a small peptidomimetic library for compounds that disrupt six-helix bundle formation and behave as fusion inhibitors.\textsuperscript{43} Competitive inhibition of the HR1-HR2 complex formed between Fe-env2.0 and C18-Aib-LY with small-molecule HIV-1 fusion inhibitors known to bind the deep hydrophobic pocket formed by the HR1 region produced expected results with $K_i$’s that were in agreement with the IC$_{50}$’s in the literature.\textsuperscript{43} In vitro syncytium inhibition assays gave IC$_{50}$’s that correlated to each positive hit from the fluorescence recovery assay. The method was then used to screen a library of 200 compounds, and resulted in the identification of three new low-molecular-weight gp41 inhibitors, with quantitative binding affinities of ~8 $\mu$M.

The methods used by Gochin and co-workers, creating a soluble, \textit{in vitro} model of a trimeric viral prefusion HR1 core promoted by metal-bipyridine chelation, could be applicable to SARS-CoV.

\textbf{\textit{β-peptides as inhibitors to protein-protein interactions}}

Peptidomimetic oligomers that are capable of higher order structure are reminiscent of proteins without their inherent vulnerability. β-peptides have a restricted conformational space as a result of their defined secondary structures in aqueous buffers, however are protease resistant, therefore providing a good template for the development of inhibitors that are therapeutic leads. Early work by Seebach, Gellman, DeGrado and
their co-workers demonstrated that β-peptides could be used for a variety of functions: β-hairpins bound somatostatin receptors with high affinity and specificity, β-peptides could inhibit cholesterol and fat uptake, had potent antibacterial activity and the ability to bind RNA. Dr. Josh Kritzer of the Schepartz laboratory created the first 14-helical β-peptide inhibitor to the protein-protein interaction of p53-hDM2 that is often misregulated in cancer. Using the scaffold β-peptide 2 (see Chapter 1, figure 1.7), he replaced the β3-HAla residues along one 14-helical face with β3-HPhe, β3-HTrp, β3-HLeu based on the Phe-Trp-Leu p53 recognition motif along the α-helix of p53 that fit into a deep pocket on the hDM2 surface. Of four initial β-peptide designs, β53-1 had an in vitro binding affinity of 0.368 ± 0.076 µM and IC50 of 94.5 ± 4.4 µM, using a fluorescence polarization assay developed by Dr. Kritzer. Dr. Kritzer, and co-workers, also created combinatorial and screening methods to identify higher affinity β53-1 analogues from a one-bead-one-β-peptide 1000 member library. Continuing work in the Schepartz laboratory by Liz Harker resulted in several very high affinity β53-1 analogues that contain unnatural amino acids and are functional in vivo in signaling apoptosis.

Dr. Olen Stephens of the Schepartz laboratory used a similar strategy and replaced the β3-HAla residues on β-peptide scaffold 2 with the residues of HIV-1 gp41 HR2, β3-HTrp, β3-HTrp, β3-HIle, that bind the deep pocket of gp41 HR1. The resulting panel of β-peptides bound a model of gp41, IZN17, with high affinity as judged using fluorescence polarization in vitro. The best inhibitor, βWW1-1, had a Kd of 0.75 ± 0.1 µM and an IC50 of 4.0 ± 0.7 µM. These β-peptides were then examined in a gp41-mediated cell-cell fusion assay used to predict the potency of inhibitors for HIV infectivity. βWW1-4 had the lowest EC50 (5.3 ± 0.5 µM), and each of the β-peptides had
EC$_{50}$ values equal if not better than those for L-peptides, cyclic D-peptides, aromatic foldamers, or small molecules.\textsuperscript{47} While not as potent as T20, these β-peptides presented a good starting place as β-peptide therapeutic leads.

β-peptide inhibitors to SARS-CoV, targeting the HR1 core, could be developed using the principles of both Dr. Kritzer and Dr. Stephens.

\textit{Design of a novel model of the SARS-CoV prefusion complex for use in vitro}

Our approach to targeting the SARS-CoV prefusion complex began with the design of a peptidyl model of the SARS-CoV S protein pre-fusion intermediate. To characterize the binding event of peptide inhibitors to SARS fusion \textit{in vitro}, it is necessary to create a trimeric, α-helical model that is soluble in a variety of physiological buffers. Two model peptide sequences were generated based upon the two accepted alignments of the heptad repeating units for HR1 residues 902-913 (Figure 2.8).\textsuperscript{3, 12, 13, 25} The “α-g” heptad repeat alignments for bDG1 (containing SARS S2 residues 902-928) and bDG2 (containing SARS S2 residues 899-928) differ in the assignment of the amino acids which face the hydrophobic core, principally through the incorporation of an aberrant “kink” in the “α-g” heptad repeat for bDG1, but not for bDG2.\textsuperscript{16} Both model peptide sequences have a seven residue N-terminal sequence that promotes three-helix bundle formation\textsuperscript{37} and a three residue linker, N-terminal to that, as a structural requirement for the metal-bipyridine.\textsuperscript{41} Each peptide is appended with the bidentate metal ion-binding ligand, 5-carboxy 2,2’-bipyridine, at the N-terminus (Figure 2.8). The addition of Fe\textsuperscript{II} leads to the formation of a hexacoordinate metal complex, driving trimeric association and initiating hydrophobic collapse into trimeric coiled-coils.\textsuperscript{41, 42}
Figure 2.8  Primary sequences of the bipyridine peptide conjugates, and two HR2 control peptides, HR2-38 and CP-1; sequence for structural requirement of metal-bipyridine in red, sequence that promotes three-helix bundle formation in blue, $\heartsuit$ = FLAG tag sequence: DYKDDDDK.
**Design of β-peptide inhibitors to SARS-CoV S protein prefusion complex**

For the development of β-peptide inhibitors, we turned to the results of a recent serine-scanning mutagenesis study that demonstrated the critical role of hydrophobic amino acids within the short helical segment of HR2. Within this motif, three residues (Leu-1168, Val-1171, and Leu-1175) pack conspicuously against the HR1 trimer grooves (Figure 2.9). With this in mind, we designed two β-peptides containing the LVL epitope in each of two possible orientations on one face of a β³-decapeptide known to possess significant 14-helical structure in aqueous solution (Figure 2.10). In addition, we noted that helical unwinding, often a result of steric clashes between large aromatic residues on one face of the helix, may allow for a better β-peptide mimic of α-helical structure. This subtle distortion of the ideal 14-helix has been observed in NMR structures of other β-peptide inhibitors. Therefore, in an effort to promote a slightly unwound 14-helix, we incorporated a β³-homotryptophan residue between the two β³-homovalines on one face of our helix to disrupt nestling of the β³-homovaline residues.

**Results**

**Experimental Goals**

The primary objective of this study is twofold. First, by creating and characterizing a trimeric, α-helical, peptide-based model of the SARS S protein prefusion HR1 region, the capacity for a variety of fusion inhibitors to bind the relatively deep grooves of HR1 can be assayed in vitro. With the Fe²⁺-bipyridine conjugate holding
Figure 2.9. Representation of iron-bipyridine conjugated to the SARS-CoV S2 HR1 region (gray); (box) detail of HR2 (green) residues packing into HR1 trimer grooves (as electron density surface)
Figure 2.10. Helical net diagrams depicting the first generation SARS-CoV β-peptide inhibitors
the trimer together, the absorption properties can additionally be exploited by the use of fluorescence quenching assays. Therefore, validation of the model provides an important contribution to the field. Second, the development of first generation β-peptide SARS-CoV inhibitors that bind the peptidyl model with tight affinity can serve as potential therapeutic leads. Through detailed characterization of these inhibitors, we can delineate the residues most improved by alteration and work towards increased potency in vivo.

Validation of the SARS-CoV S protein Prefusion Model

To evaluate the α-helical content of the bDG peptides, each model was characterized by circular dichroism (CD) spectroscopy prior to and immediately following treatment with Fe$^{II}$. Fe$^{II}$ coordination induced significant α-helical structure in bDG2 that was consistent with the formation of a Fe(bDG2) complex across a range of concentrations (Figure 2.11). By contrast, the CD spectrum of bDG1 showed identical α-helical content with or without iron treatment (Figure 2.11). The aberration in the CD seen at higher wavelengths is due to induced left-handed chirality forming Λ-isomers from the iron-bipyridine complex with the metal ligand (Figure 2.11).36 A thermal denaturation was performed on both Fe(bDG) complexes at trimer concentrations of 20-30 μM, over a range of 4 °C to 95 °C (Figure 2.12). CD scans of the Fe(bDG) complexes were taken at 222 nm over the temperature range. Fe(bDG2) had a melting temperature of ~45 °C, whereas Fe(bDG1) had a ~54 °C melting temperature. Hysteresis was seen in both cases, however more pronounced with Fe(bDG2) which did not show any renaturation upon decreasing the temperature. The reverse melt of Fe(bDG1) had a reannealing temperature of ~40 °C. Both Fe(bDG) complexes were further characterized
Figure 2.11. CD spectrum in 25 mM Tris 25 mM sodium acetate buffer at pH 6.2 and 25 °C. Left: orange: bDG2, red (dark to light): Fe(bDG2) 28 μM, 13 μM, 3 μM, 0.9 μM, inset: aberration in MRE at high wavelength as a result of the metal-bipyridine coordination. Right: light blue: bDG1, blue (light to dark): Fe(bDG2) 35 μM, 17 μM, 5 μM, 2 μM, inset: aberration in MRE at high wavelength as a result of metal-bipyridine coordination.
Figure 2.12. CD spectrum of thermal denaturation of Fe(bDG1) in red and Fe(bDG2) in blue from 4 °C to 98 °C in 25 mM Tris 25 mM sodium acetate buffer pH 6.2.
by analytical reverse-phase HPLC and ESI-MS, which confirmed trimerization of Fe(bDG2), but did not detect Fe(bDG1) trimers. All subsequent in vitro experiments were performed using the fully identified Fe(bDG2).

To test the ability of this complex to represent the HR1 trimeric coiled-coil, we employed a FRET quenching assay to monitor the direct binding of specific peptide inhibitors HR2-38 and CP-1 (Figure 2.13). Each of these peptides was modified on a cysteine residue at the C-terminus with the Lucifer Yellow (LY) fluorophore, selected as a fluorescent donor due to its high quantum yield and superior spectral overlap with the Fe$^{II}$-bipy absorption band. When LY modified peptides were incubated with Fe(bDG2), FRET quenching of LY emission by the Fe$^{II}$-bipy of Fe(bDG2) was observed. Titration experiments in which various concentrations of Fe(bDG2) were added to a constant concentration of LY appended HR2 peptides revealed that both CP-1Cys-LY and HR2-38Cys-LY bound Fe(bDG2) well with equilibrium dissociation constants of 2.5 $\pm$ 0.3, and 2.7 $\pm$ 0.4 $\mu$M, respectively (Figure 2.13). As increasing amounts of the LY-appended peptides bound Fe(bDG2), the fluorophore and Fe(bipy) complex were brought into close proximity and the fluorescence from the fluorophore was quenched.

**Characterization of the $\beta$-peptide SARS-CoV inhibitors**

All $\beta$-peptides were determined as consistently 14-helical by CD at concentrations varying from $\sim$15$\mu$M to $\sim$185 $\mu$M. Each of the peptides were also determined as monomeric at 120 $\mu$M by analytical ultracentrifugation. The FRET quenching assay used for validation of the Fe(bDG2) model was used to examine the equilibrium binding affinity of $\beta$LVL1 and $\beta$LVL2 to Fe(bDG2). $^{LY}$Cys$\beta$LVL1 and $^{LY}$Cys$\beta$LVL2 bound the
Figure 2.13. FRET quenching assay; blue: CP-1Cys-LY, yellow: HR2-38Cys-LY, black: LY_{\text{cys}}\beta_{\text{NEG}}.
target complex with dissociation constants of $456.7 \pm 215.1$ nM, and $390.1 \pm 146.4$ nM, respectively (Figure 2.14).

**A $\beta^3$-homoalanine scan of the tightest binding $\beta$-peptide inhibitor**

To assess if this affinity was due to the LVL recognition epitope, we prepared six variants of the higher affinity ligand, $\beta$LVL2, in which one residue from either the recognition (LVL) or hydrophobic (VWV) faces was changed to $\beta^3$-homoalanine (Figure 2.15). Variant $^{LY}$Cys$\beta$LVL2A3 disrupted binding slightly ($K_D = 605.1 \pm 176.6$ nM), $^{LY}$Cys$\beta$LVL2A6 completely disrupted binding, and $^{LY}$Cys$\beta$LVL2A9 significantly improved binding ($K_D = 98.40 \pm 29.12$ nM) (Figure 2.16). $^{LY}$Cys$\beta$LVL2A8 showed slight improvements to binding ($K_D = 129.4 \pm 61.87$ nM) while $^{LY}$Cys$\beta$LVL2A5 made no significant changes ($K_D = 301.3 \pm 142.7$ nM) (Figure 2.17). $^{LY}$Cys$\beta$LVL2A2 was poorly behaved in solution so it was not incorporated into the binding data analysis. No considerable fluorescence quenching was observed with the negative control molecule, $^{LY}$Cys$\beta$NEG (Figure 2.13). See Table 2.1 for a summary of the binding data.

**Cross-talk: further evaluation of the $\beta$-peptide inhibitors and the FRET assay**

To ensure that the FRET quenching assay provided a good measure of the affinity of the $\beta$-peptides to the target Fe(bDG2), a number of additional tests were performed. Also, some analysis of the $\beta$-peptides binding other macromolecular targets and of other $\beta$-peptides binding Fe(bDG2) was performed. The first assay involved incubating $^{LY}$Cys$\beta$LVL1 and 2 with bDG2, the monomeric peptide with the SARS-CoV S1 HR1 sequence, prior to treatment with iron. While some fluorescence quenching was
Figure 2.14. top: FRET quenching assay: \(^{L^\text{Yey}}\beta\text{LVL}1\) (purple) and \(^{L^\text{Yey}}\beta\text{LVL}2\) (red); bottom: wavelength CD spectra of \(\beta\text{LVL}1\) (left) and \(\beta\text{LVL}2\) (right) in 25 mM Tris 25 mM sodium acetate buffer pH 6.2 at 25 °C. dark blue: 25 µM, light blue: 50 µM, yellow: 100 µM, pink 200 µM.
Figure 2.15  Helical net diagrams depicting each of the β-peptides, including the parent molecules, the β³-HAla scan, and negative control molecule.
Figure 2.16. FRET quenching assay: alanine variants of the βLVL2 hydrophobic face (L<sub>1</sub>Y<sub>ε</sub>YβLVL2A6 not shown); see identification of curves in Figure 2.15.
Figure 2.17. FRET quenching assay: alanine variants of the $\beta_{LVL2}$ recognition face ($^{15}$LVcys$^{\beta_{LVL2A2}}$ not shown); see identification of curves in Figure 2.15.
observed, the “binding” curves were not in a sigmoidal fashion, leading to large error in fitting (Figure 2.18). In general, binding was greatly diminished as compared to the β-peptides binding Fe(bDG2): 1.06 ± 1.03 μM for $^\text{LY}$-$\text{Cys}^\beta$ $^\text{LVL1}$ and 3.94 ± 1.74 μM for $^\text{LY}$-$\text{Cys}^\beta$ $^\text{LVL2}$.

The second experiment performed was a look at positive control CP-1Cys-LY and β-peptide $^\text{LY}$-$\text{Cys}^\beta$ $^\text{LVL1}$ incubated with iron-coordinated bipyridine with no peptide sequence (Fe(bipy)$_3^{2+}$). Neither α- nor β-peptide appeared to bind Fe(bipy)$_3^{2+}$, and a binding curve from FRET quenching was not obtained in either case. However, the signal of CP-1Cys-LY was quenched lower than what was expected for free CP-1Cys-LY fluorescence (Figure 2.19).

Much like the above described experiment, Lucifer Yellow Iodoacetamide, the fluorophore compound alone untethered to a peptide sequence, was examined in the FRET quenching assay with Fe(bDG2) titrated in the concentration range used for the α- and β-peptide binding assays. At first glance, the fluorescence from the LY Iodoacetamidene appeared to be quenched with increasing concentration of iron-containing target, however when the concentration range of Fe(bDG2) over which the β-peptides bound was zoomed in more closely, it was found that the fluorescence quenching observed was nowhere near the order of magnitude of quenching that occurs when the binding assays are performed on the β-peptides (Figure 2.20).

Final analysis included binding studies of both $^\beta$ $^\text{LVL}$ peptides to other known protein targets, such as IZN17$^{40}$ (a model of HIV-1 gp41 fusion core) and hDM2$^{52}$ (an oncprotein involved in apoptosis) and evaluation of an hDM2 β-peptide inhibitor binding to Fe(bDG2). Fluorescence polarization was used to determine the binding of
Figure 2.18. FRET quenching assay evaluating the ability of $^{\text{LY}}$cys$^{\beta}$LVL1 (purple) and $^{\text{LY}}$cys$^{\beta}$LVL2 (red) to bind bDG2 not coordinated by Fe(II).
Figure 2.19  FRET quenching assay evaluating the ability of CP-1Cys-LY (left) and LYcysβLVL2 (right) to have their fluorescence quenched by Fe(bipy)$_3^{2+}$ in the absence of peptide binding sequence.
Figure 2.20 FRET quenching assay evaluating the ability of Fe(bDG2) to quench fluorescence from Lucifer yellow iodoacetamide (in orange) with no attached peptide binding sequence.
both βLVL peptides to the IZN-17 and hDM2 targets (see the Experimental section of Chapter 1 for a description of the fluorescence polarization assay). \( \text{LY-Cys}\beta\text{LVL1} \) bound IZN17, however due to errors in the binding data, a poor curve fit was obtained and the calculated \( K_D \) was \( 2.7 \pm 2.3 \) mM, obviously not a reasonable description of the binding event (Figure 2.21). \( \text{LY-Cys}\beta\text{LVL2} \) bound IZN17 with a sub-micromolar affinity of \( 698 \pm 237 \) nM. Likewise, both βLVL peptides bound hDM2 with high affinity, although since the data did not plateau, there was also a poor curve fit and the affinity appeared artificially high: \( K_D = 56.6 \pm 41.1 \) nM for \( \text{LY-Cys}\beta\text{LVL1} \) and \( 220 \pm 138 \) nM for \( \text{LY-Cys}\beta\text{LVL2} \) (Figure 2.21). As both β-peptides appeared to bind hDM2 well, the β-peptide ligand for hDM2, \( \beta53-1 \), was labeled with LY and evaluated for binding Fe(bDG2) using the FRET quenching assay. \( \text{LY-Cys}\beta53-1 \) bound to Fe(bDG2) with a dissociation constant of \( 610 \pm 330 \) nM (Figure 2.22).

**Enzyme Linked ImmunoSorbent Assay (ELISA) Competition experiments**

To assess binding affinities of fluorophore free β-peptides, a competition Enzyme Linked ImmunoSorbent Assay (ELISA) was performed.\(^{53, 54}\) A streptavidin-coated plate pre-treated with a biotinylated version of CP-1, βLVL1 or βLVL2 was incubated with varying concentrations of competing unlabeled CP-1, βLVL1 or βLVL2 and a FLAG-tagged version of the Fe(bDG2) complex (Figure 2.1). Fe(bDG2GG\(\&\)) had a similar CD spectroscopy profile as the parent molecule Fe(bDG2) (Figure 2.23) over the range of concentrations examined. The fluorescent readout from the fluorogenic enzyme substrate was fit using a standard IC\(_{50}\) isotherm (Figure 2.24). The IC\(_{50}\)’s for Fe(bDG2GG\(\&\)) binding obtained by competition against biotinylated \( \beta\text{LVL1} \), biotinylated βLVL2 and
Figure 2.21. Fluorescence polarization assay to determine the ability of $^{\text{LYcys}}\beta\text{VL1}$ (purple) and $^{\text{LYcys}}\beta\text{VL2}$ (red) to bind other macromolecular targets. (left) binding to hDM2. (right) binding to IZN17.
Figure 2.22. FRET quenching assay evaluating the ability of $^{\text{Lys}}\beta^{53}$-1 (in burgundy) to bind Fe(bDG2).
Figure 2.23. CD wavelength spectrum of Fe(bDG2GG₃₈) in 25 mM Tris 25 mM sodium acetate buffer at pH 6.2, 25 °C. Purple: bDG2GG₃₈ prior to treatment with iron. Light blue: 3.75 µM, yellow: 7.5 µM, pink: 15 µM, dark blue: 30 µM.
Figure 2.24  Competition ELISA.  βLVL1 (purple), βLVL2 (red), βNEG (black) and CP-1 (blue).  Top left: Competing with biotinylated βLVL1 for binding Fe(bDG2GGΨ); top right: competing with biotinylated βLVL2 for binding Fe(bDG2GGΨ); middle bottom: competing with biotinylated CP-1C for binding Fe(bDG2GGΨ).
Table 2.1 Comparisons of binding data across α- and β-peptide studies

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_D$ [a]</th>
<th>Bt-Peptide [b]</th>
<th>$K_i$ [c]</th>
<th>IC$_{50}$ [d]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP-1</td>
<td>2.51 ± 0.31 µM</td>
<td>CP-1C</td>
<td>142 µM</td>
<td>149 ± 63.4 µM</td>
</tr>
<tr>
<td>βLVL1</td>
<td>0.46 ± 0.22 µM</td>
<td>βLVL1</td>
<td>24.0 µM</td>
<td>57.5 ± 5.25 µM</td>
</tr>
<tr>
<td>βLVL2</td>
<td>0.39 ± 0.15 µM</td>
<td>βLVL2</td>
<td>19.2 µM</td>
<td>52.3 ± 5.00 µM</td>
</tr>
<tr>
<td>βNEG</td>
<td>&gt; 24.5 µM</td>
<td>βLVL2</td>
<td>880 µM</td>
<td>2.09 ± 0.53 mM</td>
</tr>
<tr>
<td>BLVL2A3</td>
<td>0.60 ± 0.18 µM</td>
<td>βLVL2</td>
<td>496 µM</td>
<td>1.34 ± 0.13 mM</td>
</tr>
<tr>
<td>BLVL2A5</td>
<td>0.30 ± 0.14 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLVL2A6</td>
<td>&gt; 10 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLVL2A8</td>
<td>0.13 ± 0.06 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLVL2A9</td>
<td>0.10 ± 0.03 µM</td>
<td></td>
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</tr>
</tbody>
</table>

[a] Experimental value from FRET direct binding study. Each compound is appended with a Lucifer Yellow fluorophore [b] Bt: Biotinylated [c] Calculated value [d] Experimental value from ELISA competition study
CP-1 self-competition (Figure 2.24) are reported in Table 2.1. The $K_i$ values for CP-1, $\beta_{LVL1}$ and $\beta_{LVL2}$ for each competition were calculated using known equation $K_i = [I]_{50}/([L]_{50}/K_D + [P]_{50}/K_D + 1)$ that corrects for some of the non-applicable assumptions from the Cheng-Prusoff equation (Table 2.1). Each peptide has a calculated $K_i$ consistently 40-50X greater than the $K_D$ determined from direct binding FRET studies, however the relative trends in binding, $\beta_{LVL2}$ showing the best competition, $\beta_{LVL1}$ with similar, but marginally less competition, CP-1 showing about ten times less competition than the $\beta$-peptides and $\beta_{NEG}$ having the least competition are mirrored by the FRET quenching assay results (Table 2.1).

**Testing the efficacy of the $\beta$-peptide inhibitors in vivo**

$\beta_{LVL1}$ and 2 were evaluated for their ability to neutralize SARS-CoV infectivity of Vero E6 cells. This work was performed by Dr. Zhe Yan in Prof. Robert Hodges laboratory at the University of Colorado through a collaborative effort. To determine the efficacy of $\beta_{LVL1}$ and 2 as viral inhibitors, the virus was preincubated with the peptides prior to addition to the Vero E6 cells. Then, following inoculation of the cells with virus, the percent of viral infection was obtained by counting the plaque forming units visible. $\beta_{LVL1}$ showed slight inhibition with an average of $69 \pm 7.9\%$ viral infection with no DMSO in PBS buffer, and $78 \pm 11\%$ infection with 10% DMSO in PBS. $\beta_{LVL2}$ did not significantly inhibit infection in these conditions (Figure 2.25). Comparisons were made to B1-C, a positive control $\alpha$-peptide that is 92.5% effective and virus with no other treatment, which served as a negative control (Figure 2.25).
Figure 2.25. Percent of viral infection of Vero E6 cells after treatment with inhibitors. In pink, $\beta$LVL1, in red, $\beta$LVL2, in blue, positive control peptide B1-C$_{56}$, and in black, negative control virus alone with no inhibitors.
Discussion

*Identification of Fe(bDG2) as a validated model of the SARS-CoV Pre-Fusion Complex*

For bDG2, the marked change in \(\alpha\)-helicity following treatment with Fe\(^{II}\) can be rationalized by the induction of helicity from the hydrophobic collapse into three \(\alpha\)-helical peptide bundles upon Fe-bipy coordination. For bDG1, it appears to be \(\alpha\)-helical prior to iron treatment which could be a result of the slightly different sequence causing preassociation or even \(\alpha\)-helical monomers. A mixture of monomer, dimer, and trimer, without the added mass of iron was found from ESI-MS analysis of bDG1 after treatment with iron. This suggests that the “a-g” heptad repeat alignment from the bDG1 sequence does not coincide well with the three-helix bundle forming linker sequence,\(^{37}\) leading to poor trimer association. However, minor hysteresis was seen for the melting curve of Fe(bDG1), as predicted by the description of a twenty residue peptide held in a triple helix bundle by Fe\(^{II}\)(bipy) conjugation in the literature.\(^{35}\) Whereas, Fe(bDG2) did have significant hysteresis, in that the reverse of the melt did not replicate the forward melting curve. Again, the inherent differences between the two peptides comes from their differing heptad repeat alignments. If bDG1 is aggregating in helical bundles without the aid of Fe\(^{II}\) coordination, a degree of \(\alpha\)-helicity would be seen in both the forward and reverse of the melts.

Due to the fact that Fe(bDG2) was the one of the two models properly identified, only this complex was used in all subsequent binding assays. The FRET quenching assay showed that two positive control peptides, CP-1 and HR2-38, bound the Fe(bDG2) complex with affinities equivalent to each other. The CP-1 and HR2-38 peptides most
likely have similar dissociation constants due to their overwhelmingly similar sequences (they differ by only seven residues). However, for CP-1, the difference between the EC$_{50}$, from in vivo experiments, and $K_D$ is only 10-fold, whereas for HR2-38 the difference is 1000-fold, with the $K_D$ much higher than the EC$_{50}$. Still, the evidence that both peptides, with similar sequences, bind with similar affinity is in good agreement with a reasonable model of the SARS-CoV fusion complex. Since the C-terminus of each positive control molecule contained the fluorophore, for quenching to be observed this C-terminus was brought into close proximity to the N-terminus of Fe(bDG2), which edifies the antiparallel packing of HR2 to HR1 regions. Additionally, the negative control molecule, $^{1}$Lyys-$\beta$NEG, a $\beta$-peptide lacking functionalized residues, does not show appreciable binding to the Fe(bDG2) complex (Figure 2.13). Taken together, positive control peptide inhibitors binding the model with reasonable affinity and the lack of binding exhibited by a negative control, provide good evidence that Fe(bDG2) is a reasonable model of the SARS-CoV S protein prefusion complex.

$\beta$LVL1 and 2 are 14-helical and bind the SARS-CoV Prefusion complex with tight affinity

Both of the designed $\beta$-peptides had the usual 14-helical signature upon analysis using CD spectroscopy. The additional aberration in the CD spectrum of both $\beta$-peptides at higher wavelengths is indicative of $\beta$-peptides that contain $\beta^3$-homotryptophan. Analytical ultracentrifugation described both $\beta$-peptides as monomeric over the range at which the binding studies were performed. However, the calculated dissociation constant at ~50:50 monomer:N-mer equilibrium was 419.7 $\mu$M for 10mer to monomer for $\beta$LVL1
and 330.6 µM for heptamer to monomer for βLVL2. Therefore, at high concentrations, βLVL1 and 2 both form higher order aggregates. The results of the FRET quenching assay indicate that both β-peptides were binding to the target Fe(bDG2) leading to quenching of fluorescence from the fluorophore labeled β-peptides. As the β-peptides are labeled with the fluorophore at the N-terminus, evidence of fluorescence quenching implies that the orientation of β-peptides to Fe(bDG2) upon binding is parallel with both N-termini in close proximity. The affinity of the β-peptides for the SARS-CoV S protein model is greater than both the positive control peptides, which gives these β-peptides great promise as starting materials for therapeutics development.

**Contributions of individual β-peptide residues to binding affinity**

For this analysis, β3-homoalanine residues were incorporated in place of individual residues on the hydrophobic and recognition faces of the higher affinity βLVL ligand (Figure 2.15). In total, this substitution yielded six variants, five of which were analyzed as one, βLVL2A2, behaved poorly in solution. Each residue appears to contribute differently to the overall binding event between βLVL2 and Fe(bDG2).

The largest effect appears to come from replacing the β3-HTrp residue on the hydrophobic face. This substitution completely disrupts binding, which indicates that the β3-HTrp residue is critical for the binding event (Figure 2.26). Interestingly, this residue was purposely incorporated to impart physical control of the 14-helix structure, making it more “α-helix-like.” Subtle distortions that unwind the 14-helix have been shown in the work of Dr. Joshua Kritzer and Dr. Olen Stephens to correlate with the addition of large
Figure 2.26. FRET quenching assay showing the disrupted binding of $^{c\text{Yey}}\beta\text{LV1.2A6}$ to Fe(bDG2).
aromatic, hydrophobic residues and have been a structural hallmark of their best functional β-peptide inhibitors. Alteration of the two β₃-HVal residues on the hydrophobic face gave a mixture of results. βLVL2A3 slightly disrupted binding, demonstrating the importance of the larger, branched β₃-HVal residue in this position, however βLVL2A9 greatly improved binding, indicating that the smaller β₃-HAla residue would be preferred at this position. Perhaps the β₃-HVal in the 9 position creates some type of steric clash when the compound is bound to Fe(bDG2). Overall, the significance of the hydrophobic residues to affinity of the β-peptide for its target showed that the hydrophobic face contributes predominantly to binding.

A moderate effect to binding affinity was also a result of substitution of β₃-HAla for each of the residues on the recognition face. βLVL2A8 showed improvements to binding, yet another example of a smaller, unbranched side chain providing a better fit than the larger, β₃-HLeu residue that was originally present. Substitution at position 2 unfortunately could not be adequately assessed as the compound did not provide repeatable results from the binding assay and faced difficulty remaining in solution. Substitution at position 5 did not appear to alter the binding affinity by a significant amount.

In total, residues on both faces of βLVL2 were shown to make contributions to binding affinity to the Fe(bDG2) target. This lead to the conclusion that these initial β-peptide inhibitor designs may be optimized further using established combinatorial methods that vary the residues in an informed way using the results of the β₃-HAla scan as guidelines (Table 2.1).
**Cross-talk: FRET quenching assay validated and β-peptides generally exhibit binding to a variety of macromolecular targets**

There were several control experiments performed to determine if the binding assays provided a rigorous demonstration of the β-peptides to Fe(bDG2) SARS fusion model binding event. For the first, it was assumed, based on the CD spectroscopy results, that the amino acid sequence from SARS-CoV HR1 alone, with the linker sequence and bipyridine but without the presence of the chelating metal, does not contain significant helicity, nor does it form the trimeric core without induction by Fe^{II}. Surprisingly, both βLVL1 and 2 appeared to bind, and the fluorescence was quenched in a concentration dependent manner, as a result of titration with bDG2, however binding curves were not found to be sigmoidal in nature (Figure 2.18). As mentioned above, what could be interpreted as binding was greatly diminished with much error. Most likely, the presence of some alternative form of fluorescence quenching, such as light scattering, bimolecular quenching or ligand aggregation caused by hydrophobic association could have attributed to the loss in fluorescence.

In the next described experiment, CP-1Cys-LY and LY-CysβLVL1 were incubated with Fe(bipy)_3^{2+} to ensure that fluorescence quenching observed in the binding experiment is due to the fluorescence donor and acceptor being brought into close proximity due to the binding peptide sequences, not just presence of an acceptor in solution. Evidence that both fluorescently labeled positive control and β-peptide did not quench over varying concentrations of Fe(bipy)_3^{2+} substantiated this point (Figure 2.19). However, CP-1Cys-LY started at a lower fluorescence as had previously been seen in the binding assay to Fe(bDG2). This could either be the result of an old sample that had been
exposed to an excess of incident light, and therefore had quenched fluorescence or it could be a result of the inherent self association of CP-1, as the stock is stored at higher concentrations.\textsuperscript{22}

The study of LY Iodoacetamide incubated with varying concentrations of the target Fe(bDG2) was also a means of assessing fluorophore quenching in isolation of peptide binding sequences, and the possibly of the fluorophore itself binding the Fe(bDG2) target. Without the ligand peptide sequences initiating binding to Fe(bDG2), there should be no measurable fluorescence quenching. Over the range of Fe(bDG2) concentrations, LY Iodoacetamine did show some fluorescence quenching. It was not sigmoidal in shape and did not change by an order of magnitude the way the fluorescence changes in the cases of \(\beta\)-peptide and \(\alpha\)-peptide controls binding, although it does have an overall higher fluorescence intensity as it is not tethered to a peptide. Overall, it was determined that the LY Iodoacetamide does not appear to make a huge contribution to the binding of ligands to the Fe(bDG2) target (Figure 2.20).

The specificity of the \(\beta\)-peptides for this particular target was lastly examined by a look at the binding affinities of \(\mathbf{\beta LVL1}\) and \(\mathbf{2}\) to other macromolecular targets, IZN17 and hDM2. Both of these protein or protein-like structures (as in the case of IZN17 which is a trimeric, peptidyl model of the HIV-1 gp41 fusion core) have specific \(\beta\)-peptides that were tailored for high affinity binding to their specific target. Therefore, this further examination of the \(\mathbf{\beta LVL1}\) and \(\mathbf{2}\) \(\beta\)-peptides provided insight into the specificity of these designs, and also gave some insight into the similarities of \(\beta\)-peptide binding events across macromolecular targets, and perhaps similarities of the targets themselves. For IZN17, \(\text{LY-Cys}\mathbf{\beta LVL1}\) was not properly assessed, despite repetition of the
binding trials, as it could not be adequately fit to a binding curve without the top plateau (Figure 2.21). Therefore, binding was determined to have great error and only a millimolar affinity. \( \text{LY-Cys}^{\beta_{LVL2}} \), however, did exhibit binding to IZN17 with sub-micromolar affinity, although not as tight an affinity as to Fe(bDG2), thus \( \beta_{LVL2} \) does show subtle specificity to Fe(bDG2) over IZN17. For the hDM2 target, completely unrelated to viral fusion, both \( \beta \)-peptides fit to binding curves that exhibited high affinity.

Since the data collected from the binding studies of both \( \beta \)-peptides did not plateau, and a poor curve fit occurred the affinity appeared unnatural high, artificially skewed (Figure 2.21). However, both \( \beta \)-peptides did exhibit fairly high affinity to the hDM2 oncoprotein, perhaps as great as their affinity for the Fe(bDG2) target. In both cases, the \( \beta \)-peptides bound more poorly to the HIV model of the fusion core as compared to the SARS-CoV model, but well to the hDM2 protein. For interpretation of these results it was noted that different fluorescence binding techniques were utilized to obtain these comparisons.

Since both the \( \beta \)-peptides were shown to bind well to hDM2 the original developed \( \beta \)-peptide ligand for hDM2, \( \beta_{53-1^{45}} \), was evaluated for binding Fe(bDG2) in the FRET quenching assay that was used to evaluate the \( \beta_{LVL} \) peptides. This \( \beta \)-peptide, although originally designed to target hDM2, also appears to bind to Fe(bDG2), probably aided by the aromatic residues packing into the hydrophobic grooves (Figure 2.22).

*Competition ELISA provides qualitative trends in binding among the compounds studied*
The competition ELISAs were performed to assess binding of the β-peptides when no fluorescent tag is present. Since the parent Fe(bDG2) is not a natural protein, there are no existing antibodies directed toward the construct. For standard ELISA detection, the bDG2 sequence was appended with an antigenic epitope tag that can be recognized by antibodies. The FLAG tag was chosen due to its high charge and expected solubility, and is separated from the target sequence by two glycine residues. CD spectroscopy determined that the FLAG-tagged Fe(bDG2) was behaving similarly in solution to the parent model.

However, the major disparities between the $K_i$, calculated from the IC$_{50}$'s obtained by the competition ELISA, and the $K_d$ calculated from the FRET quenching assays provided a point of contention. Such disparities between these values ($K_i$ being consistently 40-50 times greater than the $K_d$) can be attributed to aberrations such as the presence of more than one class of binding sites and/or non-specific binding of the competitor, which are not corrected in the form of the equations used.$^{57-61}$ Additionally, the lower overall affinities seen here could be a result of the FLAG-tag addition. Despite similar CD spectra, perhaps the FLAG-tag altered the overall conformation of the binding pockets. Also, there are three possible binding pockets per Fe(bDG2) molecule, as it is a trimer. However, due to steric repulsion, space constraints, and the fact that only 125 nM quantities of biotinylated peptide are immobilized on the surface, most likely only one binding pocket is vacated by the competing agent that was preincubated with the target, thus leaving the other two pockets filled with competing peptide still bound. Perhaps this could interfere with primary antibody binding. Additionally, the overall stoichiometry of the compounds poses a problem: four biotins bind one streptavidin and up to three
peptides can bind one molecule of target. It is with these considerations that the differences between the FRET quenching results and ELISA competition results must be viewed.

However, the order of IC$_{50}$’s is consistent with the order of $K_d$’s: $\beta$NEG has the least affinity for Fe(bDG2) and is the worst competitor for binding Fe(bDG2GGF), whereas $\beta$LVL2 has the highest affinity and is the best overall competitor (Table 2.1, Figure 2.24).

The first generation $\beta$-peptide inhibitors show modest effects in vivo

Inhibition of plaque formation is thought to be the most stringent test of viral inhibition. In the absence of peptide inhibitor or antisera, Vero E6 cells inoculated with SARS-CoV Urbani strain have the ability to form visible plaques when overlayed with agarose. When the cells are treated with an inhibitor concomitantly with the virus, the number of plaques should decrease and that decrease can be equated with the percent viral infection when compared in parallel to the untreated viral infection. Interestingly, the $\beta$-peptide with the best in vitro binding efficacy, $\beta$LVL2, did not show any activity in this assay as viral inhibition was not seen. However, $\beta$LVL1, which was within error of $\beta$LVL2, in the in vitro assays, did exhibit some inhibitory effect (Figure 2.25). While the 30% decrease in viral infection afforded by $\beta$LVL1 is not as good as the positive control $\alpha$-peptide used by the Hodges group, it provides a starting place for future $\beta$-peptide SARS fusion inhibitor development. Additionally, the assay itself could be improved to give a more holistic picture of viral infection and inhibition. Dilutions were made of the virus:peptide mixture prior to treatment with the Vero E6 cells, which then decreases the
amount of β-peptide introduced to the cells as an inhibitor. Also, SARS-CoV has been shown to use two distinct entry pathways, depending on the presence of proteases.\textsuperscript{11} The assay as it stands only tests the endosomal mediated pathway, however with the addition of trypsin protease the cell-surface mediated method of viral entry would be tested which might actually prove a more desirable route for β-peptide inhibition. Given that β-peptide 14-helical structure is pH dependent, (see Chapter 1), inhibition of endosomal entry, which would take place at a very low pH, would not be preferable for a structured β-peptide inhibitor. Therefore, ensuing tests of $\mathbf{\beta LVL1}$ and 2 efficacy against the cell-surface mediated pathway continue in the Hodges lab.

There are several advantages that β-peptides have rather than the B1-C α-peptide control which indicate that future optimization of β-peptide inhibitors would be worthwhile. The β-peptides used in this study are only ten residues in length and are pre-structured into helices, therefore much shorter than the 20-30 residue α-peptide that is most likely unstructured in solution prior to binding. The structural stability and shorter length of β-peptides provides much less entropic cost upon binding the HR1 region. Additionally, as β-peptides are proteolytically stable due to the additional methylene group along each monomer in the backbone, the β-peptide inhibitors would most likely have a prolonged effect as compared with the easily lysed α-peptide inhibitor.

The modest success of these first generation β-peptides for neutralizing SARS-CoV provides a starting point for optimization and development of leads for future, more potent viral inhibitors.

\textit{Conclusions}
In this work the design and characterization of a functional, peptidyl model of the HR1 region of SARS-CoV S protein prefusion intermediate proved useful for *in vitro* evaluation of SARS fusion inhibitors. The development of this model, Fe(bDG2), utilized a strategy previously used to create a functional model of the HIV-1 gp41 prefusion complex. Fe(bDG2) was used to test β-peptide ligands designed to mimic the S protein HR2 domain and prevent the HR1/HR2 binding event required for viral fusion. These β-peptides, βLVL1 and 2, exhibited nanomolar dissociation constants from Fe(bDG2) and could serve as potential leads for therapeutic inhibitors of SARS-CoV. Furthermore, this strategy could be applied to develop inhibitors of other viruses with similar Class I fusion protein mechanisms such as Ebola, HRSV, and influenza.

**Experimental**

**General**

Fmoc-protected α-amino acids, HBTu, HOBt, Wang and Tentagel resin were purchased from Novabiochem (San Diego, CA). Dimethylformamide (DMF), N-methylmorpholine (NMM), trifluoroacetic acid (TFA), and piperadine were purchased from American Bioanalytical (Natick, MA). The ammonium acetate and potassium permanganate were obtained from J. T. Baker (Phillipsburg, NJ). All other reagents were purchased from Sigma-Aldrich. Mass spectra were acquired with Applied Biosystems Voyager-DE-Pro MALDI-TOF mass spectrometer (Foster City, CA). Reverse-phase HPLC was performed using a Varian ProStar HPLC system using Grace Vydac preparative (C8, 300 Å, 10 μM, 22 mm x 250 mm; C18, 300 Å, 10 μM, 22 mm x 250 mm) or analytical (Varian C8, 300 Å, 5 μM, 4.6 mm x 250 mm; Grace Vydac C18, 300
Å, 5 µM, 4.6 mm x 250 mm) columns, using water/acetonitrile gradients containing TFA (0.1% v/v). Separations were performed as indicated with flow rates of 1 mL/min (analytical), and 10 mL/min or 12 mL/min (preparative). All compounds were detected spectrophotometrically at 214 nm, 280 nm (β³-peptides, Fmoc-β³-(L)-amino acids), 426 nm (Lucifer Yellow containing α- and β- peptides), 340 nm (5-carboxy-2,2’-bipyridine, bDG peptides), and 545 nm (Fe(bDG) complexes) using the Varian Prostar PDA UV/VIS detector. α-peptides were synthesized using a Symphony/Multiplex peptide synthesizer (Protein Technologies, Tuscon, AZ). β-peptides were synthesized using a multimode CEM MARS microwave reactor (A. i. Scientific, Saxonburg PA). Circular dichroism experiments were performed on a JASCO J-810 spectropolarimeter (JASCO, Tokyo, Japan). Analytical ultracentrifugation was performed using a Beckman Coulter ProteomeLab XL-1 instrument (Beckman, Fullerton, CA). FRET assays were performed with an Analyst AD (Molecular Devices, Sunnyvale CA) spectrofluorimeter. ELISA assays were performed both with the Analyst AD and PTI Fluorimeter (Photon Technology International, Birmingham, NJ). Electrospray ionization mass spectrometry (ESI-MS) was performed using a Q-Tof Micro (Waters, Milford, MA) mass spectrometer with an off-axis electrospray ion source at 1 µL/min flow rate.

**β-peptide Synthesis (microwave method)**

Fmoc-protected β³-amino acids were prepared following methods described by Seebach and elaborated in Chapter 1.²⁶ β-peptides were synthesized (25 µM scale) using standard Fmoc chemistry and Wang resin loaded with β³-homoaspartate(OtBu) (βLVL1, 2, 2A2, 2A3, 2A5, 2A6, 2A8 and 2A9) or β³-homoglutamate(OtBu) (βNEG) as described.⁴⁸ All β-peptides were synthesized in a glass peptide synthesis reaction vessel
with fritted glass at the top and bottom and a sidearm for addition of reagents (Ace Glass, Vineland, NJ) using the multimode CEM MARS microwave reactor.\textsuperscript{63} One cycle of β-peptide elongation consisted of the following steps: The loaded resin was first swelled with DMF (1 x 20 min, allowed to shake on the tabletop shaker). The terminal Fmoc protecting group was removed with 20% v/v piperidine/DMF (1 x 4 mL) using the CEM Discover microwave reactor program: Ramps to 70 °C for 2 min., holds at 70 °C for 4 min., cools to 25 °C for 5 min. The microwave stirs the resin solution, due to the addition of a magnetic stir-bar, as it rotates and keeps 800 psi and adjusts to 400 W. The deprotected resin was then washed with DMF (15 rinses) and treated with a cocktail containing 3 equivalents of the appropriate β\textsuperscript{3}-amino acid, 2.5 eq of HBTu, 3 eq HOBt, and 8 eq diisopropylethylamine (DIEA). Coupling ensued using the CEM Discover microwave reactor program: Ramps to 60 °C for 2 min., holds at 60 °C for 6 min., cools to 25 °C for 5 min. These steps were repeated until the β-peptide sequence was complete.

For the α-Cys appended β-peptides, the final coupling required addition of 3 equivalents of the α-Cys(trt) residue. For the biotin appended β-peptides, the final coupling involved the addition of two pre-packaged microcapsules of NHS-PEO\textsubscript{4}-Biotin (Pierce, 2 mg each). Once the final Fmoc protecting group had been removed, the resin was washed with DMF (15 x 30 sec), methylene chloride (15 x 30 sec), methanol (15 x 30 sec), dried 20 min under N\textsubscript{2}, and then treated for 90 min with a cleavage cocktail composed of water (2% v/v) and tri-isopropylsilane (2% v/v) in trifluoroacetic acid (TFA). The cleaved resin was washed once with the cleavage cocktail (1 x 30 sec) and the cleaved β-peptides were collected, concentrated by rotary evaporation, washed with acetonitrile, and reconstituted in 1:1 H\textsubscript{2}O/CH\textsubscript{3}CN. The final product was lyophilized to dryness.
**β-peptide Purification and Analysis**

The success of each synthesis was assessed first by HPLC and MALDI-TOF analysis of the crude reaction mixture. β-peptides were then purified to homogeneity by reverse-phase HPLC. The identities and purities of purified β-peptides were assessed by analytical HPLC and mass spectrometry (Table 2.2). Following purification, β-peptides were lyophilized, kept at -20 °C, and reconstituted in Tris acetate buffer (25 mM Tris base, 25 mM sodium acetate, pH 6.2-6.3 by acetic acid) immediately prior to use.

**α-peptide Synthesis**

HR2-38Cys, containing residues 1149-1186 of SARS-CoV S2 HR2 with a cysteine residue appended to the C-terminus, CP-1Cys, containing residues 1153-1189 of SARS-CoV S2 HR2 also appended with a C-terminal cysteine, bDG1, containing a 10 residue N-terminal linker that promotes 3-helix bundle formation followed by residues 902-928 of SARS-CoV S2 HR1, and bDG2, containing the same 10 residue N-terminal 3-helix bundle promoting region followed by residues 899-928 of SARS-CoV S2 HR1, were synthesized (25 µmole scale) using standard Fmoc chemistry and Tentagel resin. HR2-38Cys and CP-1Cys were acetylated on the N-terminus and amidated on the C-terminus, whereas the bDG peptides were C-terminally amidated and treated with the 5-carboxy-2,2'-bipyridine molecule on the free N-terminus. Cleavage from the tentagel resin required treatment with the cleavage cocktail as described above for β-peptides. Collected peptides were precipitated out of solution by pouring over cold ether, pelleted using centrifugation, washed with cold ether and dried over N2. Crude peptide was then reconstituted in 1:1 H2O/CH3CN and the final product was lyophilized to dryness.

*Preparation of Labeled α- and β-peptides*
## Table 2.2. Mass Spectrometry data for β- and α-peptides used in this study[^a]

<table>
<thead>
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<th>Peptide</th>
<th>[M+H] calc</th>
<th>[M+H] obs</th>
</tr>
</thead>
<tbody>
<tr>
<td>βLVL1</td>
<td>1327.7</td>
<td>1329.5</td>
</tr>
<tr>
<td>βLVL1</td>
<td>1884.2</td>
<td>1888.6</td>
</tr>
<tr>
<td>βLVL2</td>
<td>1327.7</td>
<td>1329.8</td>
</tr>
<tr>
<td>LY-Cys-βLVL1</td>
<td>1884.2</td>
<td>1885.2</td>
</tr>
<tr>
<td>LY-Cys-βLVL2</td>
<td>1842.1</td>
<td>1847.1</td>
</tr>
<tr>
<td>LY-Cys-βLVL2A2</td>
<td>1856.2</td>
<td>1859.2</td>
</tr>
<tr>
<td>LY-Cys-βLVL2A3</td>
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<td>1773.0</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>LY-Cys-βLVL2A9</td>
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</tr>
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<td>βNEG</td>
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<td>1801.7</td>
</tr>
<tr>
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<td>1801.3</td>
<td>1802.3</td>
</tr>
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</tr>
<tr>
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</tr>
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<td>CP-1Cys-Bt</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Fe(bDG2)</td>
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<td>13694.0</td>
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</table>

[^a]: Values represent the calculated and observed masses for the peptides synthesized and purified in this study.
The Lucifer Yellow (LY) labeled α- and β-peptides were generated by reaction of an HPLC-purified sample of cysteine appended peptide with Lucifer Yellow Iodoacetamide (Molecular Probes, 20-fold molar excess) in sodium phosphate (50 mM) buffer with sodium chloride (150 mM) at pH 7.4. Labeling reactions were incubated with rotation for 2-4 h at RT in the dark. Biotin labeled peptides were acquired by first bringing up the peptide in water (500 µL, 300 µM), Tris-HCl, pH 8 (50 µL, 1 M), and EDTA (2 µL, 0.5 M). To this Biotin PEO Iodoacetamide was added in excess (440 µL, 9.2 mM). The mixture was incubated with rotation for 3-4 h at RT in the dark. The crude LY-labeled peptides and Biotin-labeled peptides were purified by reverse-phase HPLC and characterized by MALDI-TOF mass spectrometry (Table 2.2).

**Preparation of FeII Promoted Triple Helix Bundles**

The 5-carboxy-2,2’-bipyridine molecule was synthesized in three steps, as suggested by Prof. Martin Case (University of Vermont) (Figure 2.27). In general, for step one, 2-Acetylpyridine (0.025 mol) and I2 (0.0197 mol) were dissolved in excess anhydrous pyridine (0.309 mol) and refluxed for 6 h (Figure 2.28). The round bottom flask was dried in the oven overnight first, and fresh drierite at the top of the reflux tube. The reaction mixture was cooled to room temperature and the product collected by suction, washed with a little pyridine, and recrystallized with ethanol and addition of charcoal to give crystals upon filtration that were dried in vacuo. Using fresh I2 and pyridine, with fewer equivalents of both, especially making the I2 the limiting reagent, and recrystallizing the mother liquor all helped improve yield of this step. On the whole a 46.7% yield was accomplished (literature yield is 62%). In general, for step two, 1-(2-pyridylacetyl) pyridinium iodide (0.0031 mol), dry methacrolein (0.0034 mol) and
Figure 2.27. Overall three step scheme of the synthesis of 5-carboxy-2,2'-bipyridine from 2-acetylpyridine.\textsuperscript{64, 65}
Figure 2.28 Mechanism depicting the first step of bipyridine synthesis: 2-acetylpyridine reacting with catalytic iodine followed by reaction with pyridine to form 1-2(pyridylacetyl)-pyridinium iodide.
ammonium acetate (0.0092 mol) were dissolved in minimal formamide and stirred for 6 h at 80 °C under argon. Note this is an optimal procedure and a mistake in the original protocol in the literature did not have the appropriate grams associated with the molar amount of ammonium acetate used. Methacrolein actually has a boiling point of ~ 60 °C. Since the reaction goes through a Krönke reaction (keto-enol tautomerization followed by Michael addition of Methacrolein and later a ring closing affected by Schiff base), in order for the Methacrolein to add and not boil away the reaction was stirred at room temperature for 2 hr until starting material disappeared, as indicated by TLC in dichloromethane and methanol (Figure 2.29). At this point the reaction mixture turns a dark red color. Heating to 80 °C for another 4-6 hours ensued as the reaction mixture turned from red to orange. Following completion of the reaction, the system is cooled to room temperature, water added and the mixture extracted with diethyl ether and dichloromethane. The combined organic phases were dried and filtered and the solvent removed by rotary evaporation. The residue is purified by column chromatography (silica gel, 220-440 mesh, CH₂Cl₂/MeOH 95:5) to give a brownish oil that was dried overnight on the vacuum line and afforded 74.8% yield in accordance with the literature. In general, for step three, a mixture of potassium permanganate (0.015 mol) and 5-methyl-2,2’-bipyridine (0.0023 mol) in water (34.8 mL) was heated at 70 °C for 24 hr. After filtration of the reaction mixture and washing the brown filtrate with aqueous NaOH (1M, 3.5 mL), the combined water fractions were collected and washed three times with CHCl₃ to removed unconverted 5-methyl-2,2’-bipyridine. The aqueous solution was neutralized with HCl (2M) and concentrated. After acidifying to pH 6 the precipitate was collected, washed (EtOH 3X) and dried in vacuo. Crude products
Figure 2.29  Mechanism depicting the second step of bipyridine synthesis: making an enolate from 1-2(pyridylacetyl)-pyridinium iodide by reaction with acetate ion, then going through a Michael conjugate addition with methacrolein, leading to a Schiff base after proton transfers which then goes through a transformation to form a conjugated double bond system leading to closing the pyridine ring and final formation of 5-methyl 2,2’-bipyridine. This overall mechanism is reminiscent of the Krönke reaction.
required further purification, and preparative HPLC was performed using a C8 column and a gradient of 1-45% solvent B (acetonitrile with 0.1% TFA) over 40 min. Purified 5-carboxy-2,2’-bipyridine was coupled to the N-terminus of the growing bDG1 and bDG2 peptides using standard solid phase chemistry. 5-carboxy-2,2’-bipyridine (2-4 equivalents) was brought up in minimal DMF with HBTU (1.6-3.2 equivalents), and diisopropylethylamine (12-24 equivalents). The coupling was allowed to proceed for 2-3 hours. Following cleavage from the resin (as described above), the bDG peptides were purified to homogeneity by reverse phase HPLC and reconstituted in Tris (25 mM) / sodium acetate (25 mM) buffer pH 6.2-6.3. The concentration of the stock was determined using an extinction coefficient at 291 nm based on a literature value. The extinction coefficient for a twenty residue peptide of similar length and sequence with a C-terminal amide and no other chromophores with an N-terminal bipyridine was 17,500 M⁻¹ cm⁻¹. This was the coefficient used for bDG1, however for bDG2, which has an additional chromophore (a tyrosine) the coefficient was 17,617 M⁻¹ cm⁻¹, and for bDG2GG (two tyrosines) the coefficient was 17,734 M⁻¹ cm⁻¹. A sample of FeCl₂ 4H₂O (1/3 molar equivalent) was added to the bDG peptide solution and allowed to react for 40-60 min. The reaction was traced by UV/Vis spectroscopy every 5 min until the reaction did not appear to proceed any further. The reaction products were then analyzed directly by analytical reverse phase HPLC and ESI-MS (performed by Dr. Eugene Davidov).

**Circular Dichroism Spectroscopy**

CD spectra were acquired in a 2 mm or 1 cm pathlength cell using a JASCO J-810 spectropolarimeter at room temperature with samples in Tris/acetate buffer (25 mM Tris
base, 25 mM sodium acetate, pH 6.2-6.3 with acetic acid). Relevant parameters include a 0.5 nm data pitch, 50 nm/min scanning speed, 4 sec response, 1 nm band width, and 3 accumulations. Each bDG peptide was analyzed at the following concentrations: Each β-peptide was analyzed at the following concentrations: 185 µM, 85 µM, 40 µM, 15 µM (βLVL1), and 183 µM, 92 µM, 40 µM, 16 µM (βLVL2). Each bDG peptide was analyzed at the following concentrations: 28 µM, 13 µM, 3 µM, 0.9 µM (bDG2), 35 µM, 17 µM, 5 µM, 2 µM (bDG1) and 30 µM, 15 µM, 7.5 µM, 3.8 µM (bDG2GGΦ).

**Sedimentation Equilibrium**

Samples analyzed using analytical ultracentrifugation were prepared by dissolving HPLC-purified and lyophilized β-peptides in Tris buffer (25 mM Tris base, 25 mM sodium acetate, 150 mM NaCl, pH to 6.2-6.3) at the desired initial concentrations (30 µM, 60 µM, and 120 µM) and higher concentration for βLVL2 (180 µM, 300 µM and 500 µM). The samples were then centrifuged, with the help of Dr. Jade Qiu, as previously described.67 Samples were spun at equilibrium at 25 °C at three different speeds (42,000, 50,000, and 60,000 rpm) in an AN 60-Ti 4-hole rotor equipped with six-channel, carbon-epoxy composite centerpieces supplied by Beckman. Absorbance was monitored at 280 nm. Data were collected with a 0.001 cm step size, and successive scans were initiated at 2-hour intervals. Samples were judged to have reached equilibrium when no significant change in radial concentration was observed in three successive scans using the program Match within the Heteroanalysis software suite (available from the National Analytical Ultracentrifugation Facility website, [http://vm.uconn.edu/~wwwbiotec/uaf.html](http://vm.uconn.edu/~wwwbiotec/uaf.html)). The data were fit to a monomer-Nmer equilibrium model using Heteroanalysis software. In all cases, the β-peptides were
monomeric at the low concentrations over which all experiments were performed. To fully evaluate the degree of oligomeric association, analytical ultracentrifugation was performed on βLVL2 at higher concentrations. In all cases the same V-bar (0.79), density (1.00529) and extinction coefficient (5690 M$^{-1}$ cm$^{-1}$ at 280 nm) were used. Results from data fitting for βLVL1, βLVL2 at higher and lower concentrations can be seen in Table 2.3. The data fit with these parameters and the resulting residuals for representative data are shown in Figure 2.30.

**Forster Resonance Energy Transfer (FRET) quenching assays**

In FRET a fluorescent donor molecule transfers energy to an acceptor molecule through a nonradiative induced dipole-induced dipole interaction.$^{68}$ It is called “nonradiative” because no photons are passed between the donor and acceptor. The electric field produced by the donor arises because incident excitation light induces electrons in the donor to oscillate (or, in quantum mechanics terms, to transition). This generates an induced, electric dipole moment in the donor which then creates its own characteristic electric field. If an acceptor molecule is in the close-up electric field of the donor, its electrons will be induced to oscillate, creating a dipole moment in the acceptor.$^{68}$ In quantum mechanical terms, the transitions in the donor due to the excited state decay rapidly to the lowest excited state and then relax back to the ground state via fluorescence, nonradiative processes, or interaction with the acceptor via a dipole-dipole interaction. If the wave function of the donor and acceptor dipoles are nearly the same in energy FRET can occur at a reasonable rate.$^{68}$ In our studies the donor and acceptor pair were the Lucifer Yellow fluorophore and the Fe(bipy)$_3$ complex, respectively. Lucifer Yellow excites at 426 nm and emits at 531 nm, whereas Fe(bipy)$_3$ absorbs energy at 545
Figure 2.30  Sedimentation equilibrium analysis of samples centrifuged to equilibrium at speeds 42 (red), 50 (blue), and 60 krpm (green) at 25 \(^0\)C.  Left: \(\beta_{LVL1}\) at 120 \(\mu\)M; Middle: \(\beta_{LVL2}\) at 120 \(\mu\)M; and Right: \(\beta_{LVL2}\) at 500 \(\mu\)M.
Table 2.3. Results of global fitting for β-peptides: sedimentation equilibrium

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Stoichiometry</th>
<th>RMSD(^{[a]})</th>
<th>LnK (\pm) 1.78</th>
<th>(C_{50})^{[b]} (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>βLVL1</td>
<td>10.40 ± 0.22</td>
<td>0.00544</td>
<td>77.27 ± 1.78</td>
<td>419.7 (\mu M)</td>
</tr>
<tr>
<td>βLVL2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low conc.</td>
<td>2 (fixed)</td>
<td>0.00452</td>
<td>7.340 ± 0.17</td>
<td>648.9 (\mu M)</td>
</tr>
<tr>
<td>High conc.</td>
<td>7.3 ± 0.04</td>
<td>0.00949</td>
<td>52.88 ± 0.17</td>
<td>330.6 (\mu M)</td>
</tr>
</tbody>
</table>

\([a]\) Root Mean Square Deviation as a result of the fit; \([b]\) Calculated dissociation constant at ~50:50 monomer:N-mer equilibrium.
nm and yet is not fluorescent so it does not emit. In general, the LY fluorophore has a high quantum yield and superior spectral overlap with the Fe(bipy)$_3$. As there is good agreement between the donor and acceptor wavelengths, the assay can be performed in which increasing concentrations of Fe(bipy)$_3$ conjugated peptide are titrated into a solution containing a fixed amount of LY appended peptide inhibitor. As more and more Fe(bDG2) binds the inhibitor, the fluorescence intensity of LY decreases as it is in close proximity to a large amount of the quenching FRET partner. This assay was precedented in the literature by Gochin et al in their work on HIV-1 gp41-mediated fusion inhibition.$^{41, 42}$ Therefore, FRET experiments were performed as described$^{41, 42}$ at room temperature in 384-well plates (Corning Black Flat PS (P/N 3710), Corning, NY). For direct binding measurements, serial dilutions of Fe(bDG2) were made in the aforementioned Tris buffer, pH 6.2-6.3 and an aliquot of LY labeled peptide (600 nM) was added to each well (50 nM final concentration in a total volume of 36 µL). The binding reaction was incubated for 20 min at RT. Twenty minutes was a sufficient length of time for the binding reaction to reach equilibrium, as judged by an absence of change in fluorescence intensity values after 60 min. The equilibrium dissociation constant of an LY labeled α- or β-peptide to Fe(bDG2) complex (L to P) may be determined by fitting the fluorescence intensity data to the equation $F = F_L + ((F_{LP} - F_L)(2[L_T]))^0.5([L_T] + [P_T] + K_d - (([L_T] + [P_T] + K_d)^2 - 4[L_T][P_T]))^{0.5}$, where $K_d$ = the equilibrium dissociation constant of the L to P complex; $F_L$ = fluorescence intensity of free ligand L; $F_{LP}$ = the observed fluorescence intensity of the L to P complex; $[L_T]$ = total concentration of ligand L; and $[P_T]$ = total concentration of protein P. In all cases the value of $[L_T]$ was fixed (50 nM).

**Enzyme-Linked ImmunoSorbent Assay (ELISA) Competition Assay**

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This method is used to detect the presence of Fe(bDG2GG\(\kappa\)) that is retained to a plate of immobilized peptide after treatment with a competing peptide. A black streptavidin-coated 96-well plate (pre-blocked with SuperBlock Blocking buffer, Pierce) was washed 3 times with manual shaking for 30 sec each, with Tris (25 mM) / sodium acetate (25 mM), and Tween (0.05%) buffer pH 6.2-6.3 (Tris-T, 100 \(\mu\)L). To each well was added a solution of Cp1CBt, \[^{15}B^{\beta}LVL1\] or \[^{15}B^{\beta}LVL2\] (Bt: biotinylated, 50 \(\mu\)L, 125 nM). The plate was incubated for 1 h at room temperature, no shaking, and the wells were washed thoroughly with Tris-T buffer (2 X 30 s manual shaking, 2 X 5 min on shaker, 1 X 30 s manual shaking). During this time, a solution of Fe(bDG2GG\(\kappa\)) (1.2 \(\mu\)M monomer concentration, 15 \(\mu\)L per well) was incubated with varying concentrations of peptide competitor (ranging from about 1 mM to 2 \(\mu\)M, 15 \(\mu\)L per well) for 30 minutes at room temperature. The peptide dilutions were prepared in Tris-T buffer and the Fe(bDG2GG\(\kappa\)) stock was diluted in Tris-T with BSA (2%, final concentration per well 1% BSA). The peptide competitor plus Fe(bDG2GG\(\kappa\)) solutions were distributed into the wells (30 \(\mu\)L per well) and incubated for 1 h, no shaking. Each well was then washed as above with Tris-T. The Rabbit antiFLAG antibody (GC Genscript Corporation) was added to each well (100 \(\mu\)L per well) at a dilution of 1:250 in Tris-T with BSA (1%). After 1 h on the shaker, the wells were washed as above with Tris-T, and anti-rabbit-IgG-HRP (Santa Cruz, 100 \(\mu\)L) diluted 1:1000 in Tris-T with BSA (1%) was added to each well. A Quanta Blu (Pierce, from the Quanta Blu Fluorogenic Peroxidase Substrate kit) working solution was prepared by mixing 9 parts Quanta Blu Substrate Solution to 1 part Quanta Blu Stable Peroxide Solution and allowed to equilibrate to room temperature over 20 min. Following 1 h incubation on the shaker, the plate was removed and washed as
The Quanta Blu working solution (100 µL per well) was added and the plate was incubated for 1 h, no shaking, before the addition of Quanta Blu Stop Solution to each well (100 µL). Each well was read using the PTI fluorimeter, with an excitation wavelength of 330 nm and an emission wavelength of 401 nm. The concentration at which inhibition is half its maximum value (the IC\textsubscript{50}) was calculated using the equation 

\[ F = F_L + \left( (F_{LP} - F_L)/(1+(IC_{50}/[I])^n) \right) \]

where [I] = [competing inhibitor], n = Hill coefficient and the other entities are defined in the FRET quenching assay section above.

**Viral Neutralization Assay**

These experiments were performed by Dr. Zhe Yan of Prof. Robert Hodges laboratory at the University of Colorado. Each peptide (βLVL\textsubscript{1}, βLVL\textsubscript{2}, and a control peptide inhibitor named B1-C) was dissolved in PBS buffer (1X, pH 7.4) or PBS with DMSO (10%) to a concentration of 120 µM. The peptides were incubated at room temperature or 37 °C for one hour with SARS-CoV\textsubscript{Urbani} (2.8 x 10\textsuperscript{2} PFU first trial, 3.4 x 10\textsuperscript{3} PFU second trial). Serial 10-fold dilutions of virus/peptide mixture were inoculated onto Vero E6 cells and incubated for 1 hr at 37 °C with rocking every 15 min. The inoculum was removed from the cells and an agarose overlay was added. The cells were then incubated at 37 °C for 3 days. A neutral red staining was performed and the number of plaques was counted.

**References**


